THE EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ (PPARγ1 AND PPARγ2) IN NAÏVE AND MEMORY CD4+ T LYMPHOCYTES

by

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In the name of Allah, the most Merciful and Compassionate

All praises and gratitude is due to Allah, the lord to whom every single creature in the heavens and the earth belongs to. May peace and blessings be upon our prophet Muhammad S.A.W, his family and companions.

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LIST OF ABBREVIATIONS

β-ME	β-Mercaptoethanol
15d-PGJ_2	15-deoxyl-$\Delta^{12,14}$-prostaglandin J_2
AF-1	activation function-1
AF-2	activation function-2
AP-1	Activated protein-1
APC	Antigen presenting cells
bp	Base pair
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CO_2	Carbon dioxide
CoA	Co-activator
CoR	Co-repressor
C_t	Threshold cycle
CDR	Complementary determining region
CTLA-4	Cytotoxic T lymphocyte associated antigen-4
DAG	Diacylglycerol
DBD	DNA-binding domain
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
DN	Double negative
DNA	Deoxylribonucleic acid
DP	Double positive

EDTA	Ethylenediamine-tetra acetic acid
EtBr	Ethidium bromide
FAM	6-carboxyfluorescein
FBS	Foetal bovine albumin
g	gram
GM-CSF	Granulocyte monocyte colony-stimulating factor
h	hour
HCl	Hydrochloric acid
IκB	Inhibitor of κB
ITAM	Imunoreceptor tyrosine activation motif
kDa	kiloDalton
L	litre
LBD	Ligand binding domain
LDH	Lactate dehydrogenase
M	molar
mg	miligram
$MgCl_2$	Magnesium chloride
min	minute
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NFAT	Nuclear factor activated T cells
NF-κB	Nuclear factor κB
NHR	Nuclear hormone receptor
ng	Nano gram
NTC	No template control
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PTPRC	Protein tyrosine phosphatase receptor type C
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
R_n	Normalized reporter
RNA	Ribonucleic acid
RPMI	Roselle's Park Memorial Institute Medium
RT-PCR	Reverse transcriptase PCR
RXR	Retinoid acid receptor
TAE	Tris-acetate-EDTA
TAMRA	6-carboxyl-tetramethyl-rhodamine
TCR	T cell receptor
TBE	Tris-borate-EDTA

T_m	Melting temperature
TNF	Tumour necrosis factor
TZD	Thiazolidinedione
UV	Ultra violet

LIST OF SYMBOLS

μ	micro
<	less than
^{0}C	degree Celcius
α	alpha
β	beta
δ	delta
γ	gamma
κ	kappa
™	trademark
®	registered
ζ	zeta
ε	epselon

PENGEKSPRESAN RESEPTOR TERAKTIF PEMPROLIFERASI PEROKSISOM γ (PPARγ1 DAN PPARγ2) DALAM LIMFOSIT T CD4+ NAIF DAN MEMORI

ABSTRAK

Sel T CD4+ periferi boleh dibahagikan kepada dua kumpulan berfungsi berdasarkan ekspresi isofom di permukaan molekul yang mengandungi dua domain fosfat intrasellular dikenali sebagai CD45. Sel T memori mengekspres isoform yang mempunyai berat molekul terendah iaitu CD45RO manakala sel T naïf mengekspres isofom CD45RA (manusia) atau CD45RB (tikus). CD45 ialah protein tirosina fosfatase yang memainkan peranan penting sebagai pengantara isyarat TCR dengan mengaktifkan lck melalui defosforilasi pengawalatur Tyr^{505}. Sel T CD4+ naïf dan memori manusia berbeza dari segi keperluan untuk pengaktifan dan magnitud tindakbalas sel. Sejenis reseptor nukleus, reseptor teraktif pemploriferasi peroksisom γ (PPARγ), dilaporkan terlibat di dalam pengawalaturan aktiviti sel imun seperti makrofaj atau monosit dan limfosit T. Memandangkan peranannya di dalam pengawalaturan imun, kajian terkini dijalankan untuk menentukan tahap pengekspresan PPARγ di dalam sel T CD4+ naïf dan memori kerana tahap ekspresi PPARγ berkemungkinan berbeza di dalam isofom CD45 yang berlainan. Tambahan lagi, perbezaan corak pengisyaratan dan rembesan sitokin bagi sel T subset tersebut mungkin disebabkan oleh gabungan dengan isofom PPARγ-kemungkinan yang masih belum dieksplorasi setakat ini. Bagi mengenalpasti peranan PPARγ di dalam pengawalaturan pengaktifan sel T CD4+ naïf dan memori, ciglitazone yang merupakan agonis bagi PPARγ digunakan untuk memodulasi status pengaktifan sel T CD4+ naïf dan memori selain pengekspresan PPARγ sendiri dan sitokin terpilih. Dengan menggunakan Real-Time PCR, sel T CD4+ naïf dan memori tak teraktif tidak

mengekspres PPARγ1 dan PPARγ2 manakala sel T CD4+ naïf dan memori teraktif mengekspres reseptor tersebut pada paras yang tinggi dengan pengekspresan PPARγ2 lebih tinggi berbanding PPARγ1 di dalam kedua-dua jenis sel ($p<0.01$). Tambahan lagi, ekspresi PPARγ1 lebih tinggi di dalam sel memori teraktif berbanding sel T CD4+ naif teraktif ($p<0.05$) manakala tiada perbezaan bagi pengekspresan PPARγ2 di dalam kedua-dua sel yang teraktif. Penambahan agonis bagi PPARγ iaitu ciglitazone meningkatkan pengekspresan PPARγ1 kira-kira 61 kali dan 175 kali masing-masing di dalam sel T CD4+ naïf dan memori yang teraktif ($p<0.01$). Berbeza dengan PPARγ1, penambahan ciglitazone mengurangkan ekspresi PPARγ2 kira-kira 650 kali dan 140 kali di dalam sel T CD4+ naif dan memori teraktif ($p<0.01$). Tambahan lagi, tahap ekspresi gen TGF-β dan IL-1β adalah tinggi di dalam sel T CD4+ naif dan memori yang tak teraktif tetapi berkurangan di dalam keadaan teraktif ($p<0.01$). Gen IL-8 mengekspres pada tahap yang rendah di dalam sel T CD4+ naif dan memori yang tak teraktif tetapi meningkat di dalam keadaan sel tersebut yang teraktif ($p<0.01$). Walaubagaimanapun tiada perbezaan bagi ekspresi sitokin tersebut di antara sel T CD4+ naif dan memori di dalam kedua-dua keadaan. IL-2, IFN-γ, IL-5, IL-13, TNF-α, GM-CSF dan IL-6 hanya mengekspres di dalam sel T CD4+ naïf dan memori yang teraktif tetapi tidak dalam keadaan yang tak teraktif. Tahap pengekspresan IL-2 dan IL-13 adalah tinggi di dalam sel naïf yang teraktif berbanding sel T CD4+ memori teraktif ($p<0.01$). Berbeza dengan IL-2 dan IL-13, tahap pengekspresan IFN-γ adalah tinggi di dalam sel memori teraktif berbanding sel naïf yang teraktif ($p<0.05$). Walaubagaimanapun, tiada perbezaan di dalam pengekspresan IL-5, IL-6, TNF-α dan GM-CSF di antara kedua-dua jenis sel yang teraktif. Penambahan ciglitazone mengurangkan tahap pengekspresan TGF-β, IL-1β, IL-8, IL-2, IFN-γ, IL-5, TNF-α dan GM-CSF di dalam sel T CD4+ naïf dan memori yang teraktif. Peningkatan PPARγ1 dan perencatan ekspresi PPARγ2 di dalam sel T CD4+ naïf dan memori di dalam kehadiran

ciglitazone mencadangkan bahawa isoform PPARγ mungkin mempunyai fungsi yang berbeza dalam pengawalaturan sel T. Pengekspresan gen sitokin yang terpilih di dalam sel T CD4+ naïf dan memori yang teraktif adalah konsisten dengan kajian terdahulu. Mekanisma sebenar bagaimana PPARγ merencat pengekspresan sitokin di dalam sel T CD4+ naïf dan memori teraktif atau isoform PPARγ yang mana bertanggung jawab terhadap kesan ini masih belum dapat di pastikan. PPARγ berkemungkinan merencat pengekspresan gen sitokin di dalam sel subset yang teraktif melalui interaksi dengan NF-κB, AP-1 dan STATs yang merupakan faktor transkripsi yang penting bagi sitokin tersebut sebagaimana kajian terdahulu di dalam sel yang lain.

THE EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ (PPARγ1 AND PPARγ2) IN NAÏVE AND MEMORY CD4+ T LYMPHOCYTES

ABSTRACT

Peripheral CD4+ T cells can be divided into two functional groups based on the expression of distinct isoforms of the surface molecule that contains an intracellular two-domain phosphatase portion, known as CD45. Memory T cells express the lowest molecular weight CD45RO isoform, whereas naïve T cells express CD45RA (human) or CD45RB (mouse) isoforms. CD45 is a protein tyrosine phosphatase which plays an important role in TCR-mediated signaling through its activation of Lck by de-phosphorylating the regulatory Tyr^{505}. Human naïve and memory CD4+ T cells differ in the requirements for activation and magnitude of the cellular responses. The nuclear receptor, peroxisome proliferator-activated receptor γ (PPARγ) has been reported to be involved in regulating the activities of immune cells such as macrophages or monocytes and T lymphocytes. Given their roles in immune regulation, the current study was carried out to determine the expression of PPARγ in human naïve and memory CD4+ T cells since it is possible that PPARγ may be differentially expressed in the different isoforms of CD45. In addition, the differential signaling patterns and cytokine secretion of these subsets of T cells may require engagement with PPARγ isoforms - a possibility that has not been explored thus far. To further dissect the role of PPARγ in the regulation of naïve and memory CD4+ T cell activation, the PPARγ agonist, ciglitazone, was used to modulate the activation status of naïve and memory CD4+ T cells as well as the expression of PPARγ itself and selected cytokines. Using Real-Time PCR, unstimulated naïve and memory CD4+ T cells were found not to express PPARγ1 and PPARγ2, whereas stimulated naïve

and memory CD4+ T cells express high levels of these receptors with PPARγ2 expression being higher than PPARγ1 in both cell types ($p<0.01$). In addition, the PPARγ1 expression was higher in stimulated memory as compared to stimulated naïve CD4+ T cells ($p<0.05$) whereas there was no significant difference between PPARγ2 expression in both types of stimulated cells. The addition of the PPARγ agonist, ciglitazone significantly increased the expression of PPARγ1 by about 61-fold and 175-fold in stimulated naive and memory CD4+ T cells respectively ($p<0.01$). In contrast to PPARγ1, the addition of ciglitazone significantly decreased the expression of PPARγ2 by about 650-fold and 140-fold in stimulated naïve and memory CD4+ T cells respectively ($p<0.01$). In addition, the expression levels of TGF-β and IL-1β gene were higher in unstimulated naïve and memory CD4+ T cells but decreased in their stimulated state ($p<0.01$). IL-8 gene was expressed at low levels in unstimulated but elevated in stimulated naïve and memory CD4+ T cells ($p<0.01$). However, there were no significant differences in the levels of these cytokines between naïve and memory CD4+ T cells between both states. IL-2, IFNγ, IL-5, IL-13, TNFα, GM-CSF and IL-6 were only expressed in stimulated naïve and memory CD4+ T cells but not in their unstimulated state. The expression levels of IL-2 and IL-13 were significantly higher in stimulated naïve as compared to stimulated memory CD4+ T cells ($p<0.01$). In contrast, the expression levels of IFNγ were significantly higher in stimulated memory as compared to stimulated naïve CD4+ T cells ($p<0.05$). However, there were no significant differences in the expression of IL-5, IL-6, TNFα and GM-CSF between both stimulated cell types. The addition of ciglitazone, decreased the expression levels of TGF-β, IL-1β, IL-8, IL-2, IFNγ, IL-5, TNFα and GM-CSF in stimulated memory and naïve CD4+ T cells. The induction of PPARγ1, and suppression of PPARγ2 expression in naïve and memory CD4+ T cells in the presence of ciglitazone suggest that the PPARγ isoforms may have different functions in T cell regulation. The expression of selected cytokine genes in

activated naïve and memory CD4+ T cells is consistent with previous studies. The exact mechanism of how PPARγ inhibit cytokine expression in stimulated naïve and memory CD4+ T cells or which PPARγ isoforms is responsible for this effect remain uncertain. It is possible that PPARγ inhibit the expression of cytokine genes in these stimulated cell subsets via interacting with NF-κB, AP-1 and STATs which is important transcription factors for these cytokines as shown by previous studies in other cells.

CHAPTER ONE

INTRODUCTION

1.1 T cell development

1.1.1 Generation of T cells

T cell development occurs in the thymus (Surh and Sprent, 2005). The thymus is a multi-lobed organ consisting of cortical and medullary areas surrounded by a capsule (Figure 1.1). T cell precursors enter the subcapsular cortical areas, where they encounter networks of cortical epithelial cells (the thymic stroma) and undergo a period of proliferation. After differentiation, they migrate from the cortex towards the medulla of the thymus.

In the thymic cortex, progenitor cells derived from the bone marrow differentiate into T cell lineage by rearranging the TCRβ chain and expression of the pre-TCR complex which lead to multiple changes including, massive expansion of 'double negative' (DN) $CD4^-$ 8^- precursors by IL-7, upregulation of CD4 and CD8 on the 'double-positive' (DP) $CD4^+$ 8^+ thymocytes, and rearrangement of TCRα chain for expression of the TCR (Michie *et al.*, 2002; von Boehmer, 2004) (Figure 1.1).

DP thymocytes undergo positive selection to remove cells that have significant TCR reactivity for self-MHC/peptide complexes. After positive selection, DP cells migrate from the cortex to the medulla and differentiate into single-positive $CD4^+$ 8^- and $CD4^-$ 8^+ cells. During this differentiation process, negative selection takes over to destroy autoreactive T cells with high avidity for self components (Sprent *et al.*, 1995; Starr *et al.*, 2003). After positive and negative selection, depending on the affinity and the context of such binding,

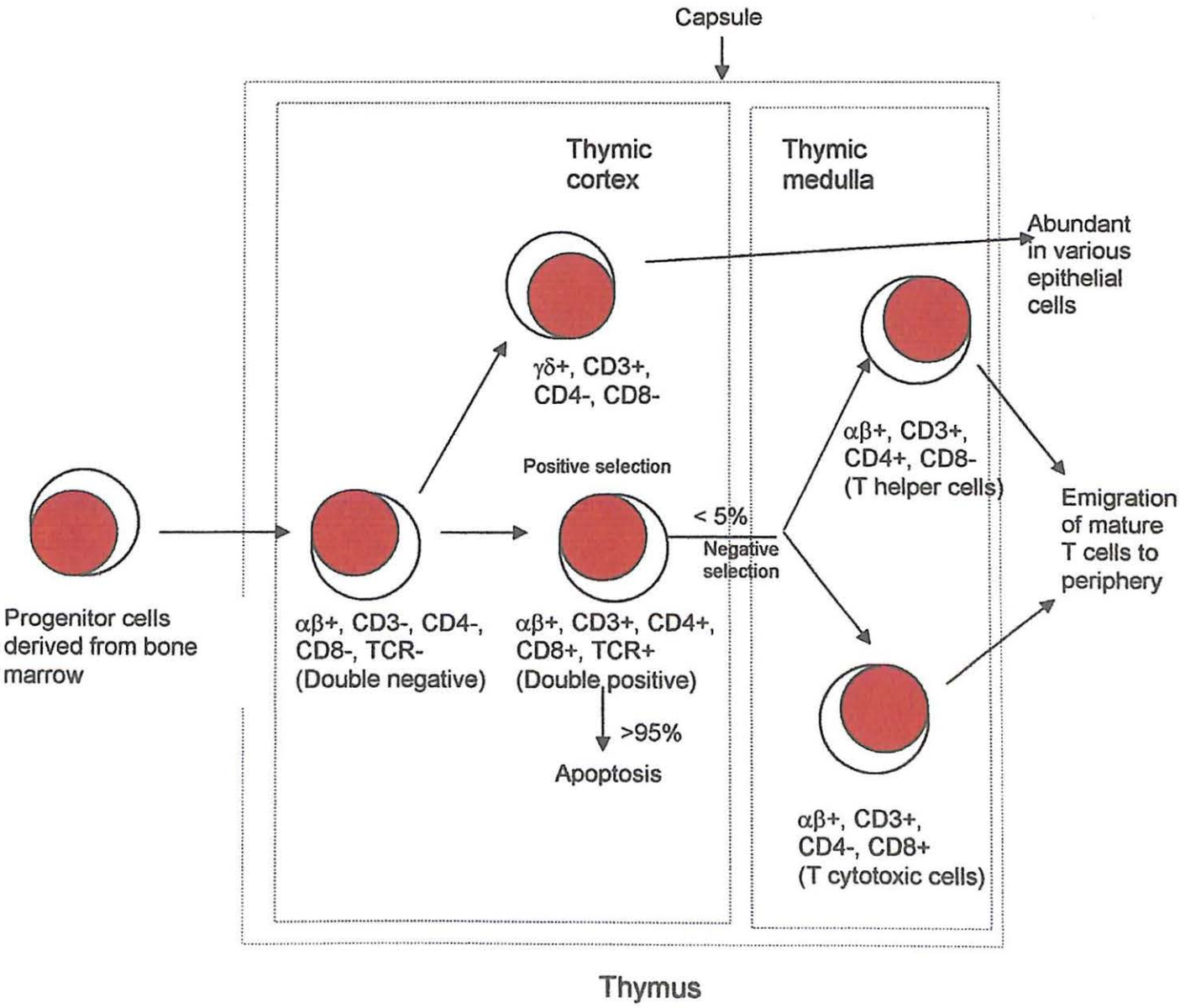


Figure 1.1 Pathways of T cell development (modified from Mehr *et al.*, 1995)

95% of developing DP cells die via apoptosis while only a small fraction (<5%) of DP cells, move into the periphery to form the T cell pool (Surh and Sprent, 2005).

In addition to T cells expressing αβ TCR, which constitute the majority of T cells in adults, there is a lineage of T cells expressing the γδ TCR. These cells are abundant in various epithelial tissues, such as the epidermis (in mice but not humans), intestinal epithelium, uterus and tongue. The γδ T cells recognize both exogenous antigens such as viral and protozoal peptides, and autoantigen such as heat shock proteins. Moreover, the peptides can be presented by either class I or class II MHC molecules, and there are several studies which suggest the γδ T cells do not require antigen to be presented by MHC molecules at all (Adams *et al.*, 2005).

1.2 T cell recognition

Approximately 90-95% of peripheral blood T cells are αβ T cells. αβ T cells are mainly divided into CD4+ helper T cells and CD8+ cytotoxic T cells. CD4+ T cells recognize their specific antigens in association with the major histocompatibility complex (MHC) class II molecules, whereas CD8+ T cells recognizes antigen in association with MHC class I molecules.

1.2.1 Antigen presenting cells

Antigens must be processed by antigen presenting cells (APCs) before presentation to T lymphocytes. The APCs which are essential for T cell activation includes dendritic cells (DCs), mature B cells and macrophages (Sille *et al.*, 2005). DCs are found in lymphoid tissues, connective tissues and epithelial cells (Bell *et al.*, 1999). DCs capture antigens in peripheral tissues and then move to lymph nodes, where they express high levels of

adhesion and co-stimulatory molecules, as well as MHC class II antigens (Bell *et al.*, 1999). DCs stop synthesis of these molecules when they migrate but express high levels of MHC class II molecules containing peptides from antigens produced by tissues where the DCs originated (Bell *et al.*, 1999).

B cells can bind to a specific antigen, internalize it and then degrade it into peptides which associate with MHC class II molecules. B cells which have high affinity antigen receptors (IgM or IgD) are the most potent APC at low concentrations of antigens because other APCs cannot capture enough antigens (Mellman *et al.*, 1998). B cells do not express co-stimulatory molecules such as B7 but can be induced to produce B7 by bacterial constituents.

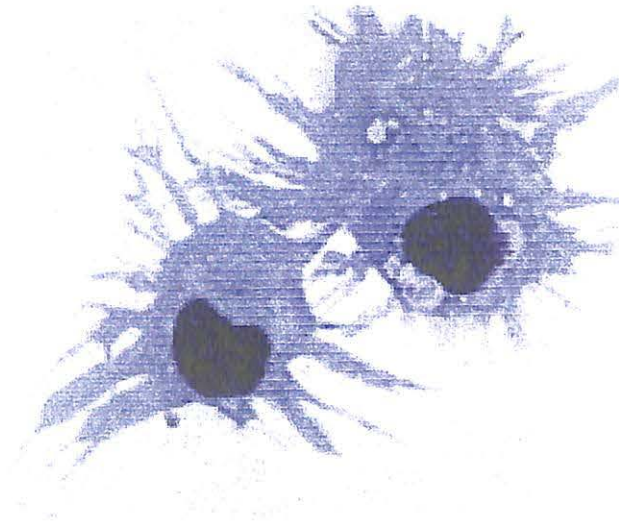
Macrophages ingest microbes and particulate antigens by phagocytosis for processing and presentation by MHC class II molecules (Kalish, 1995). High levels of MHC and co-stimulatory molecules such as B7 are induced by the uptake of bacteria which is enhanced by receptors specific to certain surface components of bacteria (Mellman *et al.*, 1998). These APCs are illustrated in Figure 1.2.

1.2.2 Antigen presentation by major histocompatability complex molecules

The MHC is involved in presentation of antigens to T cells. The human MHC class I molecules includes the HLA-A, B and C whereas the human MHC class II molecules comprise of the DR, DP and DQ (van den Elsen *et al.*, 2004). The genetic locus of MHC class I and II is presented in Figure 1.3. MHC class I and II molecules are members of the immunoglobulin supergene family which consist of multiple "immunoglobulin domains" that

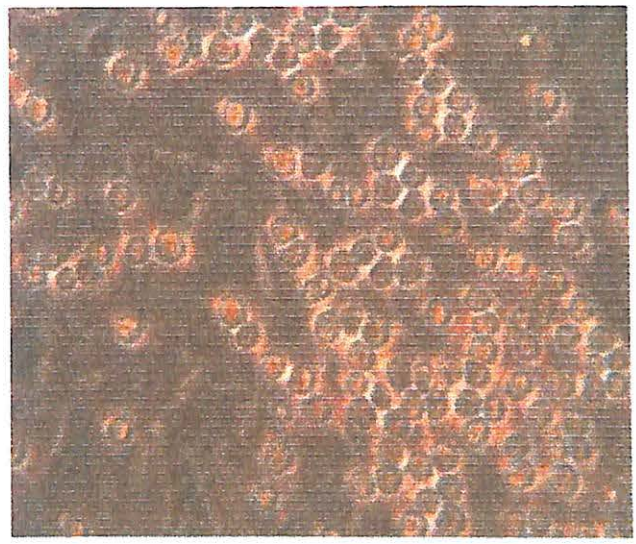
Dendritic cells

http://www.dukecancervaccines.org/images/dend.jpg



B cells

http://www.4-antibody.com/images/cells.jpg



Macrophage

http://www.healingdaily.com/detoxification-diet/macrophages.jpg

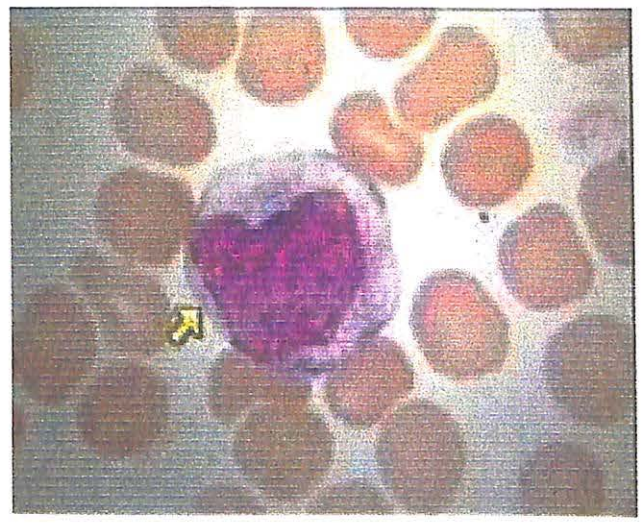


Figure 1.2 Antigen presenting cells (APCs)

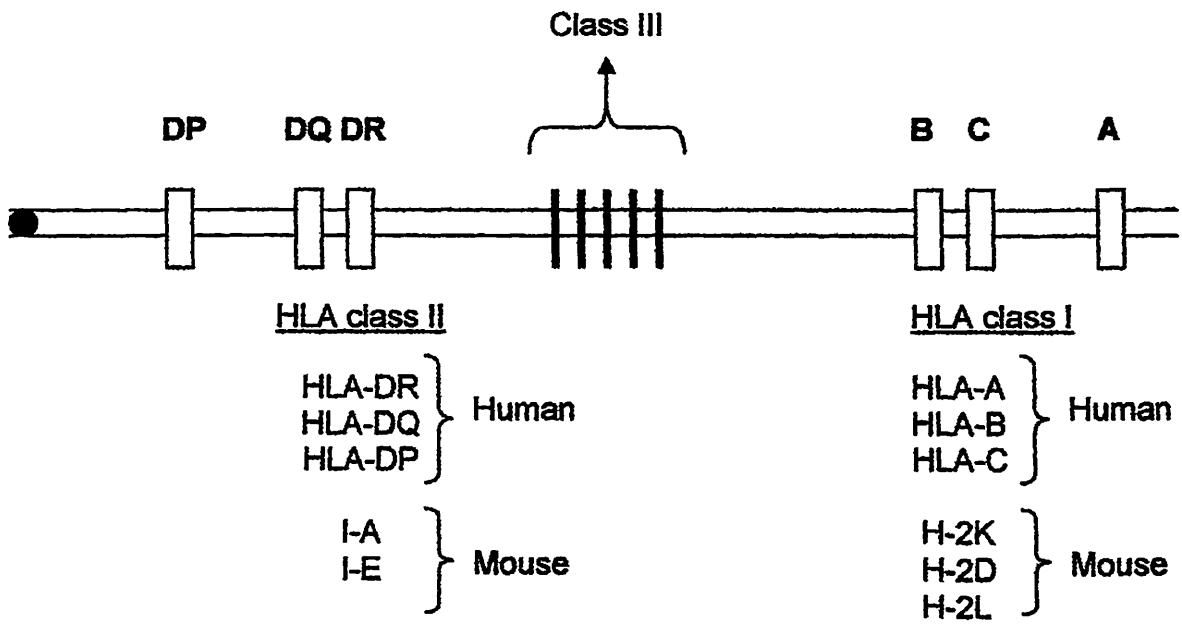


Figure 1.3 The genetic organization of MHC class I and II molecules

have similar structure and amino acid homology to the constant and variable domains of immunoglobulins (Kalish, 1995).

i) MHC class I molecules

MHC class I molecule consists of a heavy chain with three immunoglobulin domains, in noncovalent association with β_2-microglobulin (van den Elsen *et al.*, 2004) and are expressed on all nucleated cells (Kalish, 1995) (Figure 1.4). The first two domains of the alpha chain, $\alpha 1$ and $\alpha 2$ form a "groove" that binds a peptide and together with β_2-microglobulin, the three member complex allows for its stable expression on the cell surface (Anderson *et al.*, 1993). MHC class I molecules bind peptides consisting of 8 to 10 amino acids (Falk *et al.*, 1993). The peptide-binding groove contains pockets with amino acid residues which formed specific interactions with the amino and carboxylic acid terminals of the peptide (Wilson and Fremont, 1993).

ii) MHC class II molecules

MHC class II molecules are composed of two α chains and two β chains (Kalish, 1995) (Figure 1.4). The peptide-binding groove of MHC class II molecules is open at the ends and enables binding of longer peptides compared to the MHC class I molecules (Rudensky *et al.*, 1991). In the absence of stimulation, MHC class II molecules are only expressed on professional APCs such as macrophages, mature B cells, Langerhans cells and dendritic cells (Kalish, 1995). The expression of MHC class II molecules can be induced by interferon gamma on keratinocytes and endothelial cells (Kalish, 1995).

1.2.3 Antigen processing

Antigen processing involves antigen degradation into peptide fragments which are recognized by T cells via the TCR. A minority of peptide fragments from protein antigens is

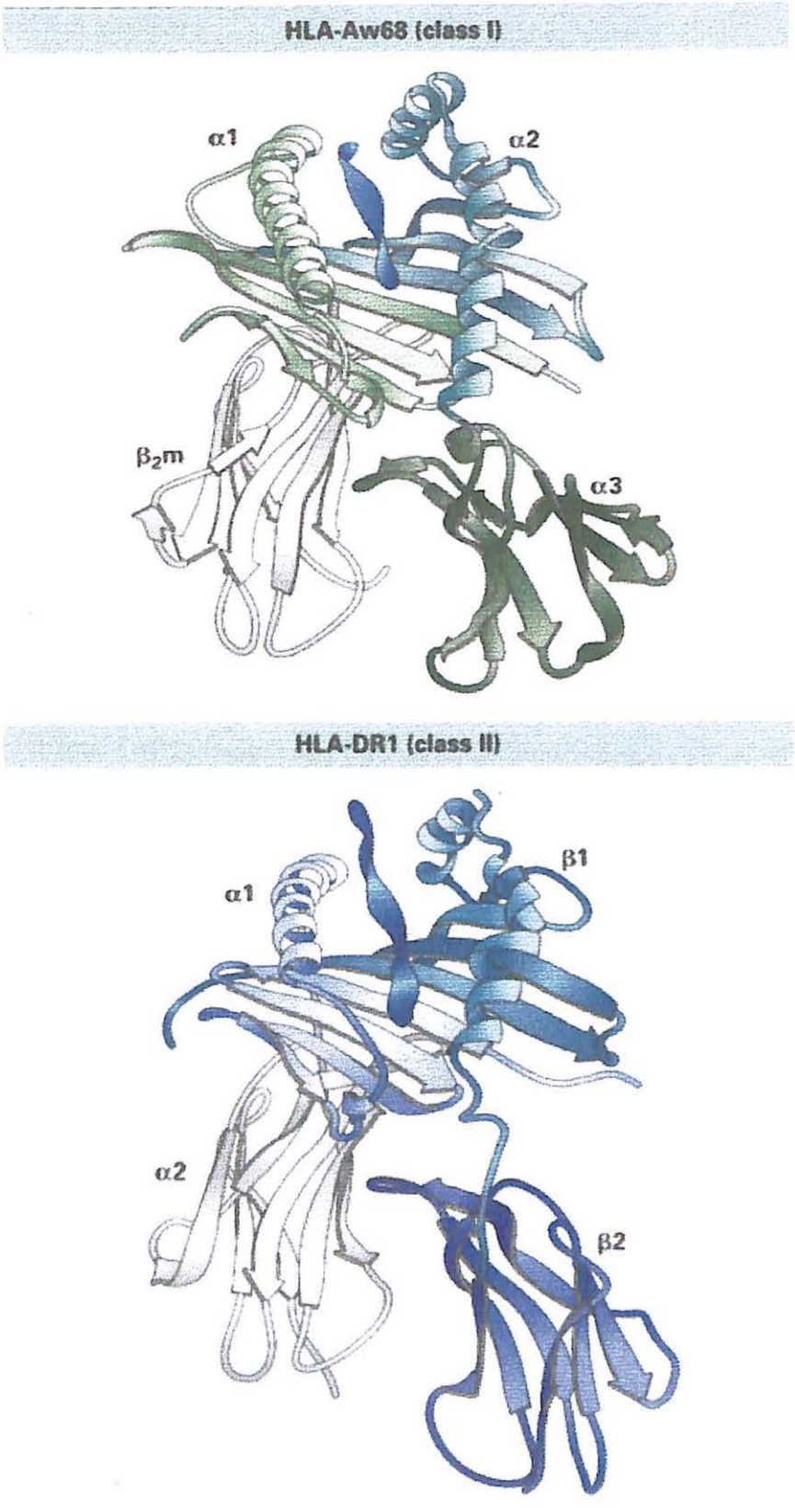


Figure 1.4 MHC molecules (adapted from Roitt *et al.*, 2001)

able to bind to particular MHC molecules. Furthermore, different MHC molecules engage with different sets of peptides.

i) Endogenous antigens

Protein antigens arising from inside the cell (endogenous antigens) are processed by the endogenous pathway for presentation to T cells. Examples of endogenous antigens are viral antigens, transplantation antigens and tumor-associated antigens (Kalish, 1995). Endogenous antigens derived from cytoplasmic proteins (Moore *et al.*, 1988) are targeted by conjugation with ubiquitin (Michalek *et al.*, 1993; Ciechanover and Schwartz, 1994) facilitating them to be degraded into peptides by the proteosome complex (Yang *et al.*, 1992). Peptides derived from the degradation of cytoplasmic proteins are transported from the cytoplasm to endoplasmic reticulum (ER) by the transporter-associated antigen processing (TAP1 and TAP2) molecules (Spies *et al.*, 1990; Attaya *et al.*, 1992; Colonna *et al.*, 1992; Spies *et al.*, 1992; Suh *et al.*, 1994).

Within the ER, a series of chaperone proteins, including calnexin, calreticulin, ERp57, and immunoglobulin binding protein (Bip) facilitates the proper folding of MHC class I and its association with β2 microglobulin. The partially folded MHC class I molecules then interact with TAP via tapasin. Newly synthesized MHC class I molecules complexed with peptides are then transported from the ER through the Golgi complex to the cell surface for recognition by CD8+ T cells (Cox *et al.*, 1990) (Figure 1.5).

ii) Exogenous antigen

Protein antigens from outside the cell (exogenous antigens) are processed by the exogenous pathway for presentation to T cells. Examples of exogenous antigens are extracellular bacteria, bacterial toxins, vaccines and allergens which include pollen and

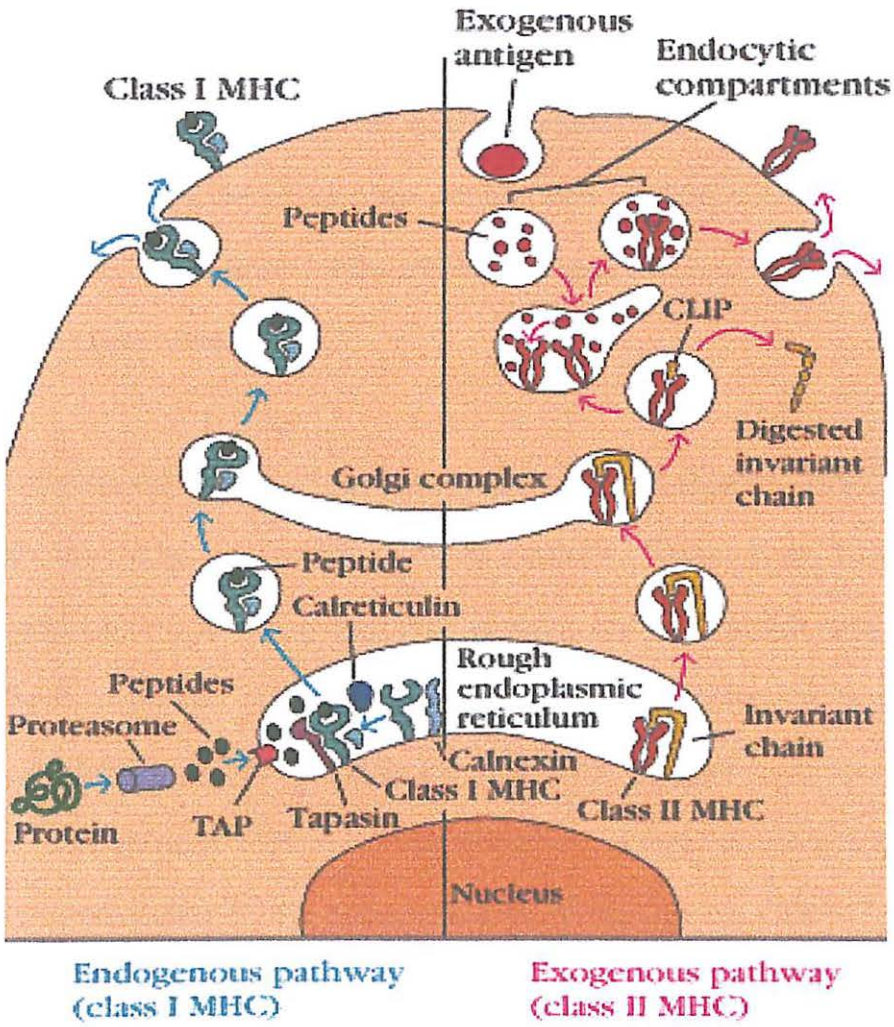


Figure 1.5 Antigens processing pathways (cited from http://www.anzies.com.au)

dust mites (Kalish, 1995). Exogenous antigens are internalized in endosomes by phagocytosis or pinocytosis. The endosomal vacuoles fused with lysosome to form lysosome/endosome compartment. This compartment has an acidic pH and contains multiple degradative enzymes such as acid proteases and cathepsins (Rodriguez and Diment, 1992) to degrade the exogenous antigen into peptides. MHC class II molecules are transported into this lysosome/endosome compartment to bind to the processed peptides (Neefhes and Ploegh, 1992).

MHC class II molecules are firstly synthesized in the ER (Kalish, 1995). The invariant chain facilitates MHC class II export from ER in a vesicle (Teyton *et al.*, 1990). This vesicle will fuse with endosome containing degraded proteins and then broken down to leave only a small fragment called CLIP which responsible to inhibit the binding of peptides to the MHC class II molecules before arrival in the endosome/lysosomal compartment (Bodmer *et al.*, 1994). Peptides generated in the lysosome/endosome compartment are presented by MHC class II molecules on the cell surface for recognition by CD4+ T cells (Fabbri *et al.*, 2003; van den Elsen *et al.*, 2004) (Figure 1.5).

1.2.4 Antigen presentation to T lymphocytes

Antigen presentation is the process by which T cells recognize antigen on the surface of an APC (Kalish, 1995). The peptide-MHC (pMHC) complex is recognized by αβ T cells through their TCR (Garcia and Adams, 2005). The TCR is a genetically recombined receptor and analogous to an immunoglobulin molecule (Garcia and Adams, 2005). It is a heterodimer consisting of α and β chain held together by disulfide bonds, each with one variable and one constant domain. However, 5% of the T cells, especially those found in the skin and mucous layers, have TCR consisting of the γ and δ chain. Vα and Vβ are the hypervariable regions of TCR, that form three complementary determining regions (CDRs)

on each chain. CDR1 and CDR2 usually bind to MHC while CDR3 usually binds to the peptide (Garcia and Adams, 2005). TCR has a very short cytoplasmic tails, which is not appropriate in signal transduction. So it is expressed on the membrane together with the signal transduction complex, CD3, as TCR complex (Figure 1.6). CD3 is composed of four invariant polypeptides called γ, ε, δ and ζ. The CD3 chains are organized as heterodimers of either $\gamma\varepsilon$ or $\delta\varepsilon$ and a homodimer of $\zeta\zeta$. The γ, δ and ε chains have negatively charged transmembrane region which forms salt bridges with positively charged transmembrane regions of TCR. The ζ chain comprises a small extracellular domain of only nine amino acids which includes the disulphide bond, a transmembrane segment including a negatively charged residue and a large cytoplasmic tail. All CD3 chains contain one immunoreceptor tyrosine activation motif (ITAM), while three copies of ITAMs are present on each of the ζ chain's cytoplasmic tail (Chan *et al.*, 1994). Following antigen binding, the ITAMs will associate with tyrosine kinase to initiate intracellular signaling cascade. CD4 and CD8 are co-receptor molecules that bind to the non-polymorphic regions of MHC molecules and enhance the binding avidity of T cells to the APC. CD4 is a monomeric protein with four domains where two distal domains that bind to the β2 domain of MHC class II. CD8 is an α and β chain heterodimer, each with one domain and a long extended region. CD8 binds to the α3 domain of MHC class I. Both CD4 and CD8 have Lck associate with its cytoplasmic tail to initiate signal transduction (Figure 1.6). The Lck or $p56^{lck}$ is a lymphocyte-specific tyrosine kinase of 56 kDa that is attached to the intracellular portions of CD4 or CD8.

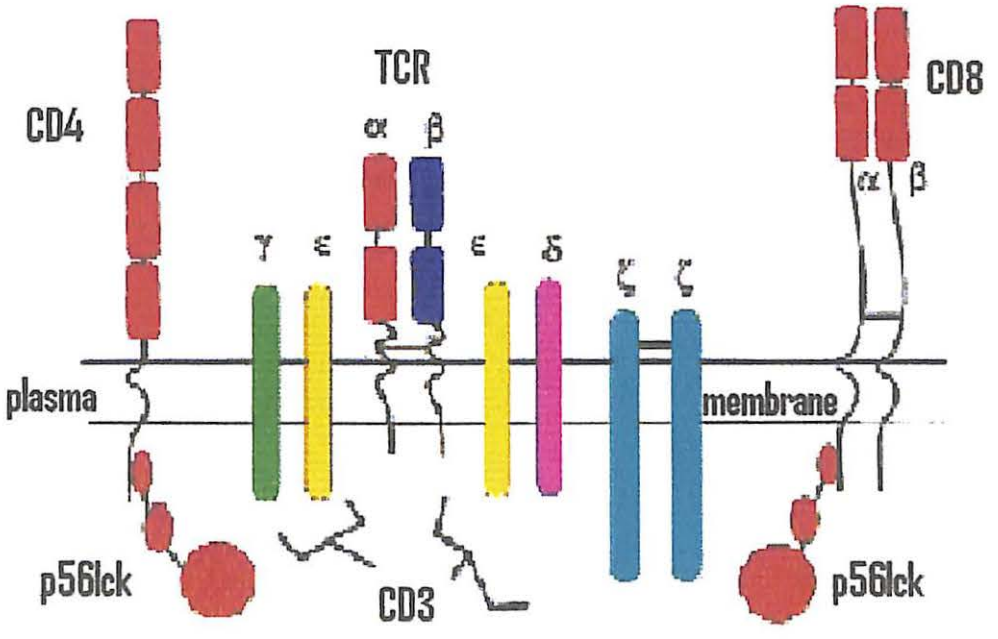


Figure 1.6 TCR complex (cited from http://www.immuno.path.cam.ac.uk)

1.2.5 Second signal-co-stimulator molecules

Co-stimulatory molecules do not bind to antigen directly but act when TCR binds to antigen. The main functions of co-stimulatory and other accessory molecules are to transduce signal for the complete activation of T cell, acts as adhesion molecules to stabilize the interaction between T cells and APCs and facilitate the migration of T cells. T cells that do not receive co-stimulation become anergic (not functioning) and this is important to ensure that self-antigen will not activate self-specific T cells that escaped negative selection (Tan *et al.*, 1993). There are perhaps many co-stimulator molecules that are involved in the T cell activation process. Some of the important ones are briefly described below.

i) B7-CD28

B7 molecules are expressed mainly by professional APCs such as dendritic cells, macrophages and mature B lymphocytes. B7-1 and B7-2 are structurally similar, single chain glycoprotein, each with extracellular Ig-like domain, a transmembrane segment and cytoplasmic tail. Co-stimulation of T cells mainly comes from the binding of B7-1 (CD80) and B7-2 (CD86) on APC to the CD28 molecule on T cell membrane (Turka *et al.*, 1990) (Figure 1.7). The binding between these molecules brings upon the expression of anti-apoptosis proteins such as Bcl-x, production of cytokines such as IL-2 and proliferation and differentiation of T cells.

ii) Cytotoxic T lymphocyte associated antigen 4 (CTLA-4) (CD152)

Activated T cells express CTLA-4 instead of CD28. CTLA-4 is also a receptor for B7 molecules. Its binding to B7 inhibits T cell function by interrupting signals transduced by CD28. Mutant T cell that lack CTLA-4 cannot be deactivated, resulting in the increase incidence of autoimmune reactions.

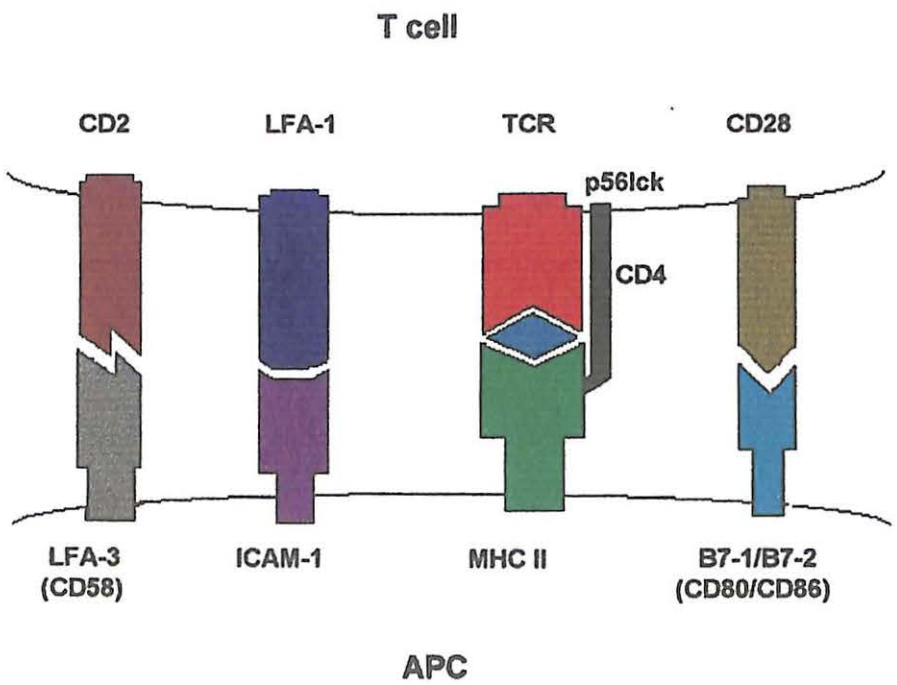


Figure 1.7 Critical molecules involved in antigen presentation (adapted from Roitt *et al.* 2001)

iii) Other accessory molecules

CD2 act as an adhesion molecule by binding to LFA-3 (CD58) molecules found on a wide range of cells (Damle *et al.*, 1992; Perlmutter, 1993) (Figure 1.7). It is involved in the interaction between T cells and APCs. CD11b/CD18 is also an adhesion molecule like CD2. It binds to the intracellular adhesion molecule-1 (ICAM-1) on APCs or target cells (Damle *et al.*, 1992; Perlmutter, 1993) (Figure 1.7). Activated CD4 cells express CD40ligands (CD40L) that binds to the CD40 molecules on B cells and macrophages. The binding induces the activation of B cell and macrophages accompanied by the induction of B7 molecule expression on APC and enhancement of proliferation and differentiation of T cells.

1.2.6 T cell activation

T cell activation is initiated by the interaction of the TCR with peptide-MHC complexes. TCR engagement triggers the tyrosine phosphorylation of the ITAM present on the TCR-associated CD3-ζ subunit by the protein tyrosine kinase (PTK) Lck (Chan *et al.*, 1994; Myung *et al.*, 2000). All ITAMs contain two sequence elements (Tyr-X-X-Leu) with potential tyrosine phosphorylation sites which is separated by seven or eight variable amino acids (Hegedus *et al.*, 1999) (Figure 1.8). When two tyrosines in a single motif are phosphorylated, ITAM forms a binding site for a cytoplasmic PTK, known as ZAP-70. This recruitment initiates ZAP-70 activation and downstream signaling cascade such as the activation of phospholipase C-γ1 and protein kinase C (PKC).

Engagement of TCR induces activation of phospholipase Cγ1 (PLCγ1), which catalyzes the hydrolysis of inositol phospholipids (PIP2) to produce inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG) which activate two distinct signaling pathways in T cells (van Leeuwen and Samelson, 1999). IP3 migrate from the cytosol to the endoplasmic reticulum,

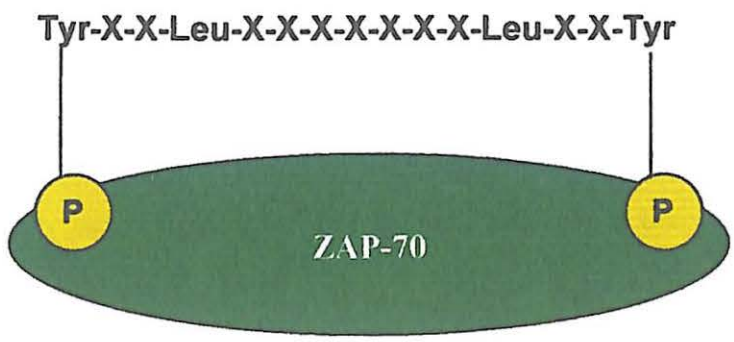


Figure 1.8 The ITAM complex

The ITAMs contain two sequence elements (Tyr-X-X-Leu) separated by 7 or 8 variable amino acids. When the tyrosine residues are phosphorylated, ITAM acts as a docking site for the tyrosine kinase, ZAP-70.

where it binds' to its receptor and initiates the release of internal Ca^{2+} stores which then cause a rapid increase in the cytosolic free Ca^{2+} ion concentration (Lewis, 2001). Cytosolic free Ca^{2+} acts as a signaling molecule by binding to calmodulin (Ca^{2+}-dependent regulatory protein) (Quintana *et al.*, 2005). This calcium-calmodulin complex activate several enzymes especially calcineurin, a Ca^{2+}-calmodulin dependent protein phosphatase which subsequently dephosphorylates the nuclear factor of activated T cells (NFAT) (Rao *et al.*, 1997). This transcription factor is an inducible regulatory complex critical for transcriptional induction of IL-2 in activated T cells, but also regulate the transcription of various genes such as cytokines, cell surface receptors and regulatory enzymes (Rao *et al.*, 1997; Martinez-martinez *et al.*, 2004). The increase in free cytosolic Ca^{2+} results in the translocation of inactive cytosolic PKC to the cell membrane. DAG activates PKC by inducing a conformational change that becomes the catalytic site of the kinase accessible to substrate (Lewis, 2001). Although DAG activates multiple isoforms of PKC, only PKCθ is required for T cell activation *in vivo* (Sun *et al.*, 2000; Pfeifhofer *et al.*, 2003). The GTP bound Ras (GTP-Ras) functions as activator of a cascade of enzymes namely mitogen-activated protein (MAP) kinases. The MAP kinase cascade consist of three different kinases, the extracellular signal-regulated kinases (ERKs) (Schaeffer and Weber, 1999), p38 Map-kinases (Han and Ueitch, 1999) and c-Jun NH2-terminal kinases (JNKs) (Davis, 2000). The activated ERK phosphorylates Elk1, which then stimulates transcription of Fos, the first component of the activation protein-1 (AP-1) (Genot *et al.*, 1996). Parallel to this pathway, the adapter protein also activates a GTP exchange protein namely Vav that acts on Rac. This Rac-GTP initiates the activation of the c-Jun by JNK at Ser-63 and Ser-73 within the transactivation domain (Whitmarsh and Davis, 1996). c-Jun heterodimerizes with c-Fos to form AP-1, the regulatory element in the IL-2 promoter that is important for early transcription of the IL-2 gene (Durand *et al.*, 1988). The brief overall intracellular signaling involved in T cell activation as described herein is summarized in Figure 1.9.

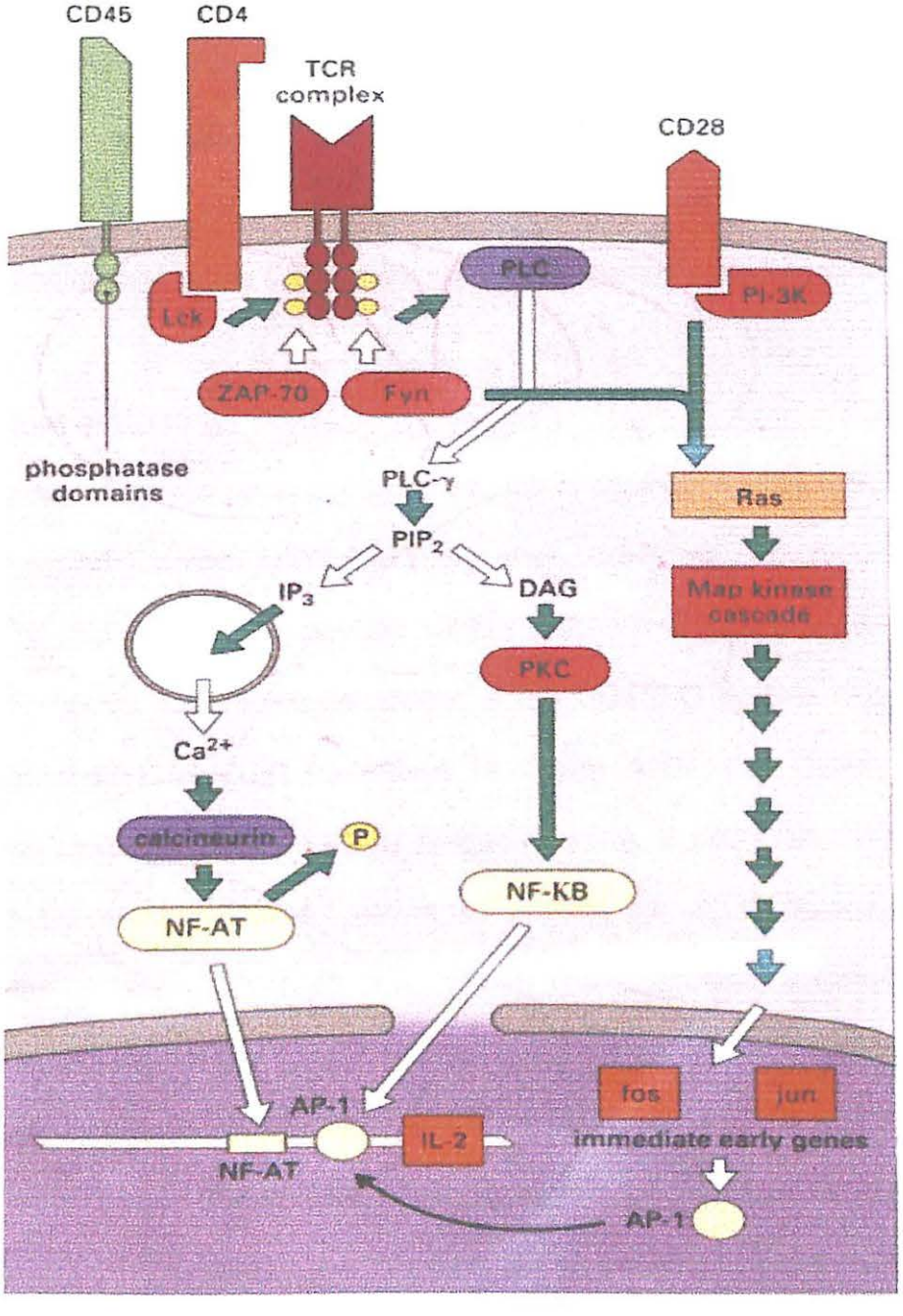


Figure 1.9 Intracellular signaling in T cell activation (adapted from Roitt *et al.*, 2001)

1.3 The protein tyrosine phosphatase, CD45

The protein tyrosine phosphatase (PTPase) CD45 is a transmembrane glycoprotein expressed on the surface of all nucleated haematopoietic cells except mature erythrocytes and platelets (Trowbridge and Thomas, 1994). CD45 consists of its splice variants namely CD45RA, CD45RB, CD45RC and CD45RO. CD45 comprises up to 10% of the T and B cell surface molecules (Sasaki *et al.*, 2001). This high level of expression coupled with the differential expression of selected variants on naïve and memory T cells and their role in immune regulation makes the CD45 molecules are important molecule to be investigated.

1.3.1 Structure of CD45

CD45 is expressed in multiple isoforms as a result of alternative RNA splicing of exon 4 to 6 (Trowbridge and Thomas, 1994; Alexander *et al.*, 1997) with a molecular weight range from 180-240 kDa. The high molecular weight isoforms contain exon 4/A, 5/B and 6/C (CD45ABC) and the low molecular weight is the CD45RO isoform (Figure 1.10). The genetic organization of CD45 is shown in Figure 1.11. The CD45 ectodomain is characterized by the three alternatively spliced exons A, B and C that are rich in serine, threonine and proline residues, a cysteine rich domain followed by fibronectin (FN) type III repeats at the N-terminus (McNeill *et al.*, 2004). The ectodomain is heavily glycosylated, mainly N-linked in the FN-III and cysteine rich regions and O-linked in the A, B and C exon encoded regions (McNeill *et al.*, 2004). The CD45RO lacks the A, B and C exon encoded region (McNeill *et al.*, 2004). The intracytoplasmic tail of CD45 is highly conserved between all mammalian species and contains the so-called D1-domain which has PTPase activity, whereas the D2-domain has no significant PTPase activity due to changes in critical amino acids required for catalytic activity (Sasaki *et al.*, 2001).

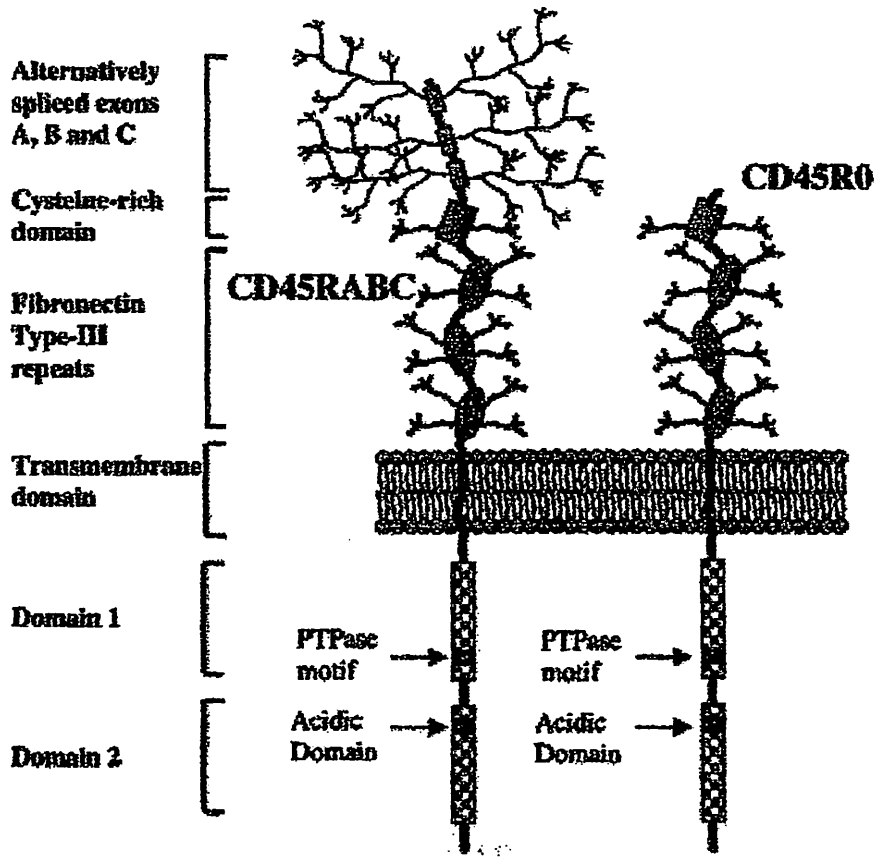


Figure 1.10 The CD45 structure (adapted from McNeill *et al.*, 2004)

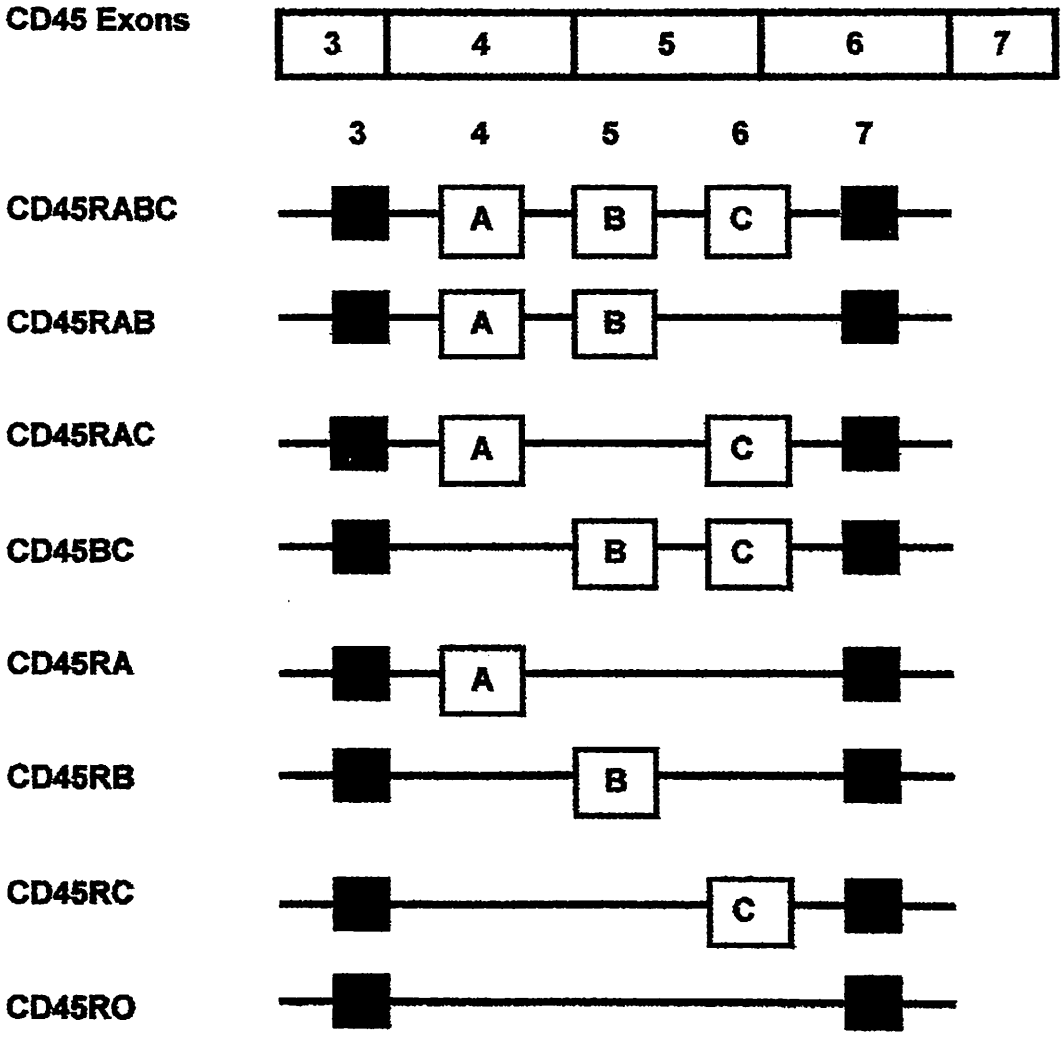


Figure 1.11 Genomic organization of CD45ABC (adapted from Fukuhara *et al.*, 2002)

1.3.2 Biological functions

CD45 is an important positive regulator of TCR and BCR mediated signaling required for the activation and development of lymphocytes (Kishihara *et al.*, 1993; Byth *et al.*, 1996). CD45 PTPase activity is required for histamine degranulation following IgE receptor cross-linking in mast cells (Berger *et al.*, 1994). CD45 expression is also involved in antigen receptor-driven thymocyte maturation and B cell selection (Kishihara *et al.*, 1993; Byth *et al.*, 1996). Mutation in the protein tyrosine phosphatase receptor type C (PTPRC) gene encoding CD45 and abnormalities in the expression of CD45 splice variants increased the incidence of severe combined immunodeficiency disease (SCID) in humans (Kung *et al.*, 2000).

1.3.3 CD45 regulates protein tyrosine kinase, Lck

An important early step in TCR signaling is the phosphorylation of ITAMs by the Src-like protein tyrosine kinase Lck (Irles *et al.*, 2002). This kinase is located in glyosphingolipid-enriched membrane (GEMs) and its activity is regulated by the opposing actions of the CD45 PTPase and the carboxyl-terminal Src kinase (Csk) (Bergman *et al.*, 1992; Thomas and Brown, 1999). Phosphorylation of Tyr^{505} in Lck by Csk leads to an Src homology domain 2 (SH2)-mediated intramolecular interaction which inhibits activation of Lck by trans-autophosphorylation of Tyr^{394} in its catalytic domain (Sicheri and Kuriyan, 1997). CD45 dephosphorylates the Tyr^{505} to relieve this inhibition (Yamaguchi and Hendrickson, 1996; Sicheri and Kuriyan, 1997) allowing the Lck to promote full phophorylation of the ITAM motifs of the CD3/ζ components and facilitate the recruitment and activation of the ZAP-70 tyrosine kinase (Leitenberg *et al.*, 1999). In addition, CD45 may maintain Lck in an "open" configuration which enhances Lck interaction and recruitment of various adapter proteins and other signaling molecules into large macromolecular complex that facilitates T

cell activation and IL-2 secretion independently of Lck kinase activity (Collin and Burakoff, 1993; Sieh *et al.*, 1993; Xu and Littman, 1993).

1.3.4 CD45 inactivate Jak family kinase

The Janus-kinase/signal transducers and activators of transcription (Jak/STAT) pathway are involved in the signaling of many cytokines (Scott *et al.*, 2002). Following stimulation of cytokine receptors, Jaks (Jak1, Jak2, Jak3 and Tyk2) are phosphorylated on their tyrosine residues and are activated to phosphorylate STATs. STATs phosphorylation by Jaks results in dissociation and homo- or hetero-dimerization and ultimately translocation to the nucleus where they can rapidly and specifically activate target genes (Ivaskhiv, 1995). CD45 dephosphorylates all Jaks and inhibit the secretion of cytokines (Sasaki *et al.*, 2001). CD45 also negatively regulates IL-13 mediated cellular proliferation, erythropoetin-dependent hematopoiesis and antiviral responses *in vitro* and *in vivo* (Sasaki *et al.*, 2001).

1.4 Naïve CD4+ T cells

Naïve CD4+ T cells are thymic emigrants which had already undergone various maturation and selection processes in the thymus and populate the peripheral blood and secondary lymphoid organs such as spleen, lymph nodes and the mucosal-associated lymphoid tissue. They have not been activated by specific antigen *in vivo* (Van Lier and Baars, 1999). These cells can be characterized by the expression of the high molecular weight isoform of CD45, CD45RA, and low levels of β1 and β2 integrins (Sanders *et al.*, 1988; Prince *et al.*, 1992; Masopust *et al.*, 2004). Naïve CD4+ T cells express CCR7 and the peripheral lymph node homing receptor CD62L that facilitate the migration of these cells to the T cell areas of the secondary lymphoid organs (Sallusto *et al.*, 1999; Masopust *et al.*, 2004; Sallusto *et al.*, 2004). Naïve CD4+ T cells require the engagement not only of TCR, but also the costimulatory receptor, CD28 for complete activation which leads to