CHAPTER I

INTRODUCTION

1.1 Statement of problems

CD4 molecule is a cell surface glycoprotein of 50-60 kDa having typical features of a type I integral membrane protein (Zola *et al*., 2007). This molecule is a member of the immunoglobulin (Ig) super-family, which contains four extracellular Ig domains. As the typical characteristics for type I integral membrane protein, CD4 molecule is composed of three components including an extracellular domain (370 amino acids), a single transmembrane region (25 amino acids), and a cytoplasmic domain (38 amino acids). Within the extracellular domain, there are four Ig-like domains, designed D1, D2, D3, and D4. The D1, D2, and D4 domains are stabilized by intradomain disulfide bonds. N-linked glycosylations can be observed in domains D3 and D4, but not in D1 and D2 (Zola *et al*., 2007). The CD4 molecule has been reported to play several biological roles and is designed as a cell adhesion receptor. Functional binding sites of the CD4 molecule to other molecules are distributed across the entire its structure. The D1 domain is demonstrated to be a receptor for glycoprotein (gp)-120 of Human Immunodeficiency Virus (HIV). Domains D1 and D2 are the binding site for major histocompatibility complex (MHC) class II, D3 domain for T cell receptor (TCR), and D4 domain for Interleukin (IL)-16. The CD4 intracellular cytoplasmic tail contains the binding sites for T-lymphocyte Lck and HIV-1 Nef (Beare *et al*., 2008; Lynch *et al*., 2006).

CD4 molecule has been demonstrated to express on surface of various cell types, including T helper cells (CD4+ lymphocytes), regulatory T cells, and monocytes (Zola *et al*., 2007; Beare *et al*., 2008; Lynch *et al*., 2006; Knapp *et al*., 1990). CD4 molecule expressed on T lymphocytes involves in the initiation of T cell activation. The function of CD4 molecule on T lymphocytes is binding to MHC class II molecule on antigen presenting cells (APC) in the interaction of TCR on T lymphocytes and peptide–MHC class II complexes on APC during the induction of adaptive immunity. This interaction is involved in the mediation of intracellular T cell signaling (Beare *et al*., 2008; Littman, 1996). Up to now, the structure, molecular interaction, and function of CD4 molecule on T lymphocytes have been well characterized. The structure and function of CD4 molecule on monocytes, however, are poorly documented. The scientists assumed that the expression and function of CD4 molecule on monocytes may be different from lymphocytes.

The expression of CD4 molecule on leukocyte surfaces was reported in several forms including monomer, homodimer, and tetramer (Beare *et al*., 2008, Moldovan *et al*., 2006, Lynch *et al*., 1999). Functions of different forms of CD4 molecule, however, are still unknown. The expression of CD4 molecule on lymphocyte and monocyte surfaces was proposed in the different forms (Lynch *et al*., 1996; 1999). CD4 molecules expressed on monocytes are in homodimer, while the expression of CD4 molecule on lymphocytes is monomer (Lynch *et al*., 1996; 1999). Up to now, anti-CD4 monoclonal antibody (mAb) specifically recognized the different forms of CD4 expressed on lymphocytes and monocytes is not available. Hence, this is the obstacle in studying the difference of CD4 molecule expressed on lymphocytes and monocytes.

Recently, in the laboratory of Prof. Dr. Watchara Kasinrerk at the Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, four clones of mAb against CD4 molecule, namely MT4, MT4/2, MT4/3, and MT4/4, were produced (Pata *et al*., 2009). Anti-CD4 mAb clones MT4, MT4/2, and MT4/3 were generated from BALB/c mice immunized with native CD4 protein or recombinant CD4 protein from mammalian cells (Pata *et al*., 2009). While anti-CD4 mAb clone MT4/4 was produced from BALB/c mouse immunized with recombinant CD4 protein from *Escherichia coli* (*E. coli*) (Pata *et al*., 2009). Since several anti-CD4 mAbs are available in our laboratory, it is an opportunity for us to employ the generated anti-CD4 mAbs for characterization of CD4 molecule expressed on lymphocytes and monocytes. In this study, by using the available anti-CD4 mAbs, we aimed to study specific reaction of the anti-CD4 mAbs to CD4 molecule on lymphocyte or monocyte surfaces, study the expression and function of CD4 molecule on lymphocytes and monocytes. The molecular characterizations and bio-functional information of CD4 molecule will offer a better understanding of the immune regulation. This may also lead to the development of new strategy or new drug for regulation of immune responses.

1.2 Literature reviews

1.2.1 Immune system

Immune system is an important system of the body for maintenance of health. It has evolved to protect the body from invading pathogenic microorganisms and also plays a role in tumor protection. Activation of the immune responses is able to generate enormous variety of cells and molecules capable of specifically recognizing and eliminating the foreign invaders. Immune recognition is remarkable for its specificity that distinguishes one pathogen from another. Furthermore, the immune system is able to discriminate between foreign molecules and self antigen (Goldsby and Goldsby, 2003; Zola *et al*., 2003; Male, 2006). The immune system consists of innate immunity, which mediates the initial protection against infections, and adaptive immunity, which develops more slowly and mediates the later, even more effective, defense against infections (Figure 1.1). The mechanisms of innate immunity provides effective defense against infections. However, many pathogenic evolved to resist innate immunity, and their elimination requires the powerful mechanisms of adaptive immunity (Eales, 2003; Chinen and Shearer, 2005; Abbas and Lichtman, 2006).

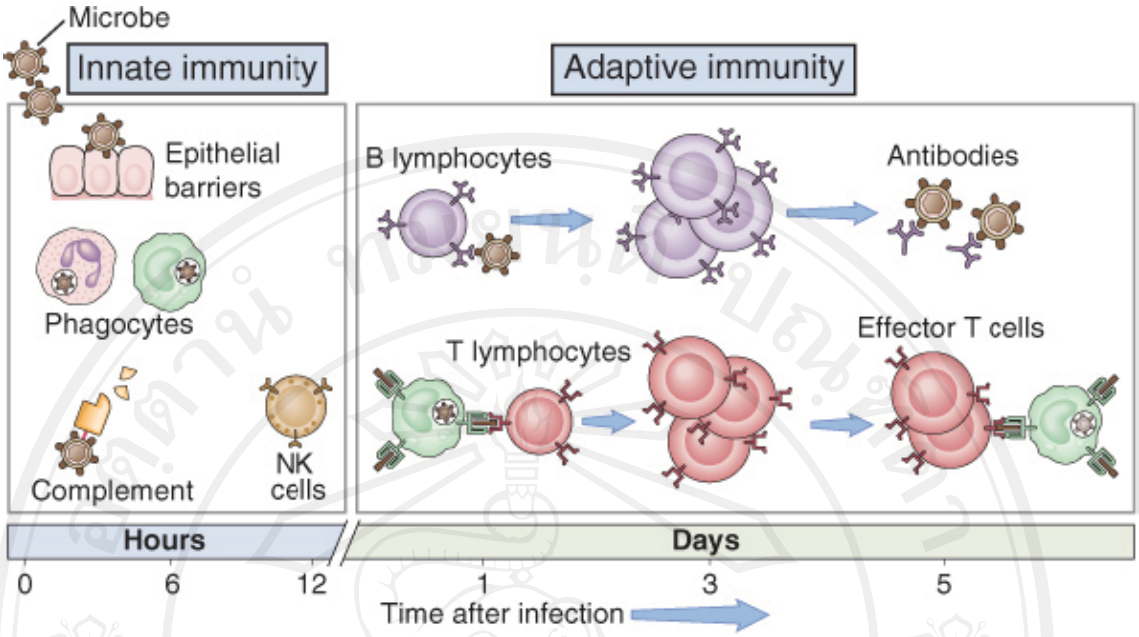


Figure 1.1 Innate and adaptive immunity. The mechanisms of innate immunity provide the initial defense against infections. Some of the mechanisms prevent infections (e.g., epithelial barriers) and others eliminate microbes (e.g., phagocytes, NK cells, and the complement system). Adaptive immune responses develop later and are mediated by lymphocytes and their products. Antibodies block infections and eliminate microbes, and T lymphocytes eradicate intracellular microbes (Abbas and Lichtman, 2006).

1.2.1.1 Innate immunity

Innate immunity provides the first line of defense mechanism against a wide range of microorganisms and allow a rapid response to invasion (Klotman and Chang, 2006; Male, 2006). The principal components of innate immunity are physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces; phagocytic cells (neutrophils, monocytes and macrophages) and natural killer (NK) cells; blood proteins, including the complement system and other mediators of inflammation; and cytokines that regulate and coordinate many of the activities of the cells of innate immunity. Innate immunity is stimulated by antigens of microbes that distinguish different groups of them. Those antigens are called pathogen-associated molecular pattern (PAMPs) (Akira *et al*., 2001; Abbas and Lichtman, 2005; Arancibia *et al.*, 2007). PAMPs are highly characteristic of potentially infectious microbes, but are not present in the host. It is the lipopolysaccharides (LPS) from Gram-negative bacteria, eubacterial flagellin, viral, and bacterial nucleic acids, fungal cell wall-derived glucans, chitins, mannans, or proteins and peptidoglycans (PGN) from Gram-positive bacteria (Sarrias *et al*., 2007). Although innate immunity can effectively combat many infections, microbes that are pathogenic, perhaps, have evolved to resist innate immunity. Defense against these infectious agents is the task of the adaptive immune responses (Abbas *et al*., 2000; Janeway *et al*., 2004; Chinen and Shearer, 2005).

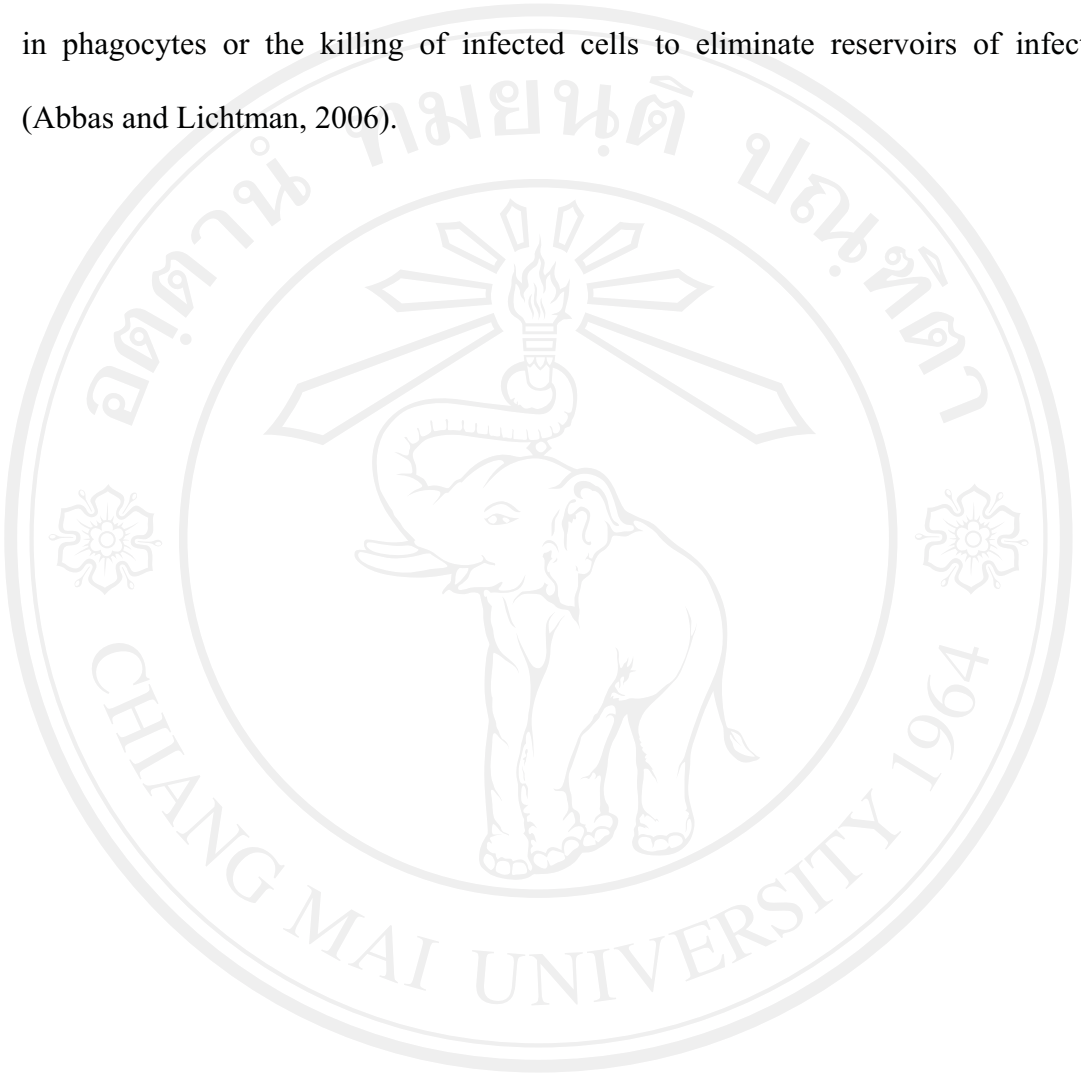
For many years it was believed that innate immunity is nonspecific and weak and is not effective in combating most infections. It was now know that, in fact, innate immunity specifically targets microbes and is a powerful early defense mechanism capable of controlling and even eradicating infections before adaptive

immunity becomes active. Innate immunity not only provides the early defense against infections but also instructs the adaptive immune system to respond to different microbes in ways that are effective at combating these microbes. Conversely, the adaptive immune response often uses mechanisms of innate immunity to eradicate infections. Thus, there is a constant bidirectional cross-talk between innate immunity and adaptive immunity (Abbas and Lichtman, 2006).

1.2.1.2 Adaptive immunity

Adaptive immunity is the next line of host defense after the innate immunity. Adaptive immune responses are trigger with microbes or substances called antigens after they pass through the innate immunity. This type of immunity refers to as antigen-specific defense mechanisms in which the process takes several days to cause microbial protection and elimination of a specific antigen. The adaptive immunity also plays an important role in elimination of tumor cells. Adaptive immune responses are mediated by lymphocytes and their products, such as antibodies and cytokines. There are two types of adaptive immunity, called humoral mediated immunity (HMI) and cell-mediated immunity (CMI) that are mediated by different cells and molecules are designed to provide defense against extracellular microbes and intracellular microbes, respectively (Figure 1.2) (Abbas *et al*., 2000; Janeway *et al*., 2004). HMI is mediated by antibodies that are produced by cells called B lymphocytes (B cells). Antibodies recognize microbial antigens, neutralize the infectivity of the microbes, and target microbes for elimination by various effector mechanisms. HMI is the principal defense mechanism against extracellular microbes and their toxins. CMI is mediated by T lymphocytes (T cells). Intracellular microbes, such as viruses and some bacteria, survive and proliferate inside the cells, where they

are inaccessible to circulating antibodies. Therefore, intracellular microbes are eliminated by a function of CMI, which promotes the destruction of microbes residing in phagocytes or the killing of infected cells to eliminate reservoirs of infection (Abbas and Lichtman, 2006).



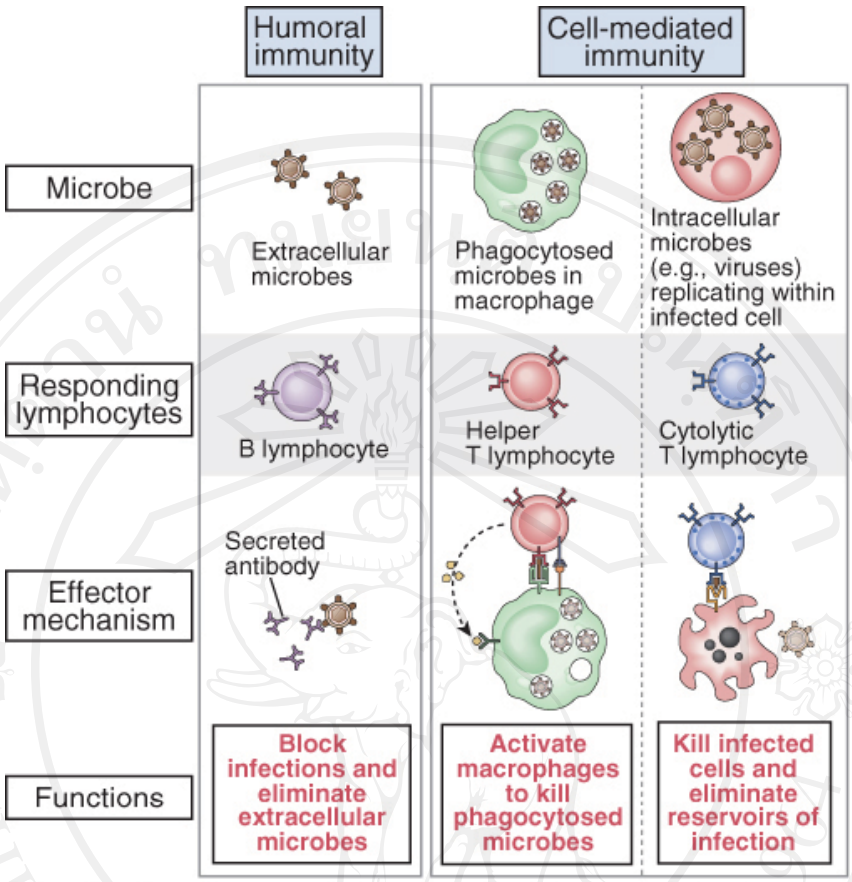


Figure 1.2 Types of adaptive immunity. In HMI, B cells secrete antibodies that block infections and eliminate extra-cellular microbes. In CMI, T cells eradicate intracellular microbes and altered cells (Abbas and Lichtman, 2006).

1.2.1.2.1 Humoral mediated immunity (HMI)

The main function of the humoral immune response in the body is to destroy extracellular microorganisms and prevent the spread of intracellular infection. HMI is mediated by proteins called antibodies, which are produced by cells called B lymphocytes. B cells recognize and are activated by a wide variety of antigens, including proteins, polysaccharides, lipids, and small chemicals. The activation of B cells results in the proliferation of antigen specific cells, also called clonal expansion, and their differentiation into effector cells that actively secrete antibodies. Antibodies are secreted into the circulation and mucosal fluids, and they neutralize and eliminate microbes and microbial toxins that are present in the blood and in the lumens of mucosal organs, such as the gastrointestinal and respiratory tracts. One of the most important functions of antibodies is to stop microbes that are present at mucosal surfaces and in the blood from gaining access to and colonizing host cells and connective tissues. In this way, antibodies prevent infections from ever getting established. Antibodies use their antigen-binding (Fab) regions to bind and block, or neutralize the infectivity of microbes and the interactions of microbial toxins with host cells. Other functions of antibodies require the participation of various components of host defense, such as phagocytes and the complement system. Antibodies use their crystallizable (Fc) regions, heavy chain constant regions which contain the binding sites for phagocytes and complement, to promote the phagocytosis or activate the complement system (Figure 1.3) (Janeway *et al*., 2004; Abbas and Lichtman, 2006).

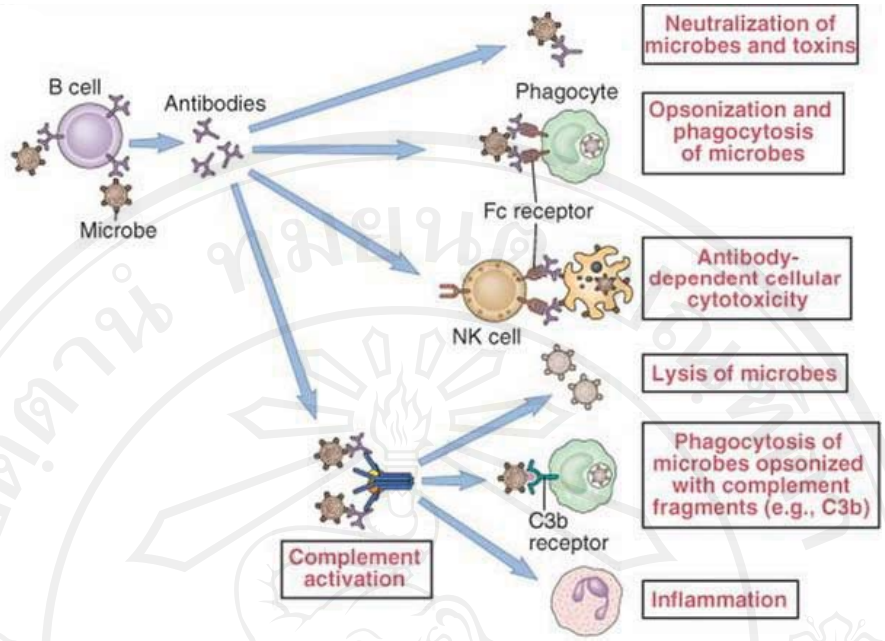


Figure 1.3 Effector functions of antibodies. Antibodies against microbes and their toxins neutralize these agents, opsonize them for phagocytosis and antibody-dependent cellular cytotoxicity, and activate the complement system (Abbas and Lichtman, 2006).

1.2.1.2.2 Cell-mediated immunity (CMI)

CMI is the adaptive immune response against intracellular microbes. It is mediated by T cells. The responses of T cells consist of sequential phases: recognition of cell-associated microbes by naive T cells, expansion of the antigen-specific clones by proliferation, differentiation of some of the progeny into effector cells and memory cells. The biochemical signals triggered in T cells by antigen recognition result in activation of various transcription factors that stimulate the expression of genes encoding cytokines, cytokine receptors, and other molecules involved in T cell responses (Figure 1.4) (Janeway *et al*., 2004; Chinen and Shearer, 2005; Abbas and Lichtman, 2006).

Two main types of T cells have been identified: CD4+ helper T lymphocytes (T_H) and CD8+ cytotoxic T lymphocytes (CTLs) are bearing either CD4 or CD8 molecules on their surface, respectively. CD4+ helper T cells differentiate into subsets of effector cells that produce restricted sets of cytokines and perform different functions. T_H1 cells, which produce IL-2 and IFN-γ, activate phagocytes to eliminate ingested microbes and stimulate the production of opsonizing and complement-binding antibodies. T_H2 cells, which produce IL-4 and IL-5, stimulate production of antibodies of B cells (Figure 1.5) (Janeway *et al*., 2004; Abbas and Lichtman, 2006). Moreover, recent studies have identified a third subset of differentiated effector CD4+ T cells. These cells have been called T_H17 cells. This cell sub-population secrete IL-17 and does not produce either IFN- γ or IL-4. The principal role of T_H17 cells appears to be the induction of inflammatory reactions. Thus, T_H17 cells may be important mediators of tissue damage in immune-mediated inflammatory diseases (Abbas *et al*., 2007).

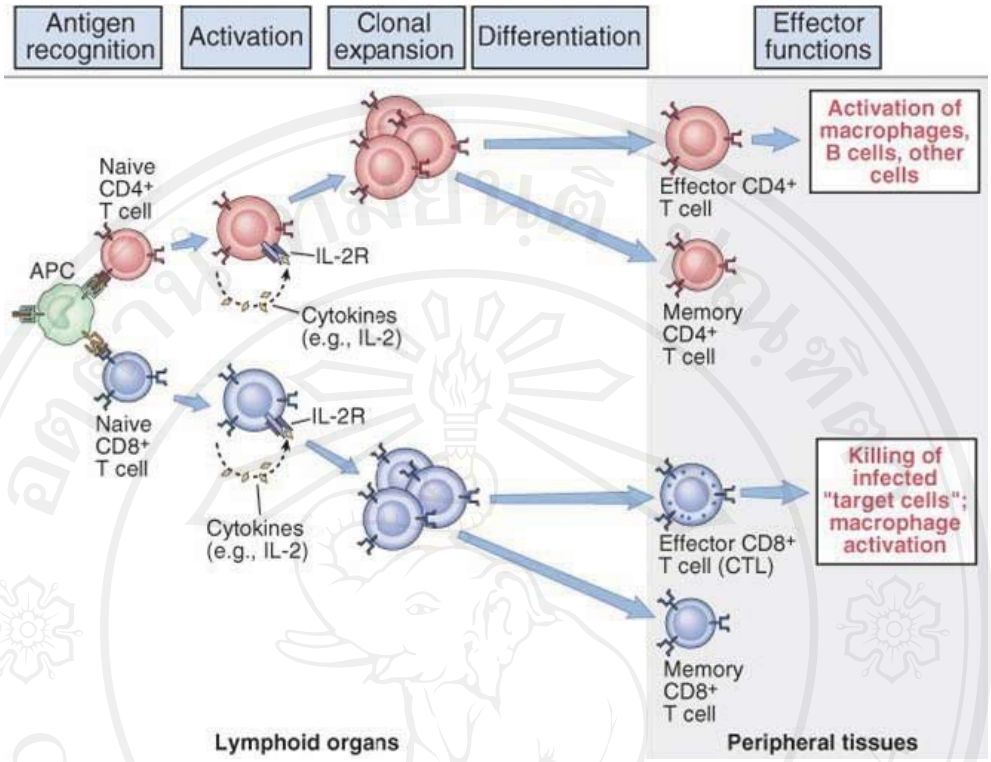


Figure 1.4 The induction and effector phases of cell-mediated immunity. The native T cells are stimulated to proliferate and differentiate into effector cells (Abbas and Lichtman, 2006).

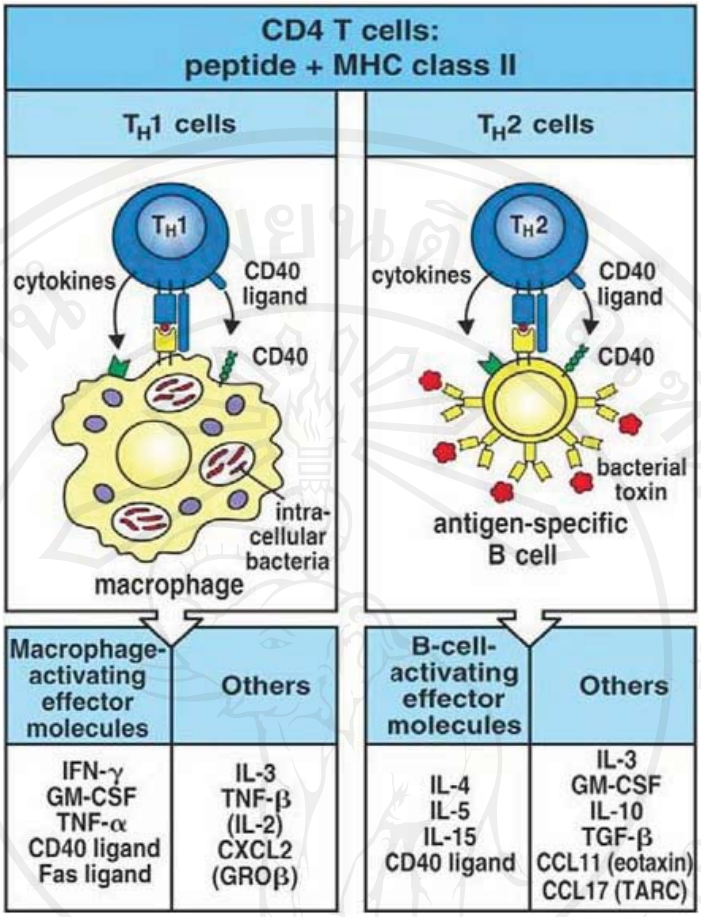


Figure 1.5 Effector functions of CD4+ lymphocytes. CD4+ helper T cells differentiate into T_H1 cells or T_H2 cells secrete cytokines which act on macrophages to increase phagocytosis and killing of microbes and act on B cells to stimulate production of antibodies (Janeway *et al*., 2004).

For CTLs, these cells recognize class I MHC-peptide complexes on the surface of infected cells and kill these cells, thus eliminating the reservoir of infection. Antigen recognition by effector CTLs results in the activation of signal transduction pathways that lead to the exocytosis of the contents of the CTL's granules to the region of contact with the targets. CTLs kill target cells mainly as a result of delivery of granule proteins, granzymes and perforin, into the target cells. Granzymes are enzymes that cleave and thereby activate enzymes called caspases that are present in the cytoplasm of target cells, and the active caspases induce apoptosis. Perforin is necessary for delivery of granzymes into the cytoplasm of the target cell. Alternatively, both perforin and granzymes may enter the target cells by receptor-mediated endocytosis, both proteins bound to a sulfated glycoprotein called serglycine. Perforin may then insert into endosomal membranes and facilitate the movement of granzymes through these membranes and into the cytoplasm. Moreover, CTLs use a second mechanism of cytolytic that is mediated by interactions of membrane molecules on the CTLs and target cells. Upon activation, CTLs expressed a membrane protein, called Fas ligand (FasL), that binds to its target protein Fas, which is expressed on many cell types. This interaction also results in activation of caspases an apoptosis of targets (Figure 1.6) (Janeway *et al*., 2004; Abbas and Lichtman, 2000; Abbas *et al*., 2000; 2007).

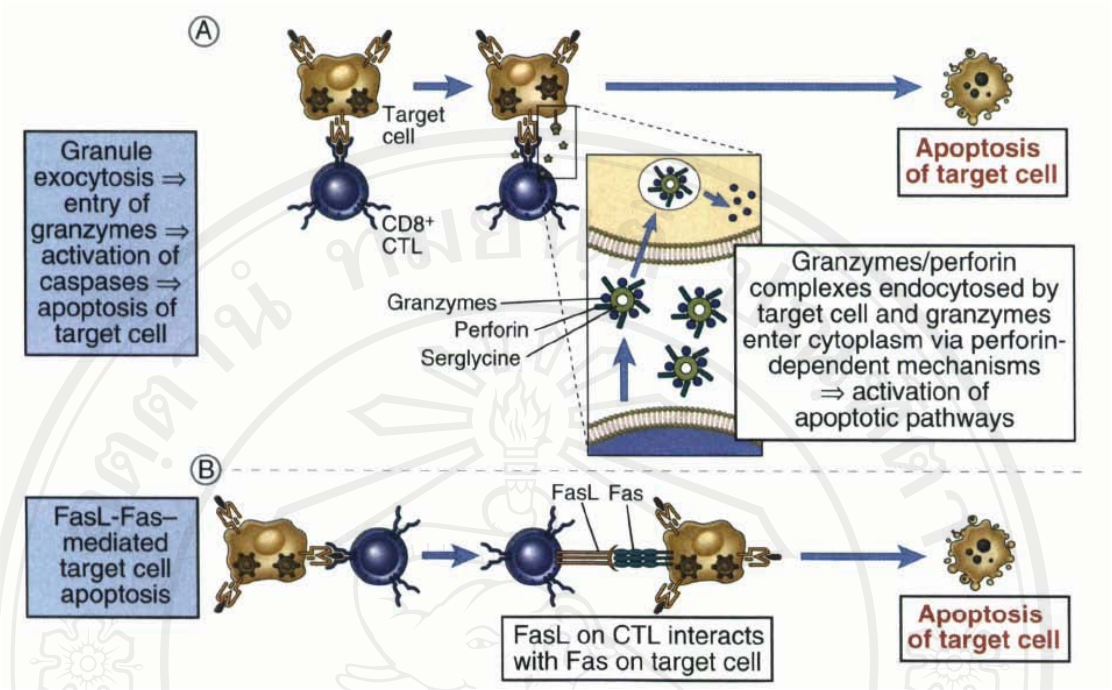


Figure 1.6 Mechanisms of cytotoxic T lymphocytes. CTLs kill target cells by two main mechanisms: complexes of perforin and granzymes (A) and Fas ligand (B) (Abbas *et al*., 2007).

1.2.2 CD4 molecule

CD4 molecule is an integral membrane glycoprotein of 55 kDa. This molecule is a member of Ig super-family and belongs to the type I integral membrane protein (Zola *et al*., 2007). CD4 molecule composes three parts: an extracellular domains, a transmembrane region, and a cytoplasmic domain. The extracellular domains contain important four Ig-like domains, including D1, D2, D3, and D4 (Figure 1.7). D1 and D3 belong to the variable Ig domains, while D2 and D4 are constant Ig domains. The structure of D1, D2, and D4 are stabilized with disulfide bonds. D3 and D4 have N-linked glycosylation (Figure 1.7) (Beare *et al*., 2008; Lynch *et al*., 2006; Zola *et al*., 2007).

The Function of CD4 molecule has been intensively investigated and suggested that this molecule is one of the adhesion cell surface receptor. CD4 molecule acts as receptors for several ligands which are both soluble and membrane proteins. Functional binding sites are distributed across the entire CD4 structure: D1 domain is a site for gp-120 of HIV, D1 and D2 domains for MHC class II, D3 domain for TCR, D4 domain for IL-16, and intracellular cytoplasmic tail for T-lymphocyte Lck and HIV-1 Nef (Beare *et al*., 2008; Lynch *et al*., 2006). Triggering of CD4 receptor by its specific ligands induce intracellular signal transductions and cellular functions.

CD4 molecule is broadly expressed on the surface of various cell types, including CD4+ lymphocytes, regulatory T cells, and monocytes (Zola *et al*., 2007). Among those cell types, the expression of CD4 molecule is considerably lower levels on monocytes. Monocytes express 10- to 20-fold less CD4 molecule than lymphocytes (Collman *et al*., 1990; Kazazi *et al*., 1989). Although, the structure of

CD4 molecule on lymphocytes has well characterized, little is known of the structure of the CD4 molecule of non-lymphocytic cells. Recently, the expression of CD4 molecule on leukocyte surfaces was reported in several forms: monomer, homodimer, and tetramer (Beare *et al*., 2008; Moldovan *et al*., 2006; Lynch *et al*., 1999). Lymphocytes and monocytes differ in their expression of CD4 have been suggested (Lynch *et al*., 1999). The CD4 molecule of adherent monocytic cells is expressed predominantly as 110 kDa covalent homodimers, but in lymphocytes mainly as 55 kDa monomers (Lynch *et al*., 1996; 1999).

For T cells, CD4 molecule is described as a 125 A°, 55 kDa linear array of monomers of four Ig-like extracellular domains, D1–D4 (Maddon *et al*., 1985). Intradomain disulfide bonds are appeared in D1, D2 and D4. Oligomers of CD4 molecule expressed on lymphocytes have also been described, which involve noncovalent dimers of D1 CDR-3 loops (Briant *et al*., 1997; Langedijk *et al*., 1994), covalent disulfide dimers of D2 (Matthias *et al*., 2002), and noncovalent (Wu *et al*., 1997) or disulfide-mediated covalent 110 kDa homodimers of D4 (Lynch *et al*., 1996; 1999; 2003). The possible contribution of disulfides in dimer formation has, however, been contentious because the intradomains D1, D2, and D3 disulfides are generally assumed to be maintained in a closed configuration (Harris *et al*., 1990). Nonetheless, the possibility exists that one or more of the disulfides can be expressed in an open or disrupted state (Matthias *et al*., 2002; Lynch *et al*., 1996; 1999) and thus permit thiol-exchange interactions.

The function of CD4 molecule expressed on T lymphocytes has been well characterized. It mediates maturation (Trobridge *et al*., 2001) but is best known for its roles in stabilizing TCR interactions with peptide–MHC class II complexes on APC,

and for mediation of intracellular T cell signaling (Wang *et al*., 2001). The interaction of TCR and CD4 on T cells and peptide–MHC class II complexes on APC during the induction of adaptive immunity is shown in Figure 1.8 (Beare *et al*., 2008; Littman, 1996; Abraham and Weiss, 2004). During T cell activation by specific antigen, large multiprotein clusters of CD4, TCR and CD3 are localized to lipid rafts and provide a focal point for intracellular signalling, through tyrosine kinase (TyK) Lck interactions with the cytoplasmic tail of CD4 (Collins *et al*., 1992; Parolini *et al*., 1999; Rudd *et al*., 1988). CD3 phosphorylation recruitment of ZAP-70, Lck autophosphorylation and protein kinase C (PKC) feedback serine phosphorylation of Lck and CD4 molecule (Glaichenhaus *et al*., 1991 Garofalo *et al*., 1998).

Not only function as the receptor for MHC class II, CD4 molecule was reported to react to several proteins. As mentioned above, functional binding sites of CD4 receptor to its ligands are distributed across the entire CD4 structure. Domain D1 is demonstrated to be a receptor for HIV-1-gp120 binding. D1 and D2 are receptors for MHC class II. D3 is the receptor for TCR and D4 for IL-16. It is suggested that D1–D4 is possibly the receptor for Human Hepatitis Virus (HHV)-7 (Lusso *et al*., 1994; Yasukawa *et al*., 1997). The intracellular cytoplasmic tail of CD4 of T cells can bind to Lck and HIV-1 Nef (Rudd *et al*., 1988; Preusser *et al*., 2001). Additional binding sites have yet to be mapped for the many other interprotein interactions of CD4, such as those with the cell surface receptor CXCR4 (Sloane *et al*., 2005). Structural change to any domain may consequently has functional ramification. Commonly, CD4 molecule expressed on both CD4+ T cells and monocytes/macrophages interact with IL-16 and for HIV-1 and HHV-7 infection (Lusso *et al*., 1994; Yasukawa *et al*., 1997; Cruikshank *et al*., 1996; Black and Pellett,

1999; Dalgleish *et al*., 1984) but whether the CD4 molecule of both cell types undergo identical ligand binding and cell response interactions is not known.

In addition to its physiologic roles in immune cells, CD4 is a receptor for HIV (Figure 1.9) (Camerini and Seed, 1990; Abbas *et al*., 2000). Infection with HIV causes AIDS by massive dysfunction of the adaptive immune system. Most of the immunodeficiency in AIDS can be ascribed to the depletion of CD4+ T cells. Recently, the CD4+ T cell count has been used to assess prognosis for progression to AIDS or death, to formulate the differential diagnosis in symptomatic patients, and to make therapeutic decisions regarding antiviral treatment and prophylaxis for opportunistic pathogens (Camerini and Seed, 1990; Janeway *et al*., 2004). Therefore, monitoring of CD4+ T cells is very important tool used for successful HIV/AIDS management and care (Stein *et al*., 1992; Phillips *et al*., 1994a; 1994b; Abbas *et al*., 2000).

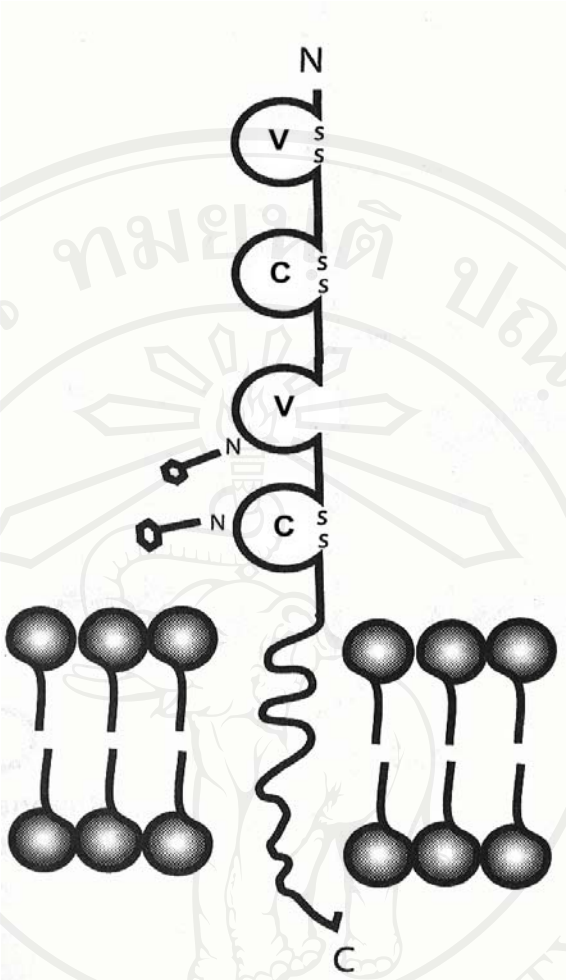


Figure 1.7 Structure of CD4 molecule. It is composed of three part: Ig-like extracellular domains, transmembrane region, and cytoplasmic domain. Ig-like extracellular domains contains four domains: D1-D4 (Zola *et al*., 2007).

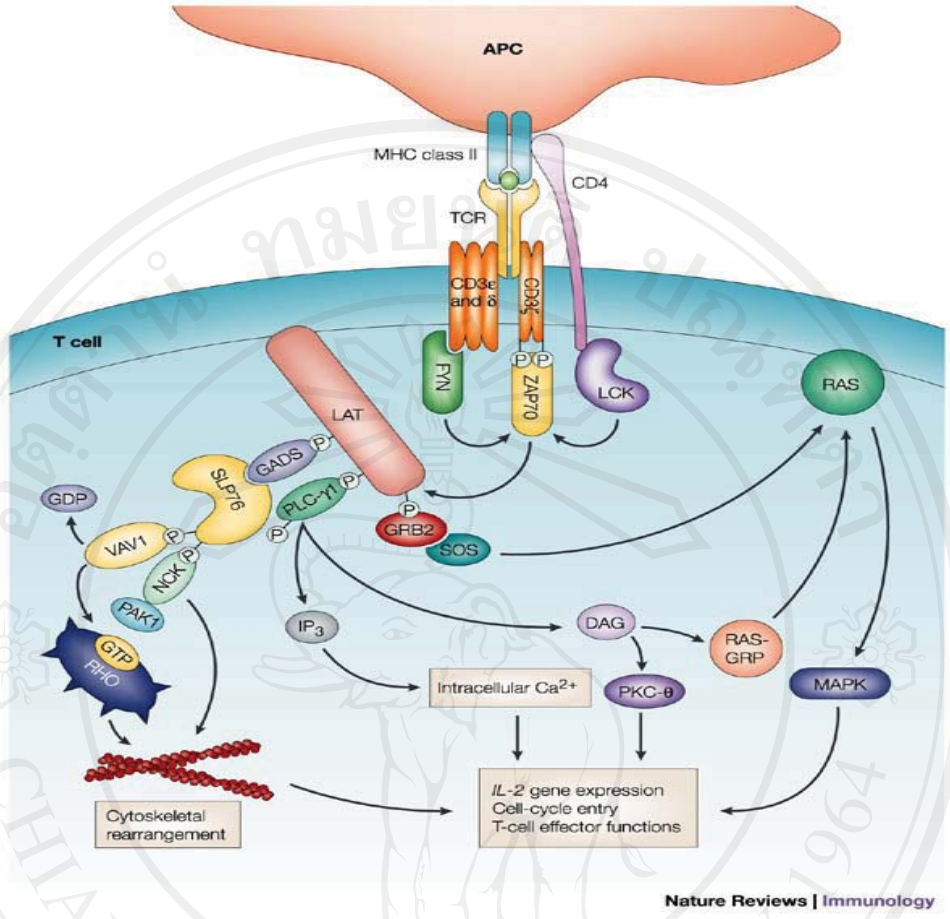


Figure 1.8 Function of CD4 molecule. It is binding to MHC class II molecule in the interaction of TCR on T cells and peptide–MHC class II complexes on APC, and mediation of intracellular T cell signaling (Abraham and Weiss, 2004).

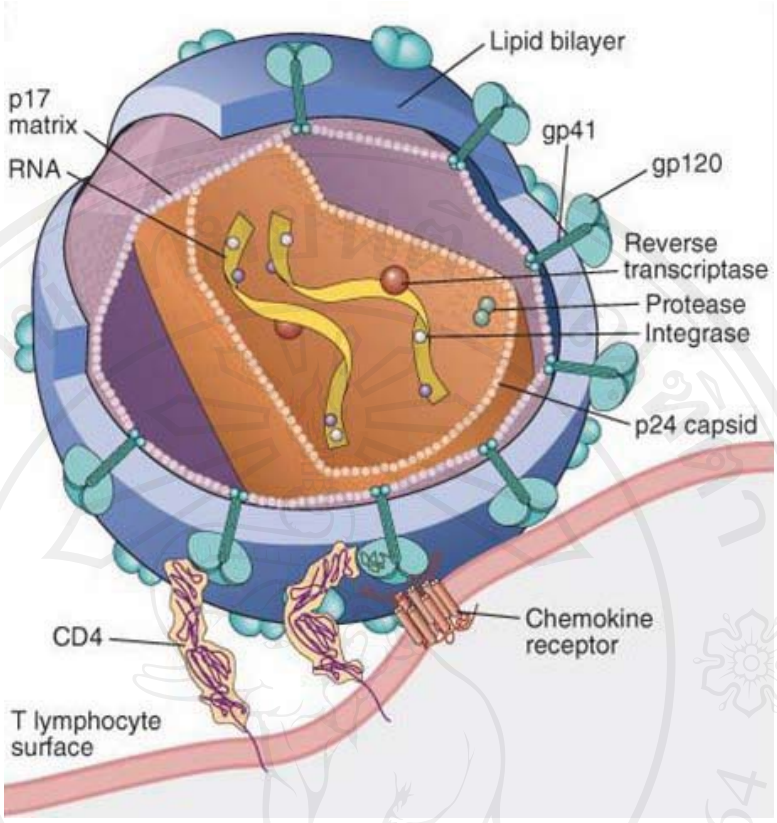


Figure 1.9 CD4 is a receptor for HIV. Infection with HIV is caused of AIDS by massive dysfunction of the adaptive immune system (Abbas *et al*., 2000).

1.2.3 Hybridoma technology and monoclonal antibody production

Hybridoma technology is the technique for production of monospecific antibody developed by Kohler and Milstein in 1975 (Kohler and Milstein, 1975). They devised a method of growing large numbers of antibody producing cells from a single B cell by fusing B cells from the antigen immunized animal with immortal myeloma cancer cells. The procedure yields an immortal cell line that produce monospecific antibodies. The generated immortalized antibody-producing cell line is called hybridoma and the produced monospecific antibodies are termed monoclonal antibodies.

The myeloma cell lines that used as fusion partner in the hybridoma technique are the cells those defected in hypoxanthine phosphoribosyltransferase (HGPRT). HGPRT is the enzyme necessary for nucleotide synthesis by a salvage pathway (Figure 1.10). As lacking of HGPRT, the myeloma cells cannot synthesize nucleotide via the salvage pathway. In normal animal cells, synthesis of DNA precursors, purine and thymidylate, are achieved by *de novo* synthesis pathway which requires tetrahydrofolate (Figure 1.10). Hence, using of antifolate drugs, such as aminopterin, the drugs block activation of tetrahydrofolate, thereby inhibits the synthesis of DNA precursors and finally rules out DNA synthesis by *de novo* pathway (Figure 1.10) (Yelton and Scharff, 1980; Goding, 1996; Abbas *et al*., 2000). Nevertheless, normal cells can still survive by producing DNA precursors from an alternative salvage pathway using HGPRT enzyme. In contrast, myeloma cells which defecting HGPRT and the salvage pathway, they cannot survive in the present of antifolate drugs (Yelton and Scharff, 1980; Harlow and Lane, 1988).

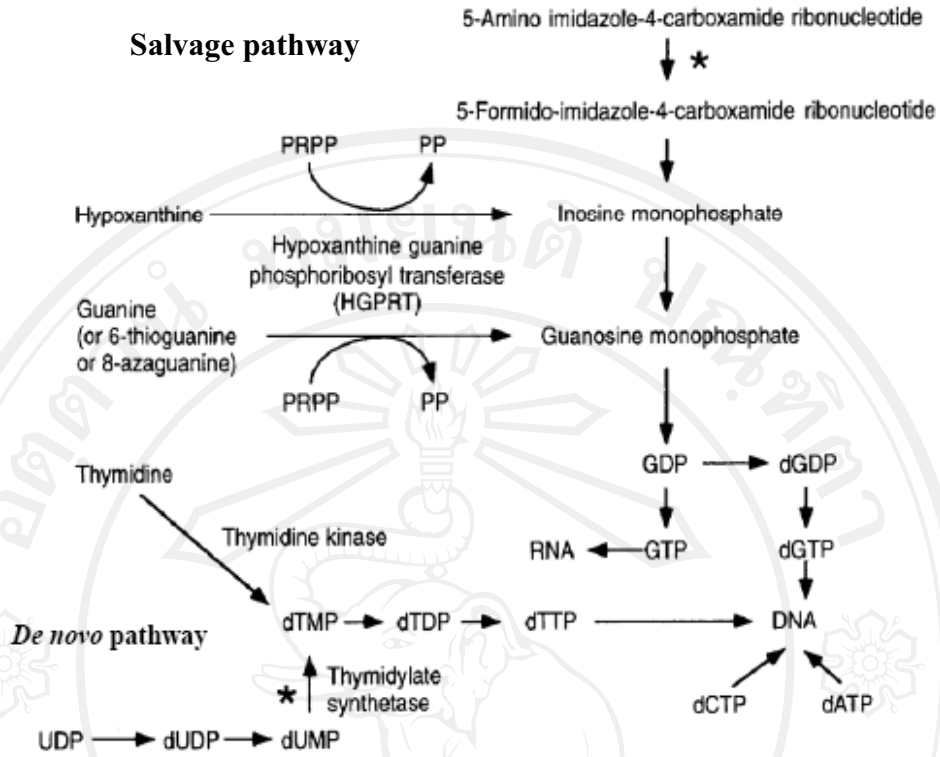


Figure 1.10 Metabolic pathways of DNA synthesis. When the *de novo* pathway are blocked with folic acid analogue (*), such as aminopterin, cell must depend on the salvage pathway (Goding, 1996).

In hybridoma technique, mouse is firstly immunized with the antigen of interest. The splenic B lymphocytes of the antigen immunized mouse are fused with the HGPRT defecting myeloma cells. The fused cells are then cultured in a selective medium that containing hypoxanthine, aminopterin, and thymidine (HAT medium). Aminopterine, a folic antagonist, presented in the HAT medium blocks the *de novo* pathway of nucleotide synthesis (Figure 1.11). By HAT medium culturing, unfused myeloma cells and fused cells that lacking of HGPRT will die (Harlow and Lane, 1988). Hybridoma cells, hybrid cells of B cells and myeloma cells, can survive in the selective medium as B cells contain HGPRT and the salvage pathway can be function. The B-myeloma hybrid cells or hybridoma cells can be proliferated in culture medium. To produce the mAbs, the hybridoma cells are then grown to allow producing the desired mAbs.

In Figure 1.12, the standard procedure for the production of mAb is illustrated. Mouse is firstly immunized by the antigen of interest. After appearing of high titer of antibody response, splenocytes of the immunized mouse are collected and fused with myeloma cells. After cell fusion, cells are placed into 96-wells tissue culture plates and cultured in selective medium (HAT medium) containing hypoxanthine, thymidine, and an antifolate drug (aminopterin). Un-fused myeloma cells and myeloma-myeloma hydrids can be selected out in HAT medium. As myeloma and myeloma-myeloma hydrids are deficient in HGPRT enzyme required for the salvage pathway of nucleotide synthesis, these cells are died in HAT medium. By the HAT medium selection, thus non-fused myeloma cells and myeloma-myeloma hydrids are died and only those cells fused to normal cells survive. In the case of the fusion of myeloma and normal cells, the outcome hybridomas can survive indefinitely in

culture medium because the normal cells supply the missing enzyme for the selection in HAT medium and the myeloma cells immortalize the hybrid cells. Un-fused normal lymphocytes can survive in culture medium for approximately 1 week then they die. Therefore, after long-term culture, only hydridomas of normal and myeloma cells grow in the selective medium. Hybridomas are then produce antibodies. To identify the hybridoma clones that produce a desired mAb, several methods, for examples enzyme-linked immunosorbent assay (ELISA) or immunofluorescence assay were employed. Since a single well may contain multiple hybridoma clones, isolation of a single hybridoma cell from a positive well is performed by cloning in either soft agar or limiting dilution. The hybridoma clones that produce a desire antibody can be expanded in culture medium in tissue culture laboratory or induced ascitic fluids in a mouse to produce large amount of mAbs (Abbas and Lichtman, 2005; de StGroth and Scheidegger, 1980; Yelton and Scharff, 1980; Galfre and Milstein, 1981).

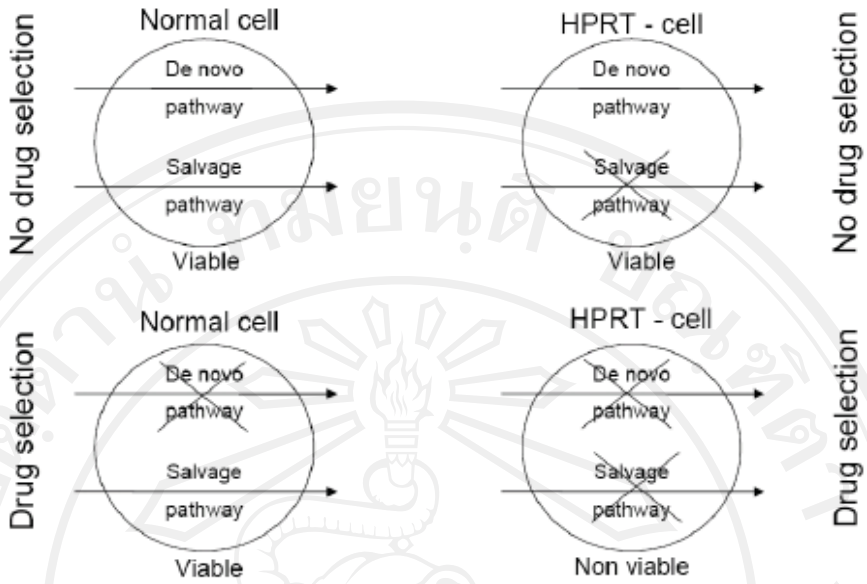


Figure 1.11 Pathway of nucleotide synthesis in the presence of antifolate drug. (Harlow and Lane, 1988).

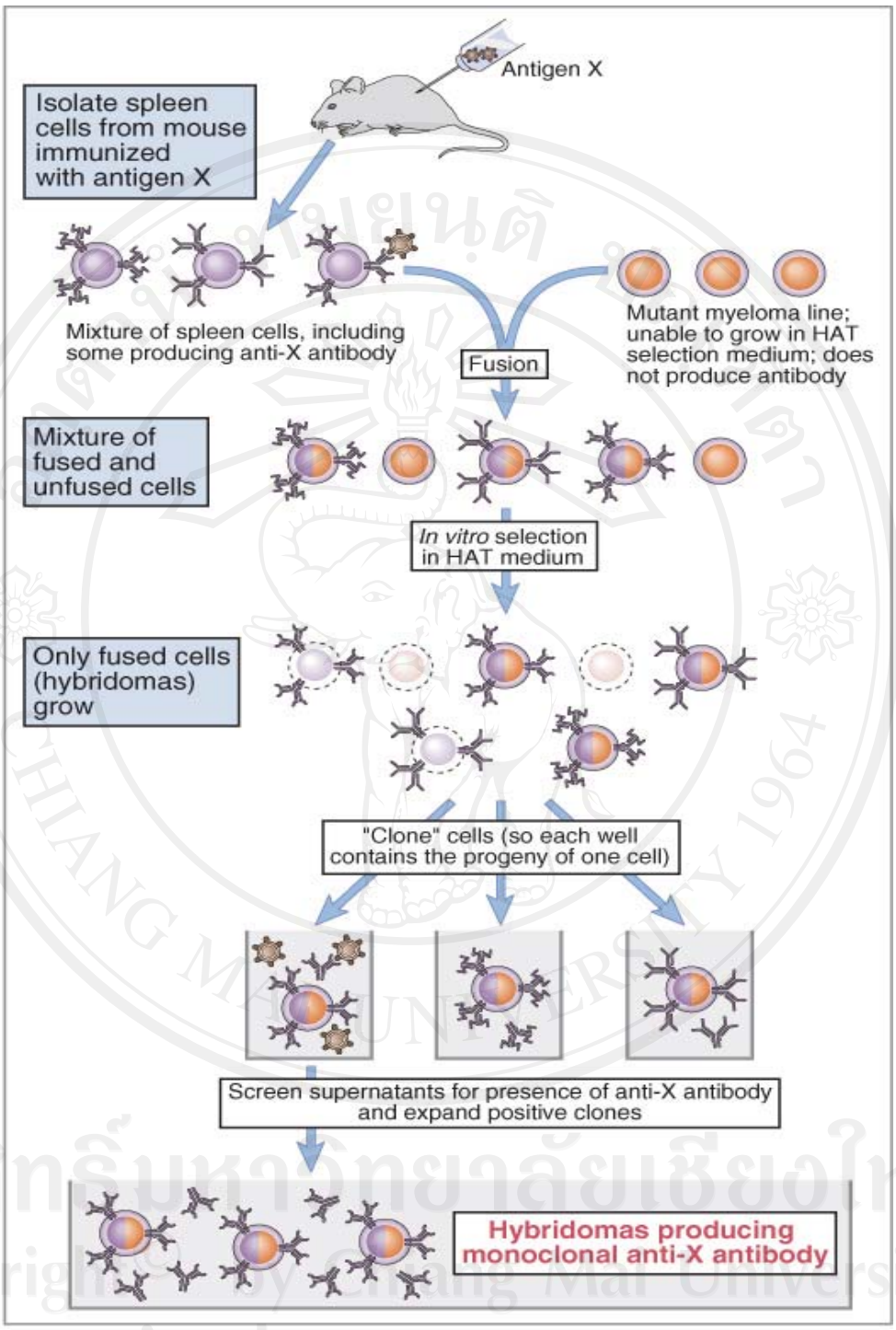


Figure 1.12 Monoclonal antibody production. (Abbas and Lichtman, 2005).

1.2.4 Production of anti-CD4 monoclonal antibodies used in this study

During the last decade, in the laboratory of Prof. Dr. Watchara Kasinrerk, effective hybridoma technique was established. By using the established hybridoma technique, various mAbs against leukocyte surface molecules were generated. Among of those mAbs, mAbs against CD4 molecule were also produced (Pata *et al*., 2009). In those studies, three different sources of CD4 immunogens were employed for CD4 mAb production. First, CD4 proteins were isolated from CD4+ lymphocytes by immunoprecipitation. The CD4 immunoprecipitated beads were used as an immunogen for mouse immunization. Second, recombinant CD4 protein-biotin carboxyl carrier protein (BCCP) fusion proteins (CD4-BCCP) were produced in *E. coli*. The recombinant CD4-BCCP fusion proteins were then isolated from other bacterial proteins by streptavidin coated beads. The CD4-BCCP beads were then used as an immunogen. Third, plasmid DNA encoding CD4 proteins were transfected into COS cells. CD4 expressing COS (CD4-COS) cells were then enriched by immunosorting. The CD4+ COS cells were then used as an immunogen. After three immunizations, by all immunization strategies, anti-CD4 antibodies could be observed in all immunized mice. By standard hybridoma technique, the CD4 mAbs could be generated from all immunization strategies. These anti-CD4 mAbs were used in this study in order to indentify the function of CD4 molecule expressed on lymphocytes and monocytes.

1.3 Objectives

1.3.1 To investigate specific reactivity of the generated anti-CD4 monoclonal antibodies to CD4 molecule expressed on lymphocytes or monocytes.

1.3.2 To compare the expression of CD4 molecules between lymphocyte and monocyte surfaces using the generated anti-CD4 monoclonal antibodies.

1.3.3 To determine the function of CD4 molecule expressed on lymphocytes and monocytes in regulation of lymphocyte activation.