CHAPTER I

INTRODUCTION

1.1 Statement of problems

The immune system is a defense mechanism to microorganisms that maintains balance of health and disease in human body. This system composes of several types of cells, organs and mediators that work together. Leukocytes are the cells which play a major role in the immune system. Leukocytes compose of lymphocytes, monocytes and granulocytes. Among lymphocytes, it can be divided into 3 sub-populations including B lymphocyte which mainly produce antibodies, T lymphocytes which control B lymphocyte, phagocytes and other aspects of the immune response, and NK cells. In particular, T lymphocytes can also be divided into multiple functional subsets which interact with each other and with other components of the immune system (Abbas and Lichtman, 2005; Male, 2006). In our body, T lymphocytes play a crucial role in orchestrating the adaptive immune response. The molecules of cell surface are demonstrated to be involved in the interactions and communications of T lymphocytes and other cells with their environment. These interactions include cell-to-cell contact with other leukocytes, stroma and endothelial cells, the detection of signals from cytokines, chemokines and hormones, and the recognition of foreign antigens presented by antigen presenting cells (Zola *et al*., 2006). In addition, recently, a myriad of functions of leukocyte surface molecules are demonstrated in

involving T lymphocyte activation, including antigen recognition, antigen processing and presentation, cell adhesion, signaling-activation, differentiation, proliferation, apoptosis, and anergy (Abbas and Lichtman, 2005; Male, 2006).

Up to the present, many of cell surface molecules that are involved in the various forms of T lymphocyte recognition, activation and effector function have been identified and characterized. Examples of these molecules and their ligands involve in the interaction of T lymphocyte and antigen-presenting cells (APCs), which are key point of T lymphocytes activation, include CD4-MHC class II, CD8-MHC class I, CD28-B7, LFA1-ICAM, CD3, CD45, and others. These molecules are involved in initial activation of T lymphocytes and quality of T lymphocyte activation. Therefore, several scientists suggested that the cell surface molecules may be a new target for therapeutic agents or immunotherapy.

Many leukocyte surface molecules were discovered and identified with 'cluster of differentiation' (CD) nomenclature. The Human Leukocyte Differentiation Antigens (HLDA) workshops have recognized some 400 molecules as leukocyte surface markers, and another 100-200 have been characterized outside the HLDA Workshops. The total number of leukocyte surface molecules, however, is estimated to be around 2,500 leukocyte surface molecules, suggesting that many molecules have remained undiscovered. Therefore, it still has undiscovered of the molecules and their function, and the knowledge of cell in immune system. At the present, several leukocyte surface molecules are being identified using specific monoclonal antibodies in many laboratories around the world. Many researchers produced monoclonal

antibodies against leukocyte surface molecules in decade year and applied for characterization of cell surface molecules (Zola and Swart, 2003; Zola *et al*., 2005; Zola, 2006; Zola *et al*., 2006; Zola *et al*., 2007).

In an attempt to identify new surface molecules of T lymphocytes, in the laboratory of Prof. Dr. Watchara Kasinrerk, many monoclonal antibodies against human leukocyte surface molecules were produced and screened. From the preliminary results, two monoclonal antibodies, named MT3 and COSA2A, are of interested. mAbs MT3 and COSA2A were demonstrated to react with a sub-population of lymphocytes. Theses monoclonal antibodies were proposed to recognized un-defined molecules. In this study, cellular distribution, biochemical characterization and functional study of molecules recognized by the mAbs MT3 and COSA2A were investigated. The information obtained from this study will leads to better understanding of lymphocyte function and the immune system.

1.2 Literature reviews

1.2.1 Immune system

The immune system is a defense system that has evolved to protect mammalians from invading pathogenic microorganisms and cancer. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating the foreign invaders. These cells and molecules act together in a dynamic network and more complexity. An immune response can be divided into two related activities-recognition and response. 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 and Swart, 2003; Male, 2006). Most immune response to infection microorganism has two phases of responses. In the earliest stage of infection, innate immunity is predominated. And later, adaptive immunity is generated by lymphocytes which have immunological memory remains within sub-population of lymphocytes, are able to eliminate the same infected microorganism with more effective and rapid response (Figure1.1) (Abbas and Lichtman, 2005; Male, 2006).

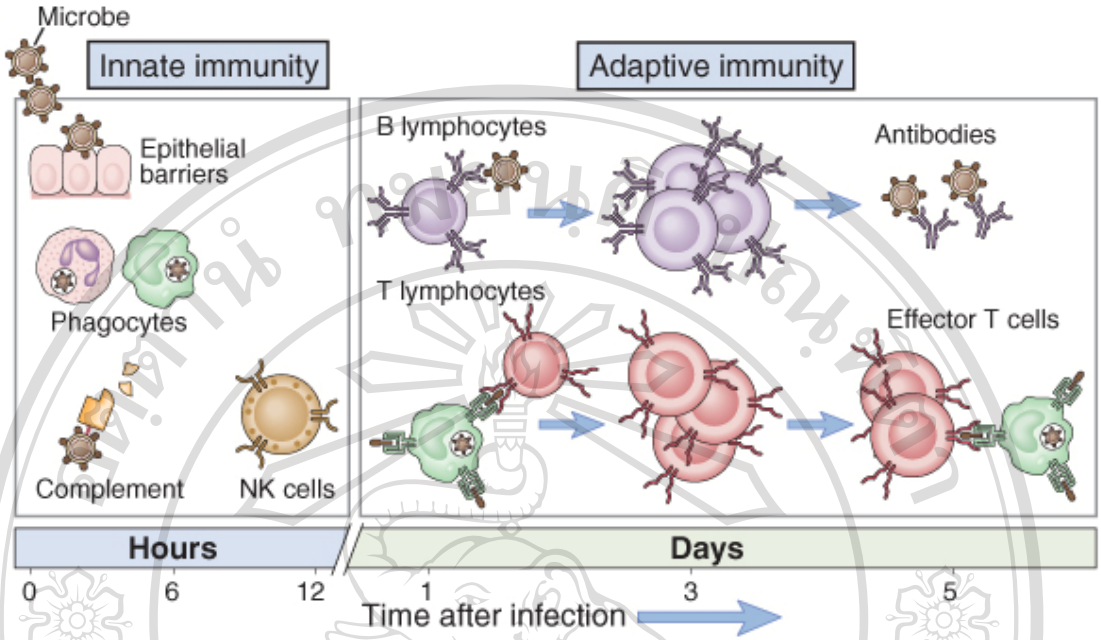

Figure 1.1 Innate and adaptive immunity. The mechanisms of innate immunity provide the initial defense against infections. Adaptive immune responses develop later and consist of activation of lymphocytes. The kinetics of the innate and adaptive immune responses are approximations and may vary in different infections (Abbas and Lichtman, 2005).

1.2.1.1 Innate immunity

Innate immune system 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 NK (natural killer) 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). The recognition of these molecules involving LPS Binding Protein (LBP), CD14 and Toll-like receptor (TLR) has been elucidated over the last years (Akira *et al*., 2001; Janeway and Medzhitov, 2002; Akira and Hemmi, 2003; Finberg *et al*., 2004; Bowie and Haga, 2005; Gust *et al*., 2007).

The innate immunity is closely interlinked with adaptive immunity. Without proper function of innate immunity, adaptive immunity can be induced. Macrophages and dendritic cells respond to phagocytosed microbes by expressing co-stimulators

and by secreting cytokines. Co-stimulators and cytokine function, together with antigen recognition, to activate T lymphocytes (Male, 2006). In addition, B lymphocytes recognize microbial antigens by their antigen receptors and recognize C3d by a receptor called the complement receptor type 1 and 2 (CR2) or CD35/CD21 (Fischer *et al*., 1996; Haas *et al*., 2004; Mitsuyoshi *et al*., 2005). Signals from the antigen receptor and CR2 (CD21) function cooperatively to activate the B lymphocytes (Tedder *et al*., 1994; Poe *et al*., 2001). Innate and adaptive immune responses are components of an integrated system of host defense in which numerous cells and molecules function cooperatively (Abbas and Lichtman, 2006).

1.2.1.2 Adaptive immunity

In contrast to innate immunity, adaptive immunity is other immune responses that are stimulated by exposure to the same infectious microorganisms. Adaptive immune responses are increase in magnitude and defensive capabilities. Adaptive immunity is specific for distinct antigens and an ability to remember and respond more vigorously to repeated exposures to the same microbe. Adaptive immunity is mediated by lymphocytes and their products (Weng, 2006). There are two types of adaptive immune responses, called humoral immunity and cell-mediated immunity (Figure 1.2). Humoral immunity 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. Humoral immunity is the principal defense mechanism against extracellular microbes and their toxins. Cell-mediated immunity 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 cell-mediated immunity, which promotes the destruction of microbes residing in phagocytes or the killing of infected cells to eliminate reservoirs of infection (Abbas and Lichtman, 2005).

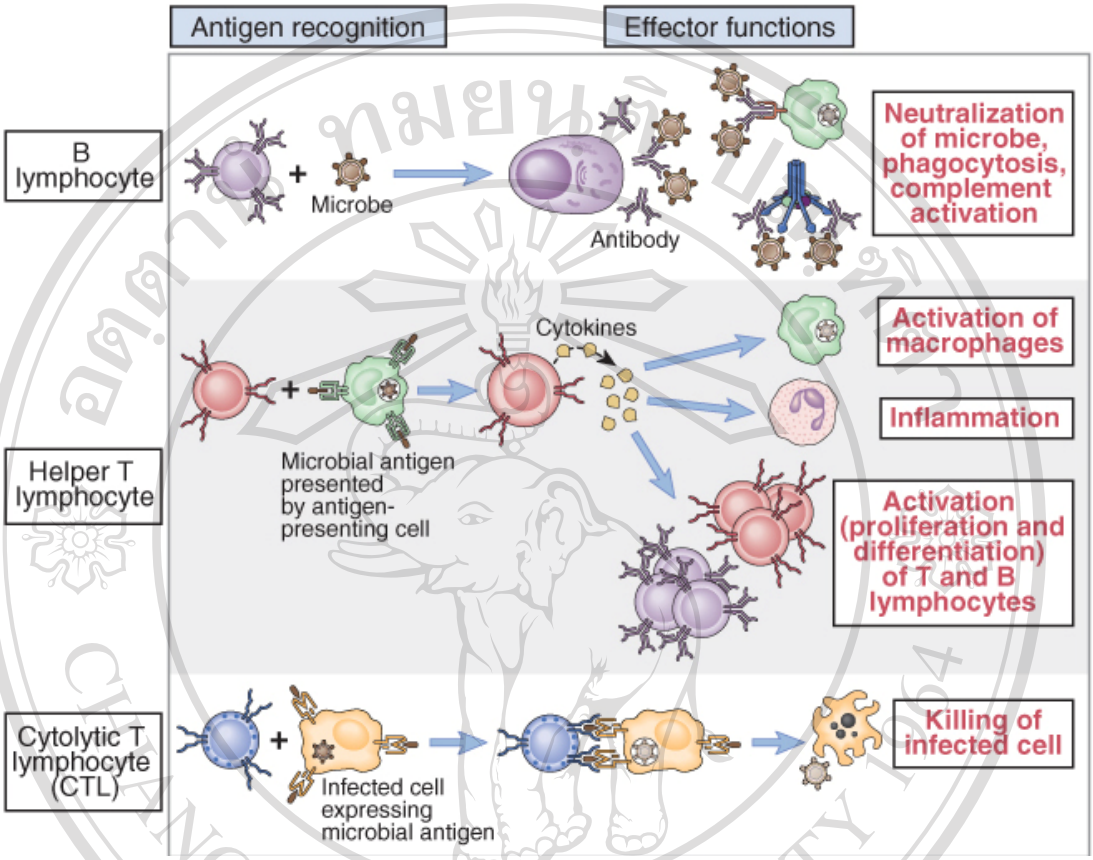

Figure 1.2 Types of adaptive immunity. In HMI, B lymphocytes secrete antibodies that block infections and eliminate extra-cellular microbes. In CMI, T lymphocytes eradicate intracellular microbes and altered cells (Abbas and Lichtman, 2005).

1.2.1.2.1 Humoral mediated immunity (HMI)

Humoral immunity is mediated by antibodies and which is responsible for defense against extracellular microbes and toxins. The antibodies can be produced by B lymphcytes and plasma cells. The effector functions of antibodies include neutralization of antigens, Fc receptor-dependent phagocytosis of opsonized particles, and activation of the complement system (Abbas and Lichtman, 2005).

1.2.1.2.2 Cell-mediated immunity (CMI)

Cell-mediated immunity (CMI) is the adaptive immune response against intracellular microbes. It is mediated by T lymphocytes. There are two main forms of cell-mediated immune reactions. In one, $CD4^+$ T helper lymphocytes recognize antigens of microbes that have been ingested by phagocytes or antigen presenting cells (APCs) and activate the phagocytes to kill the microbes. In the second type of CMI, $CD8^+$ cytotoxic T lymphocytes kill any nucleated cell that contains foreign antigens (microbial or tumor antigens) in the cytosol. Cell-mediated immune reactions consist of several steps: naive T lymphocyte recognition of antigens in peripheral lymphoid organs, clonal expansion and differentiation into effector cells, migration of the effector T lymphocytes to the site of infection or antigen challenge, and elimination of the microbe or antigen (Abbas and Lichtman, 2005).

1.2.2 Leukocyte surface molecules

Leukocytes express a large number of cell surface proteins. These molecules may be called leukocyte surface molecules. Many events regulating the development, activation, and effector functions of leukocytes are orchestrated by a complex series of leukocyte surface molecules. Some of these molecules are expressed on specific type of leukocytes, on lymphocyte subsets and are differentially expressed during lymphocyte development and activation (Barclay, 1997).

1.2.2.1 Nomenclature of leukocyte surface molecules: Cluster of Differentiation (CD) system

The leukocyte surface molecules recognized by monoclonal antibodies are called antigens, or markers. These markers can be divided into several groups such as a specific marker for a particular lineage or maturational pathway, and the state of activation or differentiation of the cells. Considerable confusion was created because these surface markers were initially named according to the antibodies that reacted with them. To resolve this, a uniform nomenclature system was adopted, initially for human leukocytes. The cluster of differentiation (CD) nomenclature was developed by the Human leukocyte differentiation antigen (HLDA) workshops. Thus, all reported leukocyte surface molecules were classified into CD designation (e.g., CD1, CD2).

The classification of leukocytes and lymphocytes by CD antigen expression is now widely used in clinical medicine and experimental immunology. These surface proteins are not merely phenotypic markers but are themselves involved in a variety of lymphocyte responses. The two most frequent functions attributed to various CD molecules are (1) promotion of cell-cell interactions and adhesion, and (2) transduction of signals that lead to lymphocyte activation. The production of monoclonal antibodies (mAbs) capable of identifying single antigens has led to the discovery of a large number of leukocyte cell surface molecules with novel activities. Human leukocyte differentiation antigens (HLDA) Workshops have proven instrumental in the identification and characterization of a large number of molecules that populate the surface of hematopoietic cells (Mason *et al*., 2001; Zola *et al*., 2003; Zola and Swart, 2003; Abbas and Lichtman, 2005).

1.2.2.2 General structure of cell surface proteins

Cell surface proteins can be classified as peripheral or integral membrane proteins. In brief, peripheral proteins are those that appear to be only weakly bound to their respective membranes and do not appear to interact with the membrane lipids, whereas the integral proteins are ordinarily more strongly bound to the membrane and exhibit functionally important interactions with the membrane lipids. It is presumed that there is a structural basis for this classification; that peripheral and integral proteins are attached to the membrane in distinctly different ways (Lodish, 2003).

(1) Peripheral membrane proteins

Peripheral membrane proteins, or extrinsic proteins, do not interact with the hydrophobic core of the phospholipid bilayer. Instead they are usually bound to the membrane indirectly by interactions with integral membrane proteins or directly by interactions with lipid polar head groups. Peripheral proteins localized to the cytosolic face of the plasma membrane include the cytoskeletal proteins spectrin and actin in erythrocytes and the enzyme protein kinase C. This enzyme shuttles between the cytosol and the cytosolic face of the plasma membrane and plays a role in signal transduction. Other peripheral proteins, including certain proteins of the extracellular matrix, are localized to the outer (exoplasmic) surface of the plasma membrane (Lodish, 2003).

(2) Integral membrane proteins

Integral membrane proteins, also called intrinsic proteins, have one or more segments that are embedded in the phospholipid bilayer. Most integral proteins contain residues with hydrophobic side chains that interact with fatty acyl groups of the membrane phospholipids, thus anchoring the protein to the membrane. Most integral proteins span the entire phospholipid bilayer. These transmembrane proteins contain one or more membrane-spanning domains as well as domains, from four to several hundred residues long, extending into the aqueous medium on each side of the bilayer. In all the transmembrane proteins examined to date, the membrane-spanning domains are α helices or multiple β strands. In contrast, some integral proteins are anchored to one of the membrane leaflets by covalently bound fatty acids. In these

proteins, the bound fatty acid is embedded in the membrane, but the polypeptide chain does not enter the phospholipid bilayer (Lodish, 2003).

1.2.2.3 Biological functions of leukocyte surface molecules

(1) Transport of small molecules

Membrane transport protein, which make up between 15 and 30% of the membrane proteins in all cells. The two main classes of membrane proteins that mediate the transfer: carrier proteins, which have moving parts to shift specific membrane across the membrane, and channel proteins, which form a narrow hydrophilic pore, allowing the passive movement primarily of small inorganic ions (Alberts, 2002).

A dynamic change in inorganic ion concentration inside the cells may play important role for leukocyte activation. In particular, ion channels and fluxes have long been suspected to play a role in lymphocyte signal transduction (Figure 1.3). Unlike nerve and muscle cells, lymphocytes lack electrical excitability, and yet patch-clamp studies have revealed a surprisingly complex biophysical phenotype, with multiple K^+, Ca^{2+}, and Cl^- channels expressed in patterns that are regulated differentially according to cell subset and state of activation. By controlling ion fluxes across the plasma membrane channels mediate changes in intracellular ion concentrations and membrane potential in response to a variety of stimuli (Lewis and Cahalan, 1990; Lewis *et al*., 1993; Lewis and Cahalan, 1995).

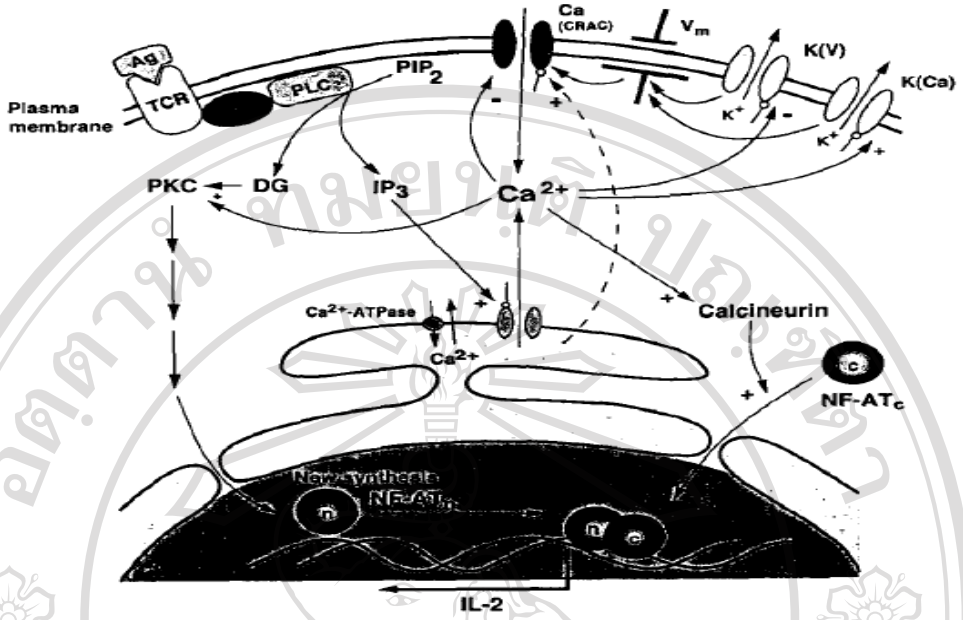

Figure 1.3 An early events in T lymphocyte activation. Selected events from antigen binding to change in an ion channel activity to IL-2 gene expression are shown. Arrow labeled with + or - indicate stimulatory or inhibitory interaction, respectively (Lewis and Cahalan, 1990; Lewis and Cahalan, 1995).

(2) Enzymatic activities

Several leukocyte surface molecules are enzyme. Some of them have enzymatically active site inside cell. These enzymes have long intracellular domain and containing kinase or phosphatase enzymatic activity such as CD45, CD115, CD117 and CD148. By contrast, ectoenzymes are membrane proteins that have their enzymatically active site outside the plasma membrane, in the extracellular environment. So, many cell surface enzymes with well-established roles in cell migration do not belong to this group of enzymes, including receptor-type protein tyrosine kinases and phosphatases (which have their catalytic domains inside cells) and many matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator.

Ectoenzymes can be classified according to their enzymatic activities that are shown in Table 1.1. Many of them are peptidases, proteases, hydrolases and nucleotidases (which hydrolyse extracellular nucleotides, NAD and NADP) or oxidases. Many ectoenzymes are type II integral membrane proteins with a short amino terminus in the cytosol or are glycosylphosphatidylinositol-linked molecules. Many ectoenzymes (such as CD26, CD38, CD73, autotaxin and vascular adhesion protein 1) are also found as soluble forms in biological fluids (Salmi and Jalkanen, 2001; Morabito *et al*., 2006).

Ectoenzymes represent almost 3-4% of the surface molecules on human leukocytes (Goding and Howard, 1998; Deaglio and Malavasi, 2006), and many ectoenzymes are also expressed on the endothelium. There are several examples in

which an ectoenzyme has a well-established role in the migration of tumour cells, fibroblasts or epithelial cells, yet its role in immune-cell trafficking remains unexplored. The potential of ectoenzyme-deficient animals that have been generated for other purposes should be fully exploited in research on leukocyte trafficking (Figure 1.4).

Table 1.1 Ectoenzymes: nomenclature and catalytic reactions (Chiorazzi *et al*., 2005; Morabito *et al*., 2006)

CD name	Catalytic activity	Name
CD39	ATP diphosphohydrolase	Nucleotidases and related enzymes
CD73	5′-Nucleotidase	
Autotaxin (CD203)	Lysophospholipase Nucleotide pyrophosphatase Nucleotide phosphodiesterase	
CD38	ADP-ribosyl cyclase NAD(P) hydrolase cADPR hydrolase Base-exchange catalyst	ADP-ribosyl cyclases and ADP-ribosyltransferases
CD157	ADP-ribosyl cyclase NAD(P) hydrolase cADPR hydrolase Base-exchange catalyst	
CD10	Neutral endopeptidase	Peptidases and proteases
CD13	Aminopeptidase	
CD26	Dipeptidyl peptidase	
CD156b	Metalloproteinase	
VAP1	Amine oxidase	Oxidases
NADPH oxidase	Oxidase	

ADPR, ADP-ribose; ADPR(P), ADPR or ADPR phosphate; ART2, ADP-ribosyltransferase 2; cADPR, cyclic ADP-ribose; cADR(P), cADPR or cADPR phosphate; cAMP, cyclic AMP; MT1-MMP, membrane-type-1 matrix metalloproteinase; NAAD, nicotinic-acid-adenine dinucleotide; NAAD(P), NAAD or NAAD phosphate; NAD(P), NAD or NADP; NH3, ammonia; VAP1, vascular adhesion protein 1.

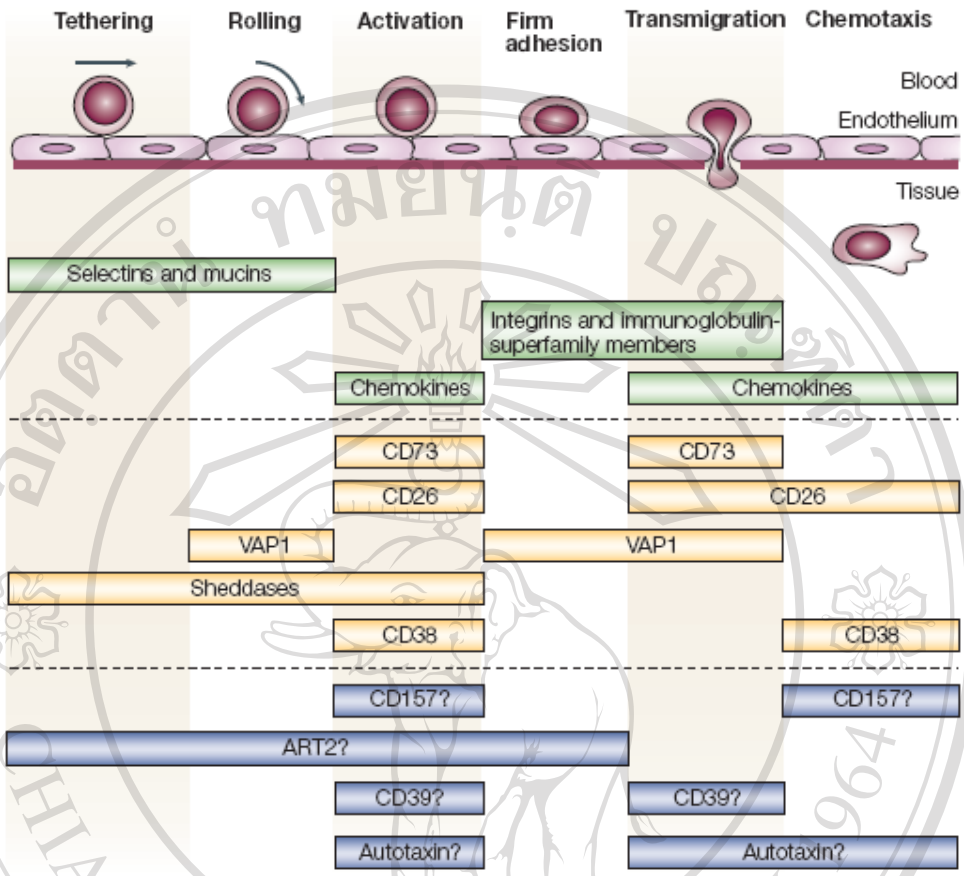

Figure 1.4 Ectoenzymes and the leukocyte-extravasation cascade. The different phases of the multistep adhesion cascade that supports leukocyte exit from the blood into the tissues, and the cell-adhesion and activation molecules that contribute to this cascade, are shown in the top panel. The centre panel shows the main steps at which the best characterized ectoenzymes in the cascade are involved. The bottom panel shows other ectoenzymes that might be involved in the cascade, as determined on the basis of substrate specificity and/or in vitro adhesion data, but the *in vivo* relevance and/or exact stage at which these ectoenzymes operate remains to be verified (Salmi and Jalkanen, 2005).

(3) Signal transduction

The challenge that faces all cells that respond to external stimuli is how the recognition of a stimulus, usually by receptors on the outer cell surface, is able to effect changes within the cell. Extracellular signals are transmitted across the plasma membrane by receptor proteins. The receptor proteins are instrumental in converting extracellular ligand binding into an intracellular biochemical event. Conversion of a signal from one form into another is known as signal transduction. The signal is converted into different biochemical forms, distributed to different sites in the cell, and sustained and amplified as it proceeds toward its various destinations. One result of intracellular signaling may be changes in the cytoskeleton and secretory apparatus. This is seen in the activation of effector T lymphocytes. Finally, the intracellular signaling can be activated the transcription factors turns on new gene expression and cell division (Alberts, 2002).

For example, Tyrosine phosphorylation is a key mechanism for signal transduction and the regulation of a broad set of physiological processes. Processes that are regulated include the following: decisions to proliferate, differentiate or die; activation of large gene-transcription programmes; cell motility and morphology; and transportation of molecules into or out of cells.

All cells of the immune system have high levels of tyrosine phosphorylation and express more genes encoding protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) than any other cell type, with the possible exception of neurons. Acute changes in tyrosine phosphorylation regulate antigen-receptor

mediated lymphocyte activation, cytokine-induced differentiation and responses to many other stimuli. Both PTKs and PTPs can have activating and inhibitory effects, and the actions of both types of enzyme are required for a physiological immune response (Abbas and Lichtman, 2005).

(4) Interactions with cells or extracellular matrix (adhesion molecules)

In general, cell differentiation, tissue formation, and coordinated dynamic interactions of cells in various processes (such as the immune response) requires direct contact between cell surfaces and between cells and the extracellular matrix. The cell adhesion molecules are required for cell contact. The first biochemical studies of cell adhesion molecules occurred more than two decades ago. There are several kinds of cell adhesion molecules, including selectins, integrins, immunoglobulin (Ig) superfamilies and cadherins.

In immune system, every steps of immune response require adhesion molecules. Both leukocyte transmigration through blood vessel and cell adhesion (or contact) all require these molecules.

Leukocyte could be getting to inflammatory site by using the adhesion molecules in three steps of rolling, activation and firm adhesion. Several kinds of adhesion molecules are required in different steps of leukocyte transmigration such as selectin and integrin (Figure 1.5). Selectin family consist of L-selectin, P-selectin and E-selectin (Springer, 1994). The interactions of selectins with their ligands enable

leukocytes to adhere to inflamed endothelium under conditions of blood flow. Integrins also participate in rolling and mediate firm leukocyte adhesion. In particular, integrins are expressed by leukocytes, such as the LFA1 (lymphocyte function-associated antigen 1) and VLA4 (very late antigen 4) and αα4 β 7-integrin. There are important for proper immune-cell function, through binding ICAM1 (intercellular adhesion molecule1) and ICAM2, and VCAM1 (vascular cell-adhesion molecule 1) and MADCAM1 (mucosal vascular addressin cell-adhesion molecule 1). For example, LFA1-ICAM interactions mediate lymphocyte trafficking to peripheral lymph nodes and attachment to APCs, whereas αα4 β 7-integrin-MADCAM1 and VLA4-VCAM1 interactions have central roles in lymphocyte migration to mucosal lymphoid organs and inflamed tissues, respectively. Leukocyte activation and arrest leukocyte are mediated by chemokines or other chemoattractants and the binding of leukocyte integrins to immunoglobulin superfamily members, such as ICAM1 and VCAM1 that expressed by endothelial cells. Leukocyte transmigration through venular walls is the final step in the process of leukocyte emigration into inflamed tissues. Immunoglobulin superfamily members including PECAM1, ICAM1, ICAM2, JAM and endothelial cell-selective adhesion molecule (ESAM), as well as the non-immunoglobulin molecule CD99 are mediated leukocytes transmigration (Ostermann *et al*., 2002; Muller, 2003; Liu *et al*., 2004; Ley *et al*., 2007).

In addition, integrins have essential roles in various processes, including leukocyte attachment to antigen-presenting cells (APCs) and cytotoxic killing. For example, LFA1–ICAM interactions mediate lymphocyte trafficking to peripheral lymph nodes and attachment to APCs. The adhesive interactions of immune cells are usually dynamic and transient. A high degree of flexibility in cell adhesion and mobility is required for a wide range of immune functions, including immune surveillance and antigen recognition.

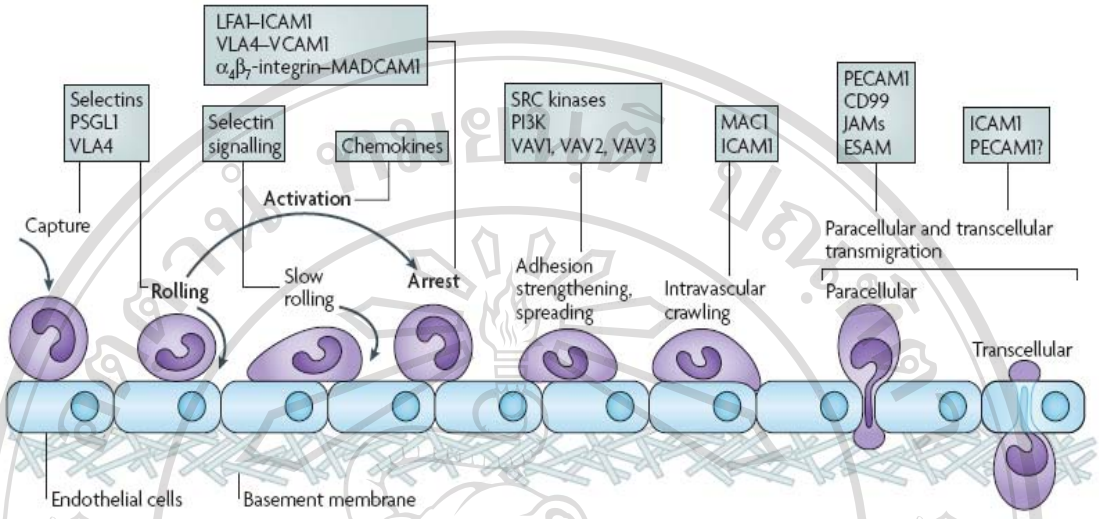

Figure 1.5 The updated leukocyte adhesion cascade. The original three steps are shown in bold: rolling, which is mediated by selectins, activation, which is mediated by chemokines, and arrest, which is mediated by integrins. Progress has been made in defining additional steps: capture (or tethering), slow rolling, adhesion strengthening and spreading, intravascular crawling, and paracellular and transcellular transmigration. Key molecules involved in each step are indicated in boxes. ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1; MAC1, macrophage antigen 1; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide 3-kinase; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (Ley *et al.*, 2007).

Lymphocytes adhere to other cells and extracellular matrix in the process of immunological recognition and lymphocyte recirculation. The interaction of lymphocytes with other cell types is critical for immune function. The most striking characteristic of lymphocyte adhesion is its regulation. Lymphocytes rapidly interconvert between a non-adherent state in circulation and an adherent and highly motile state in lymphoid and other tissues. This cycle is repeated many times over the life span of a lymphocyte. Once, T lymphocytes recognized the antigen via APCs presentation. The interaction between T lymphocyte and APCs are stabilized by an adhesive mechanism in order to generate sustained TCR signaling and T lymphocyte activation. Initial observations of T lymphocytes interacting with B lymphocytes showed that T lymphocytes physically attach to an antigen-presenting B lymphocyte and polarize towards the contact plane, thereby recruiting the adhesion receptors leukocyte function-associated molecule 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) to the contact site. The adhesion molecules may be requiring for the immunological synapse formation and controlling biological function of effector T lymphocytes (Chothia and Jones, 1997; Friedl and Gunzer, 2001; Lanzavecchia and Sallusto, 2001).

1.2.3 Lymphocytes

Lymphocytes are immune cell that can be recognized and distinguished different antigens. Lymphocytes are responsible for the two defining characteristics of the adaptive immune response, specificity and memory. Lymphocytes play important role in immune system, both CMI and HMI are mediated by lymphocytes.

These cells type consist of distinct subsets that are different in their function and protein products. Three types of lymphocyte subsets are define B lymphocytes, T lymphocytes and NK cells. B lymphocytes are the antibodies producing cells. T lymphocytes are cells that mediate cellular immunity. NK (natural killer) cells are a third population of lymphocytes whose major function is in innate immunity. Both B and T lymphocytes have clonally distributed antigen receptors. Therefore, there are many clones of these cells. Each clones of cell are specific for different antigen and express specificity receptor of the clone (Abbas and Lichtman, 2006; Male, 2006).

1.2.3.1 T lymphocytes

T lymphocyte precursors originate in bone marrow, and then migrate to thymus. Thymus is the site of T lymphocyte production and maturation. There consist of two subsets, helper T lymphocytes ($CD4^+$ T lymphocytes) and cytotoxic T lymphocytes ($CD8^+$ T lymphocytes). $CD4^+$ T lymphocytes produce cytokines that facilitate antibody production, antiviral responses, defense against large extracellular parasites and several infections. $CD8^+$ cytotoxic T lymphocytes are responsible for killing of virus-infected cells and tumor cells. Both T lymphocyte subsets play important role in adaptive immune response.

Base on leukocyte surface markers, T lymphocytes are distinguish to two subsets, $CD4^+$ helper T lymphocytes and $CD8^+$ cytotoxic T lymphocytes, that different effector functions. On the other hand, T helper lymphocytes have been indentified to two subsets that base on the pattern of cytokine production (Figure 1.6).

Type 1 (Th1) T helper lymphocytes and Type 2 (Th2) T helper lymphocytes are subset of T helper lymphocytes (Kopf *et al*., 1993). Th1 cells promote the cytotoxic effector functions of natural killer (NK) cells, $CD8^+$ T lymphocytes and macrophages. They also promote antibody-dependent cell-mediated cytotoxicity (ADCC) by supporting B lymphocyte production of IgG1a. By contrast, Th2 cells promote humoral immunity, mediated by B-cell-produced IgG4 and IgE. Two key cytokines that determine the direction of adaptive immune response are IL-12 and IL-4. Naive $CD4^+$ T lymphocytes can develop into Th1 cells responsible for cell-mediated immunity in response to interleukin 12 (IL-12). IL-12 was produced by cells of the innate immune system (Cooper *et al*., 1995; Sander *et al*., 1995). By contrast, IL-4 production induces naïve $CD4^+$ T helper lymphocytes differentiation towards Th2 pathway. Th1 cells secrete interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) and will activate macrophages to kill microbes located within the phagosome. Th1 cells also activate cytotoxic T lymphocytes to kill infected cells. In the other hand, Th1 may also stimulate B lymphocytes to secrete specific subclasses of IgG antibodies. By the way, Th2 cells secrete IL-4, 5, 10, and 13 and will mainly defend the animal against extracellular pathogens. Th2 cells can stimulate B lymphocytes to make most classes of antibodies, including IgE and some subclasses of IgG antibodies.

Thus, the decision of naïve helper T lymphocytes to differentiate into Th1 or Th2 effector cells influences the type of adaptive immune response that will be mounted against the pathogen whether it will be dominated by macrophage activation or by antibody production. The specific cytokines present during the process of helper T lymphocyte activation influence the type of effector cell produced (Abbas *et al*., 1996; O'Garra and Arai, 2000).

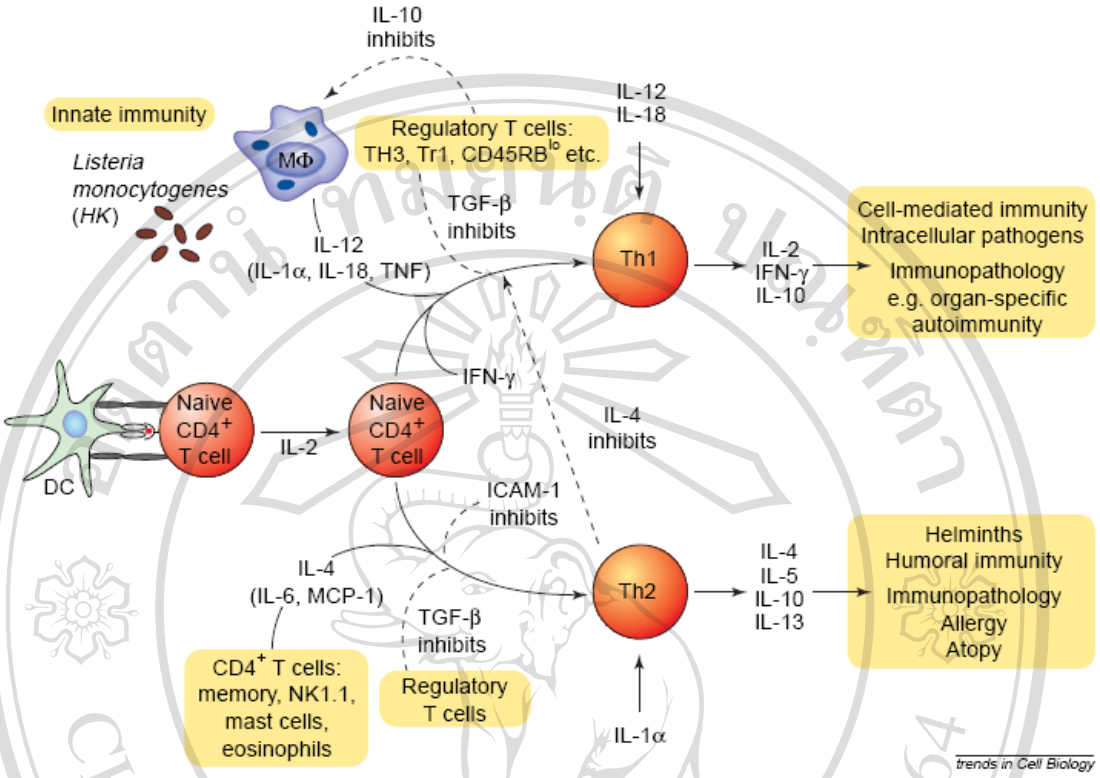

Figure 1.6 Regulation of T helper lymphocyte responses. Cytokines are major inducers of Th1 and Th2 subset development. Antigen-presenting cells (APCs), in part as a result of the cytokines that they produce, can induce the development of Th1 or Th2 cells. Th1 development is dependent on IFN-γ, and maintenance of phenotype depends on stimulation in the presence of IL-12 and IL-18. Th2 cells, dependent on IL-4 to differentiation, have been implicated in allergic and atopic manifestations. In addition, IL-4 and IL-10 have been suggested to play a role in tolerance. In the past year, it has become clear that distinct subsets of regulatory T lymphocytes are responsible for regulating both Th1 and Th2 responses and preventing the development of immune pathologies (O'Garra and Arai, 2000).

On the other hand, the cytotoxic T lymphocytes (CTLs), T lymphocytes that express CD8 on their cell surface are selected in the thymus to recognize and respond to foreign peptides that are presented in the groove of MHC class I molecules. $CD8^+$ T lymphocytes capable in response to virus-infected cells and kill any cell that expresses their target antigen via MHC class I. Thus, these cells require antigen presentation from antigen presenting cells. In some ways, our understanding of $CD4^+$ T lymphocytes help for a efficient $CD8^+$ T lymphocyte response, including clonal expansion, development of effector function and the generation of long-term memory. It means that $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes have been cooperate in immune response (Lohoff and Mak, 2005).

1.2.3.2 B lymphocytes

B lymphocytes, the cells that produce antibodies, were so called because in birds they were found to mature in an organ called the bursa of Fabricius. In mammals, no anatomic equivalent of the bursa exists, and the early stages of B lymphocyte maturation occur in the bone marrow. Thus, B lymphocytes refer to bursa-derived lymphocytes or bone marrow-derived lymphocytes. B lymphocytes differentiate into cells that actively synthesize and secrete antibodies. Some of these antibody-producing cells are identifiable as plasma cells. Memory cells express surface proteins that distinguish them from naive and recently activated effector lymphocytes. Memory B lymphocytes express certain classes (isotypes) of membrane

Ig, such as IgG, IgE, or IgA, as a result of isotype switching, whereas naive B cells express only IgM and IgD.

A subset of B lymphocytes, called B-1 cells, differs from the majority of B lymphocytes and has unique features of maturation and Ig gene expression. Many B-1 cells express the CD5 (Ly-1) molecule. B-1 cells develop earlier during ontogeny than do conventional B lymphocytes, and they express a relatively limited repertoire of V genes with less junctional diversity than conventional B lymphocytes have. Only 5% to 10% of B lymphocytes in the blood and lymphoid tissues belong to the B-1 subset (Abbas and Lichtman, 2005).

1.2.3.3 Natural killer cells (NK cells)

NK cells are specialized a subset of lymphocytes that provide a first line of defense through their ability to eliminate pathogen-infected cells and tumors. Unlike T lymphocytes, NK cells cannot mount a recall or memory response when re-exposed to the same antigenic challenge.

By surface phenotype and lineage, NK cells are neither T nor B lymphocytes, and they do not express somatically rearranged, clonally distributed antigen receptors like immunoglobulin or T cell receptors. NK cells are heterogeneous in their cell-surface phenotype, proliferative capacity and function. Mature NK cells can also be heterogeneous, and much remains to be learned about the specific subsets that are present in locations such as the liver, periphery and lymph nodes. In humans, CD56hi

NK cells, which produce large amounts of cytokines, comprise ~10% of NK cells, whereas CD56low NK cells, which express high levels of the low-affinity Fc receptor for IgG (Fc γ RIII or CD16) and are powerfully cytotoxic, comprise almost 90% of the NK cell population (Cooper *et al*., 2001; Kim *et al*., 2002).

The function of NK cells is tightly regulated by a fine balance of inhibitory and activating signals that are delivered through a diverse array of cell-surface receptors belonging to the immunoglobulin-like receptor and C-type-lectin receptor families, as well as mediated by pro-inflammatory cytokines (Moretta *et al*., 2001; Raulet *et al*., 2001).

NK cells kill infected cells and cells that have lost expression of class I MHC molecules. Those cells secrete a cytokines, mainly IFN-γ that mediate NK cells activation. NK cells play important role in defense against intracellular microbe, which infected by virus or some other intracellular microbes. They kill virally infected cells before antigen-specific CTLs can become fully active, that is, during the first few days after viral infection. Early in a viral infection, NK cells are expanded and activated by cytokines of innate immunity, such as IL-12 and IL-15, and they kill infected cells, especially those that display reduced levels of class I molecules. In addition, the IFN-γ secreted by NK cells activates macrophages to destroy phagocytosed microbes. Because NK cells can kill certain tumor cells, it has also been proposed that NK cells serve to kill malignant clones in vivo. However, tumor-associated inflammatory infiltrates typically do not contain large numbers of NK cells (Abbas and Lichtman, 2005; Degli-Esposti and Smyth, 2005).

1.2.4 Monoclonal antibody

The antibodies are produced and secreted by B lymphocyte. Many clones of B lymphocytes can be produced different antibodies that respond to different epitopes of an antigen, so called polyclonal antibodies. A monoclonal tumor of antibody-producing plasma cells was discovered in multiple myeloma patients at first time. The monoclonal antibody was produced large amounts in blood and urine of patients. Moreover these antibodies can be purified to homogeneity and analzed. In 1975, the hybridoma technique was developed by Kohler and Milstein (Kohler and Milstein, 1975; Abbas and Lichtman, 2005) This technique is based on the fact that each B lymphocyte produces a single specific antibody. The hybridoma technique was developed for immortalizing individual antibody-secreting cells from an immunized animal by producing hybridomas, each of which secreted individual monoclonal antibodies of predetermined specificity.

1.2.4.1 Production of monoclonal antibody

The hybridoma technique was developed for perform the immortal B lymphocyte. This technique requires a myeloma cell line that grows in culture medium but die in selective medium. So the myeloma cell line can be made defective in hypoxanthine-guanine phosphoribosyltransferase (HGPRT) by mutagenesis. HGPRT is an enzyme of a salvage pathway. Thus, myeloma cells that lacks of HGPRT are unable to use the salvage pathway for purine biosynthesis while the myeloma cells can use the *De novo* pathway to alive (Figure 1.7) (Goding, 1986).

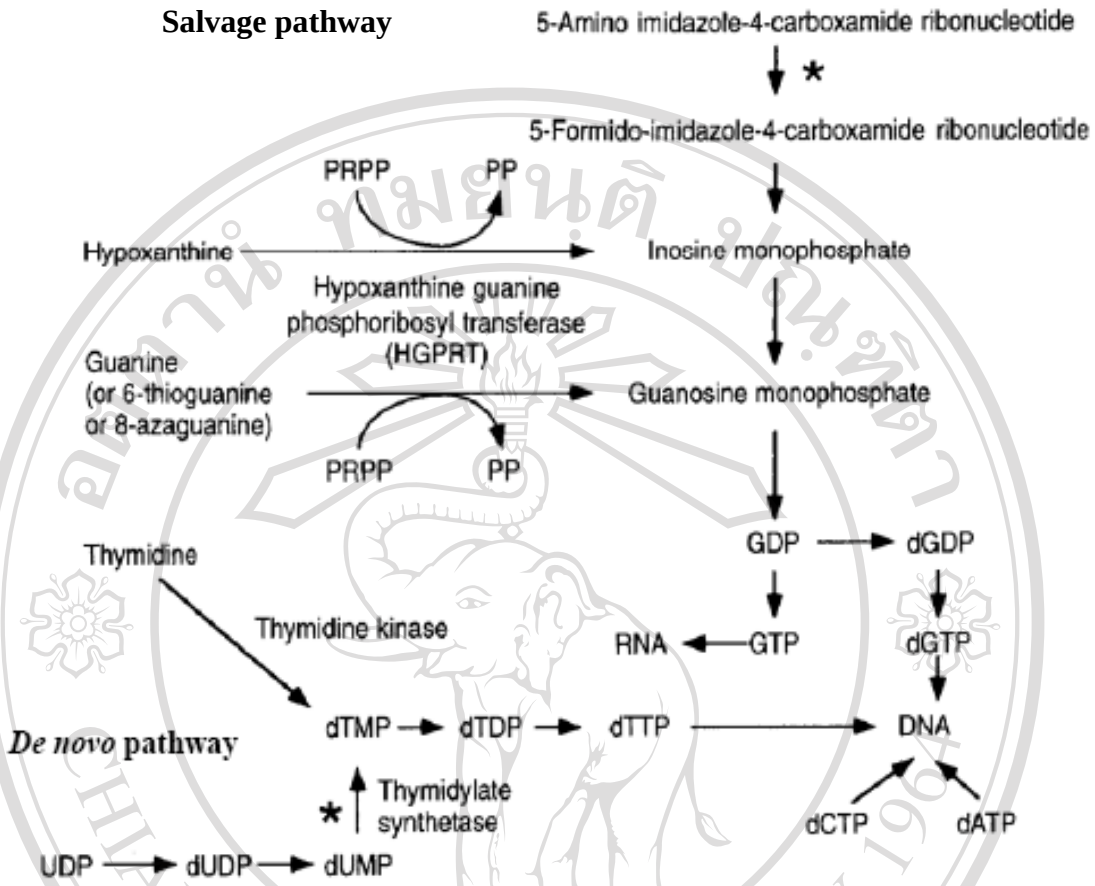

Figure 1.7 Metabolic pathways of DNA synthesis. When the *de novo* pathway are blocked with folic acid analogue (*), such as aminopterin, cell must depend on the salvage pathway (Goding, 1986).

When, splenic B lymphocytes were fused with the myeloma cells by using polyethylene glycol. The fused cells were culture in a selective medium that containing hypoxanthine, aminopterin and thymidine (HAT medium). Aminopterine is a folic antagonist that blocks the de novo pathway (Figure 1.8). Therefore, unfused myeloma cells and fused cells that have negative HGPRT will die (Harlow and Lane, 1988). Hybridoma cells can survive in the selective medium because the salvage pathway of splenic B lymphocyte partner can be function.

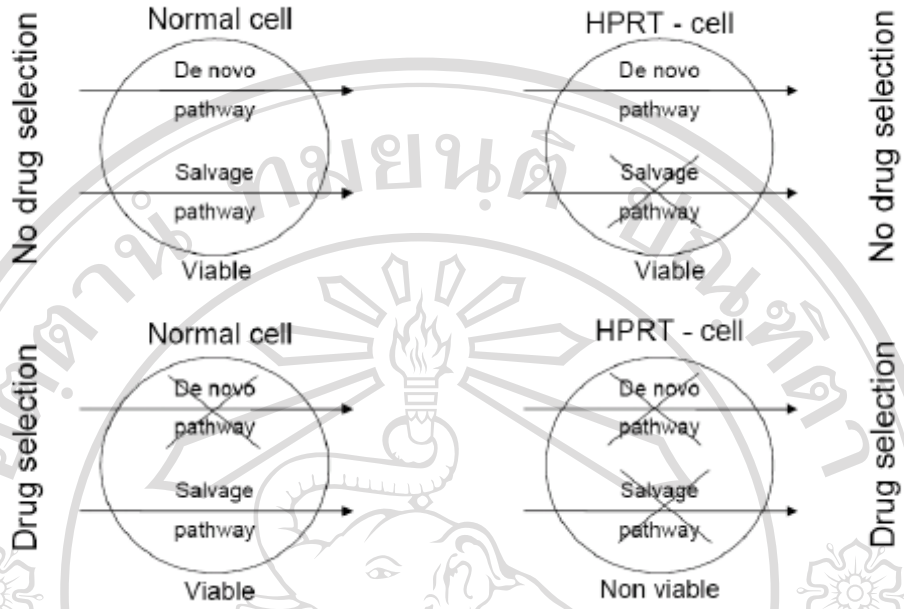

Figure 1.8 Pathway of nucleotide synthesis in the present of antifolate drug (Harlow and Lane, 1988).

To produce a monoclonal antibody as showed in Figure 1.9, a mouse is immunized with an antigen. After the titer of antibodies in mouse serum is highly significant. The B lymphocytes were isolated from spleen of immunized mouse. Then, these B lymphocytes were fused with myeloma cells. Cell fusion is achieved with polyethylene glycol. Hybrids are grown in HAT medium. Under this condition unfused HGPRT- myeloma will die and B lymphocytes will die after cultivation for 1-2 weeks. Therefore only hybrids are remained and grow. Hybridomas are assay for the production of the desired antibody. Several methods were used, for examples enzyme-linked immunosorbent assay (ELISA) and flow cytometry. The hybridoma clones that produce a desire antibody can be expanded or induced ascitic fluids in a mouse to produce large scale of monoclonal antibodies (Abbas and Lichtman, 2005).

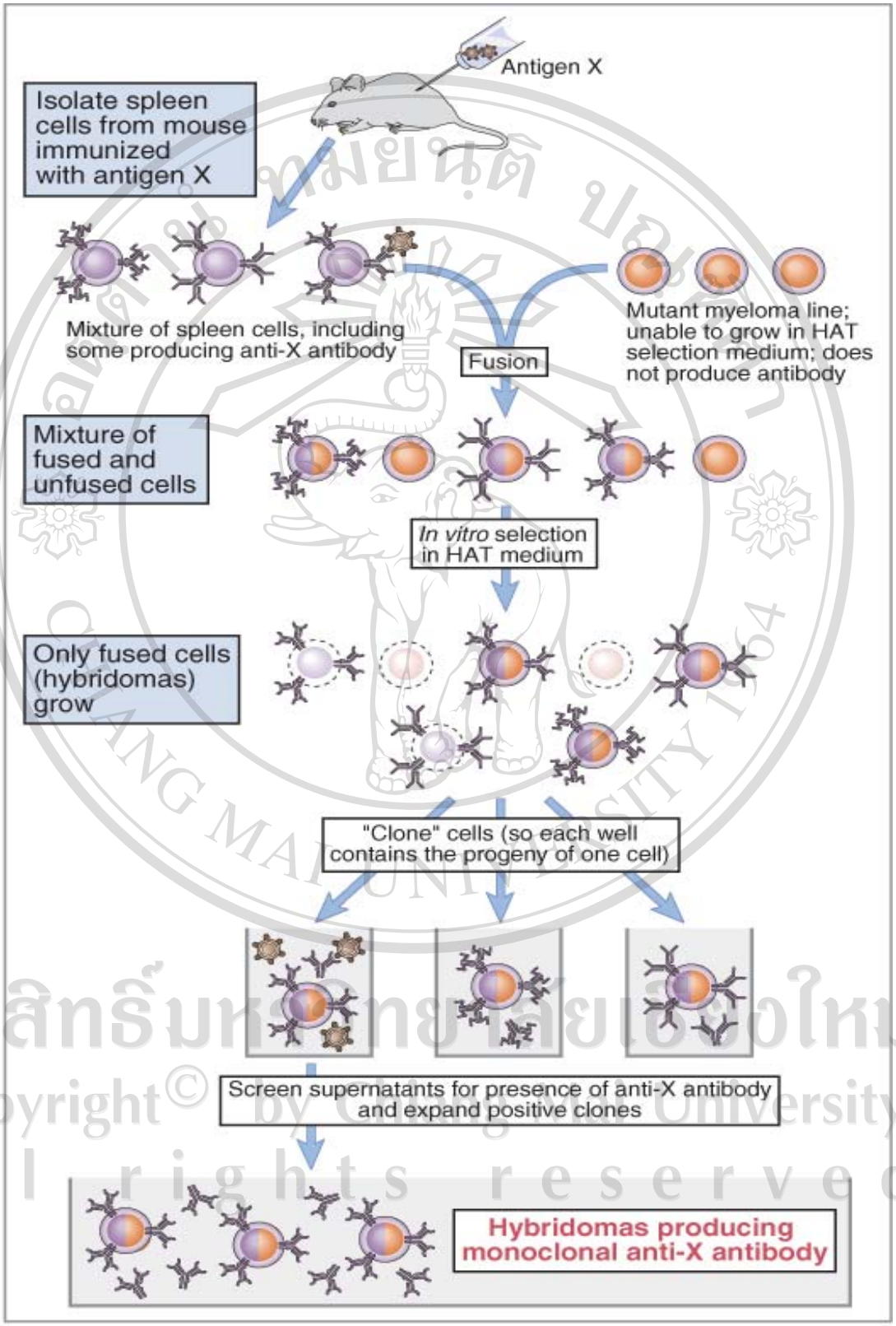

Figure 1.9 Monoclonal antibody production (Abbas and Lichtman, 2005)**.**

1.2.5 Leukocyte surface molecules in medical applications.

The immune system is complexity network that consist of the cooperation between the leukocytes, organs and soluble mediators. This system protects us from the infection and tumor. If the immune system is ineffective function or imbalance of the immune respond, these events will be a disease. The leukocytes are centrally involved in the immune response. These cells can function under the communication between cells and environment. The communicated leukocytes are upon the interaction of leukocyte surface molecules (CD) and their ligands that transmitted the different signal into the cells. Therefore the leukocytes express various CD molecules that have provided targets for research, diagnosis and therapy.

Over a century ago, Paul Ehrlich predicted that antibodies could be used as magic bullets to target and treat human disease. Now, the realized with 18 mAbs are having FDA (Federal Drug Administration) approval for therapeutic use.

The analytical applications of antibodies lead to diagnostic assays, while the preparative applications have therapeutic counterparts. Finally, antibody against a CD molecule can be used to probe, simulate, or inhibit the function of the molecule, and this also suggests therapeutic applications (Zola, 2006).

Diagnostic applications

CD molecules were used as a tool of diagnosis disease for a long time. Now, there are rapidly developing as the diagnostic kits in several diseases. The best example of the diagnostic applications of the CD antibodies is a detection of $CD4^+$ T lymphocytes in HIV infection (AIDS) because the $CD4^+$ lymphocytes are attacked, and counts of $CD3^+$ or $CD4^+$ T lymphocytes are performed frequently to monitor disease, make treatment decisions, and monitor the effectiveness of therapy. In addition CD64 that expressed on neutrophils which is increased within hours by inflammation or tissue damage. These molecules are used for diagnosis and monitor of sepsis. Not limit in infectious diseases, there are widely using the CD molecules like biomarkers especially in tumors. Leukemic disease, an abnormal in proliferation, differentiation and function of leukocytes, requires a specific detection and identification. CD45 and CD38 are useful identification type of this disease (Malavasi *et al*., 1994; Chiorazzi *et al*., 2005; Morabito *et al*., 2006).

Therapeutic applications

Antibodies have been used therapeutically for many years, starting (as far as we know) with the use of horse antisera against bacterial toxins by Emil von Behring and Shibasaburo Kitasato. In the field of CD molecules, an early success was the use of OKT3, a CD3 antibody, to reverse organ graft rejection. The number of antibodies undergoing clinical trial and late stage preclinical evaluation is even more impressive. A recent highly-publicized adverse event reminds us of the dangers, and there have been other unsuccessful trials, but the successes are impressive. About 200 antibodies are undergoing clinical evaluation, while an industry web site provides a list of many antibodies undergoing pre-clinical testing (PharmaProjects Database PJB Publications, available athttp://www.pjbpubs.com/ pharmaprojects/index.htm). The clinical applications of the monoclonal antibodies were shown in Table 1.2, 1.3 and 1.4.

Table 1.2 Clinical applications of monoclonal antibodies (Zola, 2006)

Antibody application	Clinical equivalent	Examples
Molecular identification and quantitation	Diagnostic pathology	Immunoassay of serum analytes Flow cytometric enumeration of functional T lymphocyte subtypes Immunohistochemistry
Binding and removal of molecules or cells	Antibody therapeutics	Antitoxins Anti-TNF to dampen inflammation Anti- CD20 to treat lymphoma Anti-microbials
Agonistic and antagonistic function of antibodies	Antibody therapeutics	Immunostimulatory antibodies in cancer therapy

Table 1.3 Monoclonal antibodies against CD molecules in clinical use for therapy of cancer (Zola, 2006)**.**

Specificity	Target diseases	Antibody	Antibody type
CD20	Lymphomab	Rituximab Zevalin Bexxar	Humanized Radioconjugate Radioconjugate
CD19	Lymphoma	B4	Ricin immunotoxin
CD22	Lymphoma	LymphoCide	Radioconjugate
CD52	Lymphoma, leukemia	Campath 1H	Humanized
CD25	Human T-lymphotropic virus type I (HTLV-I)-induced malignancy	Zenepax Simulect	Humanized Chimeric
CD33	Acute myeloid leukemia	Mylotarg	Calicheamicin conjugate
CD15	Acute myeloid leukemia	PM-81	Murine IgM
HER2 (CD340)	Breast cancer	Herceptin Pertuzumab	Humanized Humanized
MUC-1 (CD227)	Ductal breast tumors	BrE-3	Humanized
Transferrin receptor (CD71)	Various malignancies	42/6	Murine IgA
Carcinoembryonic antigen (CD66e)	Colorectal cancer, other epithelial tumors including lung, breast	Anti-CEA	Chimeric

Table 1.4 Monoclonal antibodies against CD molecules in clinical use or undergoing trial for therapy of immune system disorders (Zola, 2006)**.**

Specificity	Target diseases	Antibody	Antibody type
CD3	Transplant rejection	Muromonab	Murine antibody
CD4	Psoriasis	Imuclone	Humanized
CD11a	Psoriasis	Efalizumab	Humanized
CD20	Rheumatoid arthritis	Rituximab	Humanized
CD25	Transplant rejection	Basiliximab, Daclizumab	Chimeric Humanized
CD52	Inflammation	Campath-1	Humanized
CD154	Autoimmune disease	IDEC-131	Humanized

Research fields

CD molecules are widely study in this time. To understand how the immune system and leukocytes work. Many monoclonal antibodies were produced which as the magic instruments for studies immune system through the CD molecules (Zola, 2006).

1.3 Objectives

The main objective of this study is to investigate the biochemical characterization and biological function of the novel leukocyte surface molecules by using two monoclonal antibodies, MT3 and COSA2A.

The detailed objectives are as follows:

1.3.1 To study cellular distribution of molecule recognized by mAbs MT3 and COSA2A on various cell types.

1.3.2 To study on the expression of molecule recognized by mAbs MT3 and COSA2A mAb on sub-population of lymphocyte.

1.3.3 To investigate biochemical characterization and biological function of molecule recognized by mAbs MT3 and COSA2A.