IDENTIFICATION AND FUNCTION OF LEUKOCYTE SURFACE MOLECULE RECOGNIZED BY A NEWLY GENERATED MONOCLONAL ANTIBODY COS3A

Siriwan Wansook

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry

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คุณลักษณะและหน้าที่ของโมเลกุลบนผิวเซลล์เม็ดเลือดขาวที่จดจำได้โดยโมโนโคลนอลแอนติบอดี COS3A ที่ถูกสร้างขึ้นใหม่

นางสาวศิริวรรณ วันศุกร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ในการศึกษานี้โมโนโคลนอลแอนติบอดีชื่อ COS3A ถูกคัดเลือกมาหาคุณลักษณะและหน้าที่ของโมเลกุลที่จดจำได้โดยโมโนโคลนอลแอนติบอดีนี้ จากการวิเคราะห์โดยการย้อมเซลล์แบบอิมมูโนฟลูออเรสเซนส์บ่งชี้ว่าโมโนโคลนอลแอนติบอดี COS3A จับอย่างจำเพาะเจาะจงกับโปรตีนที่มีการแสดงออกโดยเซลล์ COS7 ทั้งบนผิวเซลล์และภายในเซลล์ ที่น่าสนใจคือโมโนโคลนอลแอนติบอดี COS3A ยังจับอย่างจำเพาะเจาะจงกับโมเลกุลที่มีการแสดงออกบนผิวเซลล์เม็ดเลือดของมนุษย์และเซลล์ไลน์ในกลุ่มของเซลล์เม็ดเลือดของมนุษย์ แต่ไม่พบบนผิวเซลล์เม็ดเลือดแดง จากการตกตะกอนโปรตีนโดยแอนติบอดีและวิเคราะห์ด้วย Westerm blotting บ่งบอกว่าแอนติเจนนี้มีขนาด 30-70 กิโลดาลตัน ซึ่งขนาดจะลดเป็น 25 กิโลดาลตันเมื่อมีการนำเอาน้ำตาลที่เป็น N-linked glycan ออกโดยการย่อยด้วยเอนไซม์ N-glycosidase F หรือการใช้สาร tunicamycin ซึ่งจากผลการวิเคราะห์ต่อมาด้วยเทคนิค LC/MS และการตกตะกอนโปรตีนโดยแอนติบอดีแสดงให้ทราบว่าโมโนโคลนอลแอนติบอดี COS3A จับแบบจำเพาะกับโมเลกุล CD63 ของมนุษย์

การศึกษาหน้าที่ของโมโนโคลนอลแอนติบอดี COS3A ต่อกระบวนการฟาโกไซโทซิส แสดงให้เห็นว่าโมโนโคลนอลแอนติบอดี COS3A มีผลทำให้การกิน *Escherichea coli* (*E.coli*) โดยแกลนูโลไซต์ด้วยกระบวนการฟาโกไซโทซิสลดลงอย่างชัดเจน นอกจากนี้ผลของโมโนโคลนอลแอนติบอดี COS3A ต่อการแบ่งตัวของทีลิมโฟไซต์ได้มีการศึกษาและพบว่าโมโนโคลนอลแอนติบอดี COS3A ในรูปสารละลายสามารถยับยั้งการแบ่งตัวของทีลิมโฟไซต์ที่ถูกกระตุ้นผ่าน CD3 เมื่อใช้ peripheral blood mononuclear cells (PBMCs) เป็นต้นแบบ โดยพบว่าผลของการยับยั้งนี้สอดคล้องกับการลดการสร้างสาร IL-2 การแสดงออกของตัวรับ IL-2 (CD25) และ IFN-γ ที่ผลิตโดยทีลิมโฟไซต์ แต่มีการเพิ่มการสร้าง IL-10 ที่ผลิตโดยโมโนไซต์อย่างไรก็ตามผลดังกล่าวนี้ไม่เกิดขึ้นเมื่อใช้ทีลิมโฟไซต์เป็นเซลล์ต้นแบบ เป็นที่น่าสนใจว่าเมื่อมีการตรึงโมโนโคลนอลแอนติบอดี COS3A ร่วมกับโมโนโคลนอลแอนติบอดีต่อ CD3 โคลน OKT3 บนไมโครเวลเพลท สามารถกระตุ้นทีลิมโฟไซต์ได้มากขึ้น โดยเพิ่มการแบ่งตัวของเซลล์และการสร้างสาร IL-2, IL-10 และ IFN-γ เพิ่มมากขึ้น นอกจากนี้ในการวิเคราะห์วัฏจักรของเซลล์แสดงให้เห็นว่าการเหนี่ยวนำให้มีการแบ่งตัวของเซลล์เกิดผ่านการกระตุ้นวัฏจักรของเซลล์จากระยะ G1 ไปเป็นระยะ S

จากผลการศึกษาทั้งหมดสามารถสรุปได้ว่าโมโนโคลนอลแอนติบอดี COS3A จดจำโมเลกุล CD63 ของมนุษย์ซึ่งอาจจะมีบทบาทสำคัญในขั้นตอนเริ่มต้นของการกิน *E.coli* โดยกระบวนการฟาโกไซโทซิสและการควบคุมการตอบสนองของทีลิมโฟไซต์ ดังนั้นโมโนโคลนอลแอนติบอดี COS3A จึงเหมาะสำหรับใช้ได้ทั้งในการศึกษาคุณลักษณะทางชีวเคมีและหน้าที่ของ CD63 และอาจนำไปศึกษากลไกการทำงานของ CD63 ต่อกระบวนการฟาโกไซโทซิสและการกระตุ้นทีลิมโฟไซต์ต่อไป ซึ่งความรู้ใหม่ที่ได้นี้อาจนำไปสู่ความเข้าใจบทบาทของ CD63 ในระบบภูมิคุ้มกันมากยิ่งขึ้น รวมทั้งการนำไปสู่การใช้รักษาโรคได้

สาขาวิชาชีวเคมี ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา _____

SIRIWAN WANSOOK : IDENTIFICATION AND FUNCTION OF LEUKOCYTE SURFACE MOLECULE RECOGNIZED BY A NEWLY GENERATED MONOCLONAL ANTIBODY COS3A. THESIS ADVISOR : ASST. PROF. PANIDA KHUNKAEWLA, Ph.D. 158 PP.

IDENTIFICATION AND FUNCTION OF LEUKOCYTE SURFACE MOLECULE RECOGNIZED BY A NEWLY GENERATED MONOCLONAL ANTIBODY COS3A

In this study, monoclonal antibody named COS3A was selected for identification and functional analysis of its recognizing molecule. Cellular distribution analysis by immunofluorescence staining indicated that the mAb COS3A bound specifically to a protein expressed by COS7 cell both on surface and intracellularly. Interestingly, the mAb COS3A bound specifically to a molecule expressed on the surface of various human hematopoietic cells and cell lines but not on red blood cells. Immunoprecipitation and Western blotting indicated that the antigen had molecular weight of 30-70 kDa, which was reduced to 25 kDa by elimination of N-linked glycan using either N-glycosidase F or tunicamycin. LC-MS data and immunoprecipitation indicated that the mAb COS3A bound specifically to the human CD63 molecule. Functional study on phagocytosis indicated that the mAb COS3A dramatically diminished granulocyte phagocytosis of *Escherichea coli* (*E.coli*). Furthermore, effect of the mAb COS3A on T cell proliferation was studied and found that the soluble mAb COS3A inhibited CD3-mediated T cell proliferation while peripheral blood mononuclear cells (PBMCs) were used as model. The inhibitory effect was

corresponding to down regulation of IL-2, IL-2 receptor (CD25), and IFN-γ by T cells but, enhanced IL-10 production by monocytic cells. However, these effects were not observed when purified T cells were used as model. Fascinatingly, coimmobilization of the mAb COS3A and anti-CD3 mAb OKT3 strongly enhanced T cell proliferation, secretion of IL-2, IL-10, and IFN-γ by T cells. Furthermore, cell cycle analysis indicated that induction of cell proliferation was occurred through the activation of G1/S transition.

Taken together, the mAb COS3A recognized the human CD63 molecule, which may play a crucial role in the initial step of phagocytosis of *E.coli* and in regulation of T cell responses. Thus the mAb COS3A is suitable for both biochemical and functional studies of the CD63, and may be used for further study of the mechanism of the CD63 on phagocytosis and T cell activation. The obtained knowledges may lead to better understand of the CD63 function in regulation of the immune system and in therapeutic approaches.

School of Biochemistry Student's signature _____

Academic Year 2015 Advisor's signature _____

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of Differentiation
CMI	Cell-mediated immunity
CFSE	Carboxyfluorescein succinimidyl ester
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
HMI	Humoral mediated immunity
HRP	Horseradish peroxidase
ICS	Intracellular cytokine staining
IL	Interleukin
IFN-γ	Interferon-γ
Ig	Immunoglobulin

LIST OF ABBREVIATIONS (Continued)

IL-2	Interleukin-2
IL-10	Interleukin-10
kDa	Kilodalton
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MHC	Major Histocompatibility Complex
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PE	Phyco-erythrin
PerCP	Peridinin chlorophyll protein complex
PHA	Phytohemaglutinin
PI	Propidium iodine
RPMI-1640	Roswell Park Memorial Institute medium-1640
PRR	Pattern recognition receptor
PVDF	Polyvinylidene Fluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCR	T cell receptor
TEMs	Tetraspanin enriched microdomains
WT	Wild type

CHAPTER I

INTRODUCTION

1.1 Significance of research

The immune system of human body has evolved to protect individuals against universe pathogenic microorganisms and also plays important role in tumor protection. The immune system composes of several types of molecules, cells, tissues, and organs that work together to function correctly. This system requires the coordinated expression and action of hundreds of genes and proteins. Among these, the cell-surface proteins expressed by leukocytes are of particular importance due to their critical functions they play in the network of interactions that regulate the innate and adaptive immune response. These differentiation proteins have been identified and characterized by their binding of specific monoclonal antibodies (mAbs). These cell phenotype-determining proteins are assigned cluster of differentiation (CD) numbers, which has been approved by Human Cell Differentiation Molecule (HCDM) workshops (formerly Human Leukocyte Differentiation Antigens, or HLDA). The CD nomenclature are used ubiquitously in human medical researches, immunodiagnostics and treatments, and also CD profiles are intended to be used by immunologists, biologists, pathologists, hematologists, and clinicians generally. Recently, The HCDM workshops have recognized some 500 as leukocyte cell-surface molecules, over 360 of them with CD names. In addition, it has been estimated that about 5,500 human genes encode for membrane proteins, which correspond to

approximately 26 % of the known human protein-coding genes (Diaz-Ramos, Engel, and Bastos, 2011) Certainly, many of these genes may be expressed on the cell surface, suggesting that there is still a very large number of cell surface molecules remained undiscovered and analyzed. To date, 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 and the availability of new mAbs directed against novel cell surface molecule not only has broad implications for leukocyte biology, but also for the development of new diagnostic and therapeutic (Engel, 2011; Fagerberg, Jonasson, von Heijne *et al.*, 2010; Zola, Swart, Banham *et al.*, 2007).

In collaboration with Professor Dr. Watchara Kasinrerk, whose laboratory has mainly focused on production of mAbs, the generated mAbs are used for both basic researches and medical applications. Among these, the mAb named COS3A was selected for this study. The mAb COS3A was produced by a standard hybridoma technique using CD4 expressing COS7 cell as an immunogen for antibody induction in a Balb/c mouse (Pata, Tayapiwatana, and Kasinrerk, 2009). However, this mAb was not specific for CD4 but bound strongly on a molecule on surface of COS7 cell and some human hematopoietic cell lines. Primary functional studies revealed that the mAb COS3A strongly inhibited CD3-mediated T cell activation while PBMCs were used as study model, suggesting that the molecule recognized by this mAb might play an important role in regulation of T cell response.

In this study the mAb COS3A recognizing molecule was identified. Furthermore, cellular distribution, biochemical characterization and functional study of this molecule were investigated. The information obtained from this study may

lead to a better understanding of the involvement of this molecule in the immune system, and the knowledge gained from this study may be useful for future application in basic researches, medical diagnostics or therapeutics.

1.2 Literature review

1.2.1 Immune system

The immune system of human body has evolved to protect individuals against pathogenic microorganisms and plays a role in tumor protection. The immune system comprises a variety of immune cells, as well as molecules such as cytokines, antibodies, and complement factors. Cells of the immune system are born from hematopoietic stem cells found deep in the bone marrow. Hematopoietic stem cells are immortal, capable of generating daughter cells, called progenitors that will later give rise to different type of immune cells. There are two main types of progenitors created, the myeloid progenitors and the lymphoid progenitors (Figure 1.1).Whole process of immune system can be categorized into four main important stages including; recognize, the recognition of foreign (or non-self) agents; remove, the elimination of these agents by various effector functions (this is where phagocytosis fits in); regulate, the dampening of the response to protect the body from collateral damage; remember, the remembrance of the intruding agents to be able to stop them from infecting again. Traditionally, the immune system can be broadly divided into two parts, which are innate immunity and acquired or adaptive immunity (Abbas, Lichtman, and Pillai, 2005; Kindt, Goldsby, Osborne *et al.*, 2007). Innate immunity uses the genetic memory of germline-encoded receptors to recognize the molecular patterns of common pathogens. Adaptive immunity is a complex system by which the body learns to recognize a pathogen's unique antigens and builds an antigen specific

response to destroy it (Chaplin, 2010). Moreover, whilst the innate immune response does not differ upon repeated exposure to the same pathogen, adaptive immunity allows a memory response to develop so that subsequent encounters with the same antigen are treated with increased alacrity and efficacy by the host (Figure 1.2) (Abbas *et al.*, 2005).

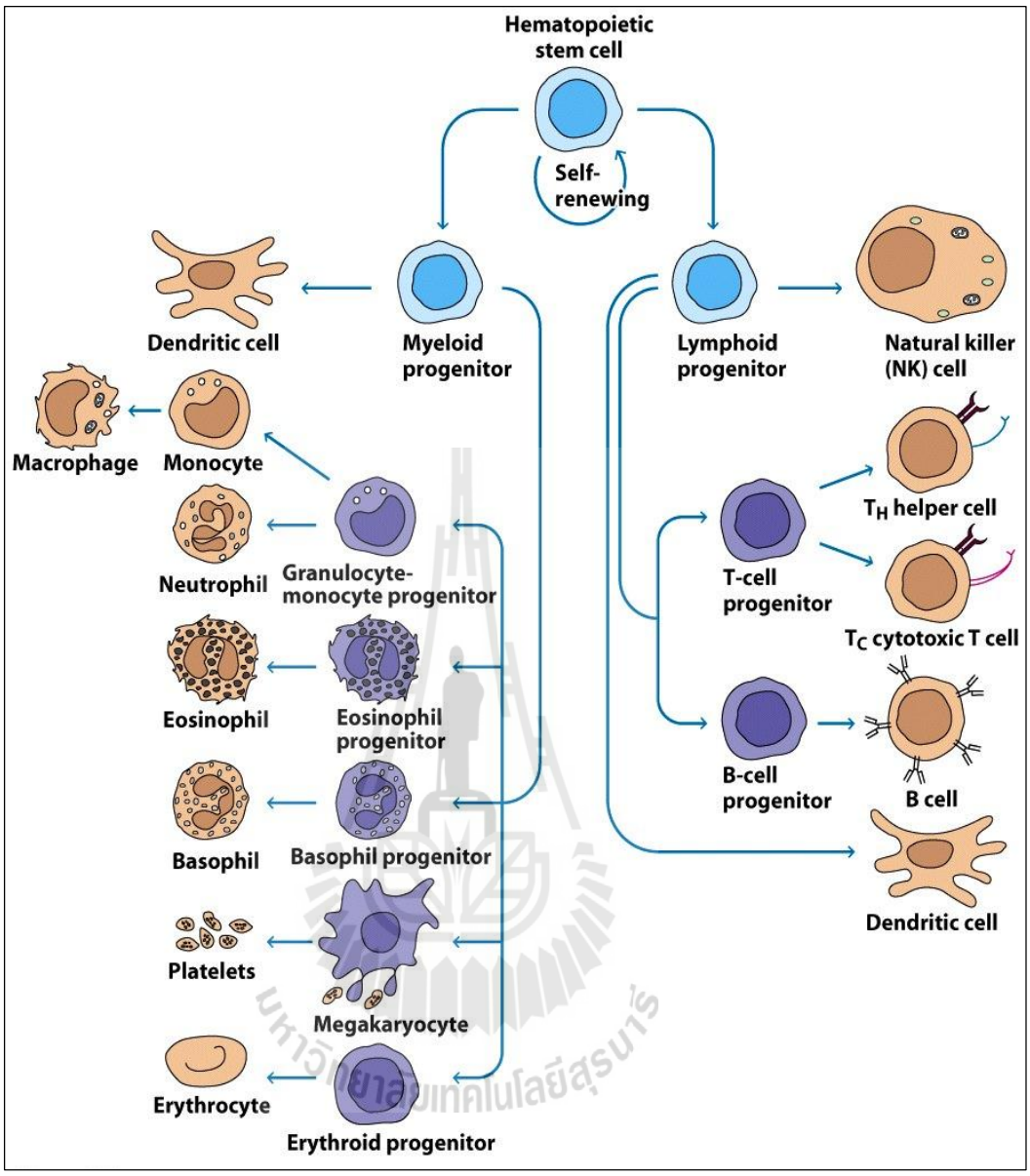

Figure 1.1 Hematopoiesis. All cells of the immune system originate from a common pluripotent hematopoietic progenitor of the bone marrow. These highly undifferentiated cells divide to produce a common lymphoid progenitor that gives rise to NK cells, B and T lymphocytes, and a common myeloid progenitor that gives rise to red blood cells, granulocytes, megakaryote (cells that produce platelets), macrophages and dendritic cells (Kindt *et al.*, 2007).

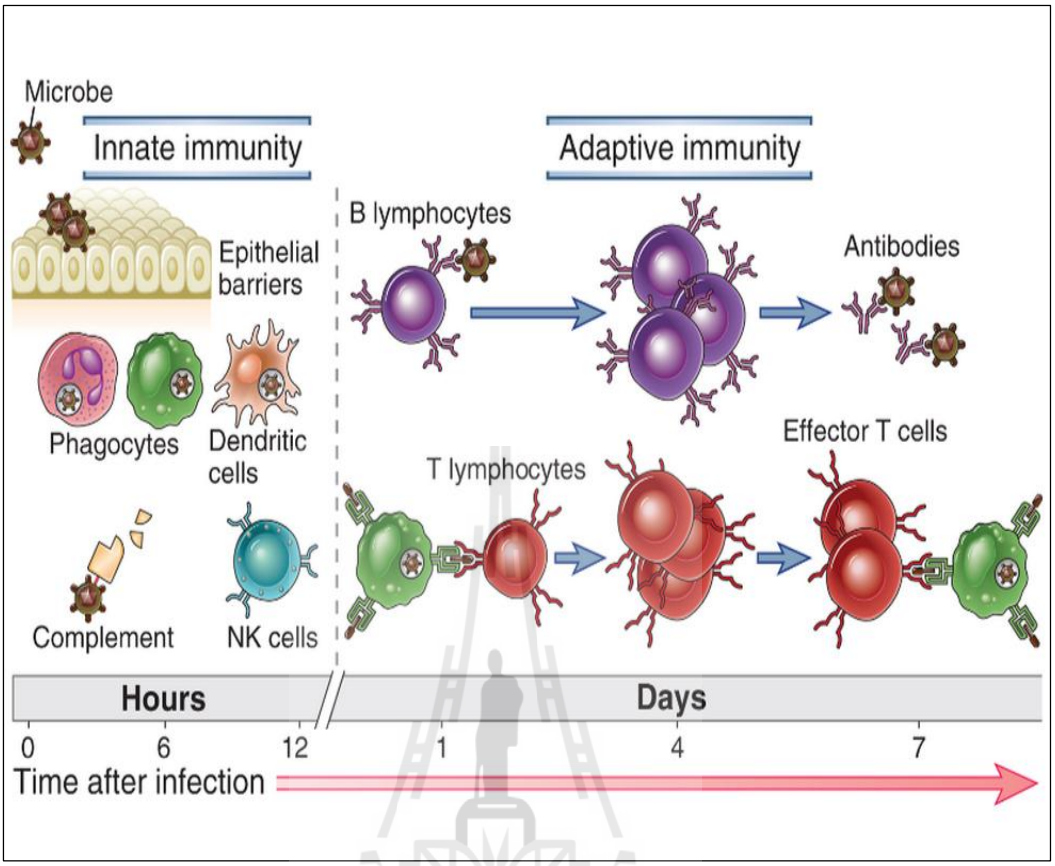

Figure 1.2 Innate and adaptive immunity. Innate immunity represents the first line of defense against pathogens. It is composed of physical, cellular and chemical barriers. Adaptive immune responses develop later and are mediated by lymphocytes and their products. The kinetics of the innate and adaptive immune responses is approximations and may be vary in different infections (Abbas *et al.*, 2005).

1.2.1.1 Innate immunity

Innate immunity is a highly effective set of conserved mechanisms used by multicellular organisms to recognize and counter the constant threat of microbial infections. There is evidence to indicate that innate responses are key to controlling most infections, as well as contributing to inflammatory responses that are central

components of disease (Finlay and Hancock, 2004). This system is ready to start from birth and constitutes our first line of defense. It includes epithelial barriers (such as skin and mucosal membranes), soluble factors (such as complement system, mediators of inflammation and cytokines) and phagocytic cells, including granulocytes, monocytes/macrophages, DCs and NK cells. The hallmark of innate immunity is the germline-encoded pattern recognition receptors (PRRs), which are able to directly activate the immune cells. These patterns are referred to pathogen associated molecular patterns (PAMPs), which are able to distinguish self from non-self and initiate the host defense or damage-associated molecular patterns (DAMPs) which released from injured cells. PAMPs are extremely conserved in microorganism, ranging from lipids and lipoprotein to proteins and nucleic acids, which are fundamental for the survival of pathogens (Akira, Takeda, and Kaisho, 2001; Akira, Uematsu, and Takeuchi, 2006; Janeway and Rosengarten Family Fund., 2001). The task of cellular and soluble components of the innate defense targets the invading pathogen tries to eradicate and present it to cells of the adaptive immune system. The various cells of innate immunity are involed in the cellular effector mechanisms. The innate immune responses set the stage for the adaptive immune response by up regulating the expression of co-stimulatory molecules and by increased cytokine production (Abbas, Lichtman, and Pillai, 2007; Janeway and Rosengarten Family Fund., 2001; Johnson, Ziegler, and Hawley, 2010).

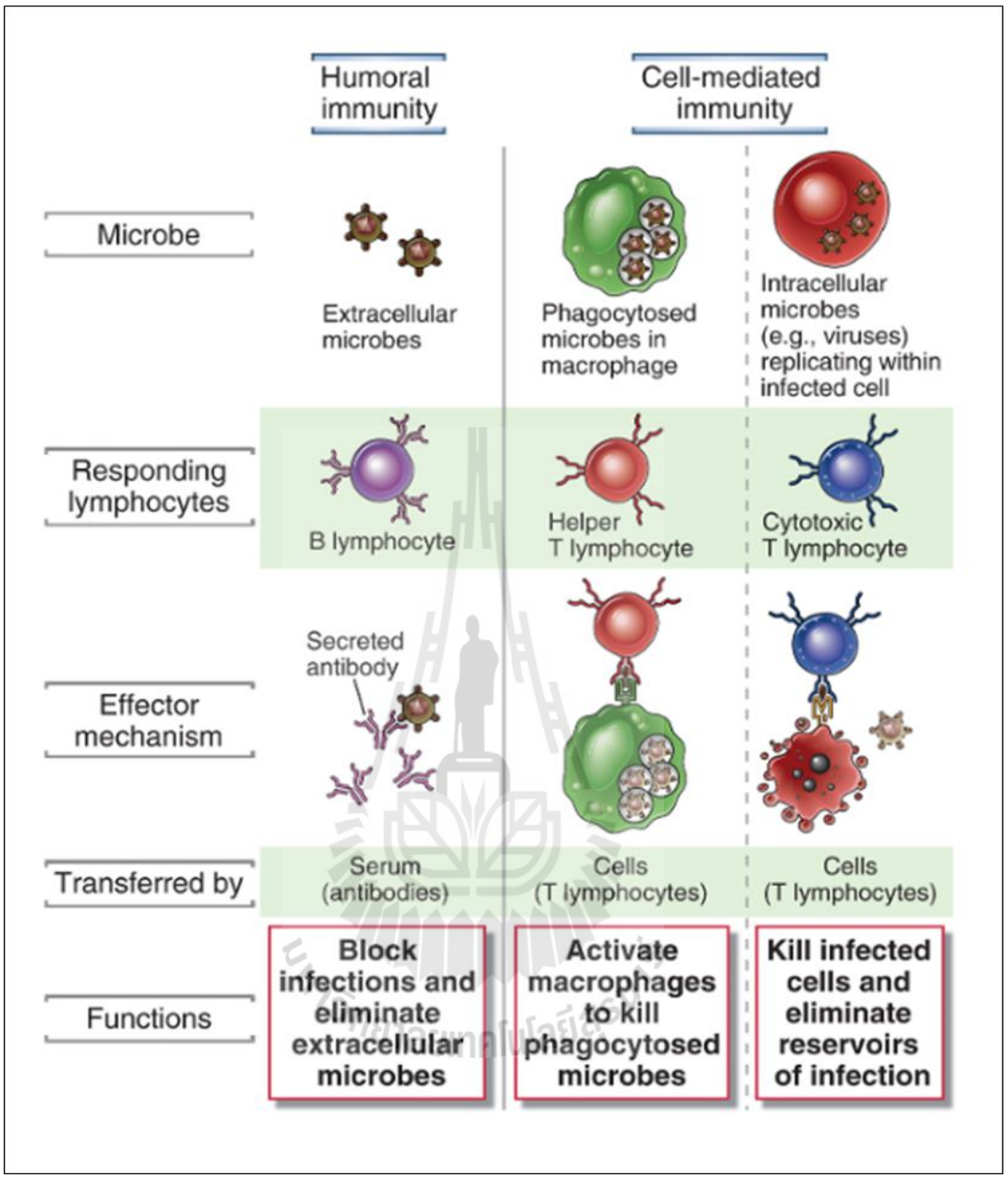

Figure 1.3 Types of adaptive immunity. In HMI, B lymphocytes secrete antibodies that block infections and eliminate extra-cellular microbes. In CMI, T lymphocytes eradicate intracellular microbes and altered cells (Abbas *et al.*, 2005).

1.2.1.2 Adaptive immunity

The adaptive immunity is different from the innate immunity. It is specific for distinct antigens, has ability to recognize the same antigen and respond more vigorously to repeat exposures. Cells that play an important role in this system consist of two broad sets of antigen-responsive cells, the B and T lymphocytes. A unique characteristic of the cells of this immune system is their epitope-specific antigen receptors, the T cell receptor (TCR) and the B cell receptor (BCR). These receptors are generated by rearrangement of gene segments and mutations during maturation that induces high diversification and specificity. In general, adaptive immune response can be divided into two types as (i) Humoral immune response, elaborated by B-lymphocytes that produce antibodies, targeting pathogens in extracellular spaces, and (ii) Cellular immune response characterized by thymus educated T-lymphocytes and their effector cytokines that specifically act on intracellular and phagocytized pathogens (Figure 1.3) (Abbas *et al.*, 2005; Paul, 2008). The most important of the adaptive immune system is specific and developed during our life-time. In addition, it gives rise to immunological memory that protects us from re-infection with same pathogen (Paul, 2011).

1.2.1.2.1 Humoral mediated immunity (HMI)

HMI components consist of B-lymphocytes or B-cells. These B-cells produce antibodies, which are capable of recognizing three-dimensional structures (3D) and thus can interact with and lead to the neutralization of pathogens in extracellular fluid (Paul, 2011). B-cells are produced in bone marrow and reside there until maturation. When they are released, they have a unique receptor on their membrane called an immunoglobulin (Ig) receptor, due to its link to a specific Ig

super-family. When a B-cell encounters an antigen, it reacts in two ways: one is the production of a memory B-cell and the other is the production of an effector B-cell. The memory B-cell also has the Ig receptor on its membrane and keeps looking for more similar antigens. The plasma cell or effector B-cell loses the Ig receptor and grows larger in size and starts producing antibodies (Abbas *et al.*, 2007; Kuby, 1997). In addition, B cells constitutively express major histocompatibility complex (MHC) class II molecules and are efficient at acquiring cognate peptide antigen, they can also act as antigen presenting cell (APC) for T cells. However, they are not the most efficient APC as they must be activated to induce expression of additional molecules such as the B7 molecules CD80, CD86, and CD40 via bidirectional signaling to promote T cell activation (Vanden Bush, Buchta, Claudio *et al.*, 2009).

1.2.1.2.2 Cell-mediated immunity (CMI)

The term CMI refers to the antigen-specific action of T cells or, T-lymphocytes. The principal role of this immunity is to detect and eliminate cells that harbor intracellular pathogens and also can recognize and eliminate cells, such as tumor cells, that have undergone genetic modifications so that they express antigens not typical of normal cells (Kindt *et al.*, 2007). The most critical function of the cell-mediated immune response is the activation and programming of T cells from their naïve/resting state to activated state, which can be devided into three major stages: activation, differentiation, and memory formation (Pennock, White, Cross *et al.*, 2013).

1.2.2 Antibody structure and function

Antibodies are Y shaped Ig molecules comprised of two light chains and two heavy chains. Each chain in turn is composed of a variable region (V) at the N-terminus of the protein and a constant region (C) at the C-terminal end of the protein. The V region comprises one heavy chain (V_H) and the adjoining V region of one light chain (V_L) from an antigen-binding site. The constant regions of light chains (C_H) have either of the two amino acid sequences named kappa and lambda. The C_H have one of five basic amino acid sequences i.e. α, δ, ε, γ, or μ. These sequences determine the isotype of the antibody molecules, and based on the isotype of the heavy chain constant region, Igs adopt one of 5 classes in humans IgG, IgA, IgM, IgD, and IgE. Among the Ig isotypes, IgG is the most abundant, making up about 75% of all Igs found in the human serum (Figure 1.4). Further, IgGs consist of four subtypes: IgG1, IgG2, IgG3, and IgG4, in decreasing order of occurrence. These subtypes differ mainly in their amino acid sequences as well as in the number of disulfide bonds between the heavy chains (Male, 2006).

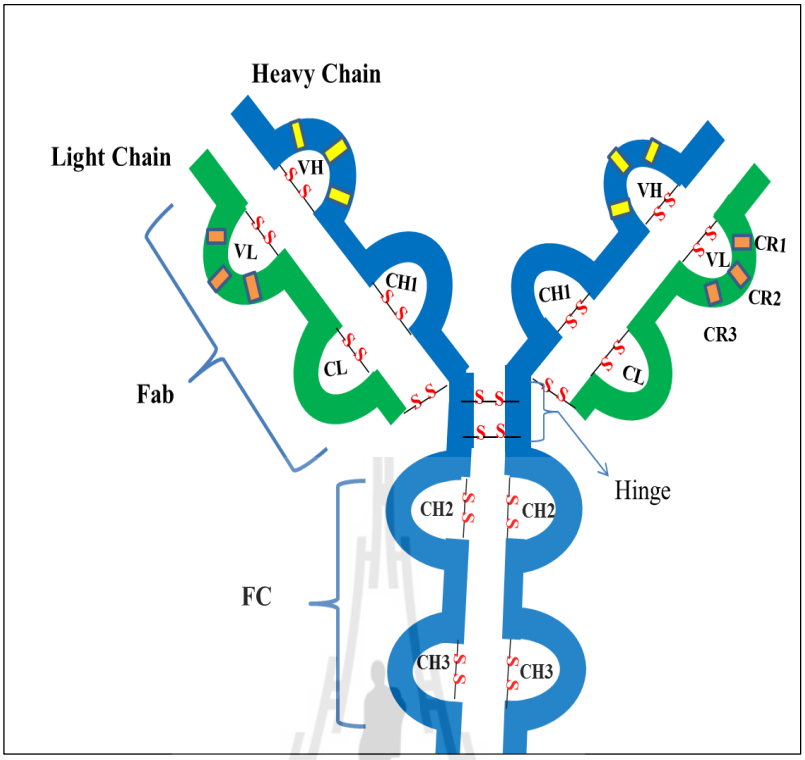

Figure 1.4 Schematic structure of the human IgG molecule. Green parts are light chains and blue parts are heavy chains. V indicates variable domains; C indicates constant domains; V_L and C_L are domains of the light (L) chain; VH, C_{H1}, C_{H2}, and C_{H3} are domains of the heavy (H) chain; the red regions in variable domains, CDR1, CDR2, and CDR3, are complementary determining regions; –S-S- indicates disulfide bond adapted from (Coico, Sunshine, and Benjamini, 2003).

The function of antibodies is to protect against infection. The mechanism can be either direct or indirect (Figure 1.5). Directly, antibodies can affect infectious agents or their toxic products by neutralization (inactivating or blocking the binding of antigens to receptors), agglutination, or precipitation (making a soluble antigen into an insoluble precipitate). Indirectly, antibodies activate components of innate resistance, including complement and phagocytes. In the body, antibodies are

generally produced as a mixed population of classes, specificities, and capacity, which can be named as polyclonal antibody. For using in scientific researches or diagnostic tests ortherapuetics, a monoclonal antibody that contains a specific binding epitope is needed. Now a day, there are common procedures to clone and generated the monoclonal antibodies, which have been used for those proposed mentioned above (Huether and McCance, 2012).

Monoclonal antibody

Monoclonal antibodies (mAbs) are monovalent antibodies which bind to the same epitope and are produced from a single B-lymphocyte clone. The production of mouse mAbs was invented by César Milstein and Georges J. F. Köhler in 1975, when they succeeded to fuse a B-cell from an immunized mouse with a mouse myeloma cell. The continuous cultures of so-called hybridomas give a renewable source of mAbs with a desired specificity (Kohler and Milstein, 1975; Liu, 2014). The hybridoma technique is the standard method for production of monoclonal antibodies and a wide range of them made which bind to protein, carbohydrate, nucleic acidand hapten antigens, and which even have catalytic activities leading to many applications including biological research, medical diagnostics and therapy. MAbs have become essential tools for a variety of laboratory techniques such as Western blotting, immunohistochemistry, immunocytochemistry, ELISA, immunoprecipitation and flow cytometric analysis (Koch, Niemeyer, Patel *et al.*, 2002). In additional, mAbs are an important tool for studies of leukocyte surface molecule, which has been proven used in several purposes include; (i) mAbs can be used to discover and identify specific molecules on cells or tissues in terms of cellular distribution, for instance, to distinguish subsets of B cells and T cells. This knowledge is helpful not only for

basic researches but also for identifying different types of leukemias and lymphomas and allowing physicians to tailor treatment accordingly; (ii) biological research, mAbs that bind to cell surface molecules and either stimulate or inhibit particular cellular functions are valuable tools for defining the functions of surface molecules, including receptors for antigens; (iii) mAbs can be used to identify genes of interest and their products (Abbas, Lichtman, and Pillai, 2010; Hiatt, 1991).

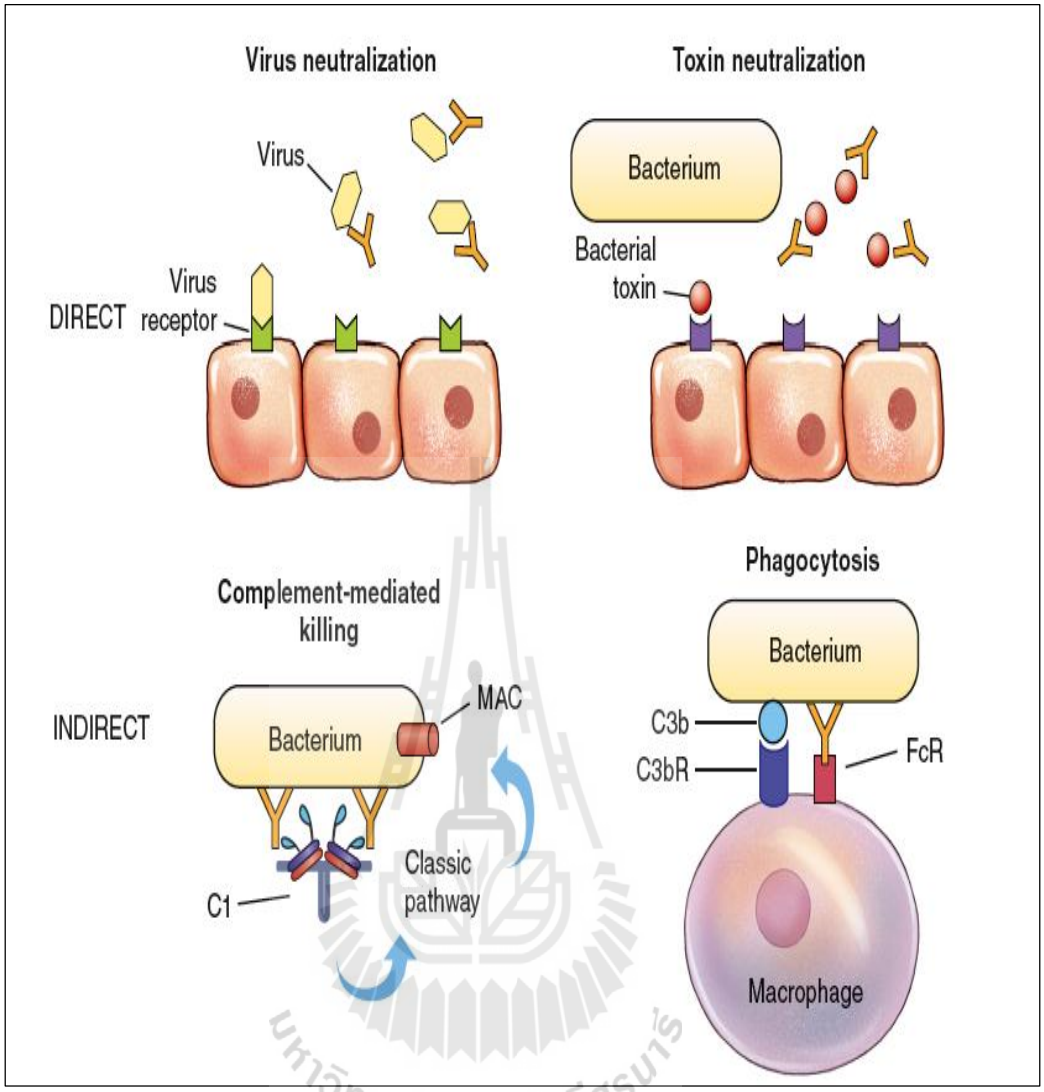

Figure 1.5 Direct and indirect functions of antibody. Protective activities of antibodies can be direct action of antibody alone or indirect activation of other components of the innate immune response. Direct means include neutralization of viruses or bacterial toxins before they bind to receptors on the surface of the host cells. Indirect means include activation of the classical complement pathway through C1, resulting in formation of the membrane-attack complex (MAC), or increased phagocytosis of bacteria opsonized with antibody and complement components bound to appropriate surface receptors (FcR and C3bR) (Huether and McCance, 2012).

1.2.3 Phagocytosis and PRRs

Innate immune mechanisms comprising the recognition of pathogens include the immune cells that are responsible for the elimination of pathogens at their sites of entry. There are several ways of these cells to take up the pathogens and this is collectible grouped under the term endocytosis. This mechanism includes receptor-mediated endocytosis, pinocytosis and phagocytosis (Beran, Potmesil, and Holub, 2011; Purves, 2004). Phagocytosis is the most important mechanism of innate immunity. This process was first described by Ilya Mechnikov in 1883 during studies on starfish larvae 408 (Mechnikov, 1988). Whole process of phagocytosis can be alienated into three main important stages including (i) recognition, (ii) internalization, and (iii) degradation (Figure 1.6A) (Flannagan, Jaumouille, and Grinstein, 2012). The cells involved in this mechanism called phagocytes including monocytes, macrophages, and granulocytes. These cells express a large number of cell surface and cytosolic receptors that allow them to recognize bacteria, fungi, parasites, viruses, and also altered self molecules (Conner and Schmid, 2003; Dale, Boxer, and Liles, 2008; Stern, Adiseshaiah, and Crist, 2012). Also, numerous studies have been reported that a phagocyte has many types of receptors on its surface that are used to bind to its ligands on the microorganisms.

The ligand-binding properties and expression patterns of these receptors suggest that they may function in one or more host-defense-related processes (Pearson, 1996). Many of these receptors have been characterized and their respective ligands have also been identified. These phagocytic receptors are expressed by phagocytic cells including scavenger and C-type lectin receptors, Toll-like receptors (TLRs) as well as receptors recognizing complement and other opsonins. In detail of

these phagocytic receptors include; (i) the scavenger receptors (SRs), including SR-A and CD36, in particular against bacterial pathogens. Moreover, important function of some SRs is to act as co-receptors to TLR, modulating the inflammatory response to TLR agonists. On bacteria, the SR ligands have commonly been reported to be lipopolysaccharide (LPS) and lipoteichoic acid, but recent advances in the field indicate that bacterial surface proteins play a more important role as target molecules for SRs than previously thought (Figure 1.6B) (Areschoug and Gordon, 2009); (ii) The C-type lectin receptors (CLRs) recognizes specific sugar structures that are present in microorganisms, but normally absent in healthy mammalian glycoproteins, though they may appear on ageing self-glycoproteins such as macrophages and dendritic cells express a variety of CLRs, including the mannose-receptor and Dectin-1 (Geijtenbeek, van Vliet, Engering *et al.*, 2004); (iii) A group of mammalian receptor proteins, homologous to the drosophila Toll proteins, have been given the name TLRs. These receptors recognize conserved molecule structures that are essential for survival of the microbe, an elegant design to prevent microbes from escaping innate recognition. Examples of the TLRs involved in recognition of bacteria include TLR2, TLR4 and TLR5. These receptors recognize bacterial cell wall components or flagella and are located at the cell surface of phagocytic cells, but may also be recruited into phagosomes (Ozinsky, Underhill, Fontenot *et al.*, 2000). In contrast, TLR9, which recognizes bacterial DNA, is only expressed intracellular (Brown, Herre, Williams *et al.*, 2003; Gantner, Simmons, Canavera *et al.*, 2003; Underhill and Gantner, 2004); and (iiii) the opsonin receptors participate in opsonisation of microbes with complement or an antibody greatly enhances their phagocytosis. Fc receptors (F_CRs) are expressed by phagocytic cells that bind to the

Fc part of immunoglobulin G (IgG) antibodies, facilitating uptake of antibody-targeted microbes or immune complexes. For example, bacteria opsonised with iC3b that are recognised and phagocytosed by innate immune cells expressing complement receptor (CR3) (CD11b/CD18) and CR4 (CD11c/CD18) (Swanson and Hoppe, 2004; Zhang, Hoppe, and Swanson, 2010).

Taken together, these types of phagocytic receptors can be engaged simultaneously during an encounter of a single pathogen, leading to the recruitment and subsequent activation of a wide variety of signaling molecules. As a result, different signaling pathways are activated and work collectively to ensure an effective elimination of the pathogen by phagocytic cells. Though a large number of receptors and molecules involved in the phagocytic process have been described, many more are expected to be discovered and investigated in order to unravel the complexity of this process. Therefore, finding the new phagocytic receptors or proteins involved in the up- or down-regulation of phagocytosis and investigating their roles in phagocytic process are essential for understanding of the pathogenesis of infectious diseases (Singboottra, Pata, Tayapiwatan *et al*., 2010).

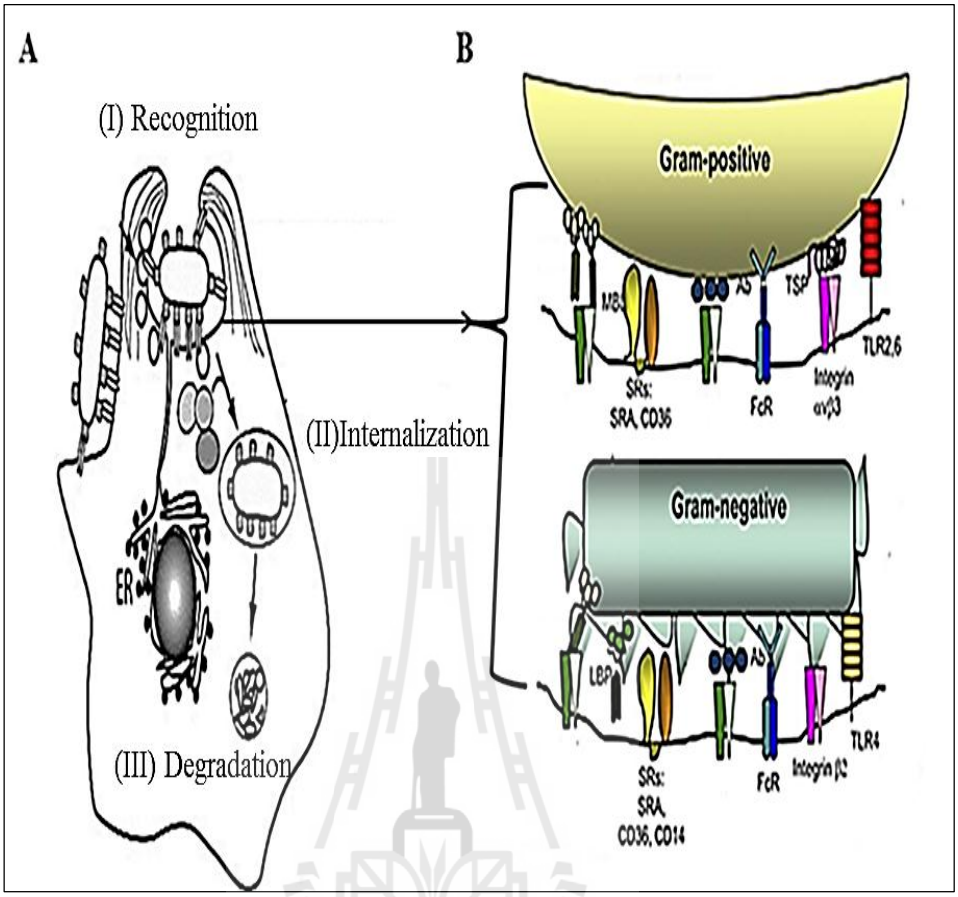

Figure 1.6 Mechanism of phagocytosis. Phagocytosis can be alienated into three main important stages including (i) recognition, (ii) internalization, and (iii) degradation. (A) The bacterium is enclosed in a vesicle and digested by a lysosome. (B) Phagocytes have specific receptors that discriminate pathogen-associated - components causing inflammatory responses. As an example, recognition of gram-positive bacteria by TLR2 and TLR6 or gram-negative by TLR4 is shown. The figure is modified from (Fallman and Gustavsson, 2005; Stuart and Ezekowitz, 2005).

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1.2.4 T cell activation

T cells are central players of the cell-mediated immune response, which help protecting the host against different pathogens. In order to complete their function, T cells need to be activated, which then lead to a variety of responses including proliferation, migration, cytokine production and even apoptosis (Kindt *et al.*, 2007). Activation of a naïve T cell is initiated by the interaction of its TCR with a peptide antigen bound to either a class I ($CD8^+$ cells) or class II ($CD4^+$cells) major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs). In addition, the TCR on T cell associates with a complex of membrane proteins collectively known as CD3 (composed of γ, δ, ε, ζ, and η chain). This interaction occurs in an immunological synapse involving various molecules, which forms between T cells and APCs. As a result of interaction between T cells and APCs, TCR-associated molecules including CD3ζ transmit a primary activating signal, which is termed signal 1 (Figure1.7) (Kershaw, Westwood, and Darcy, 2013). However, this signal alone is insufficient to fully activate T cells, which require co-stimulation that is mediated by the interaction of one or more T cell co-stimulatory receptors with their ligands expressed on APCs. Further promotion of activation and growth is provided by cytokines through the engagement of cytokine receptors on T cells to deliver what is referred to as signal 3 (Eggermont, Paulis, Tel *et al.*, 2014).

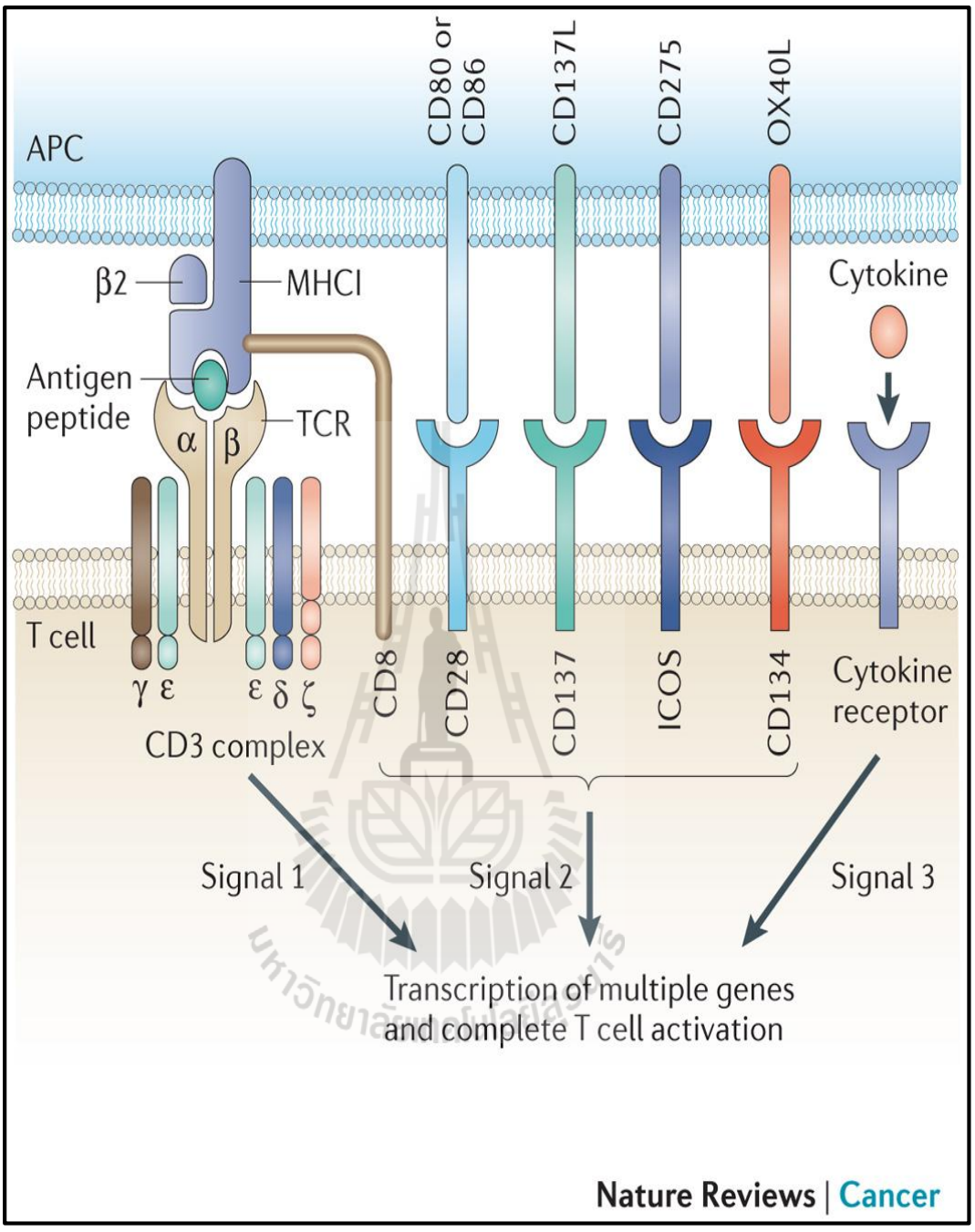

Figure 1.7 Three signals induce T cell activation. The outcome of specific antigen recognition by T cells (signal 1) is determined by co-stimulation (signal 2) and delivered by fully activated APCs and the presence of inflammatory cytokines (signal 3). Activation of naïve T cell in the presence of all three signals leads to full effector function (Kershaw *et al.*, 2013).

1.2.4.1 Costimulatory molecules

It has been widely accepted that stimulating a T cell with its cognate antigen alone does not lead to activation as a substitute results in T cell non-responsiveness or anergy to further stimulus (Pennock *et al.*, 2013). The discovery of this led to known that T cell activation requires second signal to achieve fully activated. These signals are generated by the interacting of costimualory ligands expressed on APCs with their receptors on T cell. T cell costimulatory signal are mendatory for initiation of effective immunity for host protective as well as immune-pathological process and the absence of costimulatory signals results in abortive T cell response and T cell anergy. In addition, T cell activation processes are negatively regulated by inhibitory costimulatory pathways. One of the best characterized co-stimulatory molecules expressed by T cells is CD28. This receptor interact with costimulatory ligands B7-1 (CD80) or B7-2 (CD86) on APCs, which can also transduce inhibitory signals by ligation of cytotoxic T-lymphocyte-associated protein 4 (CTLA4) on T cell (Choi, Park, and Lee, 2002; Ribot, Debarros, Mancio-Silva *et al.*, 2012). Numerous lines of evidence have suggested that CD28, which can be regarded as the most potent costimulatory receptor that promotes interleukin 2 (IL-2) production, activation of naïve T cell, T cell proliferation, differentiation, survival and entry into the cell cycle (Figure 1.8) (Kindt *et al.*, 2007). Nevertheless, mice that lack CD28 are able to raise immune responses, indicating that this molecule was in fact not the only molecule capable of providing a costimulatory signal to T cells. Clearly, the numbers of molecules other than CD28 that have been identified as providing a costimulatory function during T cell activation continues to grow. There have been shown that several protein families involved in the generation of T cell costimulatory signals

including, the tumor necrosis factor receptor (TNFR) superfamily (CD134, CD137, CD27, CD30, and DR3), the tetraspanins superfamily (CD9, CD37, CD63, CD81, and CD82), the CD2 superfamily (CD2 and CD151), the Ig superfamily (CD5, CD6, CD7, and CD47) and integrins (β2 integrin: CD11b/DC) (Leitner, Grabmeier-Pfistershammer, and Steinberger, 2010).

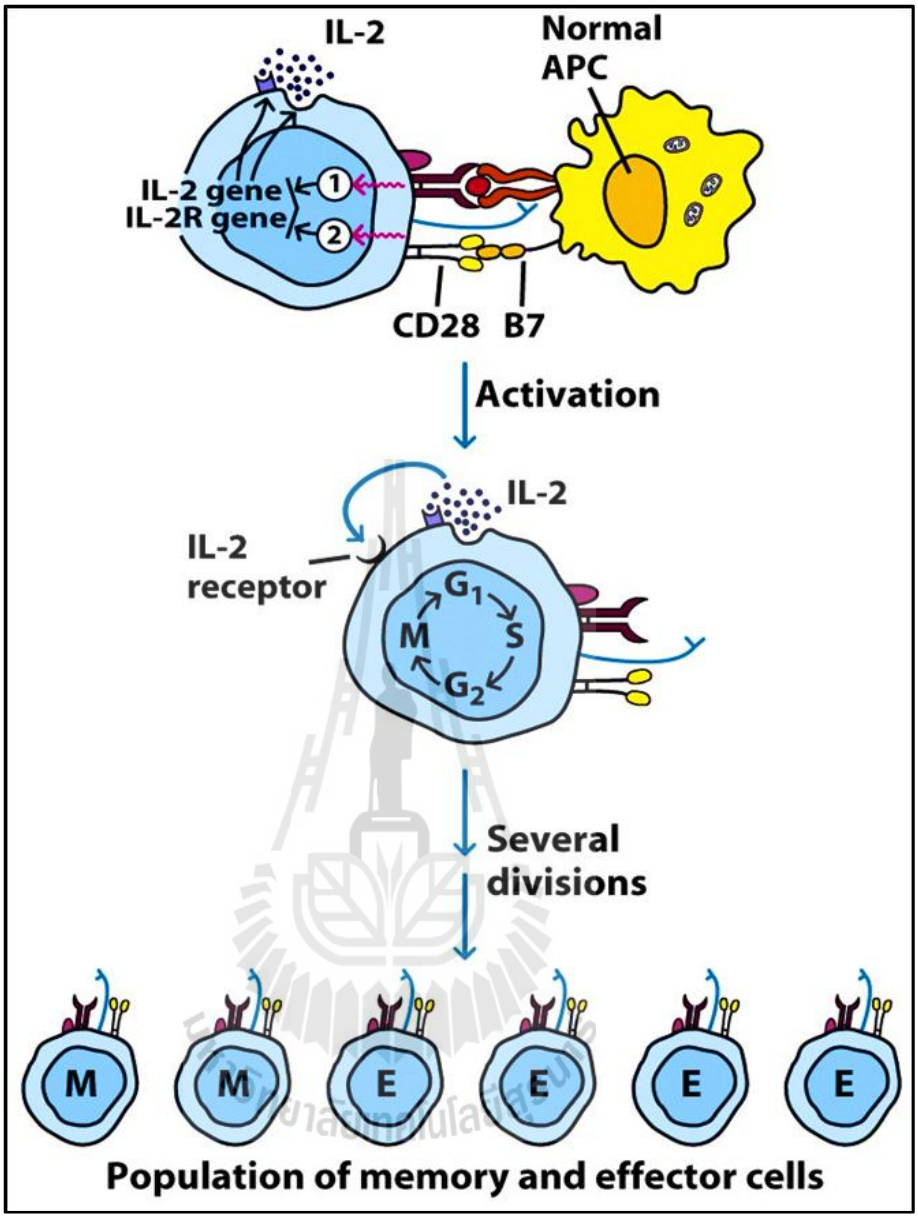

Figure 1.8 Full activation of T cell requires costimulatory signals. T cell recognition of antigen peptides presented by MHC molecule on the surface of APCs is signal 1 and costimulatory signal 2 up-regulates expression of IL-2 and the high affinity IL-2 receptor, leading to the entry of the T cell into the cell cycle and several rounds of proliferation. Some of the cells differentiate into effector cells, others into memory cells (Kindt *et al.*, 2007).

1.2.4.2 T cell regulation by cytokines

Cytokines are peptides that have a fundamental role in communication within the immune system and in allowing the immune system and host tissues cells to exchange information (Vilcek and Feldmann, 2004). There have also been reported that T cell activation and differentiation can be futher directed through binding of cytokines and cytokines receptors. These cytokines are essential for cell survival and productive immune responses, which can be secreted by APCs, $CD4^+$ T helper cells and especially $CD8^+$ T cell, (Pennock *et al.*, 2013). The most important cytokine produced by T cells early after activation, often with in 2 to 4 hours after recognition of antigen and costimulators causes production of interleukin-2 (IL-2) and expression of IL-2 receptor (IL-2R) or CD25. The IL-2/IL-2R interaction then delivers signals for clonal expansion and effector development. Therefore, IL-2 is a centeral player for T cell dependent immune response (Malek, 2008). However, there are also other cytokines that can assist in T cell activation and proliferation, include IL-12, IL-15, IL-21, and IFN-γ (Eggermont *et al.*, 2014). In addition, it has been reported that interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) exert regulatory function by suppressing T cell proliferation via the secretion of specific cytokines (Couper, Blount, and Riley, 2008; Gorelik and Flavell, 2002). IL-10 is produced by both monocytes and lymphocytes. It has been reported that this cytokine down-regulates surface expression on APC, such as CD80/86 and intercellular adhesion molecule 1 (ICAM-1), and thereby strongly inhibits antigen presenting capacity. Moreover, IL-10 was suggested to mediate the inhibition of CD28 and also inducible T-cell costimulator (ICOS) signaling pathway (Taylor, Akdis, Joss *et al.*, 2007).

Additionally, It significant increased IL-10 has been associated with increased activity of negative costimulatory signals on T cell such as programmed cell death 1(PD-1) and CTL4-A. Finally, IL-10 is effective at decreasing level of IL-2, IL-12, and IFN-γ (Sabat, Grutz, Warszawska *et al.*, 2010).

1.2.5 Leukocyte surface molecules

The human immune system is a complex machinery involving numerous proteins. Proteins located at surface of the immune cells are called leukocyte surface molecules, which were demonstrated to play key roles in all aspects of leukocyte functions (Diaz-Ramos *et al.*, 2011). Several events, such as regulating the development, activation, and effector functions of leukocytes are orchestrated by a complex series of leukocyte surface molecules. These signals then lead to the activation, differentiation, maturation, and finally control of the immune response (Barclay, Brown, Law *et al*., 1997).

Nomenclature of leukocyte surface molecule in the cluster of differentiation (CD) system

The CD system is a system of designating surface molecules of leukocytes that serves as markers of differentiation has been implemented. Such molecules can thus be used to characterize particular cell populations or subsets. The CD nomenclature system was developed by Human Leukocyte Differentiation Antigens (HLDA) Workshops, which is latter changed the name of the organization to Human Cell Differentiation Molecules (HCDM) to reflect broader objective (Zola, 2005) and also for those that have not been given a CD number. (Matesanz-Isabel, Sintes, Llinas *et al.*, 2011). The system was introduced to classify monoclonal antibodies directed against cell surface molecules of leukocytes. A statistical method called

cluster analysis was used to analyze the data and identify antibodies with similar patterns of binding to leukocytes at particular stages of differentiation in the name of the CD system. In addition, surface expression of a particular CD molecule may not be specific for just one cell or even a cell lineage. However, many are useful for characterization of cells (Barclay *et al.*, 1997). The detection of protein markers on leukocytes by CD antigen expression is now widely used in clinical medicine and experimental immunology. CD-specific monoclonal antibodies have been involved determining the functions of CD proteins, identifying the distribution of CD proteins in different cell populations in normal individuals, measuring changes in the proportion of cells carrying these markers in patients with disease (e.g. decrease in $CD4^+$ T cells is a hallmark of HIV infection) and developing therapeutic measures for increasing or decreasing the numbers or activities of certain cell populations (Zola and Swart, 2005).

1.2.6 Tetraspanins

Tetraspanins are a large family of evolutionary conserved cell-surface proteins but in some cell types are also presented in lysosomes-related organelles or secretory lysosome (Charrin, Jouannet, Boucheix *et al.*, 2014). These proteins are expressed in the wide range of organisms, including, plant (Arabidopsis), schitosome, some sponges, fungi (but not yeast), insects (Drosophila), zebrafish and mammals. This family was first described as distinct protein family in 1994 (Levy and Shoham, 2005; Wright and Tomlinson, 1994). They are comprised of small molecule (20-50 kDa) and their general structures contain four transmembrane segments (TM1–TM4) with both cytoplasmic tails at the N- and C-terminal extremities linked by one short extracellular (EC1), one short intracellular (IC), and one large extracellular (EC2)

stretches. A distinctive feature of tetraspanins compared to other four-transmembrane proteins is the conserved Cys-Cys-Gly motif and at least two other residues that contribute to disulphide bond formation in EC2. Furthermore, based on analyses of amino acid sequences of the EC2, the segment known to be the primary binding site for tetraspnin-associated proteins, there are 33 members of this family found in humans, which can be classified into four major subfamilies, including: the CD-non63 e.g., CD9, CD81, CD82, and CD151, the CD63 subfamily e.g. CD63 and TSPAN31, the uroplakin subfamily (UPK 1A/1B) and the retinal degeneration slow (RDS) subfamily (RDS-ROM) (Berditchevski, Rubinstein, and Springer Link, 2013; Hu, Song, Xie *et al.*, 2015; Kovalenko, Yang, and Hemler, 2007). The role of these molecules is relating to an organization of proteins in the cell membranes where tetraspanins direct other proteins into signal-transducing microdomains known as tetraspanin-enriched microdomains (TEMs) (Figure 1.9). The numerous of proteins associated within TEMs include integrins, growth factors, metalloproteases, signaling molecule receptors (tyrosine kinases and G-protein coupled receptors), and many of the most important membrane molecules in an immune function such as antigen-presenting molecules (MHC class I, MHC class II), molecules associated with the TCR (CD2, CD3), BCR (CD19), co-receptor (CD4, CD8), co-stimulatory molecules or PRRs. (Jones, Demaria, and Wright, 2011; van Spriel, 2011). In addition, for the majority of tetraspanins, no ligands have been discovered. Several studies have focused on looking for the partnership interaction of the tetraspanin web. Its composition is thought to be a key factor that influences tetraspanin activity by bringing molecules into a close proximity and thus encouraging cross-talk between them. The TEMs are highly dynamic with molecules constantly move into or out of

the microdomain and the changes in TEMs composition determine tetraspanin activity (Yanez-Mo, Barreiro, Gordon-Alonso *et al.*, 2009). In addition, tetraspanins have also been proposed to form functional complexes with microbial receptors in the host cell membrane. TEMs were able to serve as specialized interacting platforms for viral, bacterial, parasiticor fungal pathogens. Theere are three different mechanisms model are expected to be located under tetraspanin involvement in microbial infection of host cells (Figure 1.10) (van Spriel and Figdor, 2010). Thus, this family members are associated with diverse biological processes including membrane fusion, adhesion, motility, immune system function and microbial infection (Fanaei, Monk, and Partridge, 2011).

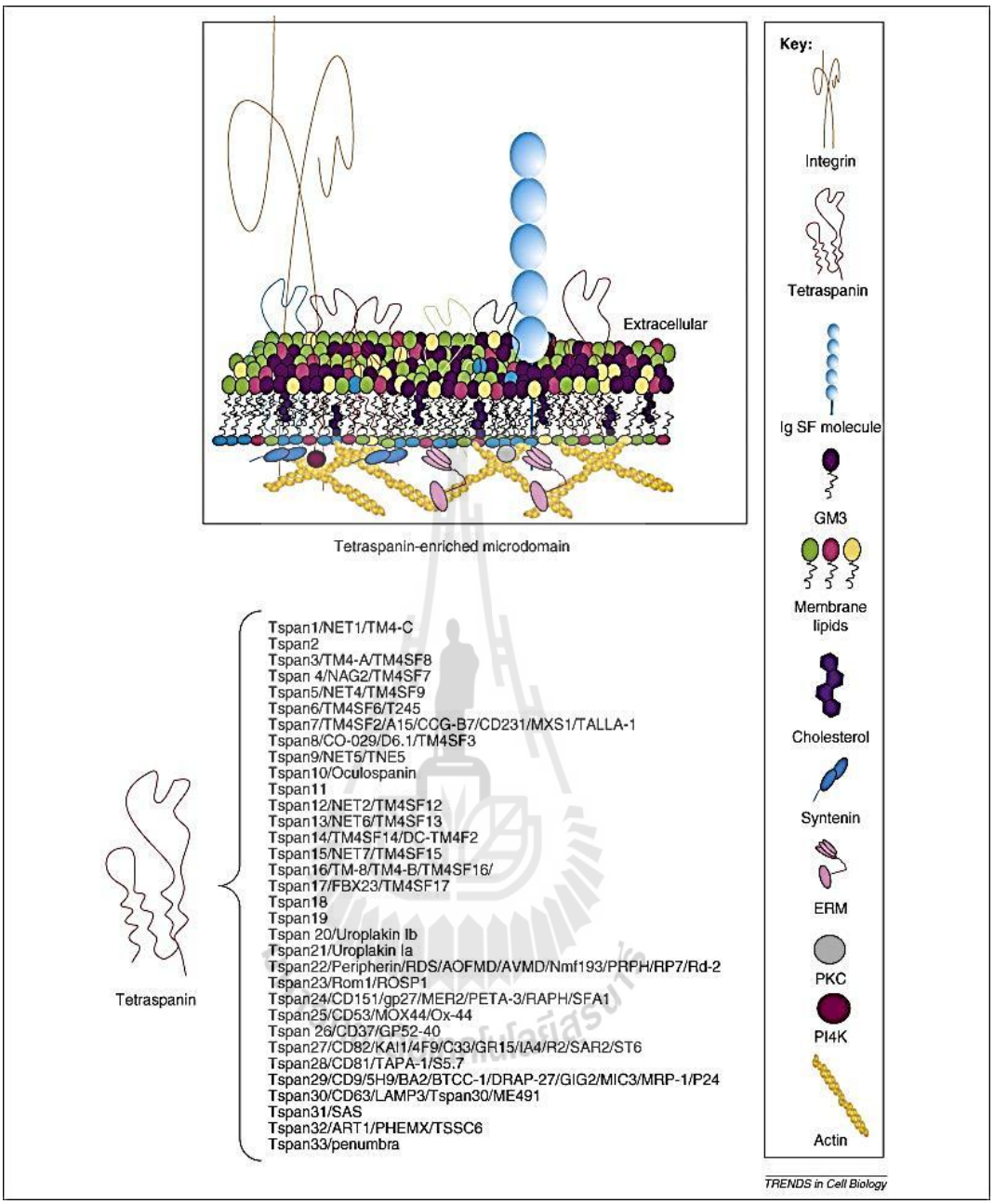

Figure 1.9 Structures of tetraspanins enriched microdomains (TEMs). Tetraspanins form clusters in the plasma membrane. TEMs are highly populated by tetraspanins as wells as associated molecule including integrins, gangliosides and Ig molecules. TEMs composition regulates signalling across the membrane (Yanez-Mo *et al.*, 2009).

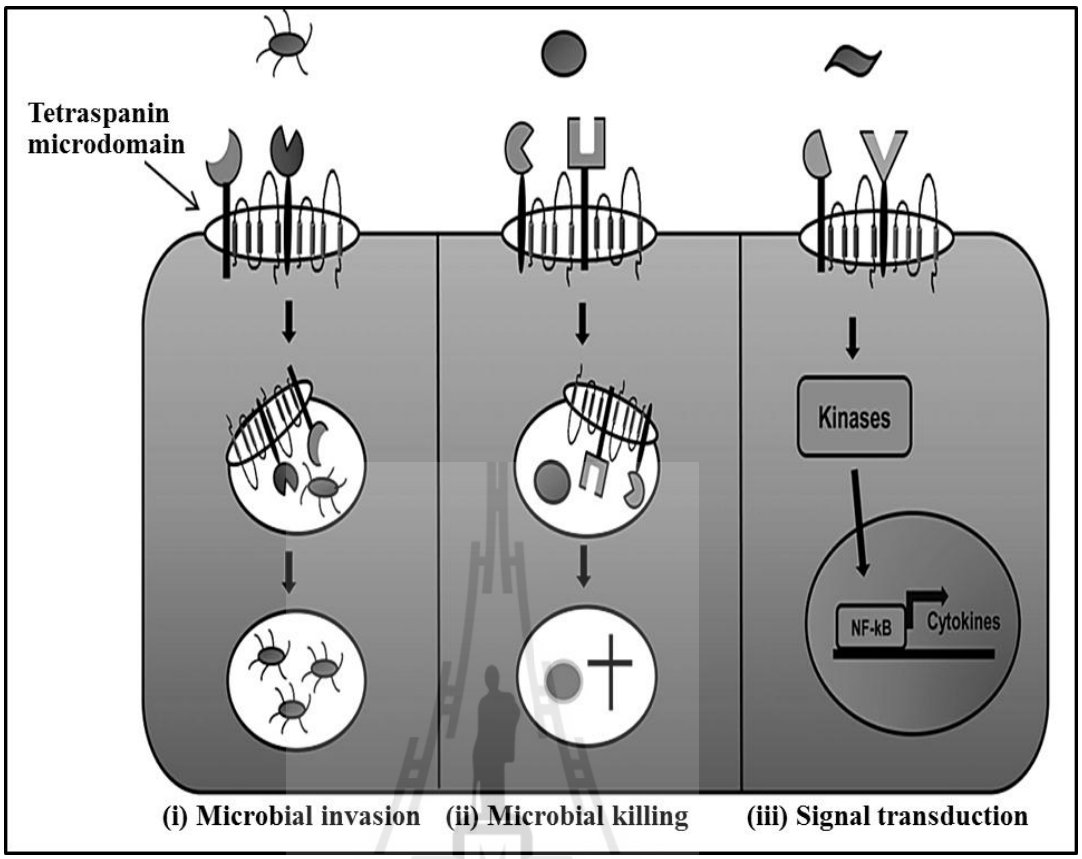

Figure 1.10 Model of three different mechanisms underlying tetraspanin involvement in microbial infection of host cells. (i) Microbes can exploit tetraspanin microdomains to enter, colonize and invade host cells. (ii) Tetraspanins may interact with phagocytic receptors that facilitate uptake and microbial killing by leukocytes. (iii) Tetraspanins can modulate signaling pathways downstream of microbial receptors that regulate gene transcription and production of cytokines by leukocytes (van Spriel and Figdor, 2010).

1.2.6.1 The CD63 molecule

Among the tetraspanins, CD63 originally identified as a protein present on the cell surface of activated blood platelet, known as platelet glycoprotein 40 (Pltgp40) and was found to be identical to ME491/CD63, an antigen associated

with human malignant (Hotta, Takahashi, and Homma, 1989; Pols and Klumperman, 2009). This molecule is also an established component of late endosomal and lysosomal membranes, known as lysosomal associated membrane protein 3 (LAMP-3) and at the plasma membrane, it cycles among these compartments (Dell'Angelica, Mullins, Caplan *et al.*, 2000). It has been found in numerous types of leukocyte cells and endo/epithelial cells, except for erythrocytes (Patnaik, Kang, Seo *et al.*, 2013). Upon cell stimulation, this molecule is mobilized to the cell surface via the exocytic pathway and gets involved in various immunophysiological processes, including cell growth, motility, signal transduction, host-pathogen interactions and cencer (Pols and Klumperman, 2009).

1.2.6.2 Structure of the tetraspanin CD63

CD63 is composed of 237 amino acids. Its gene is located on human chromosome 12q13 (Pols and Klumperman, 2009). The CD63 is tightly regulated by glycosylation with N-linked glycosylation sites, but not with O-linked glycosylation. In addition, cellular distribution studies have indicated cell-specific glycosylation patterns of the protein, as a substantial difference in the apparent size of this molecule was observed in different cell types (Ageberg and Lindmark, 2003). The molecular weight of this molecule was reported to range from 30 to 60 kDa. Moreover, the predicted molecular weight without the glycan part of the CD63 is 25 kDa (Azorsa, Hyman, and Hildreth, 1991; Tominaga, Hagiwara, Kosaka *et al.*, 2014). Althought, the complete 3D structure of human CD63 has not been determined, but CD63 structure has been reported to be a member of the tetraspanin family, consisting of four TM1-TM4 linked by EC1 and EC2 containing the CCG motif and with three potential N-linked glycosylation sites (N130, N150, and N172), short intracellular N

and C terminal tails (Figure 1.11) (Azorsa, Hyman *et al.*, 1991; Kitadokoro, Bordo *et al.*, 2001). However, the pattern of disulfide bonds has not been described by tertiary structure (3D) of this molecule (DeSalle, Mares, and Garcia-Espana, 2010).

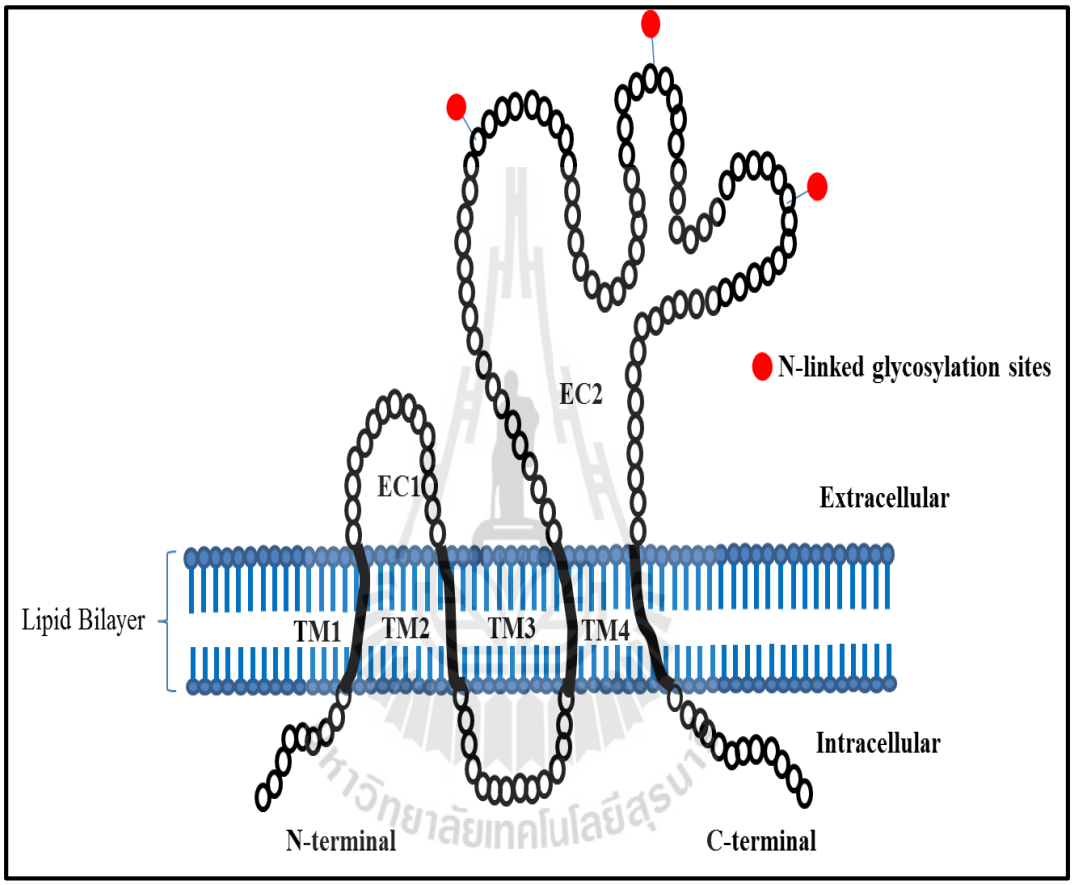

Figure 1.11 Schematic illustration of structural design of the human CD63. The short extracellular loop (EC1), the longer extracellular loop (EC2) and intracellular N-and C-terminal fragments. The transmembrane regions are highlighted in black and three N-linked glycosylation sites are depicted with EC2 region of CD63. The figure is modified from (Metzelaar, Wijngaard, Peters *et al.*, 1991; Patnaik *et al.*, 2013; Schafer, Starkl, Allard *et al.*, 2010).

1.2.6.3 The tetraspanin CD63 and partner molecules

Many studies have demonstrated that the CD63 is a component of TEMs and this molecule has diversity interacts with many different proteins, either directly or indirectly, and regulates intracellular transport and localization with in the cell (Pfistershammer, Majdic, Stockl *et al.*, 2004; Tominaga *et al.*, 2014). The interaction partners include other tetraspanins (CD81, CD82, CD9, CD151), cell surface receptors (MHC-II, CD3, FcεRI, CXCR4), several integrins (α4β1, α3β1, α6β1, LFA-1 and β2), PRRs (proHB-EGF, Dectin-1, CCR3), proteinkinases (phosphatidylinositol 4-kinase and the Src family tyrosine kinases Lyn and Hck), several subunits of adaptor protein (AP) complexes (AP-2, AP-3, AP-4) and other proteins, including L6 antigen, syntenin-1, TIMP-1, H, K-ATPase, and MT1-MMP. Presumably, the physiological functions of this tetraspanin protein are determined in large measure by its repertoire of the interacting proteins (Duffield, Kamsteeg, Brown *et al.*, 2003; Lin, Kamsteeg, Zhang *et al.*, 2008; Pols and Klumperman, 2009; Radford, Thorne, and Hersey, 1996). Several studies suggest that the interacting of CD63 and its partner molecules plays major roles in cell growth, motility, signal transduction, host-pathogen interactions and cancer. However, all physiological and signal transduction events in regulation to these association functions are not clear (Patnaik *et al.*, 2013).

1.2.6.4 The tetraspanin CD63 functions

The tetraspanin CD63 in immune system

The challenge in understanding tetraspnins function is in linking such diverse biochemical interactions with specific functions. The CD63 is know to be expressed both intracellular and surface membranes. This molecule is ubiquitously expressed

in all leukocytes such as T lymphocytes, B lymphocytes, DCs, monocytes, granulocytes, basophils, mast cells and platelets (Schroder, Lullmann-Rauch, Himmerkus *et al.*, 2009). The CD63 is also known as a biomarker for activation of several cell types including granulocytes and platelets. Though, function of the CD63 in both innate and adaptive immune response remains to be discovered. Studies of CD63 in leukocytes have currently begun to provide concrete links between partnership with specific molecules and particular functions (Jones *et al.*, 2011; van Spriel, 2011). CD63 functions in the innate immune response to the pathogenesis of a wide variety of pathogens, including bacteria, fungi, parasites, and viruses have been reported. For instance, tetraspanin CD63 found in late endosomal mutivasicular bodies (MVBs) is essential incorporated into viral particles and intracellular bacteria (Beatty, 2006; Chen, Dziuba, Friedrich *et al.*, 2008). In support of this, CD63 was first identified as gateways for Human immunodeficiency virus type 1 (HIV-1) budding at the plasma membrane and readily incorporates into HIV-1 virions. Based on experiments using mAb against CD63, or a recombinant protein containing EC2 loop of CD63 inhibits HIV infection of monocyte-derived macrophage (MDM) (Ho, Martin, Higginbottom *et al.*, 2006; von Lindern, Rojo, Grovit-Ferbas *et al.*, 2003). Moreover, the CD63 suppresses chemokine receptor CXCR4 trafficking of the cell surface, which affects HIV-1 entry into T-lymphocytes (Fu, Pan, Xie *et al.*, 2015). It has been documented that intracellular development of *Chlamydia trachomatis* depends on the interaction between CD63-positive late endocytic multi-vesicular bodies (MVBs) and the bacterial inclusion, however that CD63 itself appears not to be essential for this association (Beatty, 2006, 2008). On the other hand, the role of CD63 in inhibiting adherence of *Neisseria meningitides*,

Staphylococcus aureus, Neisseria lactamica, E. coli and *Streptococcus pneumoniae* to human epithelial cells have been observed by blocking of large extracellular loop (LEL) of CD63 using CD63 antibodies and small interfering RNAs (siRNAs) (Patnaik *et al.*, 2013). Another study demonstrated that CD63 recruitment to yeast phagosomes is dependent on phagosomal acidicfication, but occurred independently of MHC-II and LAMP-1 (Artavanis-Tsakonas, Love, Ploegh *et al.*, 2006). In basophilic granulocytes and mast cells, the CD63 interacts with the high-affinity immunoglobulin E (IgE) receptor (FcεRI) (Kraft, Fleming, Billingsley *et al.*, 2005; Sainte-Laudy, Boumediene, Touraine *et al.*, 2007). Therefore, analysis of CD63 expression in human basophilic granulocytes, the so-called basophil activating test (BAT), has been used for allergy diagnosis (Bridts, Sabato, Mertens *et al.*, 2014; McGowan and Saini, 2013; Tammaro, Narcisi, Amodeo *et al.*, 2012). Furthermore, a significant increase in CD63 surface expression was also found on neutrophils, which is observed undergoing apoptosis due either to aging or to stimulation of the Fas receptor (Beinert, Munzing, Possinger *et al.*, 2000).

CMI response is initiated by T cell engagement of peptide antigens processed by MHC I/II on the surface of APCs. Many tetraspanins interact with MHC molecules and are involved in regulation of their function (Figure 1.12) (Jones *et al.*, 2011). It has been shown that the tetraspanin CD63 is associated with MHC II molecule and these CD63-MHC class II complexes are present exclusively in MHC class II compartments (Engering, Kuhn, Fluitsma *et al.*, 2003), suggesting that this molecule is found in the complex with MHC II may be involved in the regulation of peptide loading to MHC II and present onto cell surface (Jones *et al.*, 2011). Furthermore, knocking down the CD63 in EBV-transformed

B cells increases the ability to activate $CD4^+$ T cells, an increases production of exosomes (Petersen, Odintsova, Haigh *et al.*, 2011). On T cell, there are only a few studies on function of this molecule. The CD63 is weakly expressed in resting T cell but quickly induced upon T cell activation. It has been reported that the CD63 associates with type II phosphoinositide 4 kinase in intracellular vesicles as well as infocal adhesions at the plasma membrane during T cell signaling. Binding of intact anti-CD63 mAb (11C9) or its Fab to the CD63 on T cells inhibits their responses to DCs, whereas cross-linking of the CD63 with this mAb can costimulate the activation of the TCR/CD3 complex in human T cells, suggesting that the CD63 might be involved in the delivery of a costimulatory signal (Pfistershammer *et al.*, 2004). However, the natural ligand for T cell-assosiated CD63 and molecular machanisim of the CD63 in regulation of both suppression and stimulation of T cells have not yet been identified. Function of the CD63 has also been studied in the animal model and found that the development of immune cells of the CD63-deficient mice is normal, however functional studies have not been reported. In addition, these mice exhibit morphologic changes in the collecting ducts in the kidneys, which then leads to a disturbed water balance (Schroder *et al.*, 2009). Additionally, loss of P-selectin dependent leukocyte rolling and a failure of leukocyte extravasation in a peritonitis model are found in CD63 knockout mice, indicating that the CD63 is an important component of inflammation (Doyle, Ridger, Ferraro *et al.*, 2011).

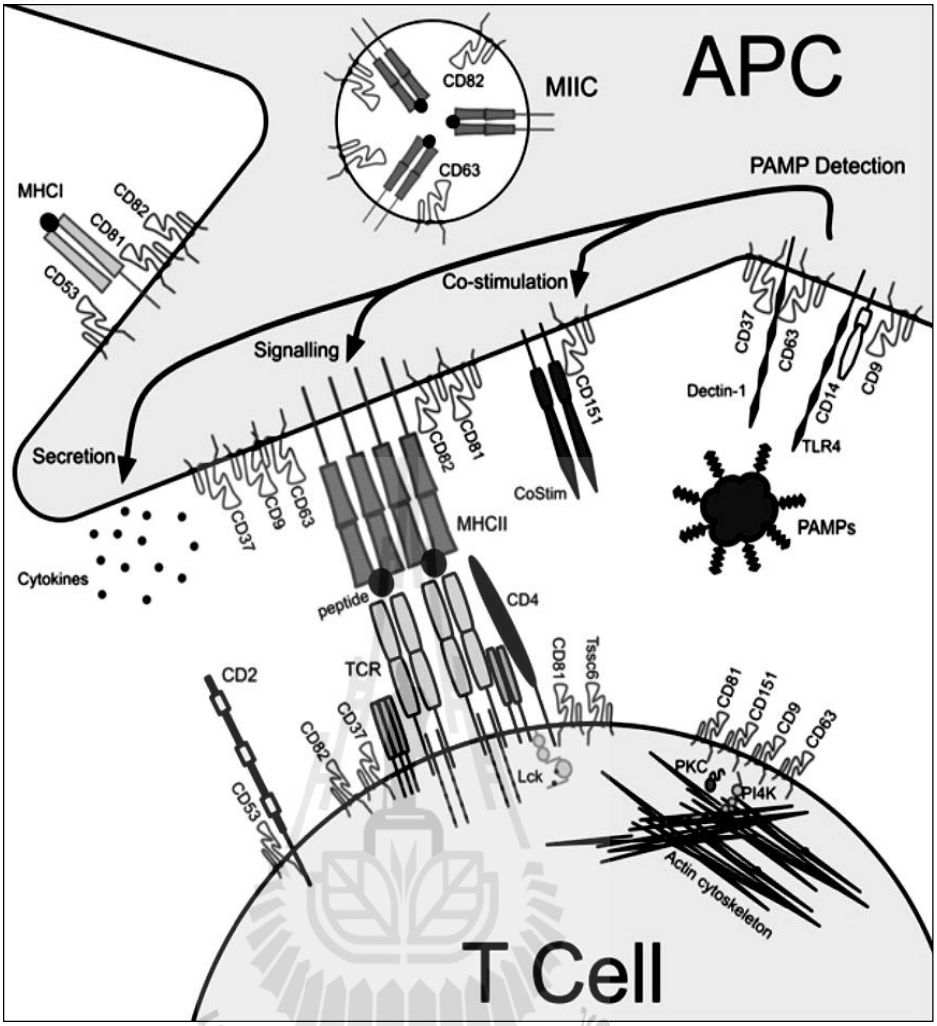

Figure 1.12 Tetraspanin CD63 in antigen presentation and T-cell activation. An APC contains MHC II compartments (MIICs) is derived following phagocytosis of exogenous pathogens and contains resident tetraspanins CD63 as well as CD82. Furthermore, CD63 or CD37 associated with Dectin-1, as PRRs partners, recognize their respective PAMPs. In the other hand, microbial phagocytosis and antigen processing occur and microbial antigen is then loaded onto MHC II. Peptide–MHC II cluster is presented on cell surface together with the CD63 and other tetraspanins (CD82, CD81, and CD9) in a close association. In addition, the CD63 also associates with PI4K, which is linked to the actin cytoskeleton (Jones *et al.*, 2011).

1.2.7 Thesis objectives

1. To study cellular distribution of the mAb COS3A recognizing molecule
2. To identify and investigate some biochemical charecteristics of the molecule recognized by the mAb COS3A
3. To study immunological function of the molecule recognized by the mAb COS3A using the mAb COS3A as a tool

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strain and vectors

Escherichea coli (*E. coli*) strain DH5α was purchased from Gibco (Grand Island, NY, USA), PQE-tri system vector was purchased from Qiagen (Valencia, CA, USA) and pEGFP-N1 vector was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA).

2.1.2 Cell preparation and cell lines

2.1.2.1 Cell lines

Human T cell lines (Molt4, SupT1, and Jurkat), B cell lines (Daudi, Ramos and Raji), myeloid cell lines (U937, KG-1a, and THP-1) and erythriod cell lines (K562) were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 40 mg/ml gentamicin, and 2.5 mg/ml amphotericin B. Monkey Kidney cells (COS7), which is non-hematopoietic cell line, were maintained in DMEM supplemented with 10% heat inactivated FCS 40 mg/ml of gentamicin, and of 2.5 mg/ml amphotericin B. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C and passaged three times a week.

2.1.2.2 Isolation of peripheral blood mononeuclear cells (PBMCs) using Ficoll-Hypaque gradient centrifugation

PBMCs were isolated by density gradient centrifugation. Briefy, heparinized whole blood from normal healthy donors were diluted 1:2 with sterile PBS and then gently overlaid onto 10 ml of Ficoll-Paque (GE Healthcare Life Sciences, Uppsala, Sweden) in 50 ml centrifuge tube and cells were centrifuged at 400 g, 25°C for 30 min without brake. The PBMCs at the interphase of plasma and Ficoll-Paque solution were carefully collected. The PBMCs were then transferred into the new centrifuge tube and washed with 10 ml of sterile PBS for 3 times by centrifugation at 1,500 rpm, 25°C for 5 min. The PBMCs were counted and adjusted into the proper medium or buffer.

2.1.2.3 T cell purification

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. Monocytes were then removed using hyperosmolar Percoll gradient (density=1.064). PBMCs (5×10^7 cells/ml) were re-suspended in 3 ml of sterile PBS before overlaying onto 10 ml of hyperosmolar Percoll solution (4.15 ml of ddH_2O, 4.85 ml of Percoll (GE Healthcare Life Sciences, Uppsala, Sweden) and 1 ml of 1.6 M of NaCl) in 15 ml centrifuge tube. The cells were isolated by centrification at 2000 rpm, 25°C for 40 min without brake. The remaining cells at the bottom of tube containing lymphocytes were then transferred into the new centrifuge tube and washed with sterile PBS pH 7.2 for 3 times by centrifugation at 1,500 rpm, 25°C for 5 min. To obtain the $CD3^+$T cells, the lymphocyte fraction were then subjected for a negative depletion of B lymphocytes, monocytes, and natural killer cells using magnetic cell sorting (MACS, miltenyi biotec, Bergisch-Gladbach, Germany). To perform magnetic

cell sorting, the cells were incubated with different anti-human mAbs specific to CD14, CD16, CD20, and CD56 for 30 min on ice. The stained cells were then washed twice with cold 0.5% BSA-PBS-2mM EDTA and centrifuged at 300 g for 5 min and were then incubation with goat-anti mouse IgG conjugated with micro magnetic beads (MACS, miltenyi biotec, Bergisch-Gladbach, Germany) for 30 min at 4°C. After, the stained cells were washed and resuspended in 500 µl of 5% BSA/2 mM EDTA in PBS and were then applied onto LD column. The column was then washed twice of 5% BSA/2 mM EDTA in PBS 2 ml. The unbound purified T cell fraction was collected into new tube. The purity of CD3 positive T cells were greater than 95% as estimated by immunofluorescence staining using FITC-conjugated anti-CD3 mAb, PE-conjugated anti-CD56 mAb, FITC-conjugated anti-CD14 mAb and PE-conjugated anti-CD19 mAb and analyzed flow cytometry.

2.1.3 Antibodies

MAb COS3A, anti-bacteriophage protein 13M (anti-13M) and CD147 mAb clone M6-1E9 are IgG2a isotype, CD3 mAb clone OKT3 (IgG1 isotype), CD14 mAb clone MT14/3 (IgG1 isotype), These mAbs were produced in the laboratory of Prof. Dr. Watchara Kasinrerk, Faculty of Associated medical science, Chiang Mai University. CD16 mAb clone LN-K16, CD20 mAb clone MEM-97, and CD63 mAb clone MEM-259 are IgG1 isotype, CD56 mAb clone MEM-188 (IgG2a isotype) were kindly provided from Prof. Dr. Vaclav Horejsi, Institute of Molecular Genetics, Prague, Czech Republic. CD28 mAb clone CD28.2 was purchased from Biolegend (San Diego, CA, USA). HRP-conjugated-rabbit-anti-mouse Igs was purchased from Dako (Glostrup, Denmark), FITC-conjugated goat F(ab')2 anti–mouse immunoglobulinsn antibodies were purchased from Chemicon (Victoria, Australia).

PE-conjugated anti-CD19 mAb clone HIB19, PE-conjugated anti-IL-2 mAb clone MQ1-17H12 and PE-conjugated anti-CD25 mAb clone BC96 were purchased from eBioscience (San Diego, CA, USA), PE-conjugated anti-CD56 mAb clone MEM188, FITC-conjugated anti-CD3 mAb clone HIT3a, PerCP-conjugated anti-CD3 mAb clone UCHT1, PE-conjugates anti-CD14 mAb clone HCD14, PE-conjugated anti-IL-10 mAb clone JES3-19F1 and PE-conjugated IFN-γ mAb clone B27 were purchased from BioLegend (San Diego, CA, USA), Alexa Fluor® 488 conjugated F(ab')2 fragment of goat anti-mouse IgG (H+L) and Dynabeads goat-anti mouse IgG were purchased from Molecular Probes-Invitrogen. Micro magnetic beads conjugated goat-anti-mouse IgG was purchased from Miltenyi Biotech (Bergisch Gladbach, Germany).

2.1.4 Chemicals and Materials

Acrylamide, ammonium persulfate, aprotonin, bis-*N*, *N*''-methylenebisacrylamide, 2-β-mercaptoethanol, bovince serum albumin (BSA), bromophenol blue, Coomassie brilliant blue G250, ethylenediamine tetra-acetic acid (EDTA), *N*, *N*′, *N*″, *N*‴-tetramethylethylenediamine (TEMED), glycerol, Iodoacetamide, lipopolysacchariles (LPS) from *E. coli* 055:B5, Propidium iodide (PI), pepstatin A, phorbolmyristate, acetate, Saponin and Tris-base were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfozide (DMSO) was purchased from Bio Basic lnc (Makham Ontario, Canada), ampicillin, phenylmethyl sulfonyl fluoride (PMSF), Triton X-100 were purchased from USB Corporation (Cleveland, OH, USA). Skimmed milk, Tryptone, yeast extract powder and agar powder were purchased from Himedia Laboratories (Marg, Mumbai, India). Cyanogen bromide-activated (CNBr) Sepharose 4B, Ficoll-Hypaque solution, Percoll

PLUS were purchased from GE Healthcare Life Sciences (Uppsala, Sweden). Prestained standards protein markers were purchased from Fermentas (MA, USA). Heparin was purchased fromLio (Ballerup, Denmark). Ammonium sulphate, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphaste, orthophosphoric acid 85%, glycerine, methanol and ethyl alcohol were purchased from Carlo Erba (Rodano, Milano, Italy). 3-Aminophthalydrazide and Paraformaldehyde were purchased from Fluka (Buchs, Switrland). Monensin, human IL-2, IL-10, or IFN-γ Enzyme-Linked Immuno-Sorbant Assay (ELISA) MAX™ Deluxe kits were purchased from BioLegen (San Diego, CA, USA), tween-20 was purchased from Scharlau chemie SA. (La Ja, Barcelona, Spain). Carboxyflorescein succinimidyl ester (CFSE), *4',6-diamidino-2-phenylindole, dilactate* (DAPI, dilactate), DNA miniprep kit, PCR purification kit and Ribonuclease A (RNase A) were purchased from Molecular probes-Invitrogen (Camarillo,CA,USA), N-glycosidase F was purchased from Roche Applied Science (Mannheim, Germany). Phytohemaglutinin (PHA) was purchased from Biochrom AG (Berlin, Germany). Tunicamycin (TM) was purchased from R&D Systems (Minneapolis, MN, USA). FACs lysis solution, FACS clean solution and FACS rinse solution were purchased from BD Bioscience (San Jose, CA, USA). Developer, replenisher and half-speed blue x-ray film were purchased from Kodak (NY, USA). T4 DNA ligase, BamH-1 and Not1 restriction endonuclease were purchased from New England Biolabs (Beverly, MA, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from PALL (East Hill, NY, USA). LD columns and MACS Separators were purchased from Meltenyi Biotech (Bergisch Gladbach, Germany).

2.1.5 Instrumentation

The instruments were used in this experiment, including of a shaking incubator (MRC, Holon, Israel), a Thermomixer comfort and centrifuge 5415 R. (Eppendorf AG, Hamburg, Germany), a microcentrifuge Denville 26OD (Denville Scientific, Metuchen, NJ, USA). AlRsi laser scanning microscope system (Nikon, Tokyo, Japan), CX21 light microscope (Olympus, New York, USA), CKX41 inverted microscope (Olympus, New York, USA), DP50 fluorescent microscope (Olympus, New York, USA), a High-speed microentrifuge CF16RXII (Hitachi, Tokyo, Japan). Biosafety laminar flow class II (Esco Lifesciences, Thailand), Forma series II water jacket CO_2 incubator and Forma -80°C ULT freezer (Thermo Fisher Scientific, USA). Protein gel electrophoresis apparatus, PCR thermocycler, Myrun DNA electrophoresis apparatus with power supply. Thermomixer, Trans-blot SD semi-dry cell transfer (Bio-Rad, USA). BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.2 Methodology

2.2.1 Cellular distribution of the mAb COS3A recognizing molecule

2.2.1.1 Indirect immunofluorescence staining

Cells (1×10^7cells/ml) or 0.3% RBCs in PBS pH 7.2 were pre-incubated with 10% human AB serum in staining buffer (1% BSA and 0.02% NaN_3 in PBS) on ice for 30 min to block cell surface Fc receptor. Fifty microliters of either mAb COS3A or isotype-matched control mAb anti-13M at a final concentration of 20 µg/ml was added into an equal volume of the cell suspension. The cells were washed twice with the staining buffer. Afterward, 25 µl of FITC-conjugated goat anti-mouseIgG at a dilution of 1:100 were added, mixed and incubated on ice for 30 min in dark place.

Finally, the cells were washed with the staining buffer for 3 times before fixing with 1% paraformaldehyde in PBS. The stained cells were then analyzed using a BD FACSCalibur flow cytometer**.** For COS7 cells, the stained cells were analyzed using an A1Rsi laser scanning microscope system.

2.2.1.2 Intracellular of staining

COS7 cells ($5x10^4$cell/ml) were grown on coverslips in DMEM supplemented with 10% FCS, 40 µg/ml gentamicin and 2.5 µg/ml amphotericin B in a humidified atmosphere of 5% CO_2 at 37°C. After one day of cultivation the adhering cells were washed, and then fixed with 4% formaldehyde in PBS pH 7.4 for 15 min at room temperature and treated with permeabilizing buffer (0.1% TritonX-100 in PBS pH 7.4) for 5 min on ice. The cells were stained by adding 100 µl of permeabilizing buffer containing either mAb COS3A or isotype-matched control mAb anti-13M at a final concentration of 20 µg/ml. After 30 min on ice, the cells were washed and then incubated on ice for another 30 min with AF488-conjugated goat anti-mouse IgG. DAPI was used for nuclear staining according to manufacturer's instruction. After washing, the stained cells on coverslips were mounted with 50% glycerol in PBS pH 7.2 on glass slides, and sealed with nail polish. The cells were examined under an A1Rsi laser scanning microscope system from Nikon.

2.2.1.3 Immunofluorescence staining of the PHA activated PBMCs

PBMCs (1×10^6cells/well) were unstimulated or stimulated with 5 µg/ml of PHA in RPMI-1640 supplemented with 10% heated inactivated FCS and antibiotic and cultured in a 5% CO_2 incubator at 37°C for 1 and 3 days. The cells were harvested and stained with 20 µg/ml of the mAb COS3A for 30 min at 4°C. After 30 min on ice, the cells were washed and then incubated on ice for another 30 min

with AF488-conjugated goat anti-mouse IgG. Finally, the cells were washed with the staining buffer for 3 times before fixing with 1% para-formaldehyde in PBS. The stained cells were then analyzed by flow cytometry.

2.2.2 Biochemical characterization of the mAb COS3A recognizing molecule

2.2.2.1 Western blotting

U937 cells (1×10^7 cells) were harvested, washed and solubilized for 30 min on ice in 1 ml of lysis buffer (1 % Triton X-100, 50 mM Tris-HCl buffer pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM Phenylmenthylsulfonyl (PMSF), 2 µM pepstatin A, 10 µg/ml aprotinin and 5 mM iodoactamide). Clear cell lysates were obtained by centrifugation at 14,000 rpm at 4°C for 10 min. The cell lysates were mixed with 5× non-reducing or reducing Laemmli sample bufferto get the final concentration of 1× buffer and were then boiled for 5 min. The proteins of cell lysates were resolved by SDS-PAGE on a 10% separating gel and then electrophoretically transferred on to PVDF membrane by semi-dry electrophoretic blotting system at 15 volts for 1 h. The membranes were blocked for 1 h in 5% skimmed milk in PBS at RT and were then rinsed twice with PBS before incubation for an additional 1 h with 2 µg/ml of the mAb COS3A or isotype-matched control mAb anti-13M at RT. The membrane were washed 5 times with washing buffer (0.1% Tween 20 in PBS) and then incubated with HRP-conjugated rabbit anti-mouse immunoglobulins in 1% skimed milk in PBS at RT for 1 h. After thorough washing, the signals were detected by enhanced chemiluminescence method.

2.2.2.2 Tunicamycin (TM) treatment

For TM treatment, U937 cells ($1x10^6$ cell/ml) were cultured in 6 well plates (2 ml/well) in RPMI-1640 medium with or without tunicamycin at a final

concentration of 5 µg/ml for 48 h of cultivations, The cells were harvested and washed three times with PBS by centrifugation at 1,500 rpm for 5 min at RT. The cells were further subjected to cell lysis and Western blotting or immunoprecipitation.

2.2.2.3 N-glycosidase F treatment

The immunoprecipitated protein was dissociated from the beads by adding of 80 µl of 20 mM phosphate buffer containing 1% SDS boiled for 5 min. The supernatant containing proteins were collected into a new tube by centrifugation at 12,000 rpm for 20 secounds. After that, 160 µl of 20 mM phosphate buffer containing 1% Triton X-100 was then added. To remove N-linked sugar five units of N-glycosidase F were added into the eluates followed by incubation for 16 h at 37°C. The deglycosylated proteins were analyzed by Western blotting.

2.2.3 Identification of the mAb COS3A recognizing molecule

2.2.3.1 Preparation of the mAb COS3A coupled beads

The CNBr sepharose beads were prepared by swirling the bead powders in 1 mM HCl solution. The slurry activated CNBr sepharose beads were washed three times with PBS pH 7.4. The activated beads were then resuspended in 1 ml of coupling buffer (0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl) containing one milligram of the mAb COS3A at RT for another 3 h. To stop the coupling reaction and an equal volume of 50 mM glycine in PBS was added and rotated at 4°C for 15 min. The mAb coupled beads were then washed twice with PBS pH 7.4 and stored at 4°C.

2.2.3.2 Cell lysate preparation

U937 cell lines ($1x10^6$ cells) in a condition of tunicamycin treatment as described in 2.2.2.2. The cells ($5x10^7$ cells) were then harvested, washed and

solubilized for 30 min on ice in 1 ml of lysis buffer (1% TritonX-100, 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 1 mM PMSF, 2 µM pepstatin A, 5mM iodoacetamide, and 10 µg/ml aprotinin). The clarified lysate were obtained by centrifugation at 16,000 g, for 20 min at 4°C.

2.2.3.3 Immunoprecipitation

U937 cell lysates were precleared with CNBr-activated Sepharose beads coated with human immunoglobulins. The pre-cleared cell lysates were then incubated with CNBr-activated Sepharose beads coated with mAb COS3A or isotype-matched control mAb anti-13M and rotated for 3 h at 4°C. After five times washing, the proteins were eluted from the beads by addition of 1× non reducing sample buffer boiled for 5 min and then resolved by 10% SDS-PAGE. The protein bands were examined either by Western blotting (2.2.2.1) or blue-silver Coomassie staning. For blue-silver Coomassie staning, the electrophoresis wet gels were fixed in fixative buffer (40% ethanol, 10% acetic acid) for 1 h at RT and washed twice with double-distilled water for 5 min. The fixed gel were then incubated with blue-silver Coomassie stain (0.12 % Coomassie brilliant blue G-250, 10% ammonium sulphate, 10% o-phosphoric acid and 20% methanol) (Candiano, Bruschi, Musante *et al.*, 2004) overnight at RT and de-stained by washing twice with double-distilled water for 10 min. The protein bands of interest were cut out from the SDS-PAGE gel and sent for amino acid sequencing by liquid chromatography mass spectrometry (LC/MS) at 1st BASE Services Company.

2.2.4 Effect of the mAb COS3A on phagocytosis

2.2.4.1 Preparation of phagocytosable particles

Green fluorescent protein (GFP) expressing bacteria were used as phagocytoable particles. To generate this bacterium, the gene coding GFP was subclone from pEGFP-N1 vector by restriction enzyme digestion and inserted into the pQE-Tri expression vector at the sites of *NotI* and *BamHI* using Quick ligase from New England Biolabs (Bever, MA, USA). The recombinant vector was then transformed into *E. coli* strain DH-5α competent cells. The transformed bacteria were grown in Luria-Bertani broth supplemented with 0.01 µM of ampicillin for 18 h at 37°C and subsequently heat killed at 60°C for 30 min. The fluorescence intensity of the heat-killed bacteria was checked by flow cytometry before use. The same strain of *E. coli*, transformed into pQE-Tri empty vector, was used as a negative control. The bacterial cell suspension used in this study had an optical density at 600 nm (A_{600}) of 0.6.

2.2.4.2 Phagocytosis analysis by flow cytometry

Phagocytosis was determined using flow cytometry as previously described (Singboottra *et al.*, 2010). Briefly, 100 µl of blood EDTA-treated whole blood was mixed with 100 µl of *E. coli* strain DH-5α expressing GFP (dilution 1:300 of cell suspension with $A_{600=0.6}$ in PBS pH 7.2) in the absence or presence of the mAb COS3A or isotype matched control mAb anti-13M at a final concentration of 20 µg/ml. The cells were incubated at 37°C or on ice for 1 h and then immediately placed on ice. Fifty microliters of RBC lysing solution containing 4.5% formaldehyde and 10% diethylene glycol in PBS pH7.2 was added and incubated on ice for an additional 10 min, then 1 ml of distilled water was added to complete the lysis. White blood cells

were collected by centrifugation at 14,000x g for 30 seconds. The cells were washed twice with PBS pH 7.2 containing 0.1% NaN_3. The cells were resuspened in PBS pH 7.2 containing 1% paraformaldehyde and phagocytosis was analyzed by using a BD FACSCalibur flow cytometer. Fractional of phagocytosis were obtained from the following equation.

$$\%\ Phagocytosis = \frac{(number\ of\ GFP\ positive\ cells\ at\ 37℃ - number\ of\ GFP\ positive\ cells\ at\ 0℃)_{with\ mAb}}{(number\ of\ GFP\ positive\ cells\ at\ 37℃ - number\ of\ GFP\ positive\ cells\ at\ 0℃)_{without\ mAb}} \times 100$$

2.2.5 Effect of the mAb COS3A on T cell activation and cytokine production

2.2.5.1 Fluorescent cell labeling using carboxyfluorescein diacetate succinimidylester (CFSE)

For cell proliferation assay, PBMCs or purified $CD3^+$T cells were washed with PBS pH 7.2 for 3 times and a final concentration of the cells was adjusted to 1 x 10^7 cells/ml in PBS pH 7.2. One hundred microliters of CFSE stock solution in DMSO were added into the suspension cells drop wise to get a final concentration of 0.5 µM. The cells were mixed by tapping and incubated for 10 min at 37°C. The lebeled cells were then washed twice with pre-cooled RPMI-1640 medium. The CFSE-labeled cells were counted and resuspened in a proper culture medium. Effeciency of the CFSE labeling was checked by using a BD FACSCalibur flow cytometer.

2.2.5.2 Effect of the soluble mAb COS3A concentration on cell proliferation

The CFSE-labeled PBMCs (1×10^5 cells/well) were stimulated with immobilized anti-CD3 mAb (OKT3; 60 ng/ml) in the presence of the soluble mAb COS3A at seven different concentrations (0.78, 1.5625, 3.125, 6.25, 12.5, 25.0, and 50.0 µg/ml) or 50 µg/ml of isotype-matched control mAb anti-13M. The cells were

cultured for 5 days in a 5% CO_2 incubator at 37°C. The cells were then harvested and cell proliferation was analyzed by monitoring the reduction in CFSE using a BD FACSCalibur flow cytometer.

2.2.5.3 Effect of the soluble mAb COS3A on cell proliferation and cytokine production in PBMCs model

The CFSE-labeled PBMCs (1×10^5 cells/well) were unstimulated or stimulated with immobilized anti-CD3 mAb (OKT3; 60 ng/ml) in the absence or presence of themAb COS3A, or MEM-259 (CD63 mAb), or isotype-matched control mAb anti-13M at a final concentration of 12.5 µg/ml for 5 days in a 5% CO_2 incubator at 37°C. The cells of each condition were harvested, washed with PBS and determined cell proliferation by monitoring the reduction in CFSE by using a BD FACSCalibur flow cytometer.

For cytokine measurement, culture supernatant were harvested after 24 h of cultivation and the concentration of IL-2, IFN-γ or IL-10 were measured using human IL-2, or IFN-γ or IL-10 ELISA MAX™ Deluxe kits.

2.2.5.4 Effect of the soluble mAb COS3A on IL-2 receptor (CD25) expression

The PBMCs (1×10^5 cells/well) were unstimulated or stimulated with immobilized anti-CD3 mAb (OKT3; 60 ng/ml) in the absence or presence of the mAb COS3A, MEM-259 (CD63 mAb), or isotype-matched control mAb anti-13M at a final concentration of 12.5 µg/ml for 2 days in a 5% CO_2 incubator at 37°C. The cells were washed twice and then surface stained with PE-conjugated anti-CD25 mAb for 30 min at 4°C. The cells were subsequently analyzed using a BD FACSCalibur flow cytometer.

2.2.5.5 Intracellular cytokine staning

PBMCs (5×10^5 cells/well) were unstimulated or stimulated with immobilized anti-CD3 mAb (OKT3; 60 ng/ml) in the presence of LPS at a final concentration 0.5 μg/ml, or mAb COS3A, or MEM-259 (CD63 mAb), or isotype matched control mAb anti-13M at a final concentration of 12.5 μg/ml. The cells were cultured in 5% CO_2 incubator at 37°C for 1 h before adding of 0.5 μg/ml monensin and further cultured for another 6 h in the same condition. After cultivation the cells were washed twice and surface stained with PerCP-conjugated anti-CD3 and FITC-conjugated anti-CD14 mAbs for 30 min at 4°C. The stained cells were fixed in 200 μl fixation buffer (4% paraformaldehyde in PBS) for 20 min at RT. The fixed cells were then washed twice with PBS and incubated for another 15 min at RT with a permeabilization buffer (5% FBS, 0.1% saponin, and 0.02% NaN_3 in PBS). The intracellular cytokines were determined by staining with PE-conjugated anti-human IL-2, or PE-conjugated anti-human IFN-γ, or PE-conjugated anti-human IL-10, for 30 min at 4°C. The cells were subsequently analyzed by a BD FACSCalibur flow cytometer.

2.2.5.6 Effect of neutralization of IL-10 on suppression of cell proliferation by the mAb COS3A

The CFSE-labeled PBMCs (1×10^5 cells/well) were unstimulated or stimulated with immobilized anti-CD3 mAb (OKT3; 60 ng/ml) in the presence of 12.5 μg/ml of the mAb COS3A in combination of the anti-IL10 mAb, or IgG_1 isotype control mAb at a final concentration of 12.5 μg/ml and the cultured for 3 days in a 5% CO_2 incubator at 37°C. The cells of each condition were harvested washed with PBS and cell proliferation was examined by monitoring the reduction of CFSE by flow cytometry.

2.2.5.7 Effect of the soluble mAb COS3A on T cell proliferation and cytokine production in T cell model

The CFSE-labeled $CD3^+T$ cells (1×10^5 cell/well) were unstimulated or stimulated with immobilized anti-CD3 mAb (OKT3; 60 ng/ml) in the absence or presence of the mAb COS3A, or mAb MEM-259, or anti-CD28 mAb, or isotype-matched control mAb anti-13M at a final concentration of 12.5 µg/ml for 5 days in a 5% CO_2 incubator at 37°C. The cells of each condition were harvested, washed with PBS and determined cell proliferation by monitoring the reduction of CFSE by flow cytometry.

For cytokine measurement, culture supernatant were harvested after 72 h of cultivation and the concentration of IL-2, IFN-γ, or IL-10 were measured using human IL-2, or IFN-γ or IL-10 ELISA MAX™ Deluxe kits.

2.2.5.8 Co-stimulation assay and cytokine production

Co-stimulation assay was performed as previously described (Pfistershammer *et al.*, 2004). Briefly, CFSE-labeled $CD3^+T$ cells (1×10^5 cells/well) were unstimulated or stimulated with immobilized anti-CD3 (OKT3; 1 µg/ml) alone or co-immobilized with 2 µg/ml of the mAb COS3A, or mAb MEM-259, or anti-CD28 mAb, or isotype-matched control mAb anti-13M for 5 days in a 5% CO_2 incubator at 37°C. The cells of each condition were harvested, washed with PBS and determined cell proliferation by monitoring the reduction of CFSE by flow cytometry.

For cytokine measurement, culture supernatant were harvested after 72 h of cultivationand the concentration of IL-2, IFN-γ, or IL-10 were measured using human IL-2, or IFN-γ or IL-10 ELISA MAX™ Deluxe kits.

2.2.5.9 Cell cycle assay

Purified $CD3^+$ T cells were cultivated as co-stimulatory assay as previously described (2.2.5.8). After 5 day of cultivation, the cells were harvested and washed with cold PBS for 3 times, the cell pellets were then, resuspended by adding 300 µl of PBS. The cells were then fixed by adding 700 µl absolute ethanol and incubate on ice 30 min. The fixed cells were washed twice and before adding 1 ml of PI solution (20 µg/ml PI, 0.1% Triton X-100, 2 mM EDTA, 60 µg/ml RNase). The cells were then analyzed by flow cytometry.

2.2.5.10 Statistics

Data analysis was performed using an unpaired *t test*. Results were considered significant if $p < 0.05$ (***, $p < 0.001$; **, $p<0.01$; *, $p < 0.05$).

CHAPTER III

RESULTS

3.1 Cellular expression of the mAb COS3A recognizing molecule

The mAb COS3A was generated during the study of three different immunogen preparation strategies for production of anti-CD4 mAbs (Pata *et al.*, 2009). This mAb, however, was not specific for CD4. As the mAb were produced using the COS7 cell transfection system, we first investigated whether the mAb COS3A reacted with COS7 cell proteins. By surface staining and intracellular staining revealed that the mAb COS3A reacted strongly both to cell surface proteins (Figure 3.1) and to intracellular protein in the monkey kidney cell line, COS7 (Figure 3.2).

Moreover, the cell surface analysis by immunofluorescence staining and flow cytometry of various human hematopoetic cells and hematopoietic cell lines including of human T cell lines (Molt4, SupT1, and Jurkat), B cell lines (Daudi, Ramos, and Raji), monocyte cell lines (U937, THP-1, and KG-1a) and erythriod cell lines (K562) were performed. Fascinatingly, the mAb bound specifically to antigens on surface of various human hematopoietic cell lines (Figure 3.3) monocytes, granulocytes, lymphocytes but not on red blood cells (Figure 3.4). These results suggested a close homology between monkey and human forms of the target molecule. Additionally, cellular expression of this molecule was strongly increased upon phytohemaglutinin

(PHA) activation for up to 72 hours (Figure 3.5) indicating that it is an activation-associated molecule.

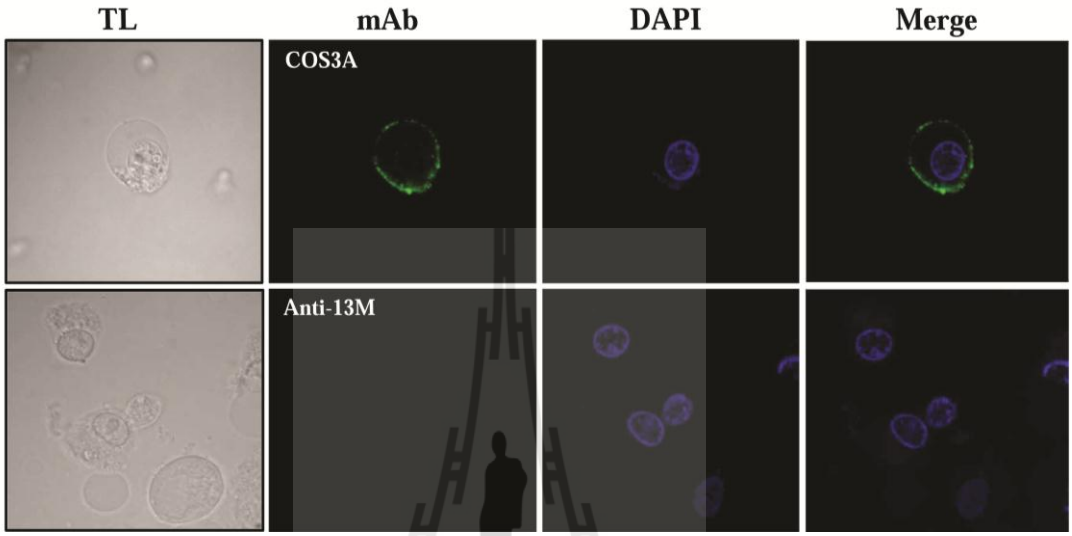

Figure 3.1 Cell surface expression of the molecule recognized by the mAb COS3A on COS7 cells. COS7 cells were stained with either mAb COS3A or an isotype matched control, mAb anti-13M by indirect immunofluorescence using AF488-labeled goat anti-mouse IgG antibody as a secondary antibody. Cell nuclei were shown by DAPI staining. The analysis was performed using an A1Rsi laser scanning microscopy system from Nikon with 100× magnification. In addition to the fluorescence a transmitted light (TL) image was recorded. A representative result from one of three independent experiments is shown.

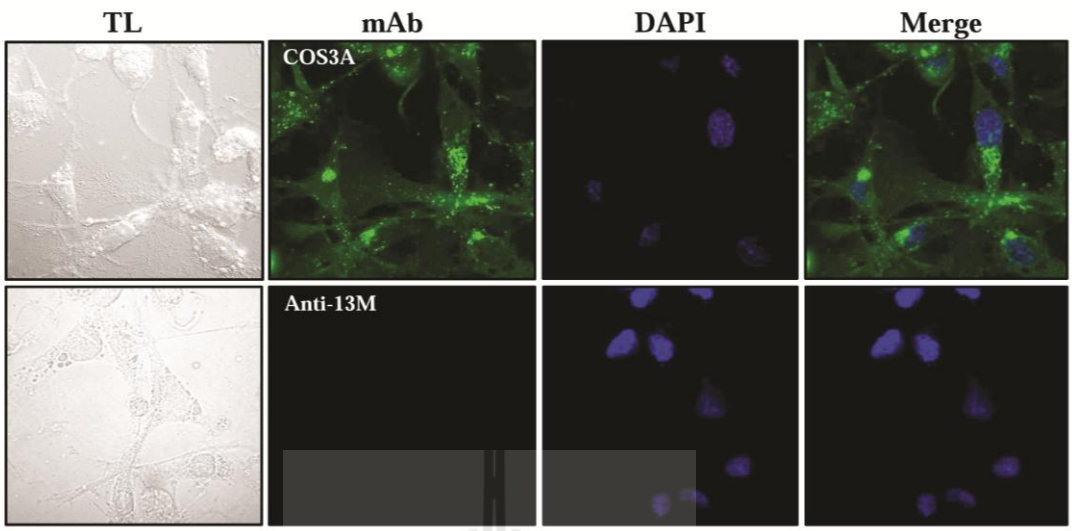

Figure 3.2 Intracellular expression of the molecule recognized by the mAb COS3A in COS7 cells. COS7 cells were fixed, permeabilized, and stained on coverslips with either mAb COS3A or an isotype matched control, mAb anti-13M by indirect immunofluorescence using AF488-labeled goat anti-mouse IgG antibody as a secondary antibody. Cell nuclei were shown by DAPI staining. The analysis was performed using an A1Rsi laser scanning microscopy system from Nikon with 100× magnification. In addition to the fluorescence a transmitted light (TL) image was recorded. A representative result from one of three independent experiments is shown.

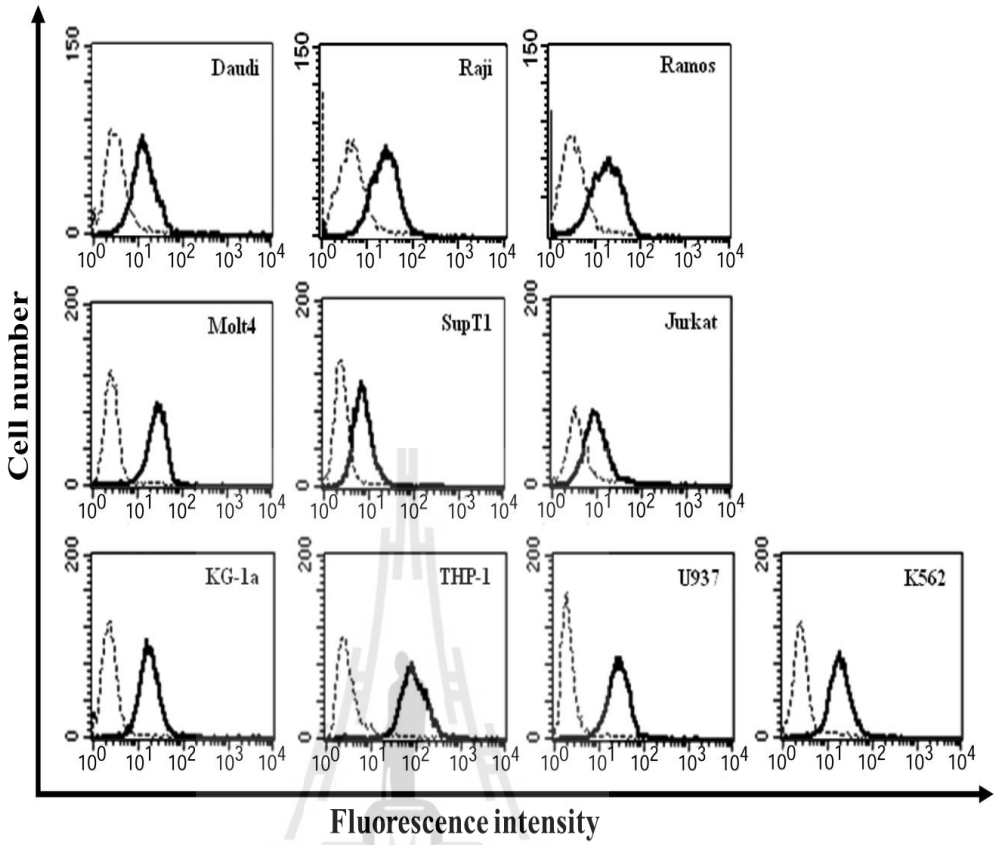

Figure 3.3 Cell surface expression of the mAb COS3A recognizing antigen on hematopoietic cell lines. Human T cell lines (Molt4, SupT1, and Jurkat), B cell lines (Daudi, Ramos, and Raji), monocyte cell lines (U937, THP-1, and KG-1a) and erythriod cell line (K562) were stained by indirect immunofluorescence with 20 µg/ml of mAb COS3A as the primary antibody. FITC-conjugated goat anti-mouse IgG antibody was used as the secondary antibody, and analyzed by flow cytometry. The results with conjugate control are shown as dashed lines, while cells stained with the mAb COS3A are represented by solid lines. A representative result from one of three independent experiments is shown.

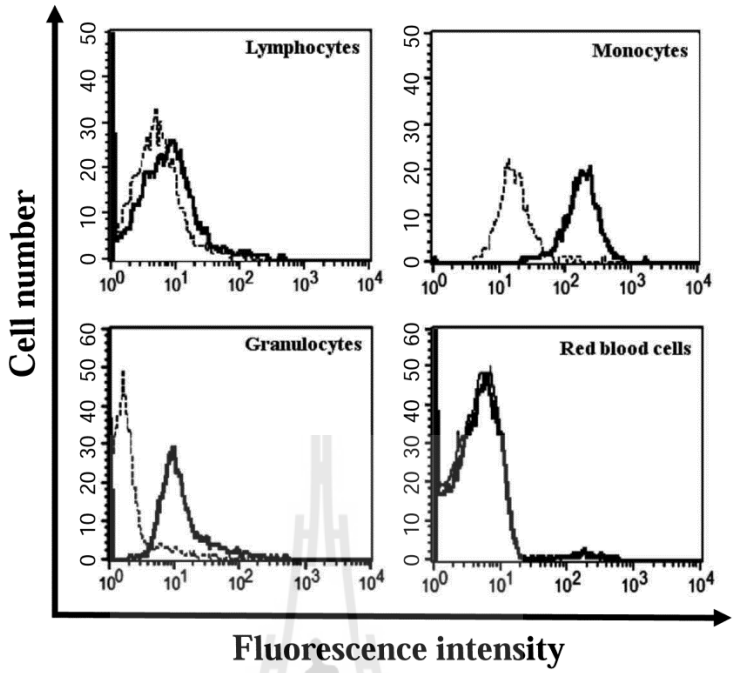

Figure 3.4 Cell surface expression of the mAb COS3A recognizing antigen on peripheral blood lymphocytes, monocytes, granulocytes and red blood cells. The cells were stained by indirect immunofluorescence with 20 µg/ml of mAb COS3A as the primary. FITC-conjugated goat anti-mouse IgG antibody was used as the secondary antibody, and analyzed by flow cytometer. The results with conjugate control are shown as dashed lines, while cells stained with the mAb COS3A are represented by solid lines, respectively. A representative result from one of three independent experiments is shown.

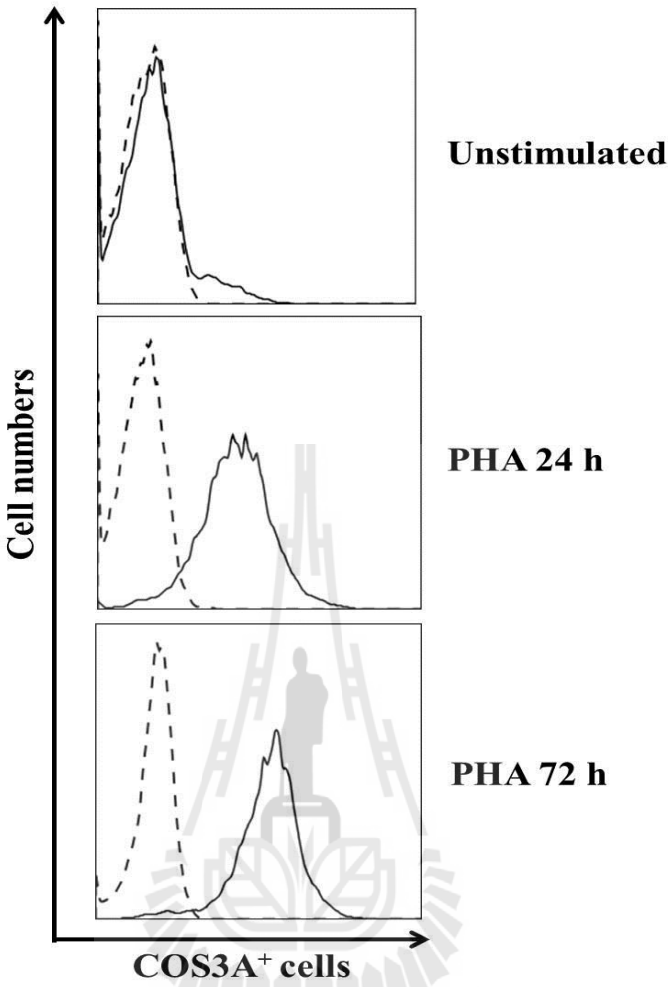

Figure 3.5 Cellular expression of the mAb COS3A recognizing antigen was strongly increased upon PHA activation. PBMCs ($1x10^6$cell/ml) were stimulated with PHA at a final concentration of 5 μg/ml at various time points. The activated cells were harvested and stained by indirect immunofluorescence with 20 μg/ml of mAb COS3A as the primary antibody, FITC-conjugated goat anti-mouse IgG antibody as the secondary antibody, and analyzed by flow cytometry. Results with conjugate controls are shown as dashed lines, while cells stained with the mAb are represented by solid lines. A representative result from one of three independent experiments is shown.

3.2 The mAb COS3A recognizes a high N-glycosylated protein

As the mAb COS3A bound to several human cells, we therefore investigated the biochemical properties of the mAb COS3A target by immunoprecipitation (IP) technique and Western blotting. First, we detected the target of mAb COS3A by performing Western blotting and used U937 cell lysate as a source of antigen. As shown in Figure 3.6 the mAb COS3A bound strongly to a broad band proteinswith molecular weight range from 30 to 70 KDa under nonreducing condition. However, the protein bands could not be detected under reducing condition. In order to the apparent smear band of the target molecule, we hypothesized that this may occur by different glycosylation moiety of sugars on this molecule. To prove our hypothesis, N-glycosylation was observed using either N-glycosidase F or by inclusion of tunicamycin, an inhibition of N-glycosylation in endoplasmic reticulum, in the cell culture medium. The broad band proteins of COS3A target molecule was converted to a sharp band of the 25 kDa by N-glycosidase-F treatment (Figure 3.7) or inclusion of tunicamycin in cell culture medium (Figure 3.8). A band found at molecular weight 50 kDa is presumed to be an imcompletely deglycosylated form of the target molecule of the mAb COS3A. Taken together, these results indicate that the target molecule is an extensively N-glycosylation protein. Moreover, the mAb COS3A recognized the protein after glycan elimination, suggesting that the mAb binds specifically to core protein of its target.

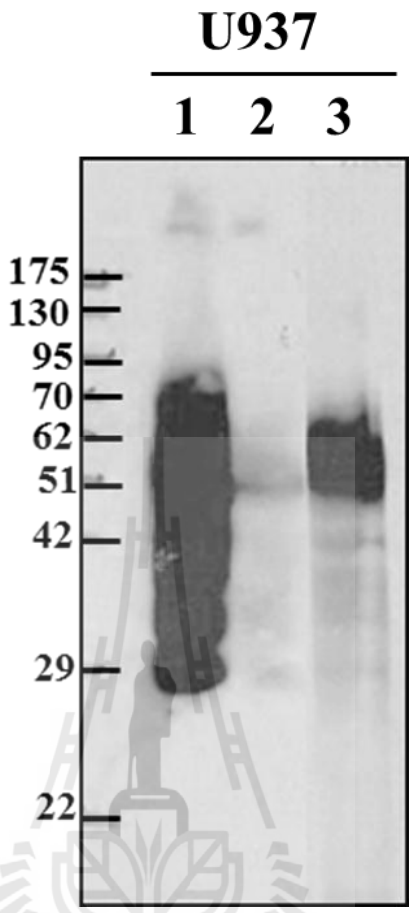

Figure 3.6 Western blotting of the mAb COS3A target molecule. U937 cell lysates were resolved under non-reducing condition by 10% SDS-PAGE and subjected to Western blotting (lane 1: mAb COS3A; lane 2: isotype-mathched control mAb, anti-13M; lane3: a positive control anti-CD147 mAb, M6-1E9). A set of representative results from one of three independent experiments is shown.

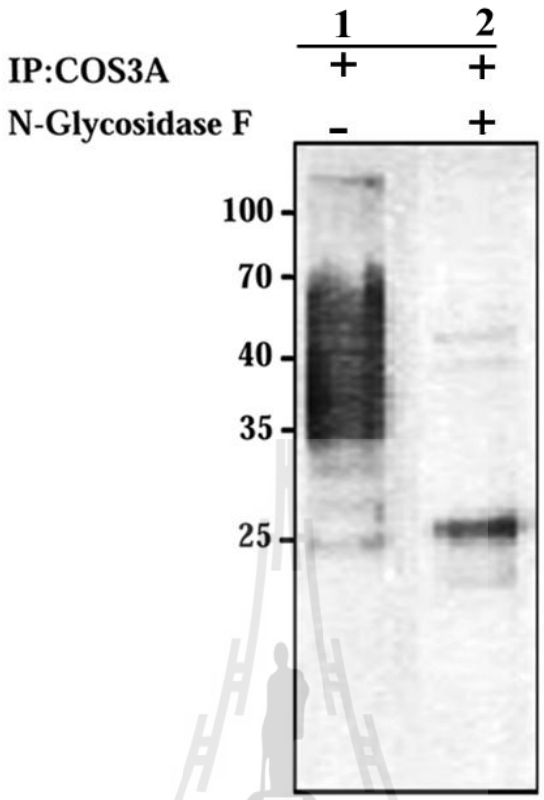

Figure 3.7 Deglycosylation of the mAb COS3A target molecule by digestion with N-glycosidase F. U937 cell lysates were subjected to immunoprecipitation using the mAb COS3A coated beads. The precipitated proteins were eluted and N-linked glycan were removed by incubation without (lane 1) or with (lane 2) five units of N-glycosidase F. The proteins were then dissolved by 10% SDS-PAGE under non-reducing condition and subjected to Western blotting using 2 µg/ml of mAb COS3A as primary antibody. A set of representative results from one of four independent experiments is shown.

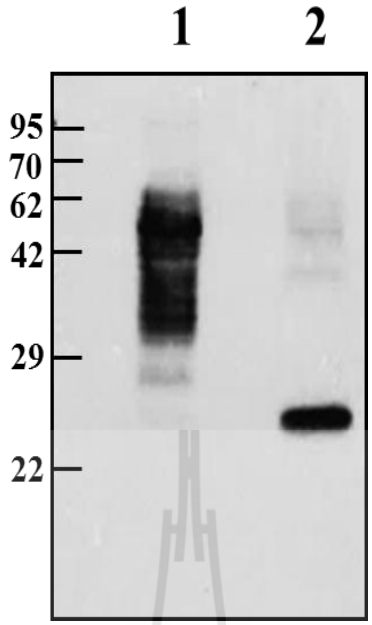

Figure 3.8 Deglycosylation of the mAb COS3A target molecule by tunicamycin treatment. U937 cells were cultured in the absence (lane 1) or presence (lane 2) of 10 µg/ml tunicamycin for 48 h at 37°C in 5% CO_2. The cells were lysed and resolved under non-reducing condition by 10% SDS-PAGE and subjected to Western blotting using 2 µg/ml of the mAb COS3A as a primary antibody. A set of representative results from one of three independent experiments is shown.

3.3 The mAb COS3A binds specifically to human CD63

To identify the target of mAb COS3A, immunoprecipitation was performed and cell lysate of tunicamycin-treated U937 cells were used as a source of antigen. The precipitated proteins from COS3A-coated beads were resolved by 10% SDS-PAGE under non-reducing condition and visualized by blue-silver Coomassie staining (Figure 3.9). Two protein bands of molecular weight about 50 kDa, an incomplete

deglycosylaed form and 25 kDa non-glycosylated form were cut out and sent for amino acid sequencing by LC/MS analysis at 1st base service company.

Tryptic fragments of these proteins were analyzed by LC/MS. The amino acid sequence of each peptide fragment was analyzed using the Mascot database, which showed that sequences of both 50 kDa and 25 kDa protein bands matched that of human CD63. The sequence coverage, match score and sequence of the identified sequences are shown in the Table To confirm the LC/MS data, immunoprecipitation and Western blotting were performed using a known anti-CD63 mAb, MEM-259, for detection of the CD63 molecule. We first used MEM-259, COS3A or a control mAb, anti-13M for immunoprecipitation from U937 cell lysates. The precipitated proteins were then subjected to Western blotting using either MEM-259 or COS3A for detection. As expected, both of the CD63 mAbs, MEM-259 and COS3A, recognized the proteins that were precipitated with both MEM-259 and COS3A-coated beads, but not protein precipitated with control mAb anti-13M (Figure 3.10). Moreover, the band of CD63 that was observed when the mAb COS3A was used for both immunoprecipitation and Western blotting was stronger than those obtained using MEM-259 mAb at the same concentration in all experiments. This suggests that the binding affinity of the mAb COS3A is higher than that of mAb MEM-259. Taken together, these results confirm that the mAb COS3A recognizes a high N-glycosylated protein, CD63 molecule.

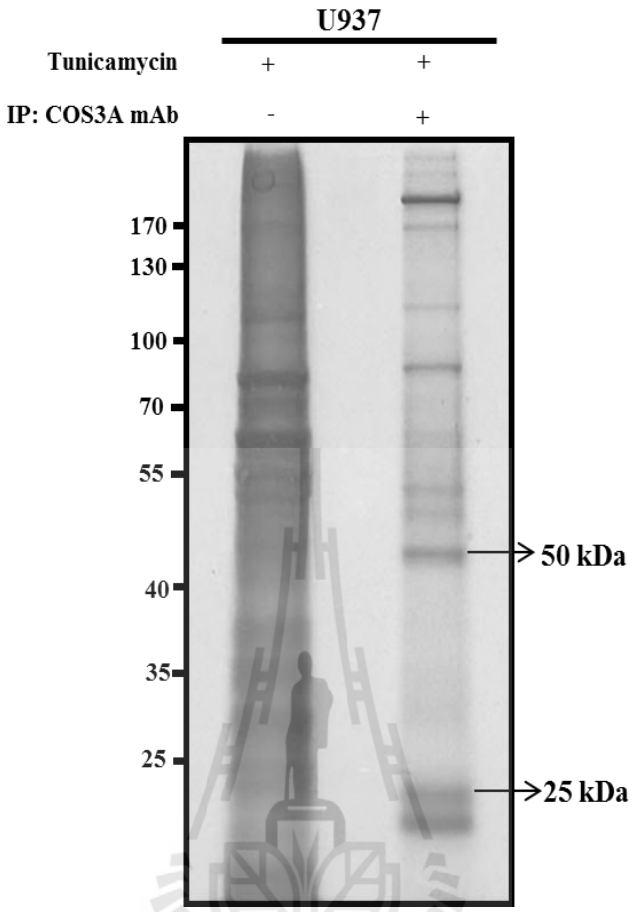

Figure 3.9 The SDS-PAGE of the precipitated proteins from the mAb COS3A coated beads. The mAb COS3A was used as a tool to precipitate its target molecule from lysates of tunicamycin-treated U937. The precipitated proteins from COS3A-coated beads were resolved in 10% SDS-PAGE under non reducing condition and stained with blue-silver coomassie staining. After staining, two protein bands of molecular weight about 50 kDa and 25 kDa were cut out and sent for amino acid sequencing by LC/MS analysis at 1st base company.

Table : Amino acid sequencing of the molecule recognized by the mAb COS3A, using LC/MS and analysis by Mascot database.

Protein band (kDa)	Access number	Matched score*	Sequence coverage (%)	Matched peptides**	Identified Protein
25	F8VV56	47	15	1 MLVEVAAAIA **GYVFRDKVMS EFNNNFR**QQM **ENYPKNNHTASILDR**MQADF 51 KCCGAANYTD WEKIPSMSKN RVPDSCCINV TVGCGINFNE KAIHKEGCVE 101 KIGGWLRKNV LVVAAAALGI AFVEVLGIVF ACCLVKSIRS GYEVM	CD63 (Homo sapiens)
50	F8VNT9	51	7	1 MAVEGGMKCV KFLLYVLLLA FCACAVGLIA VGVGAQLVLS QTIIQGATPG 51 SLLPVVIIAV GVFLFLVAFV GCCGACKENY CLMITFAIFL SLIMLVEVAA 101 AIAGYVFRDK VMSEFNNNFR QQMENYPKNN HTA	CD63 (Homo sapiens)

*Individual scores > 42 indicate identity or extensive homology ($p<0.05$)

**Matched peptides shown in Bold Red

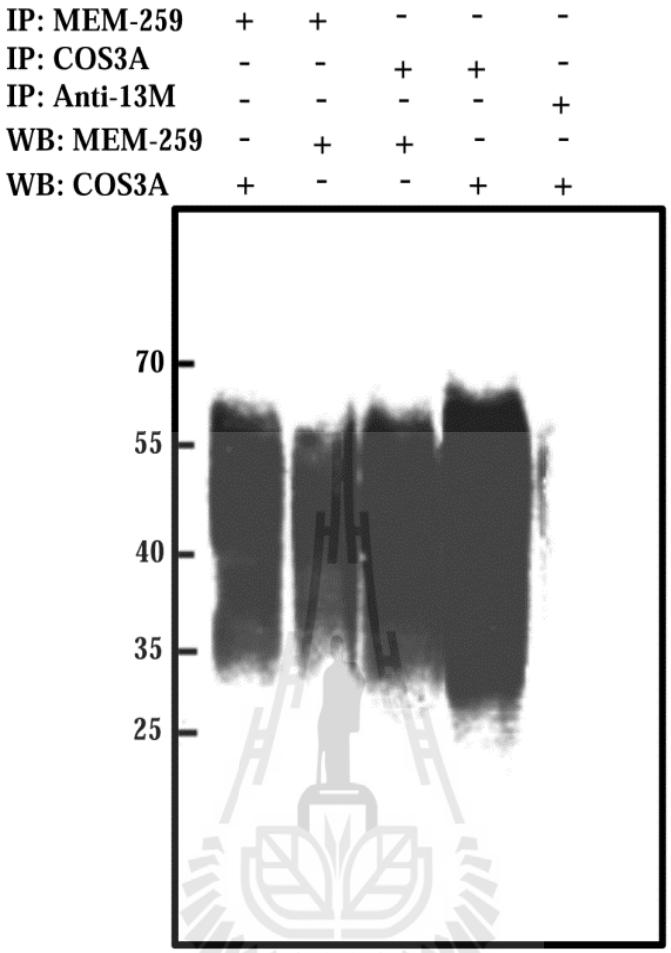

Figure 3.10 The mAb COS3A binds specifically to human CD63 molecule. U937 cell lysates were subjected to immunoprecipitation using mAb COS3A, or MEM-259 (CD63 mAb), or isotype matched control mAb, anti-13M. The precipitated proteins were then subjected to Western blotting using either mAb COS3A or MEM-259 as a primary antibody. Goat anti-mouse HRP-IgG antibody was used as a secondary antibody, and the protein bands were visualized by chemiluminescene detection system. The representative result is from one of three independent experiments.

3.4 Functional studies of the mAb COS3A

3.4.1 The mAb COS3A inhibits granulocyte phagocytosis

Phagocytosis contributes to the first line of defense against infectious pathogens, binding of the ligands on pathogens to membrane receptors of the phagocytes being an essential first step in successful phagocytosis. In assays of cellular expression levels (Figure 3.4), strong expression of CD63 was found on phagocytic cells including monocytes and granulocytes. It has been reported that CD63 is translocated from the cell surface to intracellular organelles containing phagocytosed yeast (Mantegazza, Barrio, Moutel *et al.*, 2004); furthermore, CD63 was selectively recruited to phagosomes containing a pathogenic yeast, *Cryptococcus neoformans*, in an acidification-dependent manner (Artavanis-Tsakonas *et al.*, 2006). However, since both reports related to yeast, we investigated whether surface CD63 also participates in bacterial phagocytosis. To answer this question, the mAb COS3A was used as a tool to follow the engulfment of bacterial cells via phagocytosis by granulocytes. To track the phagocytosis, *E. coli* cells expressing GFP were prepared and used as phagocytosable particle as described in 2.2.4.1. Successful construction of GFP expressing plasmid (pQEtriSystem-GFP) is shown in Figure 3.11. The recombinant pQEtriSystem-GFP vector was transformed into *E.coli* strain DH5α. The transformed *E. coli* showed strong fluorescence intensity compared to wild-type *E. coli*, which can be identified by flow cytometry (Figure 3.12) and further used in phagocytic assays.

To investigate the effect of the mAb COS3A on bacterial cells phagocytosis by granulocytes, EDTA-treated whole blood from healthy donors were incubated with *E.coli* expressing GFP on ice or at 37°C in PBS or in the presence of the

mAb COS3A or an isotype-matched control mAb anti-13M according to the method described in 2.2.4.2 and determined phagocytic activity using flow cytometry (Singboottra *et al.*, 2010). Interestingly, granulocyte phagocytosis of *E. coli* was significantly reduced when the mAb COS3A was added into the system, while the control mAb, anti-13M had no effect (Figure 3.13). These result suggested that the reduced phagocytosis is occurred through the specific binding of the mAb COS3A to the CD63, but not through the non-specific binding of the Fc receptor on surface of the phagocytes. At 0°C, the granulocytes were expected to have no phagocytic activity and the measured fluorescence originated from binding of bacteria to the surface of the granulocytes. The fractional phagocytosis is shown in a bar graph (Figure 3.14), which shows that the mAb COS3A decreased phagocytosis to 20% of control values, while there was no significant difference between the isotype-matched mAb control and PBS control. These results indicated that engagement of cell surface CD63 with the mAb COS3A blocks the interaction between ligands on *E. coli* and their specific receptor on phagocytes. Consequently ingestion of the bacterial cells was inhibited, suggesting that CD63 plays an essential role in the recognition step of phagocytosis.

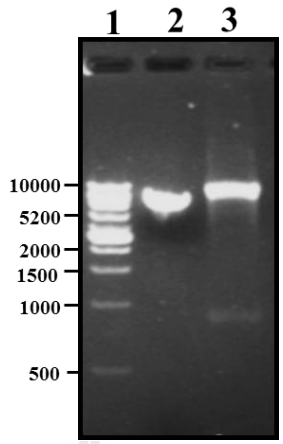

Figure 3.11 The recombinant pQEtriSystem-GFP plasmid. The pQEtriSymtem empty plasmid (lane 2) or the fragments of the recombinant pQEtriSystem-GFP plasmid (lane 3), DNA marker (lane 1) were subjected to Agarose gel electrophoresis. The 750-bp band of GFP gene was observed in lane 3 when compared with the DNA markers.

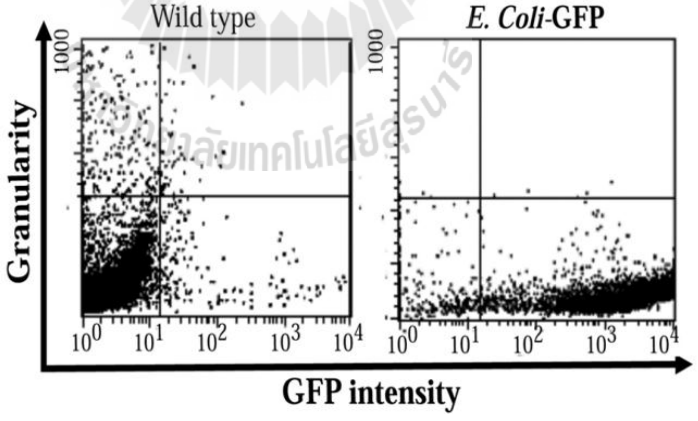

Figure 3.12 FACS profiles represent the fluorescence intensity of wild-type *E.coli* (WT) and *E.coli* expressing GFP (*E.coli*-GFP). *E. coli* strain DH5α were transformed with pQEtriSystem-GFP plasmid. Expression of the green fluorescent protein was examined by detection of the fluorescence intensity of the *E. coli* expressing GFP compared to the wild type *E.coli* using flow cytometry.

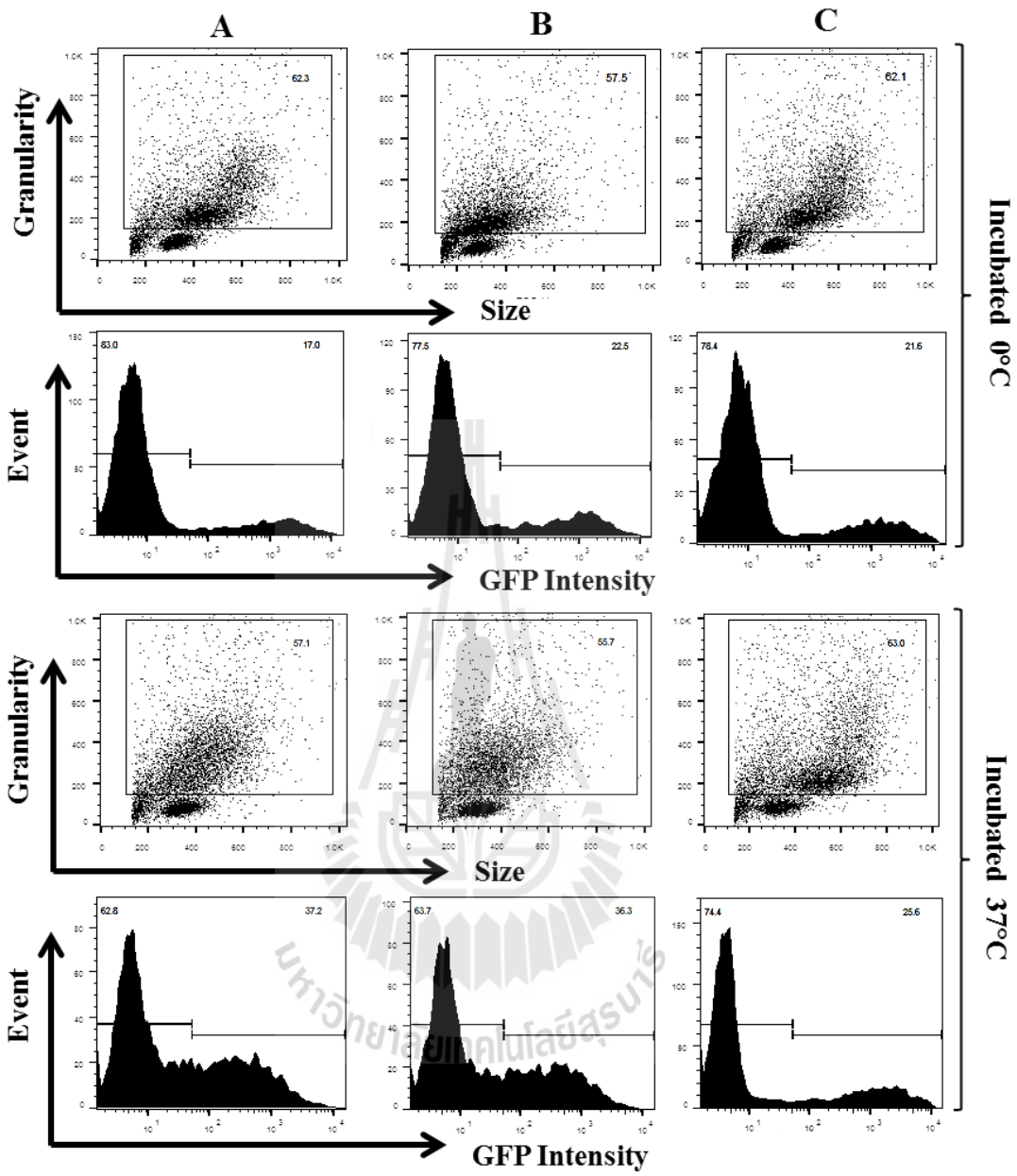

Figure 3.13 The mAb COS3A inhibits granulocytes phagocytosis. EDTA whole blood was incubated on ice or at 37°C for 1 h with *E. coli* expressing GFP in the absence of (A) or presence of 20 µg/ml of either isotype-matched control mAb anti-13M (B) or mAb COS3A (C). RBCs were lysed and granulocytes phagocytosis of *E.coli* expressing GFP was examined by flow cytometry according to the presence of GFP. The predentative result is from one of four independent experiments.

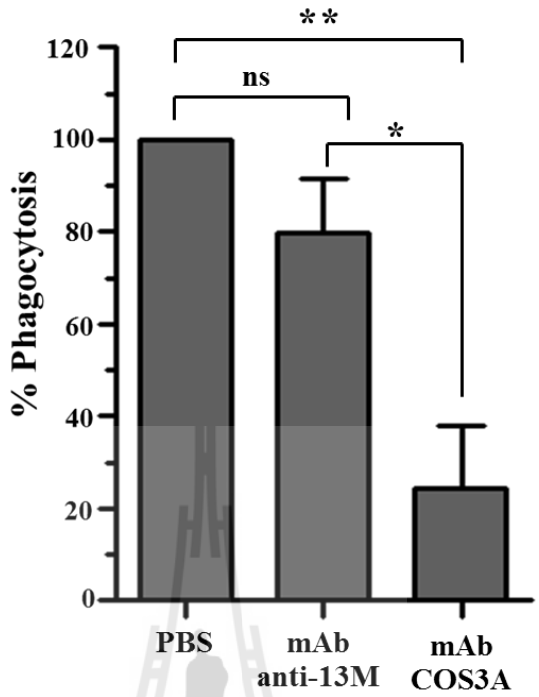

Figure 3.14 The percentage of granulocytes phagocytosis. The bar graph showing the percentage of granulocyte phagocytosis calculated from results of four independent flow cytometry experiments, according to the equation stated in the methods (ns not significant, "*" $p<0.05$, "**" $p<0.01$).

3.4.2 The soluble mAb COS3A inhibits T cell proliferation

The effect of mAb COS3A on T cell proliferation was primarily studied and found that this mAb down-regulated the T cell proliferation induced by immobilized anti-CD3 mAb OKT3 using PBMCs as target cells (Khunkaewla P, unpublished observation). We further investigated whether this inhibitory effect depends on the concentration of the mAb COS3A. To examine this phenomenon, CFSE-labeled PBMCs were stimulated with immobilized anti-CD3 mAb OKT3 in the presence of the soluble mAb COS3A at seven different concentrations or 50 µg/ml of isotype-

matched control mAb, anti-13M, and cell proliferation. The results revealed that inhibitory effect of the mAb COS3A on T cell proliferation was declined according to the decreasing of the mAb COS3A concentration while, an isotype-matched control mAb anti-13M had no effect on T cell proliferation (Figure 3.15 and Figure 3.16). These results indicated that the mAb COS3A inhibits T cell proliferation in a dose dependent manmer when PBMCs were used as model. Based on these findings, the concentration at 12.5 µg/ml was chosen for further studies on T cell activation. Furthermore, the effect of the mAb COS3A on T cell proliferation was compared with a commercially avilable anti-CD63 mAb MEM-259. CFSE-labeled PBMCs were stimulated with or without immobilized anti-CD3 mAb OKT3 in the presence or absence of mAb COS3A or mAb MEM259 or isotype-mathed control mAb anti-13M at a final concentration of 12.5µg/ml. The proliferated cells were determined for the reduction of CFSE intensity by flow cytometry. In contrast to the mAb COS3A no inhibitory effect was found while soluble anti-CD63 mAb MEM-259 was applied (Figure 3.17 and Figue 3.18). In conclusion, the mAb COS3A inhibits CD3-mediated T cell proliferation while PBMCs were used as a model.

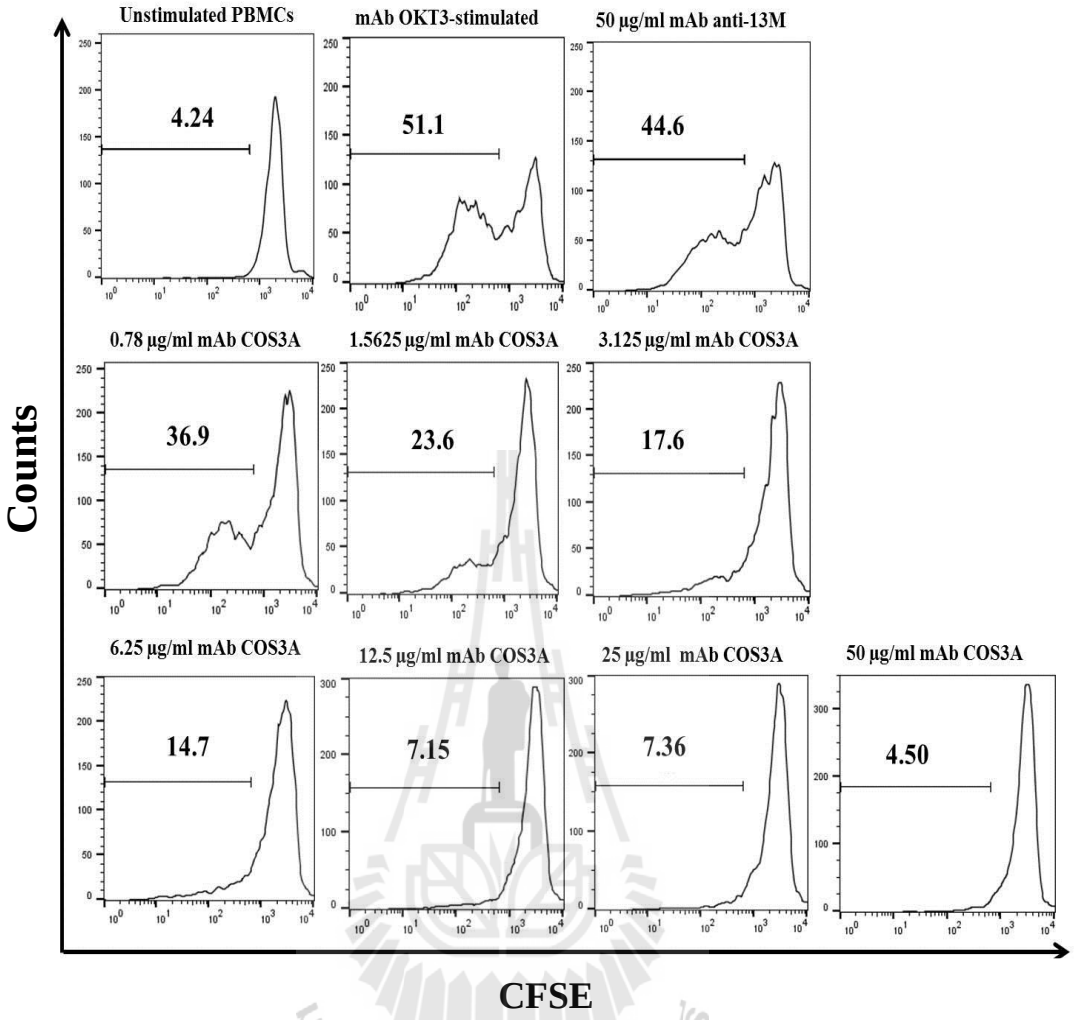

Figure 3.15 FACS profile of the inhibition of CD3-mediated T cell proliferation by the soluble mAb COS3A. CFSE-labeled PBMCs were either unstimulated or stimulated with immobilized anti-CD3 mAb OKT3. The cells were co-cultured for 5 days with or without the mAb COS3A, or isotype-matched control mAb anti-13M at different indicated final concentrations. Cell proliferation was assessed by flow cytometry are showed as histogram profile. Percentage of fluorescence intensity reduction of CFSE represents the number of the proliferated cells are indicated by a histogram mark. The results represent one of the three individual healthy donors.

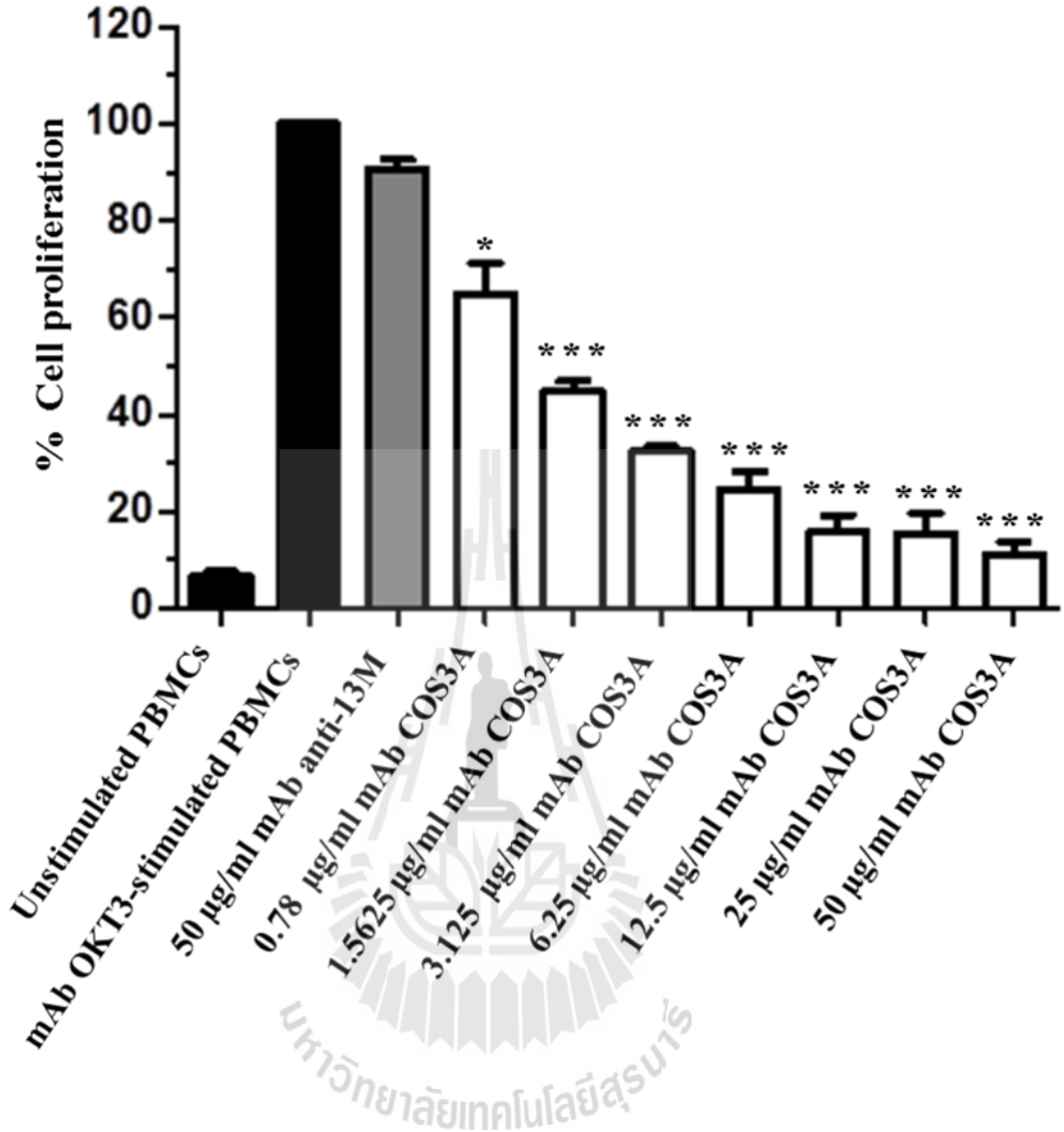

Figure 3.16 Bar graph shows the inhibitory effect of soluble mAb COS3A on T cell proliferation using PBMCs as model. The bar graph showing the percentage of cell proliferation calculated from results of three independent flow cytometry experiments ("*" $p<0.05$, "***"$p<0.001$ when compared with isotype-matched control mAb)

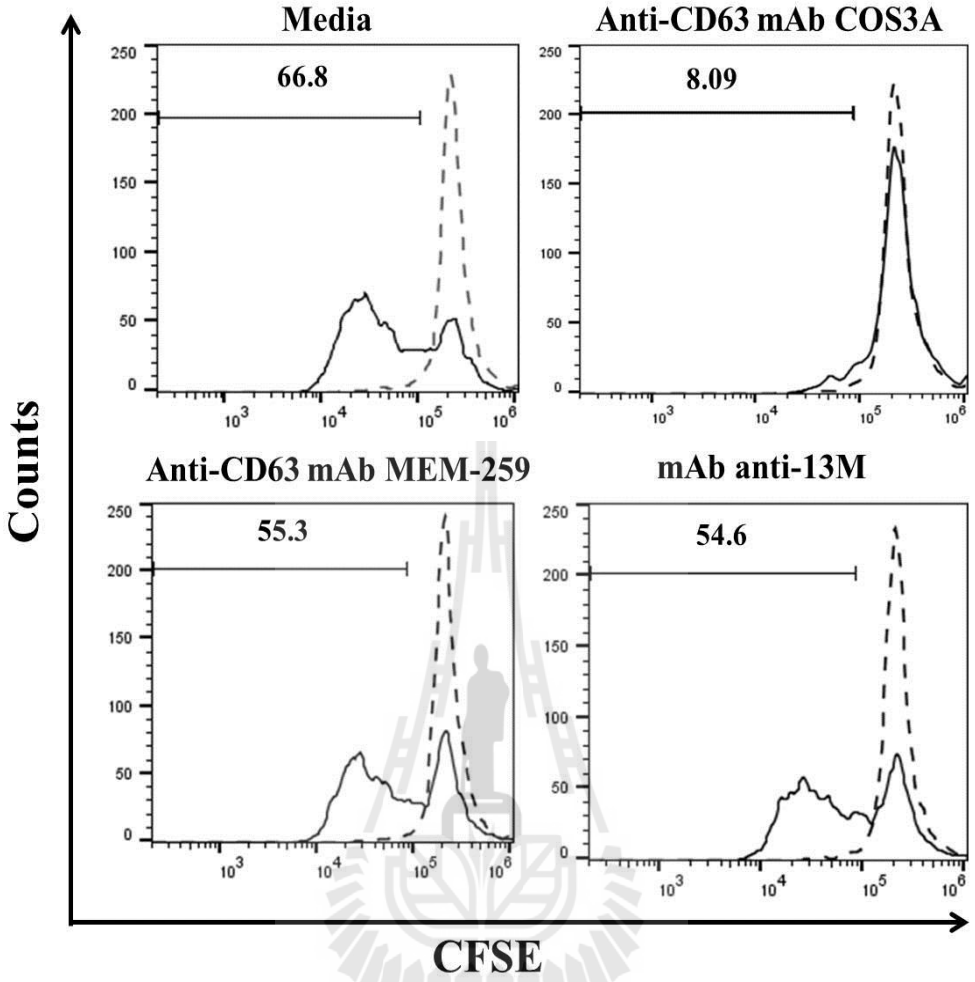

Figure 3.17 Comparison of T cell suppression between mAb COS3A and mAb MEM-259. CFSE-labeled PBMCs were either unstimulated or stimulated with immobilized anti-CD3 mAb (OKT3). The cells were co-cultured without or with the mAb COS3A, or mAb MEM-259, or mAb anti-13M at a final concentration of 12.5 µg/ml. Cell proliferation was assessed by flow cytometry. The results with unstimulated PBMCs are shown in dashed lines, while the OKT3-stimulated PBMCs are represented by solid lines. Percentage of fluorescence intensity reduction of CFSE represents the number of the proliferated cells, are indicated by a histogram mark. The results represent one of the three individual healthy donors.

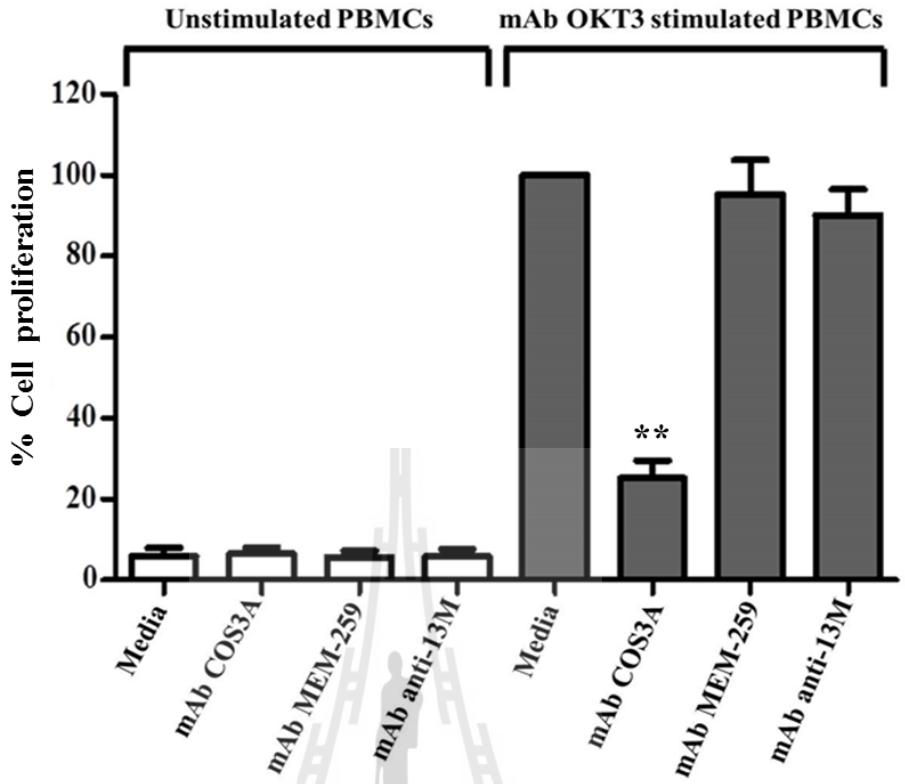

Figure 3.18 Effect of the soluble mAb COS3A on CD3-mediated T cell proliferation compared to anti-CD63 mAb, MEM-259. The bar graph showing the percentage of cell proliferation calculated from results of three independent flow cytometry experiments ("**" $p<0.01$ when compared with isotype-matched control mAb)

3.4.3 The soluble mAb COS3A inhibits CD25 expression

Primary events in the activation of T cells are the presentation of an antigenic stimulus via APC to the T cell and augmentation by co-stimulatory signals on the APC. Following this unique interaction the T cell up-regulates activation markers such as IL-2 receptor (CD25). Once IL-2 binds to IL-2 receptor (CD25) its triggers a signaling cascade, resulting in T cell proliferation (Fazekas de St Groth, Smith, and Higgins, 2004). Since the soluble mAb COS3A inhibits T cell proliferation. We

further investigated whether the mAb COS3A has effect on kinetics expression of IL-2 receptor (CD25) on surface of T cells. To do this, PBMCs were stimulated without or with immobilized anti-CD3 mAb OKT3. The cells were co-cultured with or without soluble CD63 mAbs (COS3A or MEM259) or isotype-matched control mAb anti-13M for 48 h. The cells were harvested and surface CD25 were stained by immunofluorescence staining using PE-conjugated anti-CD25 mAb. The stained cells were assessed by flow cytometry. The results showed that the mAb COS3A downregulated cell surface expression of this molecule when compared with the isotype-matched control mAb anti-13M. However, anti-CD63 mAb MEM-259 had no effect on expression of CD25 (Figure 3.19 and Figure 3.20). In summary, the mAb COS3A significantly down-regulated cell surface CD25 expression upon CD3-mediated T cell activation. These results suggested that the ligation of CD63 with the soluble mAb COS3A inhibits T cell proliferation by decreasing of IL-2 receptor, CD25, expression.

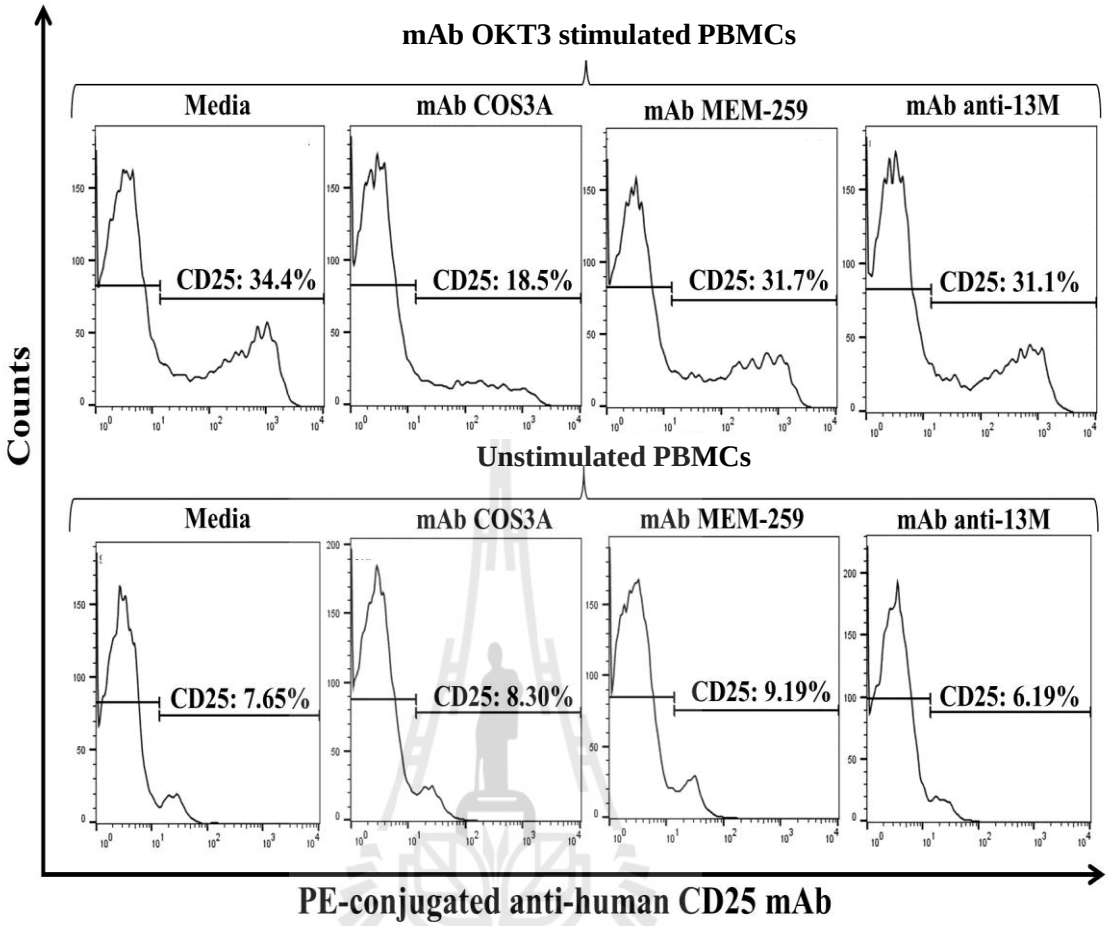

Figure 3.19 Cell surface analysis of IL-2 receptor (CD25) expression. The PBMCs were stimulated with immobilized anti-CD3 mAb OKT3 (or kept unstimulated) in the presence or absence of the mAb COS3A, or mAb MEM-259, or isotype-matched control mAb anti-13M for 2 day. The plot shows percentage of CD25 positive cells, which are indicated by a histogram mark. The results represent one of the three individual healthy donors.

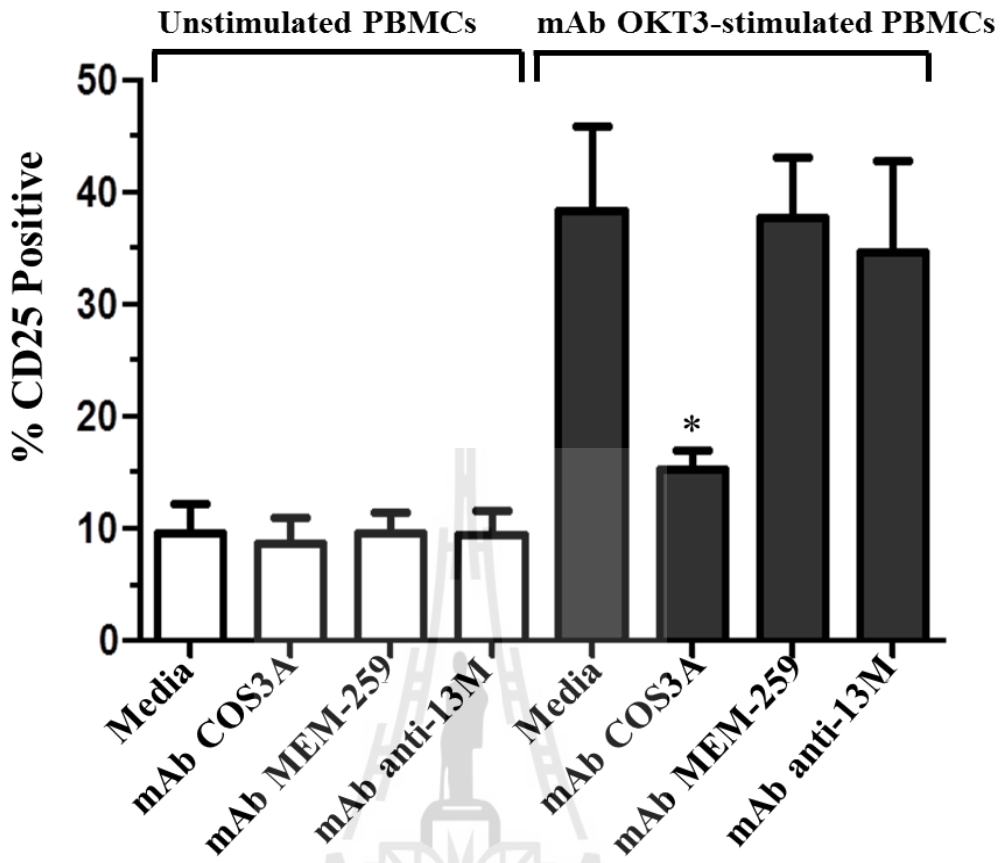

Figure 3.20 Bar graph shows the percentage of CD25 positive cells. The PBMCs were stimulated with immobilized anti-CD3 mAb OKT3 (or kept unstimulated) in the presence or absence of the indicated mAbs for 2 days. The surface expression of CD25 was analyzed by immunofluorescence staining and flow cytometry. The data represent the mean±SD of three healthy donors. "*" represents $p<0.05$ when compared to the control group (media control in OKT3-stimulated PBMCs group or isotype-matched control mAb).

3.4.4 The soluble mAb COS3A inhibited production of IL-2 and IFN-γ by T lymphocytes but enhanced production of IL-10 by monocytes during CD3-mediated T cell activation

Previous experiment indicated that the mAb COS3A inhibited CD3-mediated T cell activation and reduced surface expression of IL-2 receptor, CD25 while the PBMCs were used as a model. Because of some cytokines are involved in the regulation of T cell proliferation such as IL-2, enhancer of T cell proliferation, and IL-10, suppression of T cell proliferation. We were curious to know whether the inhibitory effect on T cell proliferation by the mAb COS3A is occured by changing the level of cytokine secretion. Thus, effects of the mAb COS3A on secretion of the IL-2 and IL-10 were analyzed. IFN-γ, a cytokine that upregulates Th1 differentiation but suppresses Th2 differentiation was also observed in this study.

To investigate the effect of mAb COS3A on cytokine production, PBMCs were activated with immobilized anti-CD3 mAb OKT3. The cells were cocultured with soluble CD63 mAbs (COS3A or MEM-259) or isotype-matched control mAb anti-13M. Secreted IFN-γ, IL-2 and IL-10 in the cell-free supernatants were harvested at 24 h post stimulation and were then immediately measured by sandwich ELISA. The results revealed that the strong reduction of IL-2 and IFN-γ secretion was observed when the cells were cocultured with the mAb COS3A, compared to those obtained from cocultivation with anti-CD63 mAb MEM-259 or isotype-matched control mAb anti-13M (Figure 3.21A-B).

In contrast to secretion of IL-2 and INF-γ, secretion of IL-10 (Figure 3.21C) was significantly increased while the mAb COS3A was presented compare to those obtained from anti-CD63 mAb MEM-259 or mAb anti-13M. In addition, the

induction of IL-10 did not come from LPS contamination in the soluble mAb COS3A, because there is no effect on induction of IFN-γ, which was upregulated upon LPS stimulation in PBMCs stimulated with only soluble mAb COS3A (Figure 3.21B).

Since IL-10 is produced by monocytes, NK-cells, Th1, and Th2 type lymphocyets, therefore we were searching for the major cell type that secretes IL-10 upon co-cultivation of the mAb COS3A by performing intracellular cytokine staining. The PBMCs were firstly stimulated with immobilized anti-CD3 mAb, OKT3, in the presence of either anti-CD63 mAbs (COS3A or MEM-259), or isotype-matched control mAb anti-13M. LPS was used as positive control, which is able to stimulate monocyte to produce IL-10 (Foey, Parry, Williams *et al.*, 1998). The cells were then subjected for intracellular staining according to the description in 2.5.5.5. The results revealed that the mAb COS3A decreased intracellular production of IL-2 (Figure 3.22) and IFN-γ (Figure 3.23) but not IL-10 (Figure 3.24) by $CD3^+$ T lymphocytes. Contrastly, intracellular production of IL-10 was enhanced in monocytes (Figure 3.25). Anti-CD63 mAb MEM-259 or isotype-matched control mAb anti-13M had no effect on production of intracellular IL-2, IFN-γ, and IL-10 either by $CD3^+$ T cells or monocytes. The results of three independent experiments were calculated and showed in bar graphs in figure 3.26. This information is clearly shown that the secretion of the IL-2 by T cells was significantly reduced compared to the controls. The secretion of the IFN-γ by T cells of individual seemed to be decreased; however, the average was not significant. The secretion level of IL-10 by T cell was not detected. Interestingly, the secretion level of IL-10 by monocytes was significantly increased.

These results suggest that down regulation of IL-2 and IFN-γ by T cells and up-regulation of IL-10 by monocytes occurred via interaction between the mAb COS3A and the CD63 on T cells and monocytes, respectively.

3.4.5 Suppression of CD3-mediated T cell proliferation by the mAb COS3A does not involve in the inhibitory function of IL-10 on T cell activation

It has been reported that IL-10 directly inhibits T cell activation and proliferation through the interruption of IL-2 production and indirectly through the blockade of the action of IFN-γ (Rowbottom, Lepper, Garland *et al.*, 1999). Furthermore, IL-10 was found as a general inhibitor of T cell proliferation and cytokine response in T cell (Akdis, Blesken, Akdis *et al.*, 1998; Maynard and Weaver, 2008). Thus we hypothesized that the increasing level of IL-10 upon cultivation with the mAb COS3A play a role on the inhibitory effect of the soluble mAb COS3A on T cell proliferation. To prove this hypothesis, anti-IL-10 mAb was used as a neutralizing antibody to capture the endogeneous secreted IL-10 in the culture supernatant as described in 2.2.5.6. The results showed that using a nuetralizing mAb against IL-10 did not improve T cell proliferation significantly compared to the control (Figure 3.27 and Figure 3.28), suggesting that endogenously produced IL-10 has no effect on suppression of CD3-mediated T cell activation by the mAb COS3A.

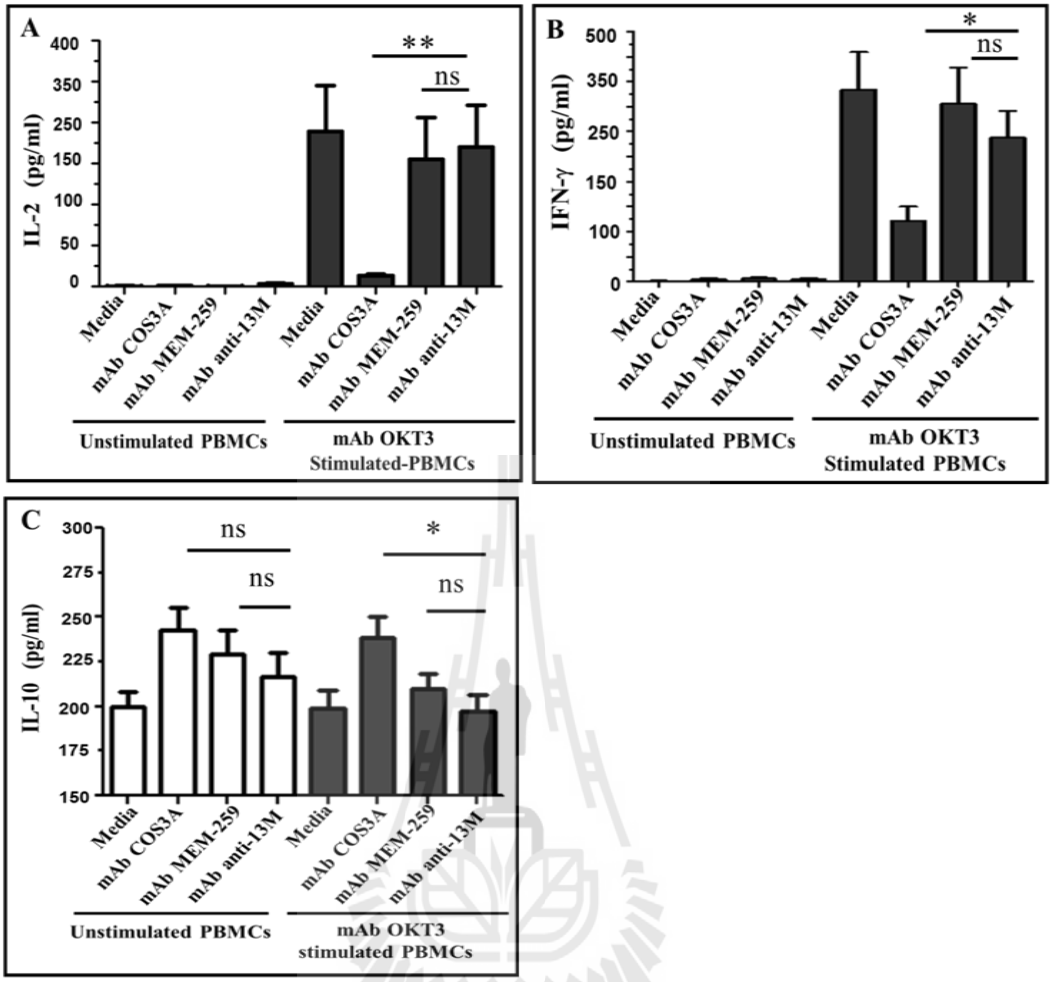

Figure 3.21 Bar graphs show effect of the mAb COS3A on a total production of IL-2 (A), or IFN-γ (B), or IL-10 (C) in supernatants were calculated from ELISA results of three independent experiments. (ns not significant, "*" $p<0.05$, "**" $p<0.01$ when compared with isotype-matched control mAb).

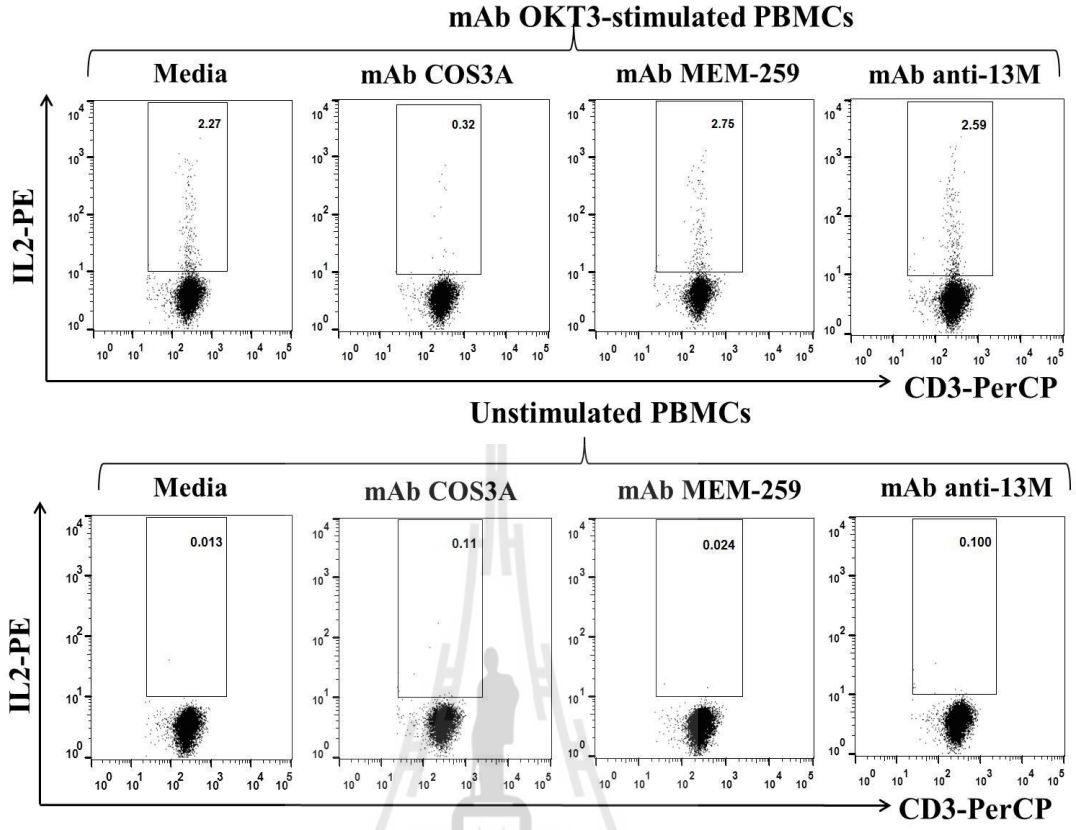

Figure 3.22 Intracellular expression of IL-2 in $CD3^+$ lymphocytes. Unstimulated PBMCs or stimulated PBMCs with immobilized anti-CD3 mAb OKT3 were cocultured with the soluble anti-CD63 mAbs (COS3A or MEM-259) or isotype-matched control mAb (anti-13M). Five µg/ml of monensin were added to the cells 1 h after cultivation and recultivation for another 5 h. The cell were intracellular stained with PerCP-conjugated-anti CD3 mAb and PE-conjugated-anti IL-2 mAb. The live $CD3^+$ lymphocytes were gated and intracellular cytokine production were analyzed using flow cytometry. A set of representative results from one of three independent experiments is shown.

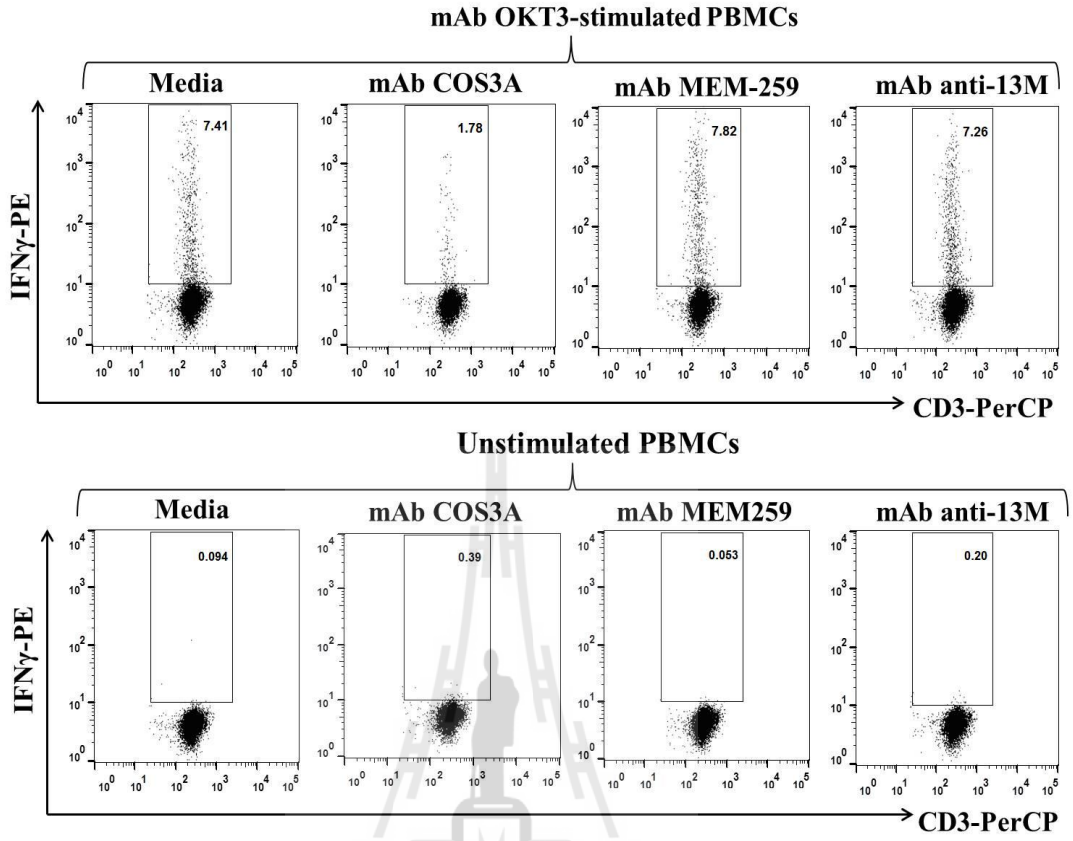

Figure 3.23 Intracellular expression of INF-γ in $CD3^+$ lymphocytes. Unstimulated PBMCs or stimulated PBMCs with immobilized anti-CD3 mAb OKT3 were cocultured with the soluble anti-CD63 mAbs (COS3A or MEM-259) or isotype-matched control mAb (anti-13M). Five µg/ml of monensin were added to the cells 1 h after cultivation and recultivation for another 5 h. The cell were intracellular stained with PerCP conjugated-anti CD3 mAb and PE-conjugated-anti IFN-γ mAb. The live $CD3^+$ lymphocytes were gated and intracellular cytokine production were analyzed using flow cytometry. A set of representative results from one of three independent experiments is shown.

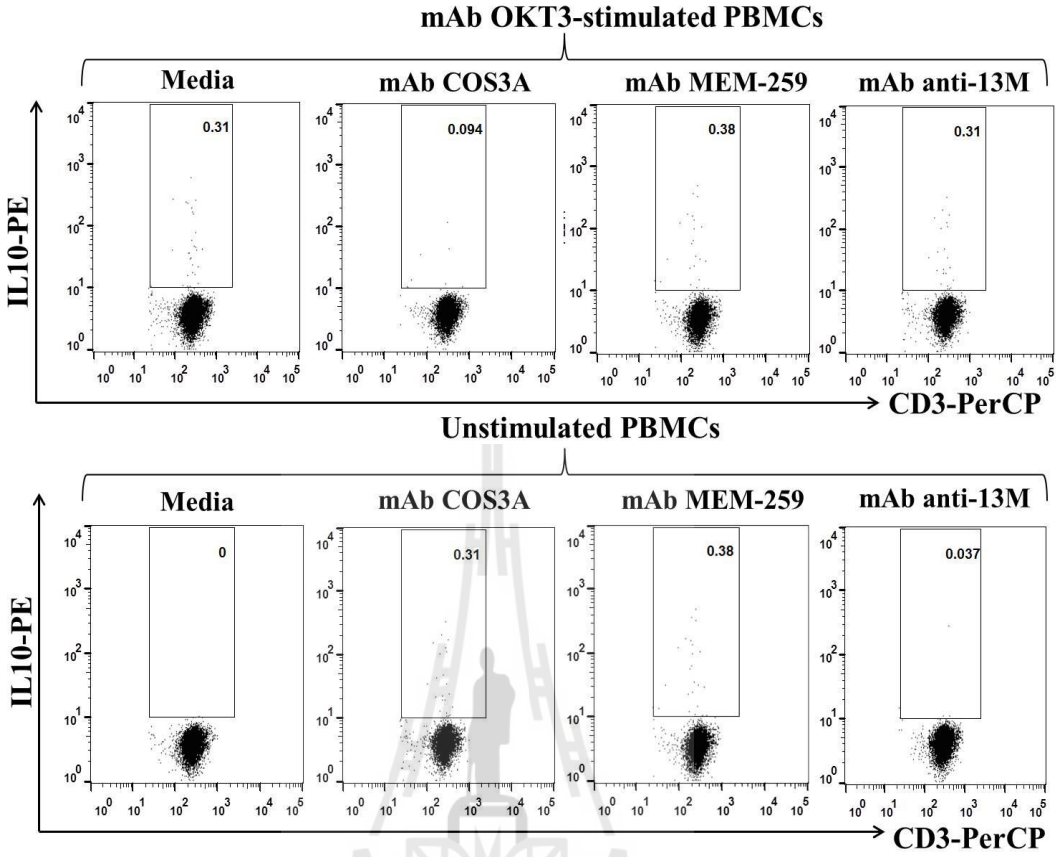

Figure 3.24 Intracellular expression of IL-10 in $CD3^+$ lymphocytes. Unstimulated PBMCs or stimulated PBMCs with immobilized anti-CD3 mAb OKT3 were cocultured with the soluble anti-CD63 mAbs (COS3A or MEM-259) or isotype-matched control mAb (anti-13M). Five µg/ml of monensin were added to the cells 1 h after cultivation and recultivation for another 5 h. The cell were intracellular stained with PerCP conjugated-anti CD3 mAb and PE-conjugated-anti IL-10 mAb. The live $CD3^+$ lymphocytes were gatedand intracellular cytokine production were analyzed using flow cytometry. A set of representative results from one of three independent experiments is shown.

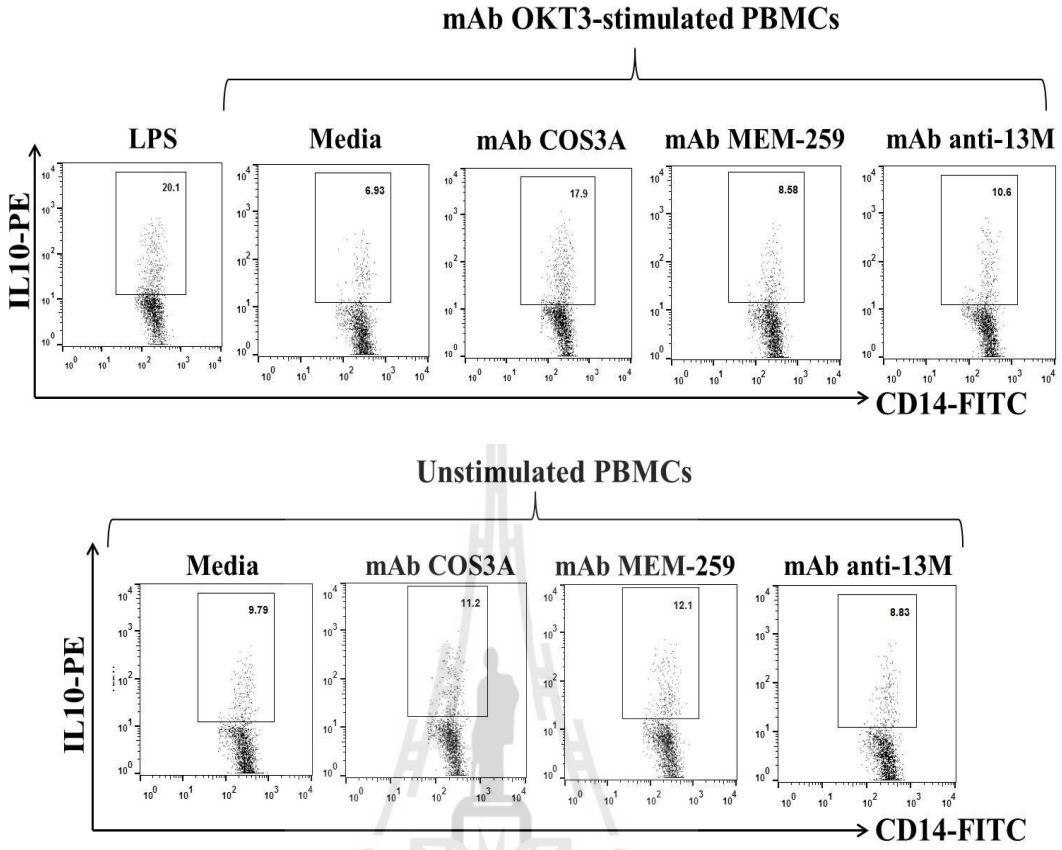

Figure 3.25 Intracellular expression of IL-10 in $CD14^+$ monocytes. Unstimulated PBMCs or PBMCs that were treated with LPS or were activated with immobilized the anti-CD3 mAb OKT3 in the presence of the soluble CD63 mAbs (COS3A or MEM-259) or isotype-matched control mAb anti-13M. Five µg/ml of monensin were added to the cells 1 h after cultivation and recultivation for another 5 h. The cell were intracellular stained with FITC conjugated-anti CD14 mAb and PE-conjugated-anti IL-10 mAb. The live $CD14^+$ cells were gated and intracellular cytokine production were analyzed using flow cytometry. A set of representative results from one of three independent experiments is shown.

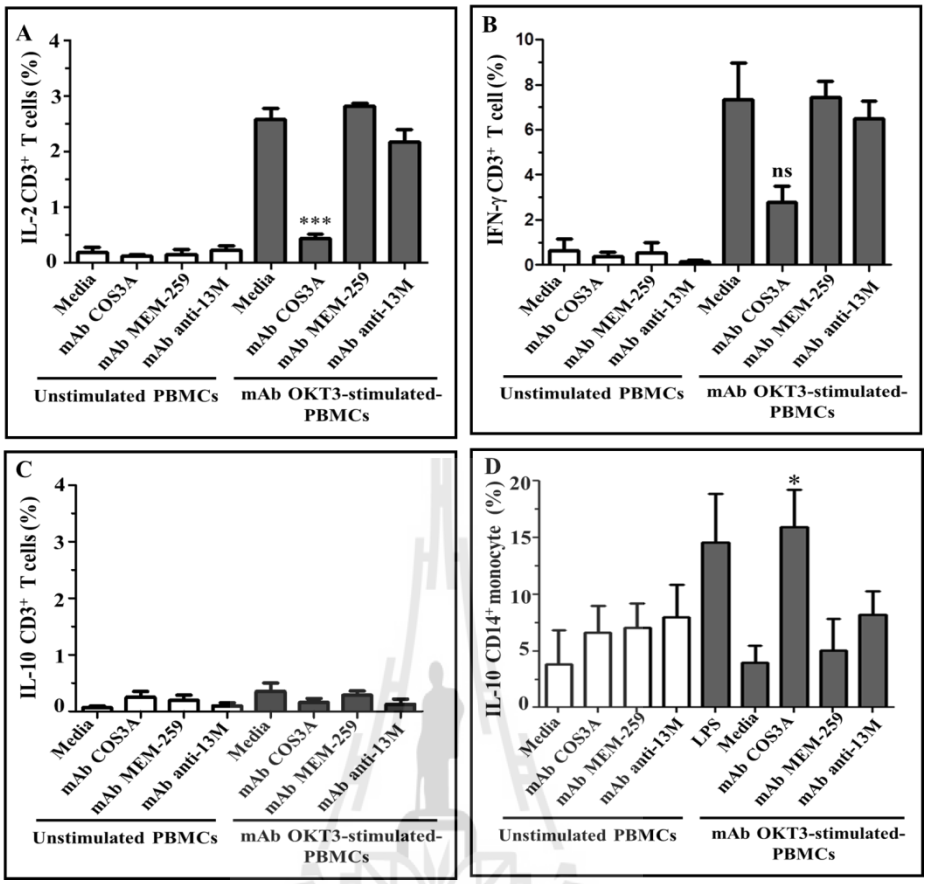

Figure 3.26 The soluble mAb COS3A decreased IL-2 and IFN-γ production in $CD3^+$T lymphocytes and enhanced IL-10 production in $CD14^+$monocytes. Bar graph showing the percentage of intracellular cytokine production calculated from results of three independent flow cytometry experiments. (ns not significant,"*"$p<0.05$, "***"$p<0.001$ when compared to media control in OKT3-stimulated PBMCs group). The average expression level of IL-2 (A), or IFN-γ (B), or IL-10 (C) in $CD3^+$ lymphocytes, or IL-10 in $CD14^+$ monocytes (D).

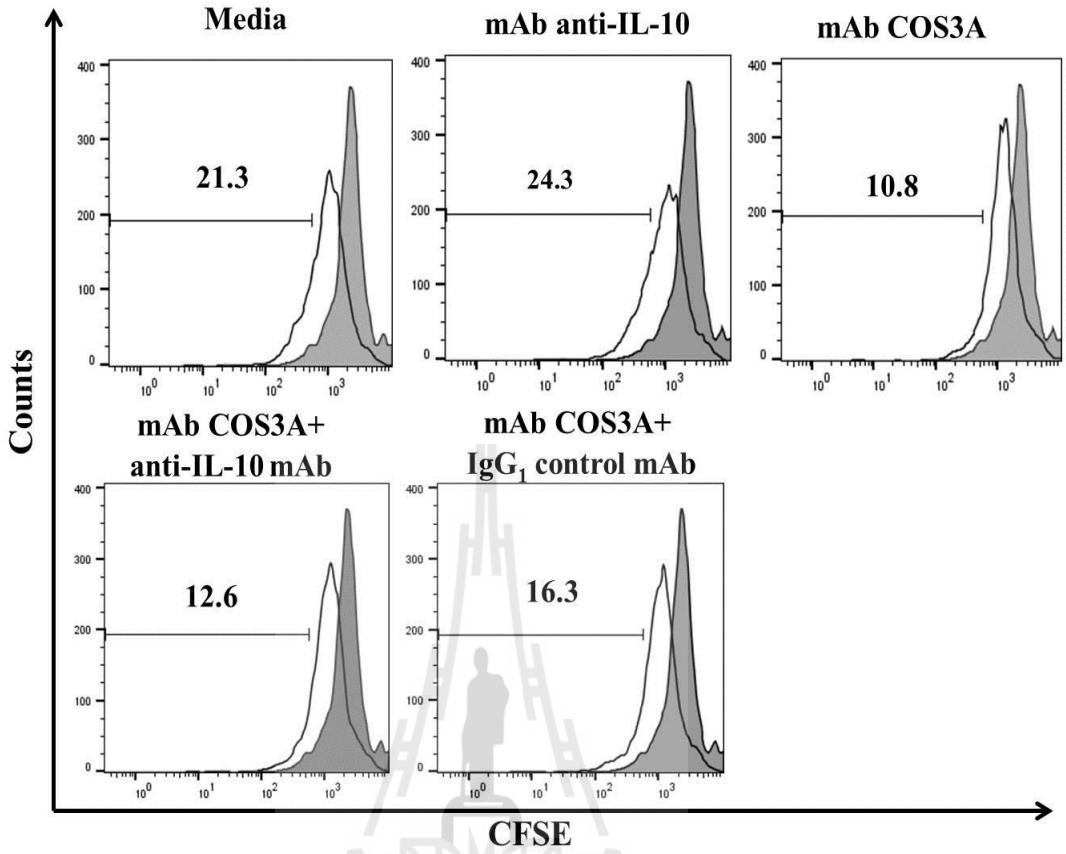

Figure 3.27 Effect of anti-IL-10 mAb on inhibitory effect of CD3-mediated T cell proliferation by the mAb COS3A. CFSE-labeled PBMCs were stimulated with immobilized anti-CD3 mAb OKT3 in the absence or presence of the indicated mAbs. The proliferation was determined on day 3 of cultivation. Grey histrogram represents unstimulated PBMCs while open histrogram represents the stimulated PBMCs. The percentage of the proliferated cells in the marker is shown. A set of representative results from one of three independent experiments is shown.

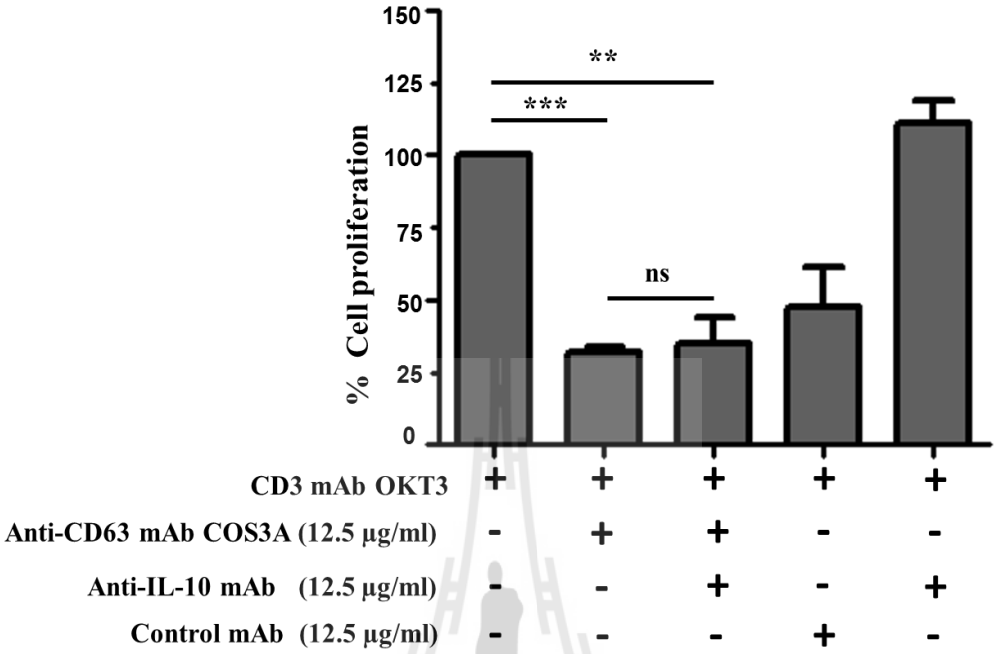

Figure 3.28 Neutralizing antibody against IL-10 could not improved the suppression of T cell proliferation by the mAb COS3A. Bar graph shows effect of anti-IL-10 mAb on inhibitory effect of CD3-mediated T cell proliferation by the mAb COS3A. The proliferation was determined on day 3 of cultivation using flow cytometry. The figure shows the percentage of cell proliferation calculated from results of three independent flow cytometry experiments (ns not significant, "**" $p<0.01$, "***" $p<0.001$ when compared with media control in OKT3-stimulated PBMCs group)

3.4.6 The soluble mAb COS3A had no effect on both purified T cells proliferation and production of IL-2, IFN-γ and IL-10 by T cells

Previous experiment showed that the mAb COS3A suppressed T cell activation while PBMCs were used and a model. We next investigested the effect of soluble mAb COS3A on CD3-mediated T cell proliferation using purified T cells as a model. CFSE-labeled T cells were stimulated with or without immobilized OKT3 mAb in the absence or presence of soluble anti-CD63 mAbs (COS3A or MEM-259) or anti-CD28 mAb, which has been uesd as soluble form, or isotype-matched control mAb anti-13M. After 5 day of cultivation the proliferation of T cells was assessed by flow cytometry. The results showed that either soluble anti-CD63 mAbs (COS3A or MEM-259) or isotype-matched control mAb anti-13M could not induced T cell proliferation as well as stimulation by immobilized anti-CD3 mAb OKT3 alone. Only engagement of the conventional costimulatory molecule, CD28 by soluble anti-CD28 mAb could induced CD3-mediated T cell proliferation. These results suggested that anti-CD28 mAb 28.2 is capable for triggering of costimulatory signal through the CD28 molecule even in soluble form. Conversely, binding of the soluble mAb COS3A failed to induced T cell proliferation while purified T cells were used as model (Figure 3.29 and Figure 3.30). Moreover, cytokine production was also observed in this study. Cell free culture supernatents were harvested after 72 h and cytokines were meased by sandwich ELISA. We found that the soluble anti-CD28 was able to induce secretion of IL-2, IFN-γ, and IL-10 by T cells. Whereas, secretion of IL-2, IFN-γ, and IL-10 by T cells were not obeserved when the soluble anti-CD63 mAbs (COS3A or MEM-259) or isotype matched control mAb anti-13M were added (Figure 3.31A-C).

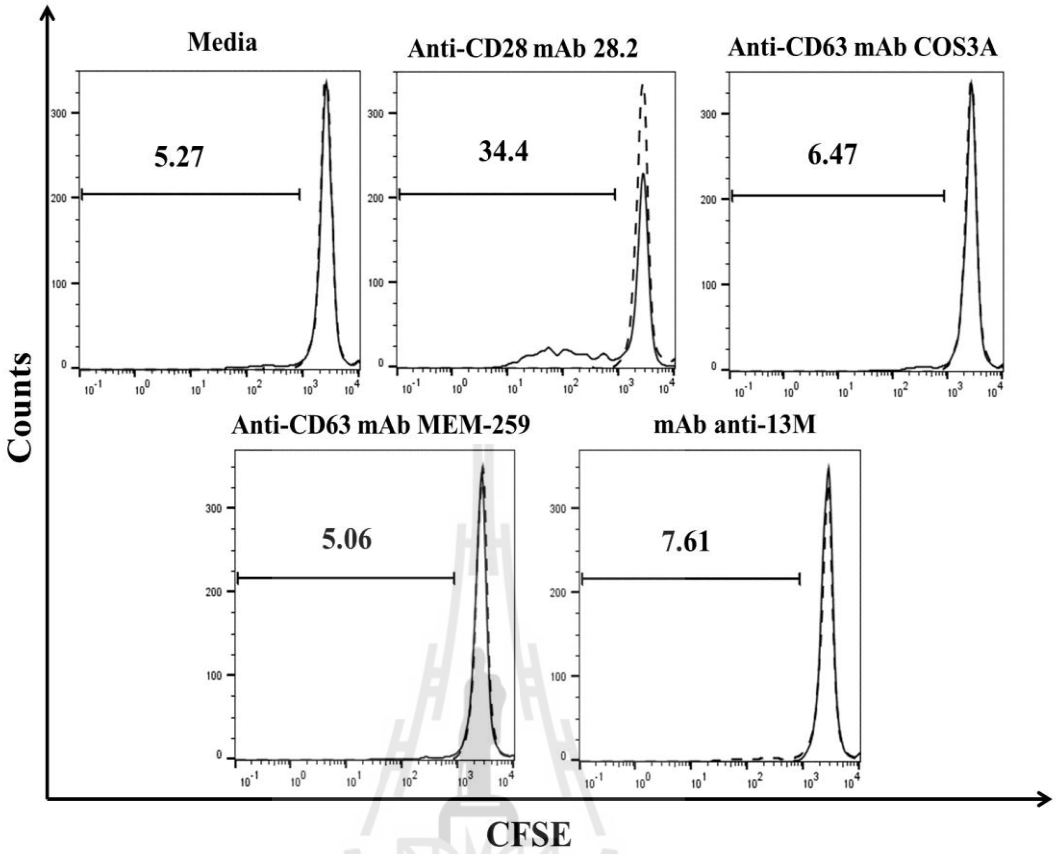

Figure 3.29 Effect of the mAb COS3A on CD3-mediated T cell proliferation using purified T cells as a model. CFSE-labeled T cells were cultivated in the OKT3-immoblized plate in the absence or presence of soluble anti-CD28 mAb 28.2 or anti-CD63 mAbs (COS3A or MEM-259), or isotype matched control mAb anti-13M. After 5 days of cultivation, T cell proliferation was assessed by flow cytometry. The dashed lines represents unstimulated T cells and the solid line represents effect of the indicated soluble mAb on CD3-mediated T cells proliferation. The results represents one of the four individual healty donors.

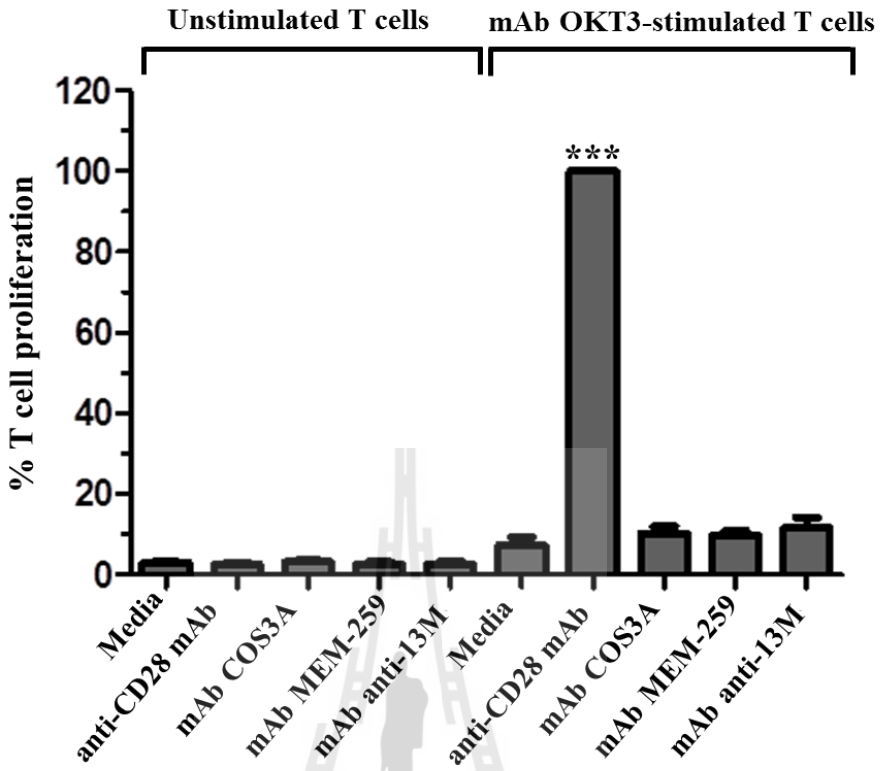

Figure 3.30 The percentage of CD3-mediated T cell proliferation in co-cultivation with the soluble mAb COS3A using purified T cell as a model. The bar graph shows the percentage of cell proliferation calculated from results of four independent flow cytometry experiments, the data represents the mean±SD of four healthy donors. "***" represents $P<0.001$ when compared with control group (OKT3-stimulated T cells group).

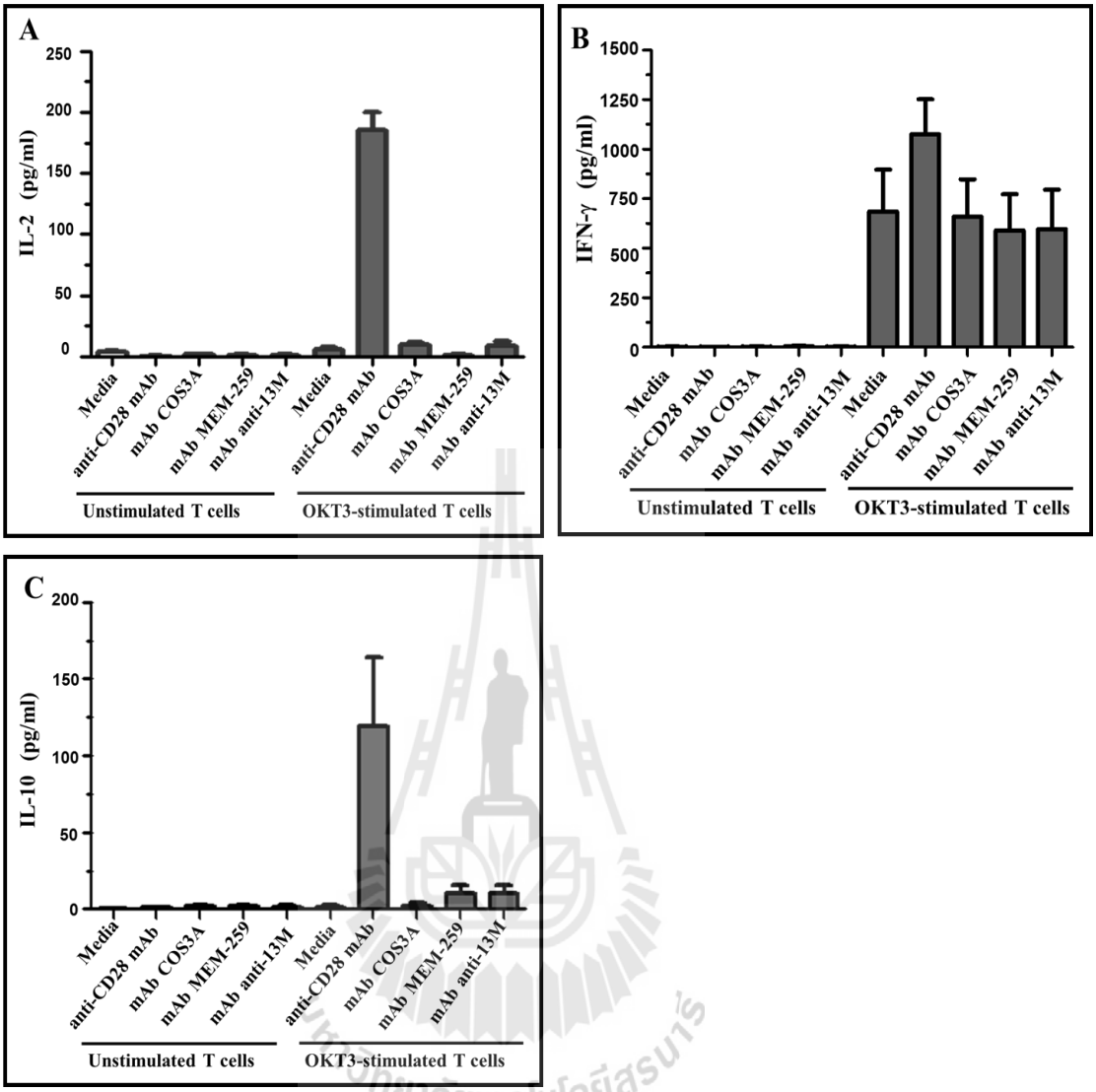

Figure 3.31 The soluble mAb COS3A could not induced production of IL-2, IFN-γ and IL-10 by OKT3-activated T cells. The purified T cells were stimulated with immobilized OKT3 mAb (or left unstimulated) for 72 h in the abesence or presence of soluble indicated mAbs. Production of IL-2 (A), or IFN-γ (B), or IL-10 (C) in the cell-free culture supernatants were analyzed by sanwich ELISA. The data represents the means±SD of triplicate culture supernatants. Data shown are representative of three independent experiments.

3.4.7 Coimmobilization of the mAb COS3A and anti-CD3 mAb OKT3 strongly enhances T cell proliferation and secretion of IL-2, IFN-γ and IL-10

Full activation of naïve T cell requires at least two distrinct biochemical signals. The first signal is typically occured through TCR/CD3 complex as a consequence of antigen recognition. The second signal is generated upon ligation of at least one of several different costimulatory molecules on T cell surface, called costimulatory signal. The importance of costimulation process is induction of high level of IL-2, prevention of anergy and enhance cell survival. In contrast, absence of this phenomenon leads to anergy (Abbas, 2003). CD28 is one of the best charecterized costimulatory molecule expressed by T cell, which interacts with CD80 or CD86 on the membrance of APC. CD63 has also been reported as an activation induced costimulatory structure on T cell, which have been obseved by coimmobolization of anti-CD63 mAb and anti-CD3 mAb (Pfistershammer *et al.*, 2004). We therefore tested whether cross-linking of CD63 with the plated bound mAb COS3A could generated a potent costimulatory signal to T cells. CFSE-labeled purified T cells were cultured in 96 well plate that had been immobilized with anti-CD3 mAb OKT3 alone or coimmobilized with anti-CD28 mAb 28.2 or anti-CD63 mAbs (COS3A or MEM-259), or an isotype-matched control mAb anti-13M. The cells were harvested for cell proliferation analysis using flow cytometry after cultivation for 5 days. The results revealed that weak proliferation of T cells was observed when the cells were cultured in the wells that immobilized with either only anti-CD3 mAb OKT3 or coimmobilization with an isotype-matched control mAb anti-13M. Strong T cell proliferation was observed when the cells were stimulated with coimmobilization of anti-CD28 mAb 28.2 and anti-CD3 mAb OKT3.

Fascinatingly, T cell proliferation that was observed when coimmobilization of the anti-CD63 mAb COS3A and anti-CD3 mAb OKT3 was stronger than those obtained using anti-CD28 as a coimmobilizing antibody. However, coimmobilization of anti-CD3 mAb OKT3 together with anti-CD63 mAb MEM-259 had no effect on T cell proliferation. No cell proliferation was observed while the cells were cultured in the plate that immobilized with either anti-CD63 mAbs (COS3A or MEM-259) or mAb anti-13M without OKT3 stimulation (Figure 3.32 and Figure 3.33). These results indicated that cross-linking of the CD63 using the mAb COS3A vigorously enhanced CD3-mediated T cell proliferation in the same way as the CD28 mAb did compared to those cells that were stimulated with only immobilized anti-CD3 mAb.

Furthermore, it has previously been suggested that an immobilized mAb interacting with CD63 induced costimulation and induction of IL-2 production in T cells (Pfistershammer *et al.*, 2004). We therefore raised the question whether the mAb COS3A also has the same effect as the previuos report. To answer this question, purified T cells were cultivated in OKT3 immobilized plate alone or in combination with the immobilized anti-CD63 mAbs (COS3A or MEM-259) or CD28 mAb, or isotype matched control mAb. After 72 h of cultivation, culture supernatants were harvested and examined for the cytokine production by ELISA. The results showed that T cells stimulated with anti-CD3 mAb OKT3 alone could not produce IL-2, IL-10 and low amount of IFN-γ. Nevertheless combination of mAbs OKT3 and COS3A induced high level of IL-2, IFN-γ and low amount of IL-10 compared to conventional T cells stimulation using combination of OKT3 and anti-CD28 mAb. The anti-CD63 mAb MEM-259 was able to induce neither IL-2 nor IL-10 production by T cells, however low level of IFN-γ secretion was observed (Figure 3.34 A-C). Two possible

reasons might be concluded from this result. First, the epitope binding of the mAb COS3A is a functional epitope of the CD63 on T cell activation, but the MEM-259 is not. Second, binding affinity of the mAb COS3A is stronger than MEM-259. Thus, increasing of the concentration of the MEM-259 may improve the costimulatory signal.

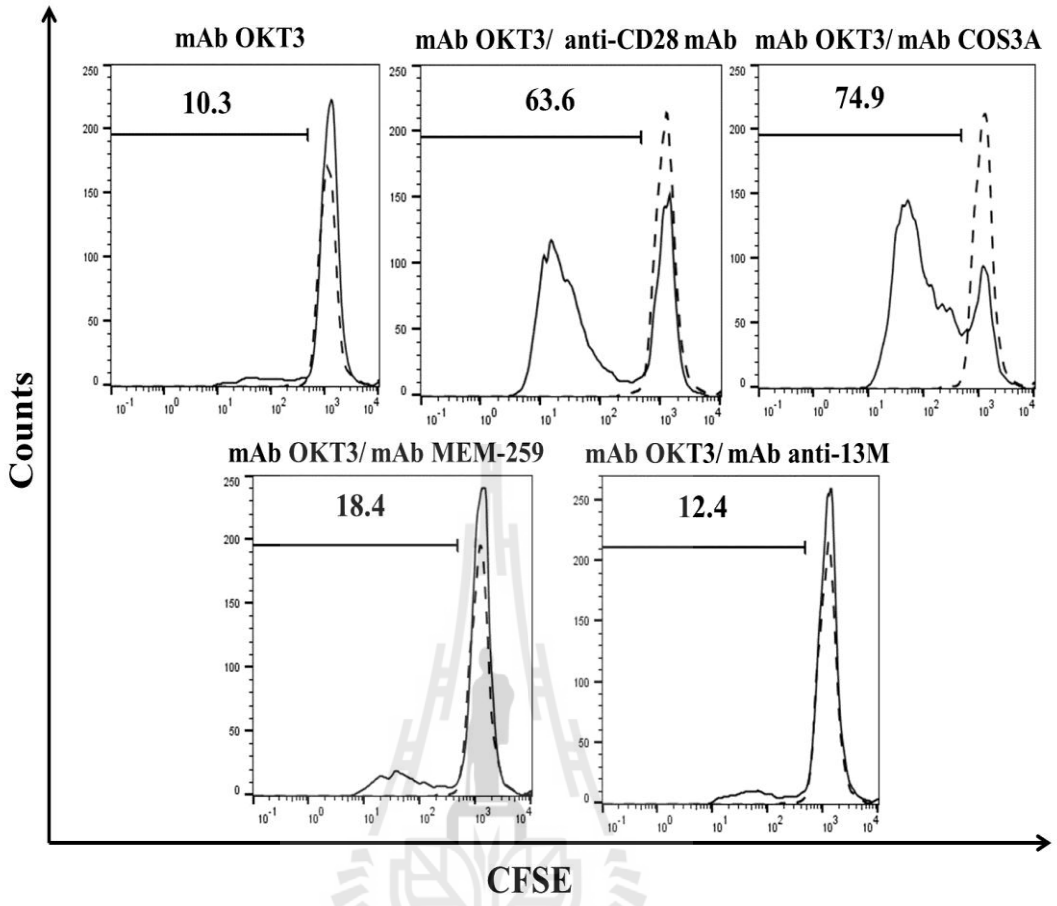

Figure 3.32 Potent T cell activation by coimmobilization of mAb OKT3 and mAb COS3A using purified T cell as model. CFSE-labeled T cells were cultivated in 96 well plate immobilized with mAb OKT3 alone or in combination with immobilized anti-CD63 mAbs (COS3A or MEM-259), or anti-CD28 mAb, or isotype matched control mAb, anti-13M). After 5 days of cultivation, the proliferation of T cells was assessed by flow cytometry. The dashed lines represent non-proliferated T cells and the solid lines represent the proliferated T cells. The number in the histogram represents the percentage of the proliferative cells in the marker lines. The results represent one of the four individual healty donors.

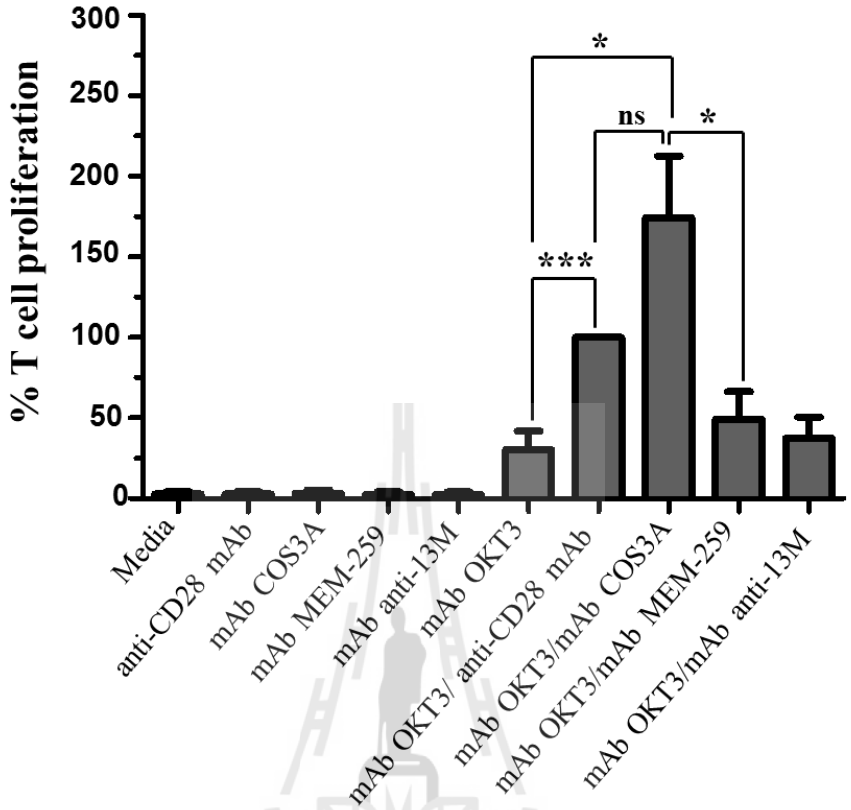

Figure 3.33 Coimmubilization of mAb OKT3 and mAb COS3A provided a potent costimulatory signal on human T cell activation. T cell proliferation was assessed by flow cytometry as shown in figure 3.32. The figure shows the percentage of cell proliferation calculated from results of four independent flow cytometry experiments. The data represents the mean±SD of four healthy donors. (ns not significant, "*" $p<0.05$, "***" $p<0.001$).

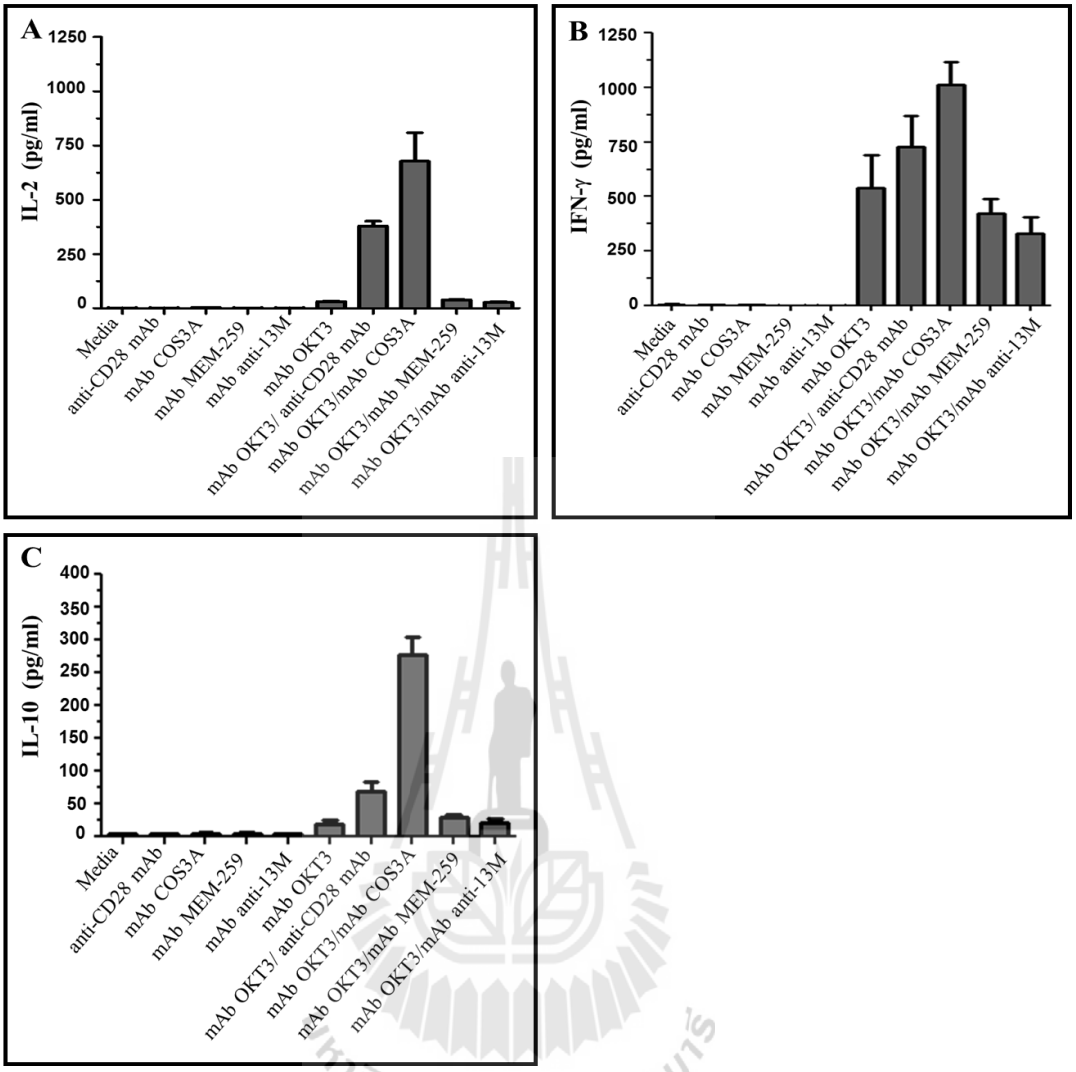

Figure 3.34 Coimmobilization of mAb OKT3 and mAb COS3A induced secretion of IL-2, IFN-γ and IL-10. Purified T cells were cultivated in 96 well plate with anti-CD3 mAb immobilized plate alone or in combination with immobilized anti-CD63 mAb (COS3A or MEM-259) or anti-CD28 mAb, or isotype-matched control mAb anti-13M for 72 h. Production of IL-2 (A), or IFN-γ (B), or IL-10 (C) in the cell-free supernatants were analyzed by ELISA. The data represents means ±SD of triplicate culture supernatants. Data shown are representative of three independent experiments.

3.4.8 Coimmobilization of anti-CD63 mAb COS3A and anti-CD3 mAb OKT3 promoted transition of cell cycle from G1 to S phase

Proliferation is a multistage process characterized by successive entering of various stages of the cell cycle (Kubsch, Graulich, Knop *et al.*, 2003). To assess the mechanisms involved in the up-regulation of the proliferative of T cells when stimulated by coimmobilized mAb COS3A and anti-CD3 mAb OKT3 cell cycle experiments were performed by analyzing the DNA content using flow cytometry. For that purpose, purified T cells were co-cultured with immobilized anti-CD3 mAb OKT3 alone or coimmobilized with other mAbs for 5 day. The results showed that cell cycle progression was remained at G1 phase when T cells were stimulated with anti-CD3 mAb OKT3 alone. Coimmobilization of anti-CD3 mAb OKT3 and anti-CD28 mAb promoted G1/S phases transition as same as using immobilized anti-CD63 mAb COS3A incombination with anti-CD3 mAb OKT3. In contrast, accumulation of the cell cycle in the G1 phase was obseved when T cells were costimulated with anti-CD3 mAb OKT3 and anti-CD63 mAb MEM-259, or isotype- matched control mAb, anti-13M (Figure 3.35A and Figure 3.35B). These results indicate that cross-linking of the CD63 via mAb COS3A and immobilized anti-CD3 mAb induced cell cycle progression into S phase, which is a critical step for T cell proliferation.

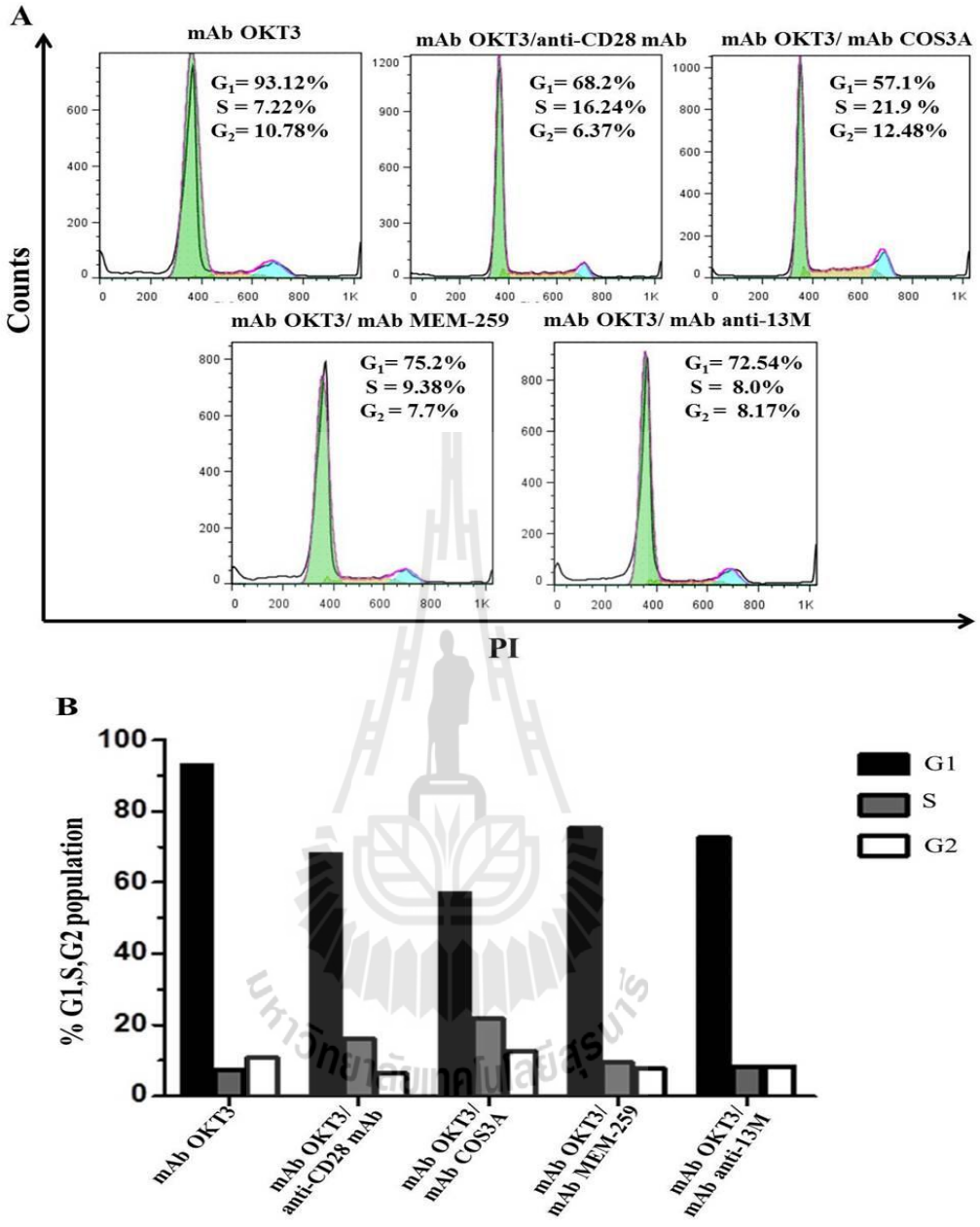

Figure 3.35 Cell cycle analysis. (A) Flow cytometry analysis showed that the percentages of the cells in S phase were increased when the cells were activated with coimmobilization of mAb OKT3 together with either anti-CD28 mAb or the mAb COS3A compared to controls. (B) The measurement of G1, S, and G2 cells was shown. bar graph are representative of mean values of percentage of G1, S, and G2 cell.

CHAPTER IV

DISCUSSION

Monoclonal antibodies are a valuable tool in many applications including in basic research, biomedical research, microbiological research and therapy. The standard method for generating monoclonal antibodies is the so-called hybridoma technique, which was established in 1975 by Georges J.F. Köhler and César Milstein (Kohler and Milstein, 1975; Liu, 2014). Using this technique, several mAbs recognizing leukocyte surface molecules were generated. In collaboration with Prof. Dr. Watchara Kasinrerk, whose laboratory has mainly focused on production of mAbs, the generated mAbs are used for both basic researches and medical applications (Kasinrerk, Tokrasinwit, Moonsom *et al.*, 2000; Kasinrerk, Tokrasinwit, and Naveewongpanit, 1998; Kasinrerk, Tokrasinwit, and Phunpae, 1999; Mahasongkram, Pata, Chruewkamlow *et al.*, 2015; Pimpha, Chaleawlert-umpon, Chruewkamlow *et al.*, 2011). A mAb named COS3A generated by his group was selected for biochemical characterization and functional analysis of its target molecule. The antibody was initially produced by a convention hybridoma technique using CD4 expressing COS7 as immunogen for antibody induction in a Balb/c mouse (Pata *et al.*, 2009). The mAb, however, was not specific for CD4. Primary functional characterization of the mAb COS3A on T cell activation found that this mAb strongly inhibited CD3-mediated T cell proliferation using PBMCs as

a model (unpublished data). For this reason, the mAb COS3A was expected to bind surface molecule on human white blood cells and was selected for identification and searching for function of its specific molecule. As the mAb was produced using the COS7 cell tranfection system, we first determined whether the mAb COS3A reacted with COS7 cell proteins. We found that the mAb COS3A bound strongly to cell surface protein and intracellular protein in the monkey kidney cell line, COS7. Cellular distribution of the mAb COS3A specific molecule was also observed by immunofluorescence staining and flow cytometry. With the exception of erythrocytes, the antibody also bound to a molecule expressed on the surface of several human hemaopoietic cells and cell lines.These results suggested a close homology between monkey and human forms of the target molecule. Additionally, up-regulation of this molecule upon PHA activation was observed, suggesting that the mAb COS3A target is an activation-associated molecule.

Biochemical analysis revealed that the molecule recognized by COS3A has molecular weight of about 30-70 kDa and amino acid sequencing by LC/MS subsequently indicated that this molecule is human CD63. Immunoprecipitation and Western blotting using two anti-CD63 mAbs, MEM-259 and COS3A, clearly showed that the mAb COS3A binds to CD63-purified protein using MEM-259, while MEM-259 was able to bind to the COS3A-purified protein. These results confirm the specific binding of the mAb COS3A to the CD63. Moreover, the band of the CD63 that was observed when the mAb COS3A was used for both immunoprecipitation and Western blotting was stronger than those obtained using MEM-259 at the same concentration in all experiments. This suggests that the binding affinity of the mAb COS3A is higher than that of mAb MEM-259.

Also the FACS profile of cellular distribution obtained using the generated mAb were the same as those described in previous reports for the human CD63 (Horejsi and Vlcek, 1991; Pfistershammer *et al.*, 2004; Tarrant, Robb, van Spriel *et al.*, 2003).

CD63 (TSPAN30, Granulophysin, Melanoma antigen, ME491, platelet glycoprotein 40 kDa, sometime ambiguouslylamp-3) was the first member of the tetraspanin family that had been cloned in 1988 from human cancer cell (Azorsa *et al.*, 1991; Pols and Klumperman, 2009). This molecule has been found in ubiquitously expresses and localizes within the endosomal system and on the cell surface. It is expressed on a wide variety of tissues and cell types. There have also been showed to play a role in activation of several cell types including platelets, neutrophils, eosinophils, basophils, monocytes and T lymphocytes (Skubitz, Campbell, and Skubitz, 2000; Sturm, Bohm, Trummer *et al.*, 2004; Takeda, Tachibana, Miyado *et al.*, 2003).

The human CD63 is comprised of 237 amino acid residues with predicted molecular weight of 25 kDa. Although, the complete 3D structure of human CD63 has not been determined, but CD63 structure has been reported that it comprises of four putative transmembrane domains, a small (EC1) and a large (EC2) extracellular domain, short cytoplasmic N- and C- termini, and three N-linked glycosylation (Metzelaar *et al.*, 1991; Monk and Partridge, 2012). By removing the N-linked glycan, N-glycosidase F treatment reduced the molecular weight of this protein to 25 kDa, and the same result was obtained using tunicamycin to block post-translational protein N-glycosylation in the endoplasmic reticulum. The biochemical properties found in this study correlate well with those of the CD63 molecule

(Azorsa *et al.*, 1991; Engering *et al.*, 2003). As the mAb COS3A was able to bind with both glycosylated and non-glycosylated forms of CD63, suggesting that the antigenic epitope locates on the core protein of the extracellular domains.

Tetraspanins family proteins are known as a diverse functional proteins and can form extended microdomains within the plasma membrane or in interaction with one another as well as with specific receptors and signaling proteins, which probably can serve as specialized binding platform for viral, bacteria, parasitic or fungal pathogens (van Spriel and Figdor, 2010; Yunta and Lazo, 2003). The CD63 belongs to the tetraspanin protein family, which is ubiquitously expressed and is localized both within the endosomal system and on the cell surface (Pols and Klumperman, 2009; Radford *et al.*, 1996). Although, the molecular function of the CD63 is not clear, nonetheless it has been reported to play an important role in several fundamental cell processes including intracellular protein trafficking and migration under normal as well as pathological condition. Furthermore, the CD63 is known as marker for activation of several cell types such as platelet, mast cell and basophil (Azorsa *et al.*, 1991; Schafer *et al.*, 2010; Tammaro *et al.*, 2012; van Spriel and Figdor, 2010). A significant increase of CD63 was found on activation of basophil and this is now used in allergy testing (Bridts *et al.*, 2014; McGowan and Saini, 2013). However, information about the involvement of CD63 in phagocytosis is limited.

Phagocytosis has been acknowledged as a critical component of the innate and adaptive immune response. Whole process of phagocytosis can be devided into three main important stages including (i) recognition, (ii) internalization, and (iii) degradation (Flannagan *et al.*, 2012). The cells involved in this mechanism

called phagocytes including monocytes, macrophages, and granulocytes. Unlike macropinocytosis, phagocytosis involves the recognition and binding of ligands on pathogens by cell surface receptors, so called a receptor-mediated event. Two types of phagocytosis have been described; the first is opsonin-dependent phagocytosis, in which antibody or complement opsonizes the pathogen and promotes its phagocytosis via Fcγ or complement receptors on phagocytes. The second type is opsonin-independent phagocytosis, in which phagocytes recognize the microorganism via pattern-recognition receptors, such as scavenger and mannose receptors. The CD63 was reported to be specifically recruited to phagosomes internalizing *S cerevisiae* (Mantegazza *et al.*, 2004) and *Cryptococcus neoformans*, a phathogenic yeast (Artavanis-Tsakonas *et al.*, 2006). Further evidence for CD63 is essential recruited to phagosomes internalizing *Mycobacterium tuberculosis* in macrophages (Seto, Matsumoto, Tsujimura *et al.*, 2010). Since phagosomes are plasma membrane-derived, thus the primary composition of phagosomes must vary depends on the selective engagement of surface molecule. Many pathogens exhibit various surface molecules which do not exist in higher organism. These molecules can be sensed by several phagocytic receptors (so called PRRs) such as mannose receptor, CD14, and CD36 which selectively bind to mannose unit of polysaccharides present on surface of some yeast cells, lipopolysaccharide of bacterial cell wall, and plasmodium falciparum-infected erythrocytes, respectively (Flannagan *et al.*, 2012). Although, a large number of phagocytic receptors and the molecules involved in phagocytosis have been defined however it does not cover the complete spectrum of infectious agents that phagocytes confront. The CD63 is abundantly found in late endosome, lysosome, and mature phagosome, which are essential for the acquisition

of microbicidal functions by phagosomes (Flannagan *et al.*, 2012; Mantegazza *et al.*, 2004). Furthermore, CD63 expressed in late endosomes is essential for development of intracellular bacteria *Chlamydia trachomatis* (Beatty, 2006, 2008). Although, it has been reported that the role of CD63 in blocking adherence of several bacteria species including *Neisserise meningitides, Staphyphylococcus aureus, Neisseria lactamica, E. coli,* and *Steptococcus pneudomonia*e to human epithelial cell have been observed with the help of antibodies that generated against a large extracellular domain of CD63 (Green, Monk, Partridge *et al.*, 2011). However, functional role of the CD63 in recognition process of bacterial phagocytosis by granulocytes has not been identified. In this study, we observed the effect of the anti-CD63 mAb COS3A on granulocyte phagocytosis of *E. coli* using flow cytometry analysis, and found significant obstruction of the induction of phagocytosis compared to the controls. And we show here for the first time that the CD63 may play an important role in the recognition step of bacterial phagocytosis through an opsonin-independent pathway. There are two possible reasons for this. First, the CD63 may act as a phagocytic receptor for its natural ligand expressing on *E. coli*, therefore binding of the mAb COS3A to the CD63 molecule blocked the natural ligand-receptor interaction which eventually leads to failure of phagocytosis (Figure 4.1A). Second, the CD63 has been reported to interact with several partner proteins in the cell membrane, so it may not act as a phagocytic receptor at all, but binding of this molecule by the mAb COS3A may trigger negative signals that indirectly inhibit the internalization of *E.coli* by granulocytes (Figure 4.1B). This observation opens the question of whether the CD63 acts as a phagocytic receptor or is involved in

the regulation of the phagocytic process, especially for the gram-negative bacterium *E. coli*, and further study is required to elucidate the actual mechanism.

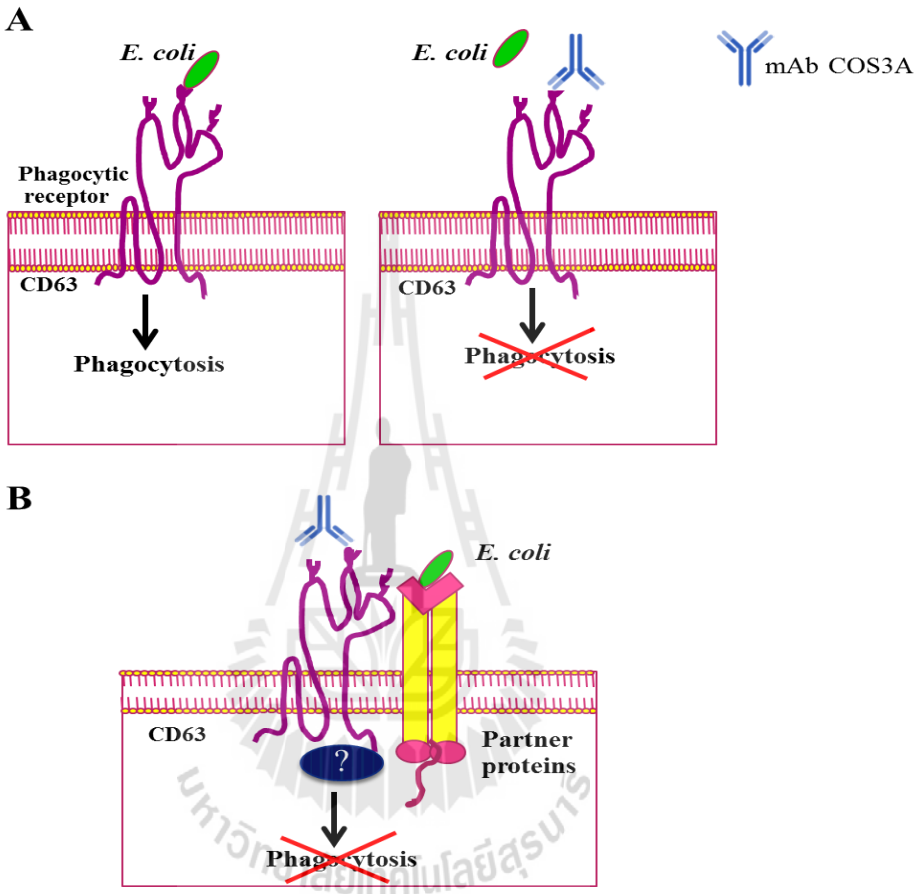

Figure 4.1 Model of two possible mechanisms of inhibitory effect of the mAb COS3A on phagocytosis of *E. coli* by granulocytes. (A) The CD63 may act as a phagocytic receptor for its natural ligand expressing on *E. coli*, therefore binding of the mAb COS3A to the CD63 molecule blocked the natural ligand-receptor interaction which eventually leads to failure of phagocytosis. (B) The CD63 may not act as a phagocytic receptor at all, but binding of this molecule by the mAb COS3A may trigger negative signals that indirectly inhibit the internalization of *E.coli* by granulocytes.

Back to our first interest of the mAb COS3A was the preliminary results that showed the inhibitory property of this mAb on T cell proliferation. We thus further investigated the effect of the mAb COS3A on T cell proliferation using either PBMCs or T cells as models in this study. T cells are central players of the adaptive immune response, which help to protect the host from infectious agents. In order to perform their function, T cells need to be activated, a process that could lead to a variety of responses including proliferation, migration, cytokine production and even apoptosis (Lever, Maini, van der Merwe *et al.*, 2014; Smith-Garvin, Koretzky, and Jordan, 2009). In this study cell proliferation and cytokine production assay were observed. Fully activation of T cells composes of 2 signals. Signal 1 is the initial interaction between the antigen-specific T cell receptor and APCs. Signal 2 is the signal that mediated by the interaction of one or more T cell co-stimulatory receptors with their ligands expressed on APCs. CD28 is proposed as the major costimulatory molecule receptor on naïve T cell. This receptor binds to two co-stimulatory molecules, B7-1 (CD80) and B7-2 (CD86) expressed on antigen presenting cells. Further extra-promotion of activation and growth is provided by cytokines through the engagement of cytokine receptors on T cells to deliver what is referred as signal 3 (Pennock *et al.*, 2013). Characteristic features of an effective costimulation process promotes IL-2 production, survival and entry into the cell cycle (Bour-Jordan and Blueston, 2002).

The human tetraspnin CD63 is weakly expressed on lymphocytes, however up-regulated on T cells upon activation.This may indicate that this molecule has some involvements in T cell biology (Pfistershammer *et al.*, 2004). However, there are a few reports about the involvement between the CD63 and T cell activation.

It has been shown by Pfistershammer group that soluble CD63 mAb inhibits DC induced T cell proliferation but, cross-linkage of the CD63 together by its specific mAb with TCR-triggering induced strong T cell activaton and IL-2 production (Jang and Lee, 2003; Pfistershammer *et al.*, 2004). They claimed that the CD63 may have a regulatory function in co-stimulatory or induced inhibition of TCR activation. However, the molecular mechanism is not clear.

In this study, PBMCs were used as our first model to study the effect of anti-CD63 mAb COS3A on Tcell proliferation. We found that triggering of the CD63 by the soluble mAb COS3A dramatically reduced the threshold of CD3-mediated T cell proliferation in a dose-dependent manner. The mAb COS3A exhibits similar effect on T cell activation as shown by using the soluble anti-CD63 mAb 11C9 (Pfistershammer *et al.*, 2004). In addition we found that the inhibitory effect of the soluble mAb COS3A on T cell proliferation is corresponding to the decreasing of IL-2 and its receptor (CD25) as well as IFN-γ production by T cells. But the secretion of the suppressive T cell response cytokine, IL-10, by monocytic cells was enhanced (Figure 4.2A). IL-10 is produce by both monocytes and lymphocytes. This cytokine has effect on decreasing level of IL-2 and IFN-γ (Sabat *et al.*, 2010). Nonetheless, our study revealed that the inhibitory effect of the mAb on T cell proliferation was not reversed by addition of neutralizing anti-IL-10. This result clearly showed that suppression of CD3-mediated T cell proliferation by the mAb COS3A does not involve in the suppressive function of IL-10 on T cell activation. Another possible mechanism on this inhibitory effect is that the mAb COS3A blocks the interaction of costimulatory ligands expressing on APCs to the CD63 on T cells. This hypothesis can be supported by previous observation that presence of the CD63 mAb did not

affect T cell activation mediated by CD3/CD28 coated beads (Pfistershammer *et al.*, 2004). Moreover, the CD63 associates with MHC II molecule and plays an important role in MHC II sorting, clustering and organization on T lymphocytes (Mantegazza *et al.*, 2004). Knocking down the CD63 in EBV-transformed B cells increased the ability to activate $CD4^+$ T cells and increased production of exosomes (Petersen *et al.*, 2011). However, no costimulatory ligand on APCs that binds directly to the CD63 on T cell has been defined. Taken togther the results suggested that the novel CD63 mAb COS3A has the potential to inhibit CD3-mediated T cell activation, however presence of the APCs is needed.

To be more specified on T cells, purified T cells were used as another study model on the effect of the mAb COS3A on T cell activation. In this model we found that coimmobilization of the mAb COS3A together with TCR/CD3-triggering induced strong T cell activation. Nonetheless, the soluble mAb COS3A had no effect on T cell activation, both cell proliferation and cytokine production pattern of the CD3-mediated T cell activation (Figure 4.2B). Moreover, the efficient of CD63-mediated costimulatory signal on T cell via the mAb COS3A induced a strong secretion of IL-2 and IFN-γ production. These finding suggest that immobilization of the mAb COS3A to plastic surface is required for strong cross-linking achieved to induce the CD63 costimulation in T cell. In addition, preliminary results on cell cycle analysis revealed that cross-linkage of the mAb COS3A together with TCR/CD3-triggering induce cell cycle transition of T cells from G1 to S phase (Figure 4.2C). Our data obtained for the CD63 in regulation of T cells are quite similar as were observed in the previous report using anti-CD63 mAb 11C9 (Pfistershammer *et al.*, 2004). In addition, another commercially available anti-CD63

mAb MEM259, which has not been investigated on T cell activation, was also included in this study. However, this mAb has no effect on T cell activation as shown by using the mAb COS3A; in both PBMCs and T cell model. This may suggest that either anti-CD63 mAb COS3A recognizes a distinct epitope of the mAb MEM-259 on the CD63 molecule, or binding affinity of the mAb COS3A is higher than those of the mAb MEM-259.

In addition, CD63 is incorporated into tetraspanin microdomains, it is conceivable that the CD63 causes a similar deregulation of these microdomains followed by diminished antigen presentation as observed for CD37 and Tssc6 (Gartlan, Belz, Tarrant *et al.*, 2010). Alternatively, the CD63 may have a regulatory function in co-stimulation or induced inhibition of TCR activation. Further study is required to elucidate the actual mechanism of the CD63 on T cell response.

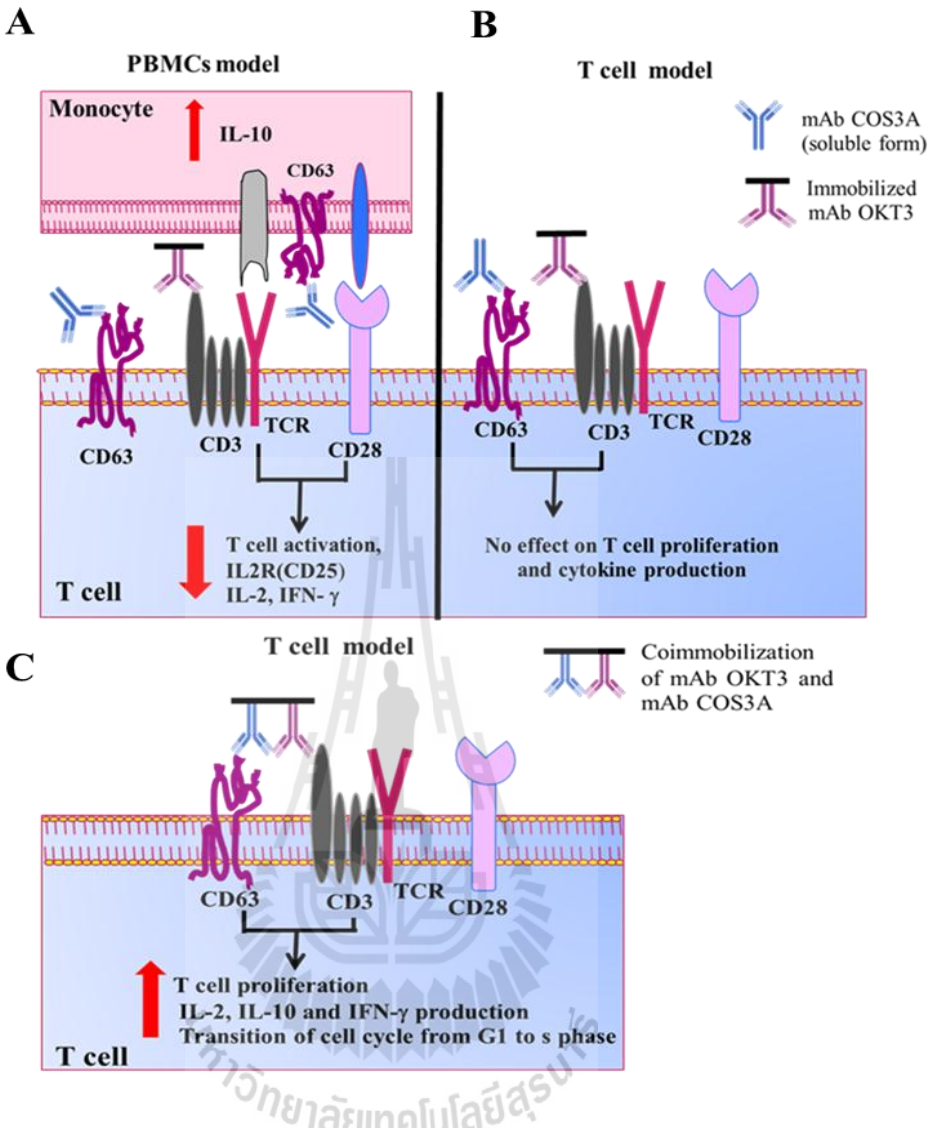

Figure 4.2 Effect of the anti-CD63 mAb COS3A on T cell activation. (A) The soluble mAb COS3A significantly inhibits CD3-mediated T cell proliferation using PBMCs as model. The suppression phenomenon is corresponding to decrease production of IL-2 and its receptor (CD25) as well as IFN-γ by T cells, but enhance IL-10 production by monocytic cells. (B) The soluble mAb COS3A had no effect on T cell proliferation and cytokine production. (C) Coimmobilization of the mAb COS3A and anti-CD3 mAb OKT3 strongly enhanced T cell proliferation, secretion of IL-2, IL-10 and IFN-γ by T cells. Furthermore, cell cycle analysis indicated that induction of cell proliferation was occurred through the activation of G1/S transition.

CHAPTER V

CONCLUSION

In conclusion, we have identified a novel mAb specific for the human CD63, named COS3A. The antibody is suitable for both biochemical and functional studies of the CD63 molecule. In addition, the mAb COS3A significantly inhibits phagocytosis of *E. coli* by granulocytes, and we show here for the first time that the CD63 may play an important role in the recognition step of bacterial phagocytosis. This observation opens the question of whether the CD63 acts as a phagocytic receptor or is involved in the regulation of the phagocytic process. Furthermore, the effect of the mAb COS3A on T cell activation was studied. We found that binding of intact mAb COS3A significantly inhibits CD3-mediated T cell proliferation using PBMCs as model. The suppression phenomenon is corresponding to decrease production of IL-2 and its receptor (CD25) as well as IFN-γ by T cells, but enhance IL-10 production by monocytic cells. This inhibitory phenomenon was not reversed by addition of neutralizing anti-IL-10 mAb. In the purified T cell model, a soluble of mAb COS3A has no effect on anti-CD3-triggered T-cells, whereas cross-linkage of the CD63 with the mAb COS3A is needed for costimulatory activation of CD3-mediated T cell proliferation. The efficient CD63-mediated costimulatory on T cell activation induced a strong production of IL-2, IL-10, and IFN-γ. Furthermore, cell cycle analysis indicated that induction of cell proliferation was occurred through the

activation of G1/S transition. These findings lead to the question of whether the CD63 acts as co-stimulatory signal or induced inhibition of TCR/CD3 activation.

Further study is needed to elucidate the actual mechanism of the CD63 on T cell response. Indeed, identification of putative CD63 ligand remains crucial for a better understanding of how does the CD63 function in the immune response and also its clinical applications.

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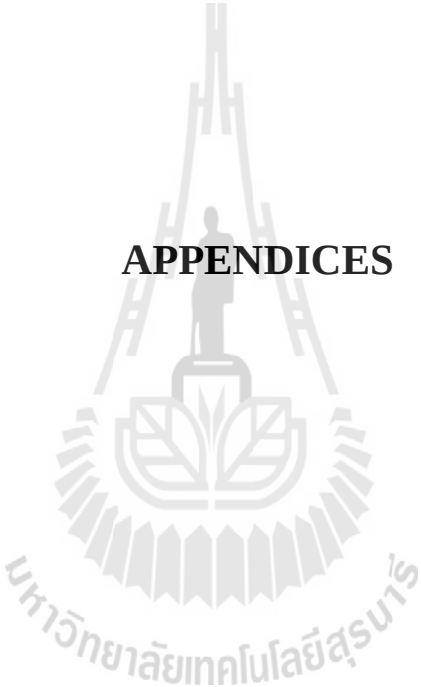
APPENDICES



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APPENDIX B

OUTPUT FROM THESIS

1) Publication

Wansook, S., Pata, S., Kasinrerk, W., and Khunkaewla, P. (2016) Biochemical and functional analysis of COS3A, a novel CD63-specific monoclonal antibody. **Asian Pac J Allergy Immunol**, March 20, doi: 10.12932/ AP0735. (IF 2014 = 0.971)

2) Poster presentations

1. Wansook S., Pata S., Mahasongkram K., Chruewkamlow N., Kasinrerk W., and Khunkaewla P. Effect of newly generated CD63 monoclonal antibody on T cell activation. 10th International Symposium of the Protein Society of Thailand (PST), July 15-17, 2015. Bangkok, Thailand.
2. Wansook S., Pata S., Mahasongkram K., Chruewkamlow N., Kasinrerk W., and Khunkaewla P. Cellular distribution, biochemical characterization, and functional analysis of COS3A recognizing molecule. The 7th Asia Oceania Human Proteome Organization (AOHUPO) Congress and 9th International Symposium of the Protein Society of Thailand (PST), August 6-8, 2014. Bangkok,Thailand.
3. Wansook S., Pata S., Mahasongkram K., Kruakhamlaw N., Kasinrerk W., and Khunkaewla P. COS3A molecule; cellular expression, biochemical

characterization and function on T cell activation. The Federation of immunological societies of Asia Oceania (FIMSA) advanced training course 2013: molecule and cells of innate immune system, October 22-25, 2013. Chiang Mai.Thailand.

4. Wansook S., Pata S., Mahasongkram K., Kasinrerk W., and Khunkaewla P. COS3A, a high glycosylated leukocyte surface molecule plays a role on cell activation. The 5th Congress of the Federation of National Societies of Biochemistry and Molecular Biology in the Asian and Oceanian Region (FAOBMB), November 25-29, 2012. Bangkok, Thailand.
5. Wansook S., Pata S., Mahasongkram K., Kasinrerk W., and Khunkaewla P. Cellular expression and biochemical identification of leukocyte surface molecule recognized by newly generated monoclonal antibody COS3A. The 5th Congress of the Federation of Immunological Societies of Asia-Oceania (FIMSA), March 14-17, 2012. New Delhi, India.

CURRICULUM VITAE

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2009-2016	School of Biochemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand **Ph.D. candidate in Biochemistry**
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