FUNCTIONAL STUDY OF T LYMPHOCYTE SURFACE MOLECULE RECOGNIZING BY MONOCLONAL ANTIBODY CARA

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คำสำคัญ: โมโนโคลนอลแอนติบอดี, โปรตีนบนผิวเซลล์เม็ดเลือดขาว, การกระตุ้นทีเซลล์ ไซโตไคน์, ฟอสโฟไทโรซีน

เม็ดเลือดขาวมีบทบาทในระบบภูมิคุ้มกัน ทำหน้าที่ป้องกันและตอบสนองต่อสิ่งแปลกปลอมผ่านกลไกการสื่อสารระหว่างเซลล์ โดยอาศัยโมเลกุลบนผิวเซลล์เป็นสื่อกลาง โมเลกุลดังกล่าวบางชนิดได้รับการระบุและศึกษาแล้ว แต่มีโมเลกุลอีกหลายชนิดที่ยังคงไม่ทราบลักษณะและหน้าที่อย่างชัดเจน ตั้งแต่มีการพัฒนาเทคนิคไฮบริโดมา ทำให้สามารถผลิตแอนติบอดีจำเพาะต่อแอนติเจนหรือเรียกว่าโมโนโคลนอลแอนติบอดี เป็นเครื่องมือสำคัญในการระบุและศึกษาคุณลักษณะของโมเลกุลต่าง ๆ บนผิวเซลล์ งานวิจัยนี้มุ่งเน้นการศึกษาโมโนโคลนอลแอนติบอดีชนิดหนึ่งที่ผลิตขึ้นภายในห้องปฏิบัติการ คือ โมโนโคลนอลแอนติบอดีโคลน CARA ที่จำเพาะต่อโมเลกุลหนึ่งบนผิวทีเซลล์ของมนุษย์ที่ยังไม่สามารถระบุชนิดได้ ผลการทดลองเบื้องต้นที่ยังไม่ตีพิมพ์ชี้ให้เห็นว่าโมโนโคลนอลแอนติบอดี CARA สามารถจำแนกประชากรย่อยของทีเซลล์ที่ไม่เคยมีการรายงานมาก่อนและไม่อยู่ในกลุ่ม $CD4^+$, $CD8^+$ หรือทีเซลล์ชนิดควบคุม (regulatory T cell) นอกจากนี้ ของเหลวส่วนที่เป็นของเหลวเหนือตะกอนที่ได้จากการเพาะเลี้ยงเซลล์ไฮบริโดมาที่ประกอบด้วยโมโนโคลนอลแอนติบอดี CARA แสดงผลการยับยั้งการกระตุ้นทีเซลล์ผ่าน CD3 ทั้งแสดงให้เห็นถึงศักยภาพในการควบคุมการทำงานของทีเซลล์ อย่างไรก็ตาม ผลของโมโนโคลนอลแอนติบอดี CARA ที่ผ่านการทำให้บริสุทธิ์ต่อการกระตุ้นทีเซลล์ยังไม่เคยมีการศึกษา งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อระบุเป้าหมายโมเลกุลที่จำเพาะของโมโนโคลนอลแอนติบอดี CARA และศึกษาผลของแอนติบอดีดังกล่าวต่อการกระตุ้นและการทำงานของทีเซลล์ โดยใช้เซลล์เม็ดเลือดขาวชนิดโมโนนิวเคลียร์ในเลือดส่วนปลายของมนุษย์ (PBMCs) เป็นแบบจำลองในการศึกษาทางหน้าที่ และใช้ทีเซลล์สายพันธุ์ Molt4 เป็นแบบจำลองในการศึกษาเชิงโมเลกุล ผลการวิเคราะห์ชนิดของไอโซไทป์ด้วยวิธี Capture ELISA และ RT-PCR พบว่าโมโนโคลนอลแอนติบอดี CARA มีองค์ประกอบของหน่วยย่อยโพลีเปปไทด์สายหนักชนิดแกมมา 3 (γ3) และสายเบาชนิดแคปปา (κ) ซึ่งโมโนโคลนอลแอนติบอดี CARA สามารถผลิตและทำให้บริสุทธิ์ได้ด้วยวิธีโครมาโทกราฟีแบบแอฟฟินิตีโดยใช้โปรตีน L โดยแอนติบอดียังคงความสามารถในการจับจำเพาะต่อแอนติเจนไว้ได้และถูกนำมาใช้ตลอดการศึกษา ในการศึกษาผลของโมโนโคลนอลแอนติบอดี CARA ต่อการเพิ่มจำนวนของทีเซลล์ที่ถูกกระตุ้นด้วย anti-CD3 ใช้การย้อมสีด้วย CFSE

ร่วมกับการวิเคราะห์โฟลไซโตเมตรีและประเมินผลการผลิตไซโตไคน์โดยวิธี sandwich ELISA รวมถึงการแสดงออกของโมเลกุลบนผิวเซลล์และไซโตไคน์ถูกวิเคราะห์ด้วยวิธีการย้อมฟลูออเรสเซนต์โฟล-ไซโตเมตรีและกล้องจุลทรรศน์แบบสแกนด้วยเลเซอร์ สำหรับการศึกษาสัญญาณภายในเซลล์ วิธีเวสเทิร์นบลอตถูกใช้ในการตรวจสอบการฟอสโฟรีเลตของโปรตีนไทโรซีน ผลการศึกษาแสดงให้เห็นว่า โมเลกุลที่โมโนโคลนอลแอนติบอดี CARA จับ มีการแสดงออกแบบไม่สม่ำเสมอในเซลล์ Molt4 โดยสามารถแบ่งออกเป็น 3 กลุ่ม ได้แก่ $CARA^{neg}$ $CARA^{low+}$ และ $CARA^{high+}$ ทั้งยังไม่พบการแสดงออกร่วมของโมเลกุลที่โมโนโคลนอลแอนติบอดี CARA จับได้ กับ CD3 ในเซลล์ Molt4 และทีเซลล์ที่ถูกกระตุ้น นอกจากนี้โมโนโคลนอลแอนติบอดี CARA มีผลยับยั้งการเพิ่มจำนวนของทีเซลล์ที่ถูกกระตุ้นผ่าน CD3 อย่างมีนัยสำคัญในลักษณะขึ้นกับความเข้มข้นหลังจากเพาะเลี้ยงเป็นเวลา 3 และ 5 วัน เมื่อเปรียบเทียบกับแอนติบอดีควบคุมที่มีไอโซไทป์เดียวกัน ผลดังกล่าวยืนยันด้วยการแสดงออกที่ลดลงของ CD69 และ CD25 ซึ่งเป็นตัวบ่งชี้การกระตุ้นทีเซลล์ในระยะต้นและปลายตามลำดับ ยิ่งไปกว่านั้นโมโนโคลนอลแอนติบอดี CARA ยังลดการผลิตไซโตไคน์ ได้แก่ IL-2, IL-4, IL-10, IL-17A, IFN-γ และ TNF-α อย่างมีนัยสำคัญหลังจากเพาะเลี้ยง 24 ชั่วโมง เมื่อเปรียบเทียบกับกลุ่มควบคุม อย่างไรก็ตาม การย้อมภายในเซลล์แสดงให้เห็นว่ามีเพียง IL-2 เท่านั้นที่มีการผลิตลดลง สุดท้ายผลของโมโนโคลนอลแอนติบอดี CARA ต่อสัญญาณการส่งผ่านของทีเซลล์ผ่าน CD3 ถูกตรวจสอบด้วยเวสเทิร์นบลอตโดยพบสัญญาณฟอสโฟไทโรซีนที่เพิ่มขึ้นอย่างชัดเจนในโปรตีนที่มีน้ำหนักโมเลกุลประมาณ 63–65 kDa ซึ่งสอดคล้องกับ ZAP-70 ซึ่งเป็นโมเลกุลสำคัญในการส่งสัญญาณผ่าน TCR บ่งชี้ว่าโมโนโคลนอลแอนติบอดี CARA อาจมีบทบาทในการควบคุมการส่งสัญญาณภายในเซลล์ในระยะต้น อย่างไรก็ตาม จำเป็นต้องมีการศึกษาต่อไปเพื่อยืนยันความจำเพาะของโมเลกุลที่ส่งสัญญาณ จากผลการศึกษาสามารถสรุปได้ว่าโมโนโคลนอลแอนติบอดี CARA มีศักยภาพในการยับยั้งการกระตุ้นและการผลิตไซโตไคน์ของทีเซลล์ที่ตอบสนองต่อการกระตุ้นด้วย CD3 ซึ่งอาจเกิดจากกลไกการส่งสัญญาณภายในเซลล์ผ่านการฟอสโฟรีเลตของโปรตีนไทโรซีน แม้ว่าโมเลกุลเป้าหมายของโมโนโคลนอลแอนติบอดี CARA ยังไม่สามารถระบุได้อย่างชัดเจน แต่คุณสมบัติในการควบคุมการกระตุ้นของทีเซลล์และลดการผลิตไซโตไคน์สำคัญ เช่น IL-2 บ่งชี้ถึงศักยภาพในการประยุกต์ใช้เพื่อควบคุมการตอบสนองของภูมิคุ้มกัน โดยเฉพาะในภาวะที่มีการกระตุ้นทีเซลล์มากเกินไป เช่น โรคภูมิต้านตนเอง หรือโรคภูมิคุ้มกันต่อต้านร่างกายผู้รับการปลูกถ่าย (GVHD)

สาขาวิชาเคมี ลายมือชื่อนักศึกษา วราพรรณ ศันธุชัย

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา

WARAPAN PANCHAI : FUNCTIONAL STUDY OF T LYMPHOCYTE SURFACE MOLECULE RECOGNIZING BY MONOCLONAL ANTIBODY CARA. THESIS ADVISOR : ASSOC. PROF. PANIDA KHUNKAEWLA, Ph.D. 113 PP.

Keyword: monoclonal antibody, leukocyte surface molecule, T ell activation, cytokine, phosphotyrosine

Leukocytes are a crucial role in the immune system through their surface molecules that mediate cell-cell communication. However, some of these molecules remain unidentified. Since the hybridoma technique was invented, monoclonal antibodies (mAbs) have been produced and have become essential tools for identifying and characterizing molecules over the years. This study focused on an in-house mAb clone CARA, which targets an unidentified molecule exclusively expressed on human T cells. Preliminary unpublished findings suggest that mAb CARA can classify a previously unknown T cell subpopulation, distinct from $CD4^+$, $CD8^+$ nor regulatory T cells. Furthermore, culture supernatant containing mAb CARA from hybridoma cell cultivation exhibited suppressive effect on CD3-mediated T cell stimulation, indicating potential immunomodulatory effects on T cell biology. Nonetheless, the effect of purified mAb CARA on T cell stimulation remains unexplored. Therefore, this study aims to identify the molecular target of mAb CARA and investigated its immunological functions in T cell stimulation. Human peripheral blood mononuclear cells (PBMCs) were for functional analysis, while the Molt4 T cell line for molecular characterization. Isotyping via capture ELISA and RT-PCR indicated that mAb CARA contains gamma3 ($\gamma 3$) heavy chains and kappa (κ) light chains. A high yield of purified mAb CARA, retaining its reactivity, was successfully obtained using protein L affinity chromatography and utilized throughout the study. The effect of mAb CARA to the proliferation on CD3-mediated T cell was assessed using a proliferation assay based on carboxyfluorescein succinimidyl ester (CFSE) and flow cytometry analysis while, total cytokine was evaluated using sandwich ELISA. Immunofluorescent staining, the expression of cell surface molecules and cytokines were analyzed using flow

cytometry and a laser scanning microscope. The intracellular signalling was studied through phosphotyrosine Western blotting. The cellular expression study revealed that the mAb CARA-recognizing molecule is heterogeneously expression among the population of Molt4 cells, which could be classified these cells into three subpopulations, including $CARA^{neg}$, $CARA^{low+}$, $CARA^{high+}$. Additionally, this molecule does not colocalize with the CD3 molecule on both in Molt4 and activated T cells. The effect of the mAb CARA to activation of T cell was investigated and demonstrated that mAb CARA significantly hinders proliferation of CD3-mediated T cell in a dose-dependent manner after 3 and 5 days of cultivation compared to the matched isotype control mAb. This effect was confirmed by the decreased expression of CD69, an early activation marker, and CD25, a marker of late T cell activation, and cytokines, including IL-2, IL-4, IL-10, IL-17A were significantly reduced, accepted IFN-γ and TNF-α after 24 h of cultivation compared to the matched isotype control mAb Nonetheless, cytokine staining showed a marked decrease in IL-2 only. Finally, Western blotting revealed altered tyrosine phosphorylation patterns. Remarkably, the most prominently enhanced signal was observed in a protein with a molecular weight of approximately 63-65 kDa in the presence of mAb CARA, consistent with phosphorylated ZAP-70, a key molecule in TCR signaling. These findings suggested that mAb CARA modulates early TCR signaling events. However, further investigation is necessary to confirm its specificity. In conclusion, mAb CARA effectively suppresses T cell activation and cytokine production in response to anti-CD3 stimulation, likely through intracellular signaling via tyrosine phosphorylation cascades. Although the specific molecular target of mAb CARA remains unidentified, its ability to selectively target activated T cells and reduce key cytokine production, such as IL-2, indicates therapeutic potential in T cell-driven conditions such as autoimmune diseases or graft-versus-host disease (GVHD).

School of Chemistry Student's Signature Warapan Panchai

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LIST OF ABBREVIATIONS

AF	Alexa flour
Ag-Ab	Antigen-antibody interaction
$AIRE^{+}$	Autoimmune regulatory protein
APCs	Antigen-presenting cells
BSA	Bovine serum albumin
CD4	Cluster of differentiation 4 (helper T cells)
CD8	Cluster of differentiation 8 (cytotoxic T cells)
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CH	Constant heavy chain
CL	Constant light chain
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding region
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FITC	Fluorescein isothiocyanate
GVDH	Graft-versus-host disease
HRP	Horseradish peroxidase
HSCs	Hematopoietic stem cells
ICOS	Inducible T cell costimulator
ICS	Intracellular cytokine staining
IFN	Interferon
IgA	Immunoglobulin A

LIST OF ABBREVIATIONS (Continued)

IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
Igs	Immunoglobulins
IL	Interleukin
IP	Immunoprecipitation
ITAMs	Immunoreceptor tyrosine-based activation motifs
iTreg	Inducible T regulatory
LAT	Linker for activated T cells
Lck	Lymphocyte-specific protein tyrosine kinase
MAbs	Monoclonal antibodies
MHC	Major histocompatibility
mTECs	Medullary thymic epithelial cells
Nck	Non-catalytic region of tyrosine kinase
NF-KB	Nuclear factor-kappa B
NK-cells	Natural killer cells
nTreg	Naturally occurring T-regulatory
Numb	Inhibitor of Notch- signaling
PAbs	Polyclonal antibodies
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PBST	Tween 20 in phosphate buffer saline
PCR	Polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein
PRRs	Pattern recognition receptors
PRS	Proline-rich sequence
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
S-S	Disulfide bond
STAT	Signal transducer and activator of transcription
TCM	Central memory cells
TCR	T cell receptor
TEM	Effector memory T cell
TEMRA	Effector memory cell re-expressing CD45RA T cells
Tfh	Follicular T helper
TGF-B	Tumor growth factor-beta
Th	T helper
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
Treg	T regulatory
VH	Variable heavy chain
VL	Variable light chain
WASP	Wiskott-Aldrich syndrome protein
Zap-70	Zeta-chain-associated protein kinase 70

CHAPTER I

INTRODUCTION

1.1 Significance of research

Leukocytes are cellular components that are present in blood at a rate of approximately 1%. These cells play a crucial role in the immune system, as they are involved in both innate and adaptive immunological responses, as well as inflammatory and cellular responses to infection, illness and injury (Tigner, Ibrahim and Murray, 2020). Leukocyte surface molecules have been demonstrated to be essential for all aspects of leukocyte function including maturation, differentiation, migration, and antigen response by cell-cell communication through their surface molecules (Abbas, Lichtman and Pillai, 2019). Several molecules have been identified and characterized; however, their functions are not fully understood. It is also thought that some of these molecules are still waiting for identification.

T cell progenitors are known to originate in the bone marrow, subsequently entering the thymus where they differentiate through selection processes (Koch and Radtke, 2011) to become mature T cells. T cells can be categorized into three main types based on the expression of their surface markers including T helper cell ($CD4^+$ T cell), cytotoxic T cell ($CD8^+$ T cell) and regulatory T cell ($CD25^+$ T cell). In addition, these cells can also be categorized into subsets based on their protein marker profiles, comprising both naïve and memory T cells. The classification of these subsets is determined by CD27 (a T cell costimulatory molecule) and CD45RA. The distinction between central and effector memory is determined by the surface expression of CCR7 and CD45RA (Appay et al., 2008).

The classification of T cells into activated and memory T cells is based on the surface expression of CD45RO (Bembridge et al., 1995). There is also a reverse expression of CD45RB on CD45RA and CD45RO cells.

Upon the process of T cell activation and progression from a naïve to a memory state, CD45RB is known to undergo downregulation (Hove and Slors, 2004). Therefore, the naïve, effector, and memory stages of T cell maturation can be separated by the expression level of CD45RB in comparison to other CD45 isoforms.

The activation of T cells represents a critical event in the initiation and regulation of the adaptive immune response requiring at these distinct signals for full T cell activation. Signal 1 begins when the T cell receptor (TCR) recognizes and binds to a specific peptide presented by major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs) (Bromley et al., 2001). Costimulatory molecules B7.1 (CD80) or B7.2 (CD86) on antigen-presenting cells (APCs) bind to CD28 on T cells, delivering signal 2 for activation. The final stage of T cell activation requires cytokines secretion to deliver signal 3 to direct T cell differentiation into distinct types of helper T cell subset according to the cytokine profiles. For instance, cells expose to the cytokine IL-12 which promote naïve T cell differentiate into Th1 (Tai et al., 2018). CD3 is the component of the TCR that is responsible for transmitting activation signals into the cell. These signals initiate a process that results in T cell proliferation and differentiation. Following full activation, T cell expansion is initiated, with cells undergoing division, differentiation, and secretion of effector molecules. Effector T cells comprise two distinct subsets: $CD8^+$ cytotoxic T cells and $CD4^+$ effector T cells. The latter can further differentiate into various types of T helper (Th) cell subsets, including Th1, Th2, Th9, Th17, Th22 and regulatory T (Treg) cell, or follicular T helper (Tfh) cell, based on distinct cytokine profiles (Appay et al., 2008).

In collaborating with Prof. Dr. Watchara Kasinrerk, Department of Medical Technology, Chaing Mai University, whose laboratory has produced several mAbs to leukocyte surface molecules using conventional hybridoma technique. Those mAbs have been used as tools for identification, characterization and functional studies of their specific molecules such as mAb clone MT4 (IgM isotype) specific for CD4 molecule (Kasinrerk, Tokrasinwit and Naveewongpanit, 1998), mAb clone M6-1D4 (IgM) specific

for CD147 molecule (Kasinrerk, Tokrasinwit and Phunpae, 1999), mAbs clone MT99/1 and MT99/2 specific for CD99 molecules (Khunkaewla et al., 2007). MAb clone P-3E10 has been used for biochemical and functional characterization of its specific molecule and found to recognize Na, K ATPase β3-subunit and the mAb has also been shown to inhibit CD3-mediated T cell proliferation. This mAb was later accounted as CD298, which is the first CD discovered by Thai researchers (Chiampanichayakul et al., 2006). As well as mAb clone MT3 to TCR/CD3 complex associated protein called MAL protein play an essential role in T cell activation and can be used to determine subpopulation of naïve T cell and memory T cell (Mahasongkram et al., 2015).

MAb clone CARA (IgG3) is one of interest, it specific to an unidentified molecule on human T cells, which will be named CARA molecule. According to unpublished preliminary results revealed that the CARA molecule is heterogeneously expressed on the cell surface of human T cell line, Molt4. Thus, the cells can be divided into $CARA^{+}$ Molt4 and $CARA^{-}$ Molt4. Interestingly, it has been shown that only 20 % of T lymphocytes express this molecule on their cell surface. The mAb clone CARA may classify an unknown novel T cell subpopulation, which belongs to neither $CD4^{+}$, $CD8^{+}$ nor regulatory T cells. Expression of the CARA molecule was found on CD45RO and CD45RB positive cells and heterogeneously expressed on the cells with CD45RBlow population but was not found on CD45RA positive cells. Therefore, CARA molecule is expressed on effector and memory T cells but not on naïve T cells.

According to the preliminary results and available mAb, this study tried to identify the evaluation of the effect of mAb clone CARA on T cell biology including expression of T cell activation markers, T cell proliferation, cytokine production and cell signaling. The obtained information will provide valuable impact of this mAb on T cell biology in the context of regulating cell-mediated immune responses.

1.2 Objectives

1.2.1) To study the effect of mAb CARA on suppression of CD3-mediated T cell activation; cytokine production profile and intracellular signaling.

1.2.2) To identify the CARA recognizing molecule and its biochemical properties.

1.3 Literature review

1.3.1 Immune system

The immune system comprises a highly organized network of cells and soluble factors that collectively function to protect the host from pathogenic infections. There are two main different types of responses consisting of innate immune responses and adaptive immune responses.

1.3.1.1 Innate immunity

The immune system, in its innate state, fulfils a variety of functions that are of critical importance in the protection of the body against micro-organisms and tissue injury. In some cases, the term of nonspecific immune system is employed. This type of immune system is rapidly activated by microbes with the aim of preventing, controlling or eliminating infections. The innate response is typically initiated upon the identification of microbes by pattern recognition receptors (PRRs). These PRRs can detect molecules that are characteristic of the pathogen (Kumar, Kawai and Akira, 2011).

1.3.1.2 Adaptive immunity

The adaptive immune system is employed to denote the acquired immune system or specific immune system. This system is comprised of a number of systemic cells and processes which have the capability to eliminate pathogens or prevent them from growing. The adaptive immune system contains various cell types, these include antigen-specific T cells. These T cells are activated to proliferate via the action of antigen-presenting cells (APCs). B cells can be functionally classified based on their differentiation into memory B cells, which provide long-term immunity, and plasma cells, which are responsible for producing antibodies (Marshall et al., 2018).

As demonstrated in Figure 1.1, the adaptive immune responses can be divided into two types including humoral immunity and cell-mediated immunity. Together, these mechanisms allow the human body to mount a targeted immune response against pathogens, such as bacteria, viruses and toxins.

The process of humoral immunity is facilitated by antibodies, which are produced by B lymphocytes. The function of the immune system is to serve as the primary form of defense against extracellular microorganisms and their toxins. This is achieved by binding bacteria and toxins to releasing antibodies (Rabb, 2002).

The phenomenon known as cell-mediated immunity is primarily facilitated by T lymphocytes. This immune response is distinct from one which relies on antibody effector function, instead involving the activation of macrophages and natural killer (NK) cells. These cells promote the eradication of microbes within phagocytes and the destruction of infected cells, thus serving to eliminate reservoirs of infection (Rabb, 2002).

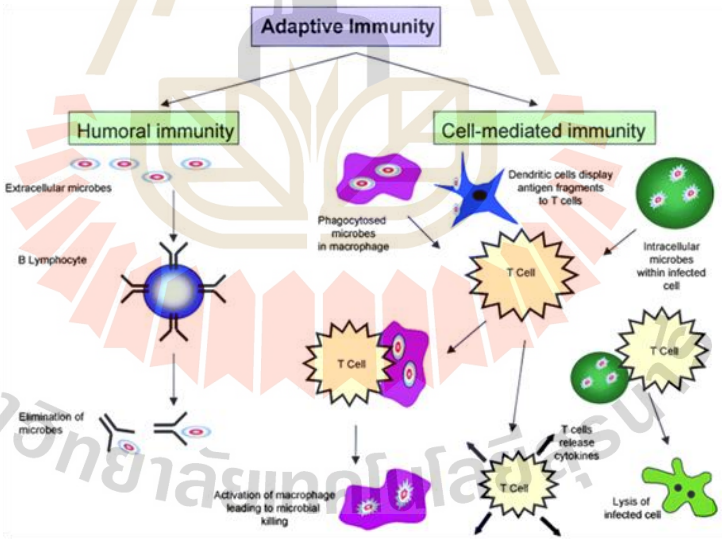

Figure 1.1 Adaptive immunity. Adaptive immunity is categorized into humoral and cell-mediated responses. Humoral immunity involves the production of antibodies by B lymphocytes, which target and neutralize extracellular pathogens. Cell-mediated immunity, on the other hand, is driven by T lymphocytes that become activated upon encountering antigens presented by dendritic cells (Rabb, 2002).

1.3.2 Leukocytes

Leukocytes are responsible for defending the body against infectious diseases and foreign invaders. It is widely accepted that all leukocytes derive from multipotent hematopoietic stem cells (HSCs) located in the bone marrow (Monga, Kaur and Dhanda, 2022). These cells develop into specific cell types under the influence of various growth factors generally known as cytokines. The cytokines work in a hierarchical and synergistic manner to promote leukocyte proliferation, differentiation, maturation, and specific function. By controlling the physiological interaction between the various cell types, Cytokines play a critical role in modulating inflammatory responses, as well as in the development and maintenance of immune function (Bordin, Heddle and Blajchman, 1994). Leukocytes can be separated into three main subtypes including granulocytes, lymphocytes, and monocytes.

1.3.3 T lymphocyte

T lymphocytes or T cells, originate in bone marrow before migrating to the thymus, where they undergo selection and differentiation to become mature naïve or regulatory T cells. Thereafter, the cells, which have become naïve and regulatory in nature then leave the thymus and migrate to secondary lymphoid organs or in circulation. Following the encounter with antigens, these cells will undergo activation and differentiation into memory or effector T cells, or both cell types.

A crucial step in development of T cells is the formation of a functional T cell receptor (TCR). Each T cell undergoes random gene rearrangement to produce a unique T cell receptor (TCR), which allows the immune system to recognize diverse antigenic peptides. This process must be tightly regulated to ensure that TCRs can bind foreign antigens presented by MHC molecules but not react against self-antigens. Proper selection ensures immune protection while preventing autoimmunity.

1.3.3.1 T cell development in thymus through positive and negative selection

T cell progenitors enter thymus as double negative cells and undergo the double positive there. T cells were distinguished into $CD4^+$ or $CD8^+$ T cells through the positive selection process. The duration of this process is between three and four days in the thymic cortex (Ross et al., 2014). At this stage, double-positive ($CD4^+/CD8^+$) T cells enter the thymic cortex, where they are exposed to self-antigens, which are presented on MHC molecules located on the surface of cortical epithelial cells. A crucial survival signal will be delivered to thymocytes that interface successfully with only either MHC-I or MHC-II. The cells that bind to MHC-I will be differentiated into single positive $CD8^+$ T cells due to deletion of gene encoding for $CD4^+$ T cells, vice versa, cells that bind to MHC-II, will be differentiated into single positive $CD4^+$ T cells. For those that are unable to interact or interact with a very strong binding affinity will not get a survival signal and will eventually undergo apoptosis from neglect as shown in Figure 1.2 (Parkin and Cohen, 2001).

After positive selection, thymocytes will move to the cortex-medulla border of the thymus for their negative selection process. At this area, MHC of medullary thymic epithelial cells (mTECs) will present self-antigen to the entrance single positive thymocytes (Hinterberger et al., 2010). Self-antigens expression at mTECs is subject to regulation by the autoimmune regulator protein ($AIRE^+$), which is encoded by the AIRE gene. This ensures the appropriate expression of self-antigens from all tissues of the body on their MHC class I peptides. Since $CD4^+$ T cells have undergone positive selection by interacting with MHC class II molecules, APCs containing MHC class II are necessary for negative selection of $CD4^+$ T cells. Some mTECs can present self-antigen on MHC class II molecules by thymic dendritic cells phagocytosis, which makes them become AIRE-APCs. Thymocytes that engage with the self-antigen too strong experience an apoptotic signal leading to cell death. Some of these cells are developed into Treg cells. The remaining survival cells will differentiate into naïve T cells that finally leave the thymus (Pekalski et. al, 2017). This procedure is a crucial part of central tolerance to prevent the development of self-reactive T cells that can cause autoimmune disorders in the host as shown in Figure 1.2 (Parkin and Cohen, 2001).

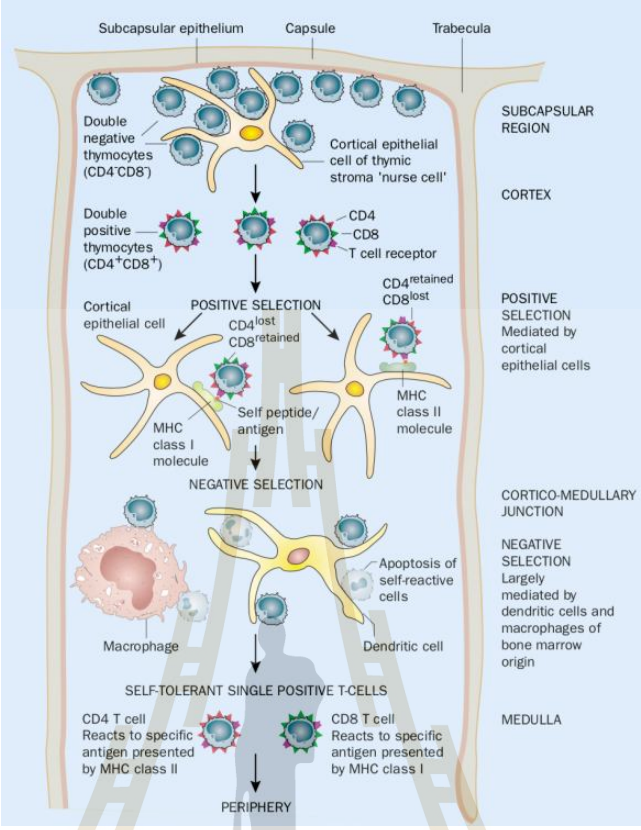

Figure 1.2 Positive and Negative Selection in the Thymus. Developing T cells that recognize self-MHC undergo positive selection in the thymic cortex, then move to the cortico-medullary junction, where self-reactive T cells are removed by negative selection. This ensures mature T cells can respond to foreign antigens while remaining tolerant to self (Parkin and Cohen, 2001).

T cells can be divided into two primary types including $CD4^+$ T cells and $CD8^+$ T cells. $CD4^+$ T cells have been identified as playing a pivotal role in regulating effective immune responses to pathogens. Following interaction with the antigen-MHC class II complex, naïve $CD4^+$ T cells become effector cells and specialize into distinct subtypes, primarily based on their cytokine production profiles (Luckheeram et al., 2012). $CD4^+$ effector T cells are subdivided into distinct subtypes, including Th1, Th2, Th9, Th22, Th17, regulatory T cells (Tregs), and T follicular helper cells (Tfh) (Omman and Kini, 2019) as illustrated in Figure 1.3 (Golubovskaya and Wu, 2016). $CD8^+$ T cells are activated and differentiate into effector cytotoxic T cells when their TCRs recognize

specific antigens, which is presented by an APC via the MHC class I molecule. This cell performs a crucial function in eradicating infected or malignant cells by releasing cytokines, chiefly TNF-α and IFN-γ, which exhibit anti-tumor and antiviral microbial characteristics.

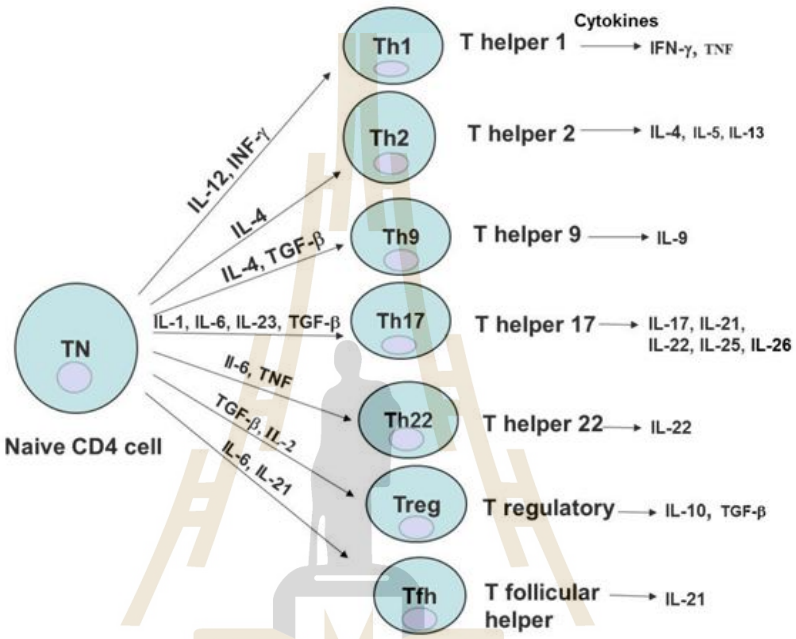

Figure 1.3 $CD4^+$ Helper T Cell Subsets. Several types of $CD4^+$ T cells subsets are generated from naïve precursors in response to specific cytokines, each defined by a characteristic cytokine profile (Golubovskaya and Wu, 2016).

1.3.3.2 Classification of naïve effector and memory T cells

Naïve T cells are differentiated into effect and memory T cell subsets is one of an essential aspects of T cell mediated immunity. The differentiation of these cells is identified by the expression of specific surface molecules, including CD45 and CCR7, a chemokine receptor that facilitates cell migration to the lymph nodes. The classification of naïve T cells is determined by the surface expression of a long CD45RA isoform of the cell surface tyrosine phosphatase CD45, CCR7, CD27 and CD28 ($CD45RA^+CCR7^+CD27^+CD28^+$). Upon encountering an antigen, the cells undergo a process of loss of CD45RA and differentiation into central memory (TCM) cells,

characterized by the expression of CD45RA, CCR7, CD27, and CD28. Furthermore, evidence suggests that these cells also express low levels of certain molecules, including CD57, Programmed cell death protein 1 (PD-1 or CD279), CX3CR1 (a chemokine receptor) and CD45RB (Lim et al., 2006) However, high levels of CD7, IL-7R and CD62L have also been observed. The initial finding pertained to the CD45 isoform, CD45RO, which was found to be a marker for both memory and effector T cells. However, the alteration of CD45RO to CD45RA has been documented (Beverley, 1992). CD45RA expression is frequently employed as a means of distinguishing between naïve, memory and effector cells. Further stimulation of the cells resulted in their differentiation into a population of effector memory (TEM) cells, characterized by the expression of CD45RA, CCR7, CD27, and CD28 antigens as shown in Figure 1.4 (Appay et al., 2008). It has been established that a subset of TEM re-express CD45RA and are designated as effector memory cell re-expressing CD45RA T cells (TEMRA) (Laphanuwat, Gomes and Akbar, 2023). Naïve, central memory, and effector T cells differ in their surface markers, which determine their functions, homing potential, and tissue localization.

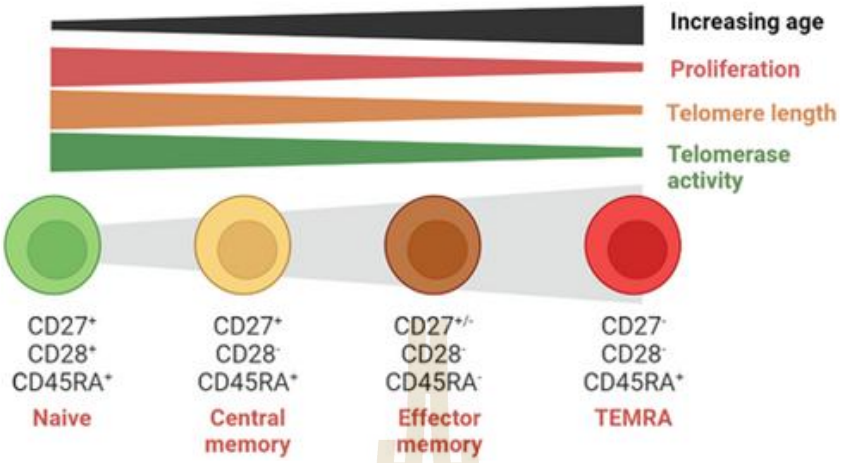

Figure 1.4 T cell differentiation during aging. Naïve T cells differentiate into memory and effector cells after activation. With aging, the number of $CD45RA^+$ effector memory T cells (TEMRA) increases. During this process, lose CD27, CD28, and CD45RA during maturation, but re-express CD45RA when they become senescent with limited ability to divide. (Laphanuwat, Gomes and Akbar, 2023).

1.3.3.3 T cell activation

T cell activation required at least 2 signals for fully activation. Signal one is the antigen-specific signal triggered by binding of T cell receptor (TCR) interaction with its natural ligand, a peptide presented by an MHC molecule of APCs. The peptide antigen held in the MHC class I and MHC class II on APCs will be presented to TCR on $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells, respectively (Gwyer Findlay et al., 2014).

Signal two refers to the co-stimulatory signal required for full T cell activation. It is generated through interactions between surface molecules on T cells and APCs. In $CD4^+$ T cells, the co-stimulatory receptor CD28 binds to B7.1 (CD80) or B7.2 (CD86) on APCs, leading to the production of IL-2 and subsequent T cell proliferation.

To regulate this response, CD28 stimulation also induces the expression of CTLA-4 (CD152), an inhibitory receptor. CTLA-4 competes with CD28 for binding to B7 molecules and, due to its higher affinity, effectively inhibits T cell activation.

Although cytotoxic T cells are less dependent on CD28, they still require co-stimulatory signals from other receptors, such as CD70 and 4-1BB (CD137), to become fully activated. Additional co-stimulatory molecules such as ICOS, OX40, and 4-1BB

also support T cell survival and their function as shown in Figure 1.5. These receptors are expressed on activated T cells and are triggered by their specific ligands, which are upregulated on APCs only after pathogen recognition. This ensures that T cells are stimulated only in response to real threats.

If a T cell recognizes its antigen via the TCR but does not receive co-stimulation, it becomes unresponsive, helping prevent inappropriate immune activation. After receiving both the antigen-specific signal (signal 1) and the co-stimulatory signal (signal 2), T cells require a third signal cytokines to guide their functional specialization. In Th cells, these cytokines direct differentiation into specific subsets including IL-12 promotes Th1, IL-4 promotes Th2, and IL-6 or IL-23 promote Th17. Each subset has specialized roles in coordinating immune responses within tissues (Gwyer Findlay et al., 2014).

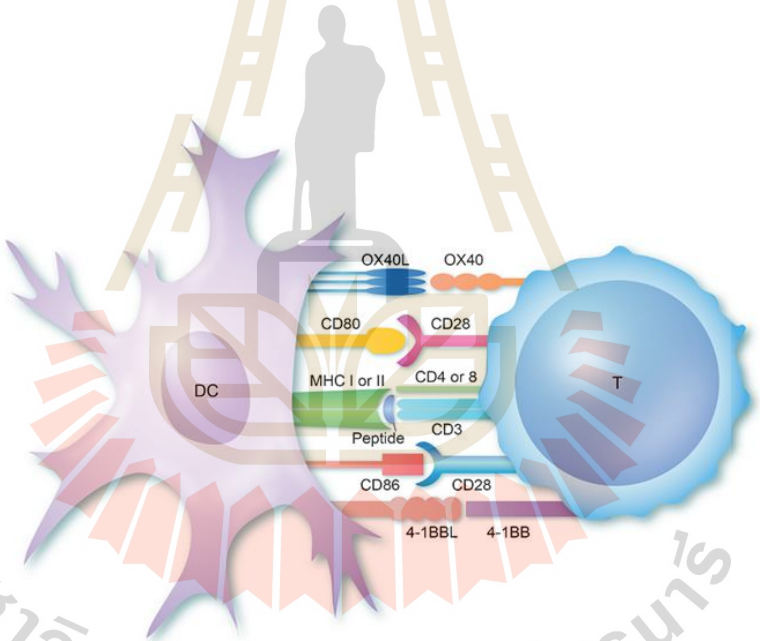

Figure 1.5 Early T cell activation. Activation of T cells begins via the binding of the TCR to a peptide, MHC complex presented by a dendritic cell, with co-receptor engagement by CD4 or CD8 depending on the T cell subtype. Co-stimulatory signals from CD80, CD86, OX40L, and 4-1BBL then drive full activation and effector function (Gwyer Findlay et al., 2014).

1.3.3.4 T cell signaling via TCR/CD3 complex

The activation of T cells is initiated by the recognition of a foreign peptide via MHC complex on an APC by the TCR-CD3 complex. This binding event triggers a series of intracellular signaling pathways that govern cytokine secretion, promote cell survival and proliferation, and drive differentiation, thereby shaping the functional outcome of the T cell response.

The TCR-CD3 complex consists of the antigen-binding TCR$\alpha\beta$ heterodimer, non-covalently associated with invariant signaling subunits consist of CD3$\varepsilon\gamma$ CD3$\varepsilon\delta$ and CD3$\zeta\zeta$. The intracellular domains of these subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs), which are rich in tyrosine residues that serve as targets for phosphorylation by tyrosine kinases.

Activation begins when the TCR binds to the peptide-MHC complex and is stabilized by co-receptor binding (CD4 or CD8), both of which are associated with the Src-family tyrosine kinase Lck (Lymphocyte-specific protein tyrosine kinase). Lck phosphorylates the ITAMs on the cytoplasmic tails of the TCR and CD3 subunits. This phosphorylation recruits the tyrosine kinase ZAP-70 (zeta-chain-associated protein kinase 70), which binds via its SH2 domains to the phosphorylated ITAMs and becomes activated.

Activated ZAP-70 phosphorylates downstream adaptor proteins, comprising linker for activated T cells (LAT) and SH2 domain containing leukocyte protein of 76 kDa (SLP-76). These proteins are essential for propagating TCR signals that lead to T cell activation, differentiation, and proliferation (Courtney, Lo and Weiss, 2018). Upon TCR activation, ligation of the TCR-CD3 complex induces a conformational change in the CD3$\zeta\zeta$ chains, exposing a proline-rich sequence (PRS) in their cytoplasmic tails. This structural change allows recruitment of tyrosine kinase (Nck), adaptor protein non-catalytic region to the PRS. Nck, in turn, can associate with the kinase Lck, facilitating its localization to the TCR-CD3 complex where it contributes to ITAM phosphorylation. Additionally, Nck recruits Wiskott-Aldrich syndrome protein (WASP), which plays a critical role in actin cytoskeleton reorganization during T cell activation. Conversely, the adaptor protein called inhibitor of Notch-1 signaling (Numb), a negative regulator

of Notch-1 signaling, can bind to CD3ε and promote TCR degradation, thereby reducing TCR signaling intensity (Ngoenkam, Schamel and Pongcharoen, 2018).

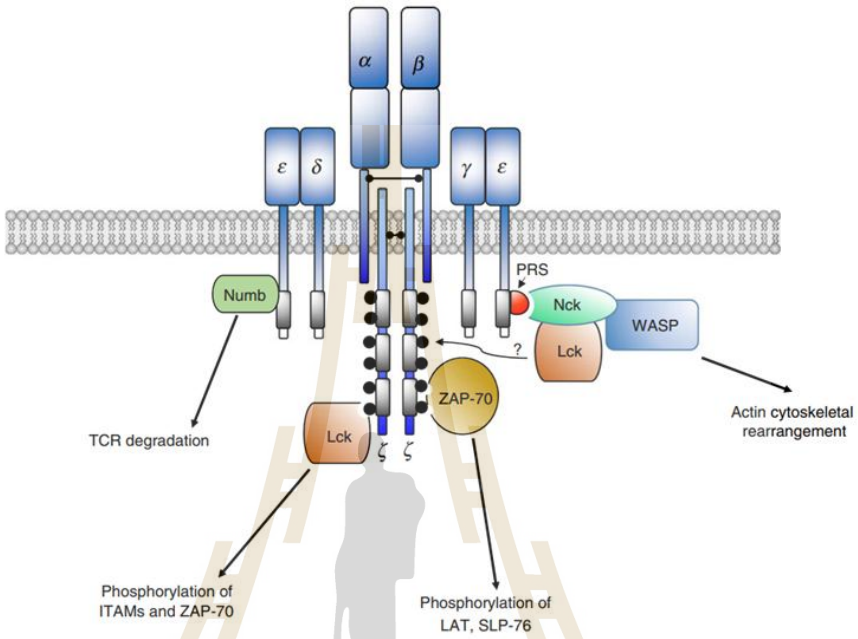

Figure 1.6 Key signaling proteins at the TCR–CD3 complex. TCR–CD3 engagement triggers a conformational change in CD3, exposing a PRS that recruits Nck. Nck helps bring Lck to the complex, enabling ITAM phosphorylation. Lck also directly binds phospho-ITAMs and phosphorylates ZAP-70, which is then activated. Nck-associated WASP regulates actin polymerization, while Numb binds CD3ε to promote TCR degradation and downregulate signaling (Ngoenkam, Schamel and Pongcharoen, 2018).

1.3.3.5 Activation markers of T cell

T lymphocytes are well known as an important role in the immune system. There are many different types of T cells, and it can be able to distinguish them accurately to study their function. Using flow cytometry, various surface markers are used to identify the different types of T cell. When T cells are activated, they will display surface markers that signify their activation status.

CD69 is an original marker known as an early activation inducer molecule. CD69 expression is upregulated when T cells are activated by TCR signaling. Additionally,

CD69 will enhance surface expression through $\alpha_4\beta_1$ integrin, one of the key cell adhesion receptors involved in mediating interactions between the extracellular matrix and cells (Humphries, Byron and Humphries, 2006) or via IL-2, IL-4, IL-12, IFN-α and IFN-γ cytokines through their receptors (Poloni et al., 2023).

CD25 (IL-2Rα) is one of the cell surface markers used to identify T cells, since CD25 is presents on 0.5-6 % of $CD4^+$ T cells. This key is that CD25 is always required along with another marker to identify cells that are specific to an antigen. (Poloniet. al, 2023). TCR signaling through the nuclear factor-kappa B (NF-κB) cause CD25 to be up-regulated, and the frequency of signaling is directly proportionate to its strength. IL-2 induced activator of transcription 5 (STAT5) and signal transducer, which peaks 48 h after activation, mediates CD25 maintenance (Shatrova et al., 2016).

1.3.4 Cytokine production

During T cell activation, through TCR and cytokines, is an essential process in the functioning of the immune system. Naïve $CD4^+$ T cells, the fundamental building blocks of this system, can undergo differentiation into at least four main types of Th cells consist of Th1, Th17, Th2, and inducible T regulatory (iTreg) cells. These cells have an integral role in coordinating adaptive immune responses to a range of microorganisms, thus ensuring optimal protection against foreign pathogens. (Zhu and Paul, 2008).

Their characteristic production of cytokine profiles and functions allow for differentiation. Th1 was induced by IL-12 and there are two cytokines of IL-2 and IFN-γ are important for differentiation of Th1. The Th1 cells mainly generate inflammatory cytokines such as TNF-α, and IFN-γ which trigger macrophage activation and are crucial for protective immune responses against intracellular viral and bacterial infections (Romagnani, 1999).

Th2 cells are essential for the expulsion of extracellular parasites like helminths through the secretion of IL-4, IL-5, IL-13, IL-9, and IL-25. IL-4 acts as on both B and T cells, it is a crucial role in controlling the antibodies production, inflammation, and hematopoiesis, as well as in the development of effector T-cell responses (Brown and Hural, 2017). IL-13 is synthesized by Th2, mast cells, and NK T cells. It influences

epithelial cells, monocytes, fibroblasts, and B cells. The important impacts of IL-13 include B-cell growth and differentiation, stimulation of isotype switching to IgE, and inhibition of the pro-inflammatory cytokine's generation. Moreover, IL-13 collaborates with IL-4 to generate biological effects linked to allergic inflammation and to provide defense against parasites (Vaillant and Qurie, 2022).

Th17 cells manage fungi and extracellular bacteria by producing IL-17f, IL-17a, and IL-22. The principal effects of IL-17 are the IL-6 secretion and additional pro-inflammatory cytokines. It boosts the functions of antigen-presenting cells. It promotes the synthesis of chemokines by endothelial cells (Vaillant and Qurie, 2022). Due to its role in promoting chronic inflammation, IL-17A has been recognized as a key contributor to the autoimmune diseases pathogenesis and is now targeted in therapies for conditions including rheumatoid arthritis, multiple sclerosis (MS), and psoriasis (McGinley et al., 2020).

Induced T-regulatory (iTreg) cells and naturally occurring T-regulatory (nTreg) cells play a critical role in maintaining immune tolerance and controlling lymphocyte homeostasis, activation, and function (Zhu and Paul, 2010). Natural Tregs mediate their suppressive effects on conventional T cells primarily through the cytokines IL-10, IL-35, and tumor growth factor β (TGF-β) (Workman et al., 2009). While the cytokine environment during antigen encounter is the primary factor influencing Th cell differentiation, factors such as the characteristics of the cognate antigen and its TCR affinity, as well as available co-stimulants that affect early cytokine generation, can also impact Th cell destiny.

Transcription factors are essential for helping Th cells develop into specific types and for controlling the production of cytokines. Each cell type needs two main types of transcription factors including master regulators and STAT (signal transducer and activator of transcription) proteins. The expression level of master regulators governs their activity, while cytokines activate STAT proteins through changes like phosphorylation. Some STAT proteins also help turn on the master regulators. Often, STAT proteins and master regulators work together to directly control cytokine gene expression. The key transcription factors for each Th cell type are T-bet and STAT4 for

GATA-3, Th1 and STAT5 for RORγt, Th2 and STAT3 for Th17, and Foxp3 and STAT5 for iTreg cells (Zhu and Paul, 2010).

1.3.5 Antibody

Antibodies, also known as immunoglobulins (Igs), are glycoproteins produced by B lymphocytes that play a central role in the adaptive immune response by specifically recognizing and neutralizing foreign antigens. A single antibody contains of four polypeptides, two heavy chains (~55 kD) and two light chains (~25 kD), linked via disulfide bonds to form a Y-shaped like structure (~150 kD) as shown in Figure 1.7. Two mains important regions of an antibody are variable region and constant region that take responsibility for specific binding to antigen and antibody receptor on cell surface for its effector function, respectively. Based on papain digestion, antibody can be fragmented into fragment antigen-binding (Fab fragment) and fragment crystallizable (Fc fragment), while pepsin digestion generates an N-terminal fragment of Fab with the hinge region, called F(ab')2 with small fragments of Fc parts (pFc) Figure 1.8 (Boushaba, Kumpalume and Slater, 2003).

The Fab fragment consists of a constant and a variable domain of either heavy chain or light chain located at the N-terminus of the Ab structure. This region specifies the idiotype of the antibody and provides the antigen-binding affinity necessary for its functional activity upon antigen engagement. The fragment crystallizable (Fc) is located at the C-terminal fragment of an antibody. This part of the Ab can interact with a cell surface receptor called the Fc receptor to mediate the effector function of the immune response, such as complement activation, phagocytic activation or antibody isotype determination (Abbas, Lichtman and Pillai, 2019).

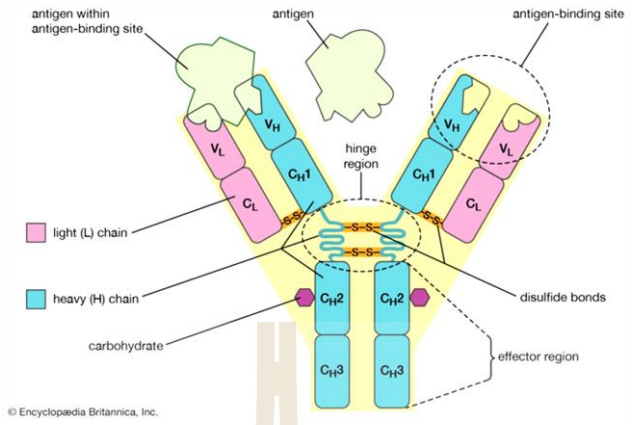

Figure 1.7 Biochemical structure of the immunoglobulin (antibody). From N-terminal of heavy chains comprise of variable heavy chains (VH) and constant heavy chains (CH1, CH2 and CH3 regions). Light chains comprise of variable light chains (VL) and constant light chains (CL) regions. These four chains are linked by disulfide bonds (S-S) (Philippidis, 2021).

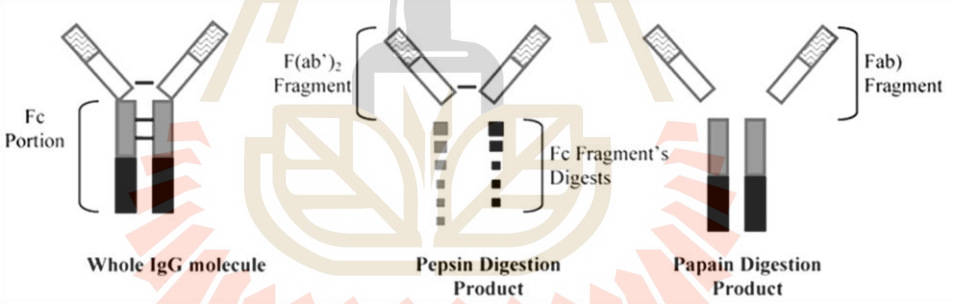

Figure 1.8 Digestion products of pepsin and papain action on the antibody molecule. Pepsin digestion of IgG molecule can be generated an N-terminal fragment of Fab with the hinge region (F(ab')2) and eliminates the Fc portion into small fragments of Fc parts (pFc) while papain digestion can be generated fragmented into fragment crystallizable (Fc fragment) and fragment antigen-binding (Fab fragment) (Boushaba, Kumpalume and Slater, 2003).

The antibodies can be classified into five predominant isotypes shown in Figure 1.9 (Perdue and Humphrey, 2025) according to their constant heavy chains include α-, δ-, ε-, γ-, and μ- chains corresponding to IgA, IgD, IgE, IgG and IgM isotype, respectively. The light chains of Ab also contain 2 isotypes including κ and λ, however, there is no significant difference between these two isotypes. Hence, an antibody isotype is totally determined by the constant heavy chains.

IgG, IgE, and IgD are monomeric isotypes of antibodies, while IgA and IgM are dimeric and pentameric isotypes, respectively. Effector functions of each antibody isotype is distinguished by their distinct biochemical structure. IgM is the first antibody to be generated. It is firstly expressed as a monomeric membrane form on surface of mature B cells and secreted as a pentamer form of the monomeric units are covalently linked via disulfide bonds. This pentamer form enables the immunoglobulin to firmly adhere to the pathogen during the first interaction. IgG is the most abundant antibody in serum. It is involved in complement fixation, toxin neutralization and bacterial opsonization. In mucosal membranes, including the gastrointestinal and respiratory tract, IgA mediates the immune response. IgE is involved in mediating type 1 hypersensitivity reactions to allergen (Schroeder Jr and Cavacini, 2010). IgD serves as an antigen receptor on B cells and is thought to be involved in their maturation, maintenance, activation, and silencing. IgD has been implicated as a regulator of humoral immune responses through the regulation of B cell selection and maintenance.

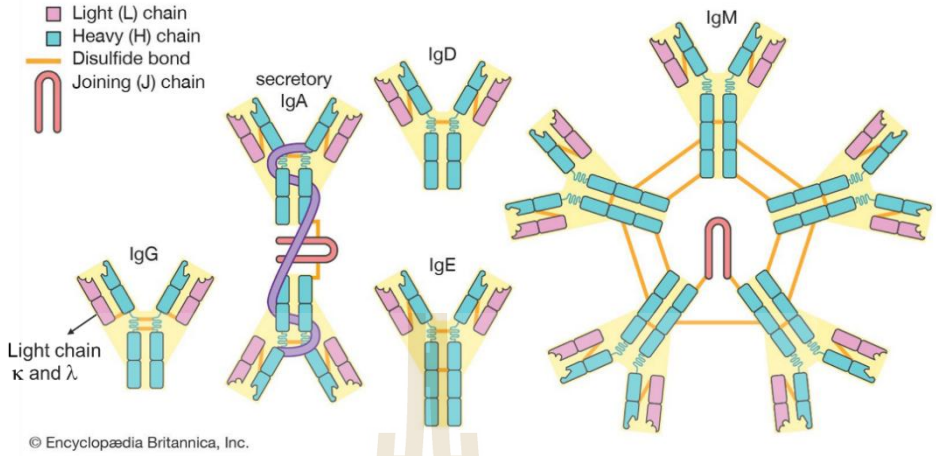

Figure 1.9 Antibody classification. The antibodies can be classified into five predominant isotypes including IgG, IgA, IgD, IgE, and IgM isotype, respectively. The light chains of Ab also contain 2 isotypes including κ and λ (Perdue and Humphrey, 2025).

1.3.6 Polyclonal antibodies (pAbs) and monoclonal antibodies (mAbs)

Polyclonal antibodies are a diverse mixture of immunoglobulins (Igs) produced by multiple clones of plasma B cells. They recognize and bind to different epitopes on the same antigen. Polyclonal antibodies are commonly generated by immunizing suitable animals, such as rabbits, due to their ease of handling and the high yield of antibody production they provide as shown in Figure 1.10. The production process can be scaled up to larger animals such as goats, sheep, etc. (Picó, 2007).

Although pAb is easy to produce in terms of producing and high production yields. However, the heterogeneity of pAb can cause high cross-reactivity among antigens and variation among each production lot may occur, which is unproper for some functional studies or therapeutics uses. Therefore, another type of antibody called monoclonal antibody (mAb), which is a homogeneity antibody with highly specific to single epitope would be of choice.

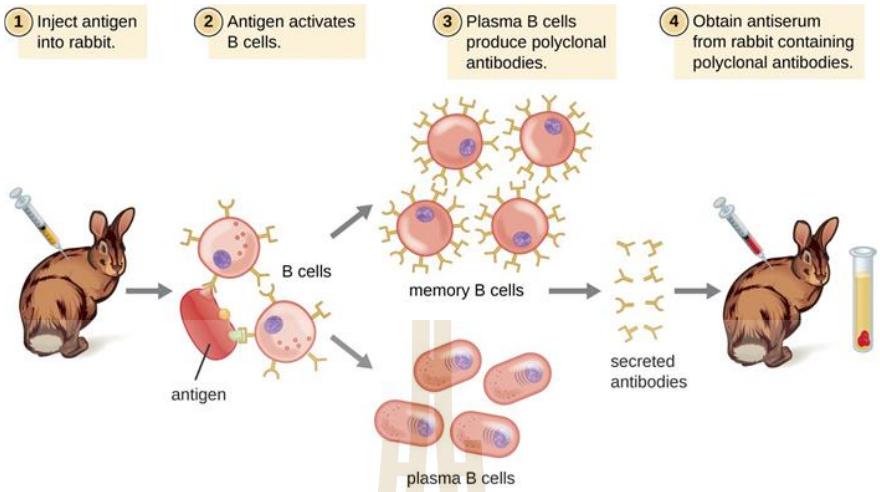

Figure 1.10 The diagram shows the production of polyclonal antibodies. An immunogen is injected into animals like rabbits, activating B cells to produce polyclonal antibodies. These antibodies are collected from the antiserum and can be further purified to remove other serum proteins (Polyclonal and Monoclonal Antibody Production, Microbiology, n.d.).

MAb is produced from hybridoma cells generated in the laboratory by fusing single clone of antibody producing cells, plasma cells and myeloma cells, thus it is named mAb. The mAb binds specifically to the epitope of an antigen. These mAbs are widely used in biomedical research and are vital for diagnosing and treating diseases such as cancer and infections.

These antibodies are made using cell lines created from animals, usually mice, that have been immunized. B cells from the immunized animal are fused with myeloma (cancer) cells to form hybrid cells, called hybridomas (Köhler and Milstein, 1975). These are placed in a special medium where only the hybridomas survive, unfused myeloma cells can't grow, and normal B cells die. The surviving hybridomas can keep growing and make antibodies. Scientists then test them to find the ones producing the specific mAb they want. These selected hybridomas are grown in culture, and the antibodies are collected and purified from the culture medium as shown in Figure 1.11 (Tanikawa et al., 2008).

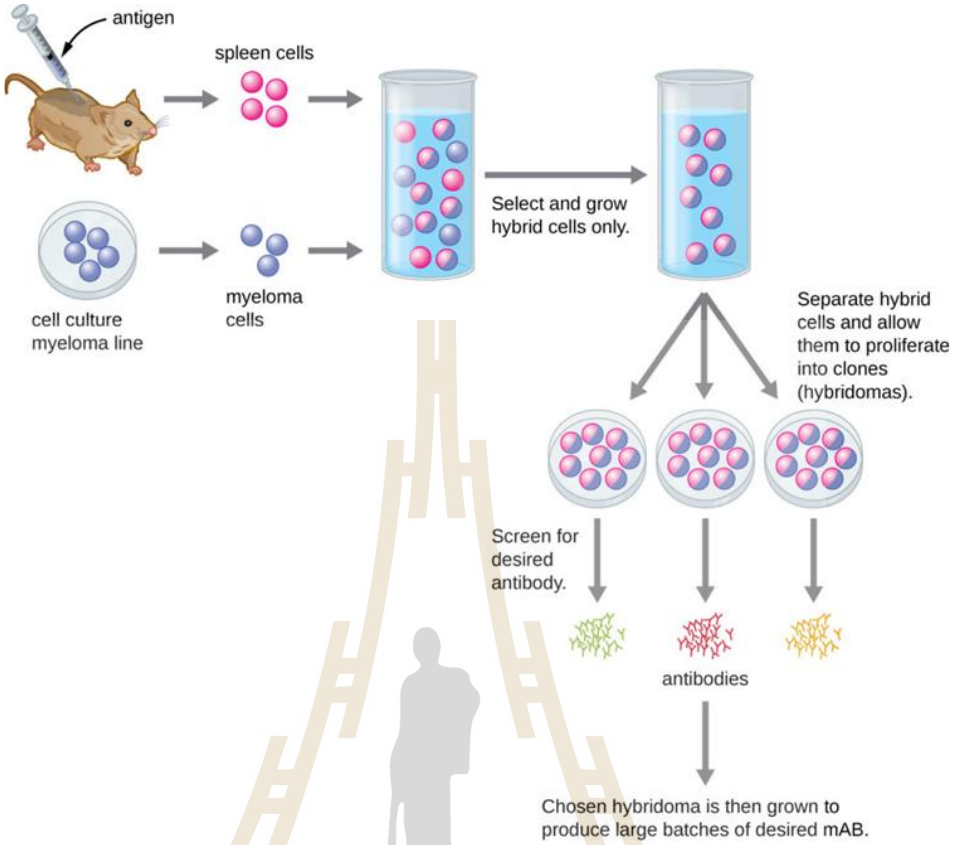

Figure 1.11 Hybridoma technique. The hybridoma technique produces monoclonal antibodies by fusing antigen-stimulated B cells from a mouse with myeloma cells, creating immortal hybridomas. These are cultured, screened for the desired antibody, and the selected hybridomas are expanded to produce and purify monoclonal antibodies (Polyclonal and Monoclonal Antibody Production, Microbiology, n.d.).

1.3.7 Antigen-antibody interactions

Antibodies bind to antigens through several weak chemical interactions, including hydrogen bonds, electrostatic forces, hydrophobic interactions, and Van der Waals forces (Van Oss, Good and Chaudhury, 1986). This antigen–antibody (Ag–Ab) interaction is highly specific. In general, the better the structural and chemical fits between the binding site of an antibody and the epitope of an antigen, the stronger the interaction. Epitopes are specific regions on the surface of antigens that are recognized by the antibody's binding site, known as the paratope. Epitopes are classified as either conformational or linear. Linear epitopes are composed of a

contiguous stretch of five to ten amino acids, usually revealed upon protein denaturation or proteolytic cleavage, as depicted in Figure 1.12 (Flaherty, 2011).

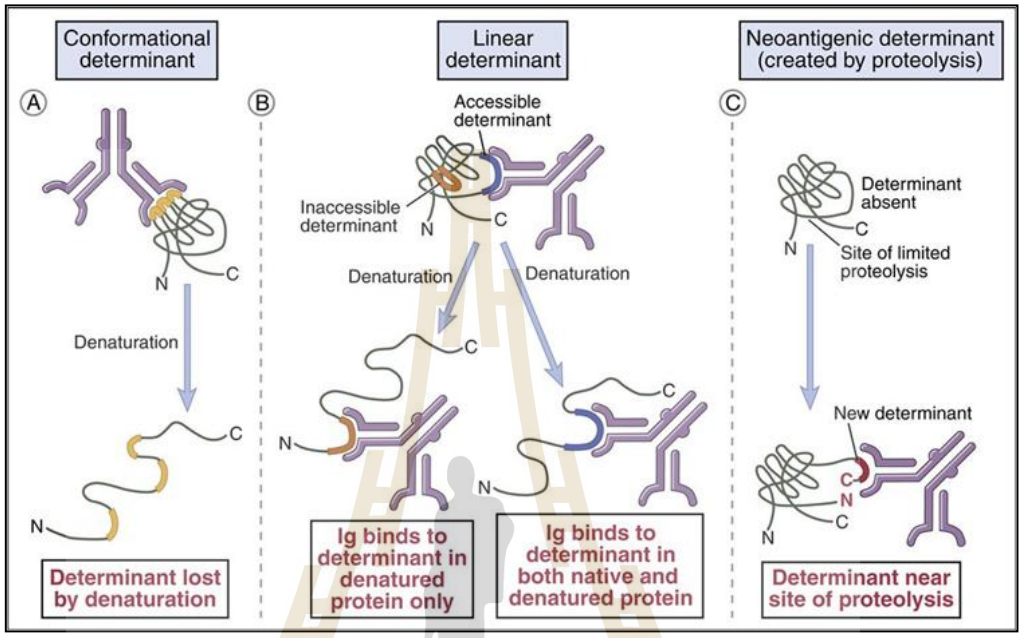

Figure 1.12 The nature of antigenic determinants. Antigenic determinants can depend on protein structure. Some are present in the native (folded) protein and lost upon denaturation (A), others are revealed only after unfolding (B), and some, called neodeterminants, result from modifications like peptide cleavage (C) (Abbas, Lichtman and Pillai, 2010).

1.3.8 Immunoprecipitation (IP) assay

Immunoprecipitation (IP) is a technique that is widely used to purify a specific protein or antigen from the mixture. The basic principle of this technique is based on antigen-antibody interaction. IP can be performed by mixing cell lysate containing interested proteins and its specific antibody to allow the formation of antigen-antibody (immune) complex. The immune complex is subsequently captured on solid support such as Sepharose or agarose beads, or magnetic beads via the specific binding of IgG Fc part of the Ab to either protein A or protein G, or protein L to kappa light chain. Following the removal of unbound proteins through washing, the immune complex components are eluted and subjected to SDS-PAGE, then analyzed using Western blotting to detect the protein of interest, as illustrated in Figure 1.13 (Lal, Haynes and Gorospe, 2005).

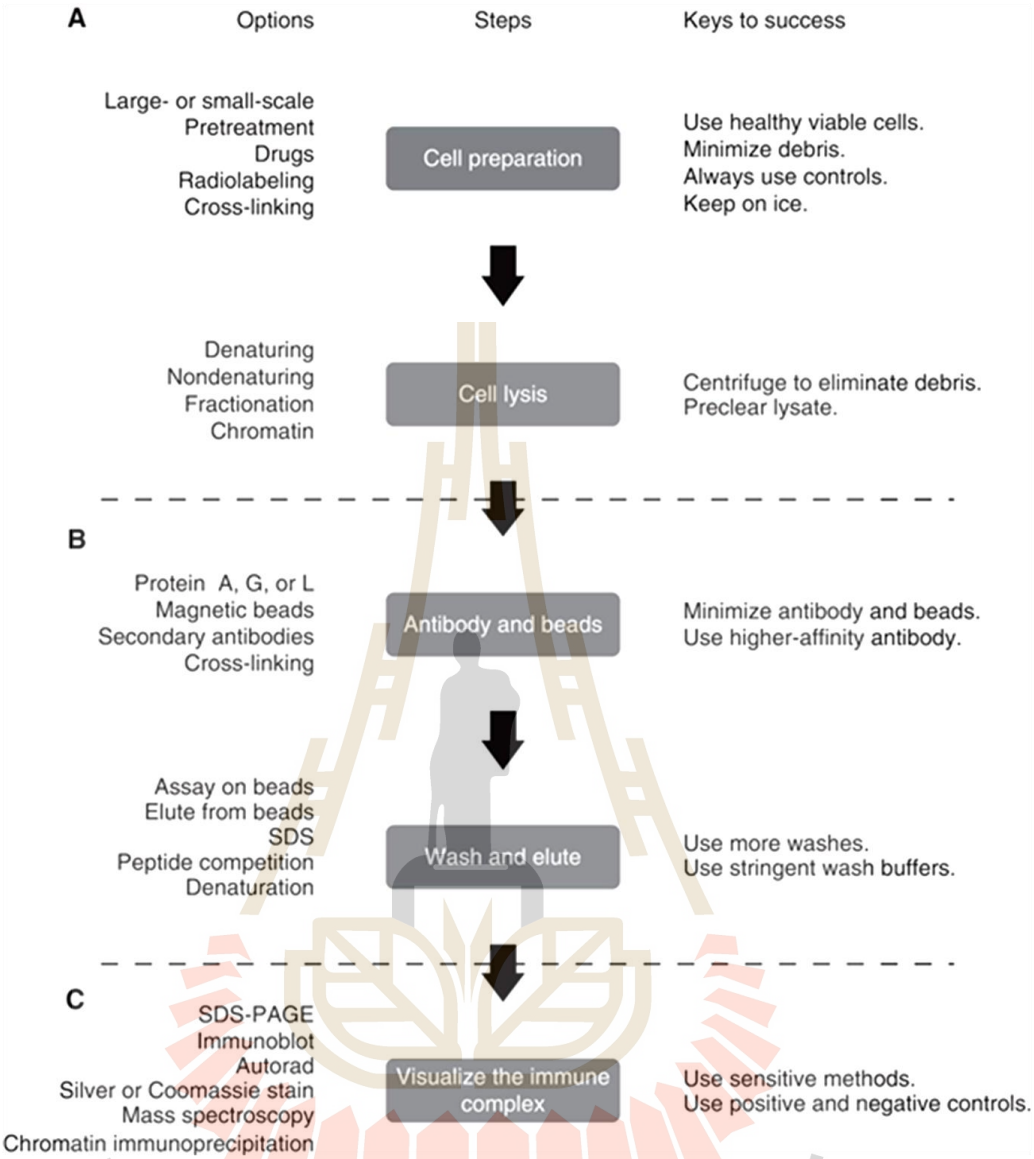

Figure 1.13 Immunoprecipitation experiment strategies. (A) The preparatory phase, (B) the antibody-antigen complex, and (C) the immune complex detection phase (DeCaprio and Kohl, 2017).

1.3.9 Intracellular cytokine staining (ICS)

Intracellular cytokine staining (ICS) is a flow cytometry-based immunofluorescence technique used to assess cytokine production within individual cells. It is particularly valuable for evaluating antigen-specific T cell responses, offering a high-dimensional readout that facilitates detailed phenotyping of cytokine-expressing T cell subsets (Maecker et al., 2005). As well as other cell types by using immunofluorescent labeling-Ab specific to cell surface marker of each cell type for example anti-CD3 Ab for T cells, anti-CD14 for monocyte and anti-CD19 for B cells etc. Cytokines are produced in cytoplasm, modified at endoplasmic reticulum (ER), and are secreted to extracellular via vesicular transportation. Thus, secretion of these cytokines from ER can be prevented by adding inhibitors such as monensin or brefeldin A allowing accumulation of cytokines in the cells. The cytokines will be then detected by their specific antibody tagged with fluorochrome and analyzed by a flow cytometer. As the cytokines are kept inside the cell, cell fixation and permeabilization of cell membrane are required. As some surface marker molecules cannot be detected by their specific Abs after cell fixation, thus cell-surface staining step may be required first (Lovelace and Maecker, 2011).

CHAPTER II

MATERIALS AND METHODS

2.1 Cells, Chemicals, Instrumentation

2.1.1 Cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized whole blood of healthy donors. Human T cell line, Molt4 was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 40 µg/ml gentamycin, and 0.25 µg/ml amphotericin B in a humidified atmosphere of 5% CO_2 at 37 °C. Hybridoma cells producing mAb CARA were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated FBS, 40 µg/ml gentamycin, and 0.25 µg/ml amphotericin B in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2 Methodology

2.2.1 Isotypic determination by captured ELISA

Isotypic determination of the mAb CARA was performed using the goat anti-mouse monoclonal antibody isotyping reagent (Sigma Aldrich) according to manufacturer's instruction. Briefly, antibodies against different isotypes of immunoglobulin (IgA, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM) were diluted in coating buffer (carbonate/bicarbonate buffer, pH 9.6) at dilution 1:100 and coated onto ELISA plate at a volume of 50 µl per well. After incubation at room temperature (RT) for 2 h, for an hour at RT, 100 µL of 2% skimmed milk in PBS pH 7.2 was added to block the plate. The blocking solution was removed, culture supernatant of hybridoma cells producing monoclonal antibody named CARA at a volume of 50 µl was added into each well and incubated at RT for an hour. After washing the plate with 0.05% Tween®20 in phosphate buffer saline (0.05% PBST) for 3 times, each well received 50 µl of Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins antibody (HRP-anti-mouse-Igs) (Dako, Glostrup, Denmark) at dilution of 1:5,000. The solution was then incubated at RT for an hour. After washing the plate with 0.05% PBST, 50 µl of tetramethylbenzidine (TMB; Merck, Darmstadt, Germany) substrate was added into each well and incubated at RT for 3 min. The reaction was terminated by adding 100 µl of 1 N HCl. Finally, measurement of the optical density at 450 nm was completed using a microplate reader (Tecan, Switzerland).

2.2.2 Total RNA extraction

Hybridoma cell producing mAb CARA ($2.5x10^6$ cells) were resuspended in 1 ml of GENzol™ reagent and thoroughly mixed by pipetting before incubating at RT for 5 min. Two hundred microliters of chloroform were added and shaker vigorously for 10 sec before centrifugation at 13,000 x g for 15 min at 4 °C. The upper layer containing total RNA was transferred into a new tube. To precipitate the total RNA, five hundred microliters of isopropanol were added into the extract solution and mixed by inverting at RT for 10 min before centrifugation at 13,000 x g for 15 min at 4 °C. The total RNA pellet was collected and washed once with 1 ml of 70% ethanol. After centrifugation let the RNA pellet dry approximately 10 min at RT. The total RNA pellet solubilized in

30 µl of RNase-free sterile water and incubate at 60 °C for 15 min. The total RNA concentration was determined using ND-1000 Nanodrop spectrometer (Thermo Scientific, USA) at wavelength 260 nm. The RNA purity was examined by resolving in 1.2% agarose gel electrophoresis and visualized using a gel documentation system (Bio-Rad, USA) using SYBR green (Thermo scientific) as a gel stain.

2.2.3 Reverse transcription polymerase chain reaction (RT-PCR)

The synthesis of the complementary DNA (cDNA) was completed using a M-MuLV cDNA synthesis kit (Vivantis, Malasia) in accordance with the instructions provided by the manufacturer. In short, the synthesis was executed by employing two distinct 10 µl reaction mixtures, each consisting of 1 µg extracted RNA, M-MuLV, 2 µM of reverse transcription primer designed to detect mouse immunoglobulin either for kappa light chain (mIGk) (5'TTGTCGTTCACTGCCATCAATC3'), or lambda light chain (mIGL) (5' GGGGTACCATCTACCTTCCAG 3') (Meyer et. al, 2019), 2 µM dNTPs, and nuclease-free water to reach the final volume. After incubating at 72 °C for 3 min and further chill on ice for 3 min, the mixture of 10X Buffer M-MuLV (2 µl), 100U/µl Mu-MV reverse transcriptase, 6 µM of template-switch oligo –
(5' AAGCAGTGGTATCAACGCAGAGTACATGRGRGR 3') were added.

The reaction mixture was subjected to further incubation at 42 °C for an additional hour before being terminated by incubation at 70 °C for 5 min to denature the residual reverse transcriptase. The PCR was executed with a total reaction volume of 50 µl, comprising 5 ng/ml of first-strand cDNA, 0.5 µM Universal forward primer (ISPCR), 0.5 µM reverse primer (kappa, lambda) as indicated in Table 2.1 , 0.2 µM dNTP mix, 5X Phusion™ HF Buffer (10 µl), 0.02 U/µl Phusion™ Hight-fidelity DNA polymerase (Thermo Scientific, USA) and nuclease-free water to reach the final volume. These reactions were performed in a fifty microliter of master mix and incubated under the PCR cycling condition (initial denaturation at 98 °C for 30 sec, followed by 35 cycles of 98 °C for 10 sec, 57 °C for 30 sec, 72 °C for 30 sec and a final extension at 72 °C for 7 min). The PCR product was analyzed by 1.2% agarose gel electrophoresis containing SYBR safe DNA gel stain in 0.5X Tris-acetate-EDTA (TAE) buffer and visualized using a gel documentation system (Bio-Rad, USA).

Table 2.1 Mouse IgG PCR primers adapted from (Meyer et al., 2019).

Primer	Description	Sequences (5'→3')
ISPCR	Universal forward primer	AAGCAGTGGTATCAACGCAGAG
mIGK PCR	Reverse primer for kappa chain	ATCGTACACACCAGTGTGGC
mIGL PCR	Reverse primer for lambda chain	GGGATCCAGAGTTCCAGGTC

2.2.4 Monoclonal antibody purification by affinity chromatography

Cultured supernatant containing mAb CARA was harvested and purified by affinity chromatography using HiTrap™ Protein L column, linked to an AKTA START protein purification system (GE Healthcare Bio-Science AB, Uppsala, Sweden) according to manufacturer's instruction. The concentration of the purified mAb was measured using BCA protein assay kit (Thermo Scientific). The purified mAb was stored at -80 °C until use.

2.2.5 Determination of antibody activity by indirect immunofluorescent staining and flow cytometry

Molt4 cells were harvested, washed twice with 1X phosphate buffer saline (PBS) pH 7.2 and resuspended ($1x10^7$ cells/ml) in staining buffer (1%BSA-PBS-0.02% NaN_3) containing human IgG at a final concentration 2 µg/ml to block Fc receptor. After incubation on ice for 30 min, 50 µl of the blocked cells suspension was added to 50 µl of the purified mAb CARA or the isotype-matched control mAb to get a final concentration of 10 µg/ml. Cells incubated with staining buffer were used as a conjugate control. Cells were incubated on ice for 30 min before being washed twice with staining buffer to remove unbound primary antibodies. The binding of the mAb CARA and its specific molecule on cell surface were tracked staining with Alexafur-488 conjugated goat anti-mouse IgG (Goat-anti-mIgG-AF488) at a final dilution of 1:1000 in the staining buffer. After a 30 min incubation on ice, the stained cells were washed 3 times using a buffer staining. The stained cells were then resuspended in 300 µl of 1%

paraformaldehyde in PBS and analysis using a flow cytometer (Attune NxT Flow Cytometer, Invitrogen, USA).

2.2.6 Cell staining and stimulation for confocal microscopy

Slides and cover slips were cleaned with ethanol before being rinsed once with DI water. They were then sonicated for 15 min at RT in a tank containing 1 M KOH. Afterward, the slides and cover slips were washed twice with DI water and once with absolute ethanol before being dried using an air blower.

Cover slips were then coated with anti-CD3 clone OKT3 antibody (20 µg/ml) or PBS pH 7.2 at 4 °C for overnight. The cover slips were washed twice with 2% FBS-PBS and then blocked in the same solution at RT for 1 h.

One hundred microliters of suspension Molt4 cells or PBMCs ($1x10^6$ cells) in PBS were placed gently on coated area of the cover slips. After incubation at 37 °C for 5 min, 4% paraformaldehyde in PBS pH7.2 was added at volume of 100 µl to fix the cells and incubated at RT for 10 min. The fixed cells underwent a washing step with 2% FBS-PBS twice and an incubation in 2% human IgG in 2% FBS-PBS at RT for 20 min. The blocking solution was discarded, and the cells were washed twice with 2% FBS-PBS before staining by adding 100 µl of mAb CARA at final concentration 10 µg/ml and incubated at RT for 30 min. The cells were washed twice with 2% FBS-PBS, then incubated at RT with 100 µl of Alexa fluor (AF) 633-conjugated goat anti- mouse antibody in 2% FBS-PBS at a 1:100 dilution for 20 min. The stained cells were washed twice with 2% FBS-PBS before direct stained with 100 µl of FITC-CD3 mAb in 2% FBS-PBS at a 1:100 dilution at RT for 30 min. Cells were washed twice before the nuclei were counterstained by adding 100 µl of 300 nM DAPI in 2% FBS-PBS. This was followed by an incubation in the dark for 5 min. After washing twice, the staining of the cells was mounted with 50% glycerol in PBS pH 7.2 on glass slides and then sealed with nail polish. A field emission scanning electron microscope (Zeiss GeminiSEM 500, Zeiss, Germany) was used to observe the localization of CD3 and the recognition molecule of mAb CARA on Molt4 cells and PBMCs.

2.2.7 Purity determination of the purified monoclonal antibody using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

The purified mAb CARA (5 µg) were separated by 10% SDS-PAGE under both reducing and non-reducing conditions. Protein separation was performed by applying an electricity field of 130 Volts for 1 h. The separated proteins were visualized by staining the gel with Coomassie Brilliant Blue R250 solution at RT for 1 h. The staining gels were treated with de-staining gel solution I and II until appearance of a clear band with no background.

2.2.8 Carboxyfluorescein succinimidly ester (CFSE)-based proliferation assay

Peripheral blood mononuclear cells (PBMCs) ($1x10^7$ cells/ml) were labelled by incubation with CFSE at final concentration 1 µM in PBS pH 7.2 and incubated at 37 °C for 10 min in 5% CO_2 incubator. Ten milliliters of cold 10% FBS-RPMI was added to terminate the CFSE-labeled PBMCs reaction and centrifuged at 1,500 rpm for 10 min at 4 °C. After discarding the supernatants, the CFSE-labeled PBMCs were washed twice with cold incomplete RPMI at 1,500 rpm for 10 min at 4 °C before further analysis.

The CFSE-labeled PBMCs ($1x10^5$ cells) were cultivated in 96-well plate immobilized without or with anti-CD3 mAb clone OKT3 (60 ng/ml) in the absence or presence of mAb CARA at final concentration 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 µg/ml or mouse IgG3 isotype matched control at final concentration 20 µg/ml in 5% CO_2 incubator at 37 °C. After 3 days and 5 days of cultivation cells of each cultivation condition were harvested and transferred into 1.5 ml tube. T cell activation was evaluated based on cell proliferation, as determined by monitoring CFSE reduction using a flow cytometer (Attune NxT Flow Cytometer).

2.2.9 Determination of cell surface CD69 and CD25 expression using immunofluorescent staining

The PBMCs ($5x10^5$ cells) were cultivated in 24-well plate coated without or with anti-CD3 mAb clone OKT3 (60 ng/ml) in the absence or presence at final concentration 10 µg/ml of mAb CARA or mouse IgG3 isotype-matched control in 5% CO_2 incubator at 37 °C. After 24 h of cultivation, cells of each treatment condition were collected, washed and resuspended in 1%BSA-PBS-0.02% NaN_3 (staining buffer) containing 2 µg/ml of human IgG ($1x10^7$ cells/ml) to block cell surface Fc receptor. The cells were incubated on ice for 30 min before staining with cocktail antibodies: either FITC-conjugated anti-CD3 mAb and, PE-conjugated-anti-human CD25 mAb or FITC-conjugated anti-CD3 mAb and, PE-conjugated-anti-human CD69, followed by incubation on ice for an additional 30 min. The stained cells were washed once with staining buffer before resuspending in 300 µl of staining buffer before analysis using a flow cytometer (Attune NxT Flow Cytometer).

2.2.10 Total cytokines measurement using Sandwich ELISA

After 24 hours of cultivation, the culture supernatants of PBMCs in each condition were harvested. The concentration of secreted IL-2, IL-4, IL-10, IL-13, IL-17, IFN-γ, and TNF-α were determined using Human IL-2, IL-4, IL-10, IL-17, IFN-γ, TNF-α ELISA MAX™ Deluxe kits (BioLegend, San Diego, CA, USA), and the Human IL-13 uncoated ELISA kit (Invitrogen, USA) following the manufacturer's recommended protocol.

2.2.11 Intracellular cytokine staining (ICS)

PBMCs ($5x10^5$ cells) were stimulated with immobilized anti-CD3 mAb clone OKT3 (100 ng/ml) or unstimulated in presence or absence of soluble mAb CARA or mouse IgG3 isotype-matched control at a final concentration of 10 µg/ml in 5% CO_2 incubator at 37 °C. After an hour of cultivation, brefeldin A (BioLegend) was introduced into each well at a final concentration of 3 µg/ml, and the cells were then cultivated for an additional 5 h. Thereafter, the cells were harvested into 1.5 ml tube and washed twice with PBS pH 7.2 before fixing in fixation buffer (4% paraformaldehyde in PBS pH 7.2) at volume of 200 µl for 15 min at RT. The fixed cells were washed once with PBS pH 7.2 before incubating at 4 °C for overnight with human IgG at a final concentration 2 µg/ml in PBS pH 7.2, this step was done to block the Fc receptors. The blocked cells were made permeable by resuspending them in a permeabilization buffer containing 5% FBS, 0.1% saponin and 0.02% NaN_3 in PBS pH 7.2 at RT for 15 min. The permeabilized cells were stained with cocktail antibodies, including FITC-conjugated anti-CD3 mAb and PE-conjugated anti-human IL-2, or IL-4, or IL-10, or IL-17A, or IFN-γ, or TNF-α on ice for 30 min. Thee stained cells were then washed twice with permeabilization buffer before resuspension in PBS pH 7.2 and determined using a flow cytometer (Attune NxT).

The gating of T-cells for the flow cytometry analysis was based on the fluorescent signal of the FITC-anti-CD3 mAb respectively. The next step was to assess intracellular cytokine production in $CD3^+$ T-cells by detecting PE-positive cells.

2.2.12 Phosphotyrosine (pTyr) Blot

PBMCs ($1x10^7$ cells/ml) were stimulated with mAb OKT3 10 µg/ml or unstimulated in presence or absence of mAb CARA or isotype-matched control mAb at final concentration 10 µg/ml at various time points at 37 °C. Cellular activation was terminated by adding cold Tris-HCl buffer pH 7.5 (150 mM NaCl, 20 mM Tris-base, 5 mM EDTA) containing 2 mM of Na_3VO_4. Cell pellets were harvested by centrifugation at 13,000 rpm for 20 sec at 4 °C. The stimulated cells were lysed by adding 100 µl of Tris-HCl buffer pH 7.5 containing 1% Triton-X 100 and a protease inhibitor cocktail (100 mM PMSF, 0.5 M NaF, 0.5 M Iodoacetamide, 1 µg/ml aprotinin, 1 µg/ml pepstatin A), mixed well using pipette and incubated for 10 min at 4 °C. Clear cell lysates were collected by centrifugation at 13,000 rpm for 10 min at 4 °C. The protein containing cell lysates were resolved in 10% SDS-PAGE under reducing condition and electro transfer onto PVDF membrane using semi-dry blotting system. The membrane was blocked with 5% BSA in Tris-HCl pH 7.5 for 1 h at RT on a rocking shaker. The blocked membrane was rinsed once with Tris-HCl pH 7.5 before staining with HRP-conjugated anti-phosphotyrosine antibody clone 4G10 at dilution of 1:5,000 in 2.5 % BSA in Tris-HCl buffer pH 7.5. After shaking on rocking shaker at RT for 1 h, the stained membrane was washed 3 times with 0.1% Tween® 20 in Tris-HCl buffer pH 7.5 (TTB) and twice with Tris-HCl pH 7.5. The protein bands were visualized using an enhanced chemiluminescent detection system and analyzed by Amersham Image Quant™ 500 CCD imaging system (Cytiva, USA).

2.2.13 Cell surface biotinylation and cell lysates preparation

Human T cell lines (Molt4) were harvested, then washed 3 times with PBS pH 7.2 before centrifugating at 1,500 rpm at 4 °C for 5 min. Washed cells were resuspended ($4x10^7$ cell/ml) in 2 ml of 5 mM Biotin-NH-Sulfo in PBS pH 7.2, mixed well and incubated on ice for 1 h. Cell surface biotinylation was terminated by adding cold 1 mM Glycine in PBS to a final volume 50 ml. The biotinylated cells were harvested by centrifugation at 1,500 rpm, 4 °C for 5 min and washed twice with cold PBS pH 7.2. The biotinylated cells were lysed by adding 1.5 mL of Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, 50 mM Tris-base, 2 mM EDTA, 0.02% NaN_3, 1 mM PMSF, 5 mM Iodoacetamide, 10 µg/ml aprotinin, along with 1% Triton-X 100. The mixture was thoroughly mixed and incubated on ice for 30 min. Cell lysates were collected by centrifugation at 13,000 rpm, 4 °C for 10 min. The clear cell lysates were transferred into a new tube and kept on ice before use.

2.2.14 Immunoprecipitation

Five hundred microliters of the clear cell lysate were incubated with different antibodies either mAb CARA or positive control mAb at final concentration 20 µg/ml respectively. For conjugate control, no mAb will be added. The mAbs were allowed to bind to its recognizing molecules by rotation at 4 °C for 2 h. The mAbs-cell lysates mixture was added to protein L agarose beads (30 µl) and rotated at 4 °C for overnight to allow protein L bind to the antibody-protein complexes. The beads were washed 10 times with 1 ml of cold lysis buffer (Tris-HCl buffer pH 7.5) containing 1% Triton-X 100 and 3 times with cold lysis buffer by centrifugation at 13,000 rpm, 4 °C for 10 sec. After the last wash, the precipitated protein was eluted from the dried beads by adding 35 µl of 1X reduced Laemmli buffer and boiled at 95 °C for 5 min.

The precipitated proteins were resolved in 10% SDS-PAGE under reducing condition before transferring onto PVDF membrane using semi-dry blotting system. The membrane was blocked with 5% skimmed milk in PBS pH 7.2 for 1 h. The blocked membrane will be stained with HRP-conjugated streptavidin at dilution 1:5,000 in 2.5% skimmed milk at RT for 1 h. After three washing with 0.1% PBST and twice with PBS pH

7.2. A chemiluminescent detection system was used to visualize the protein bands, and they were captured with an Amersham Image Quant 500.

2.3 Statistical analysis

Statistical analysis and data management were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA) Descriptive data and results are presented as mean (±SD). Two-way ANOVA was used for data analysis, as appropriate. Statistical significance was defined as $p < 0.05$; *, $p < 0.01$; **, $p < 0.001$; ***, and not significant (ns, $p > 0.05$).

CHAPTER III

RESULTS

3.1 Monoclonal antibody CARA belongs to IgG3 with kappa light chain

Isotypic determination of the mAb CARA was performed using the goat anti-mouse monoclonal antibody isotyping reagent, antibodies against different isotypes of immunoglobulin (IgA, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM). Culture supernatant of hybridoma cells producing mAb clone CARA was used in this study. The optical density at 450 nm was determined using a microplate reader indicated that culture supernatant of hybridoma cells producing mAb clone CARA belong to IgG_3 isotype as shown in Figure 3.1

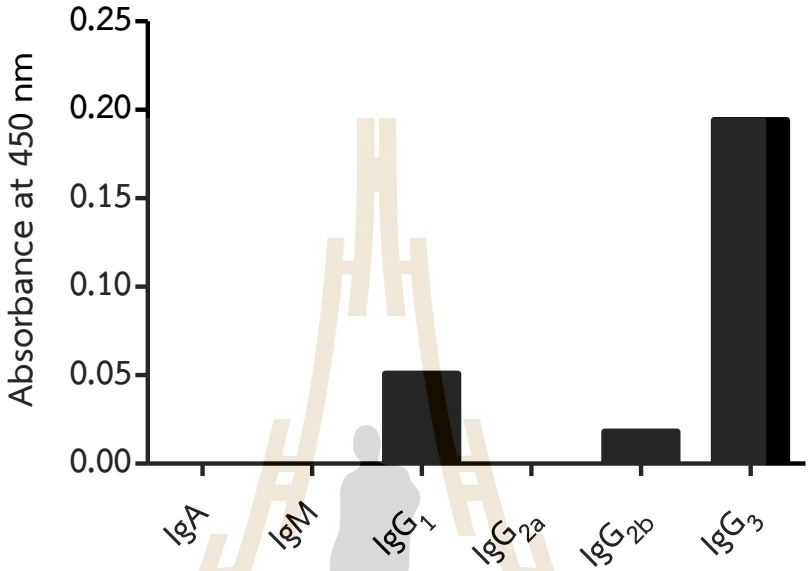

Figure 3.1 Isotypic determination of the mAb CARA. Capture ELISA; fifty microliters of mAb CARA were added into the ELISA plate coated with antibodies against different anti-mouse immunoglobulin isotypes. The detected isotype was examined by adding 50 µl HRP-conjugated rabbit-anti-mouse-Igs (dilution 1:5,000). TMB substrate was added and optical density at 450 nm was measured using a microplate reader (Tecan, Switzerland).

The total RNA was extracted from the hybridoma cell producing mAb to human T cell clone CARA. The purity of the extracted RNA was analyzed using 1.2% agarose gel electrophoresis and found clear two bans of the 28s rRNA and 18s rRNA (Figure 3.2A). This finding indicates the high purity of the extracted RNA, making it suitable for amplification of the specific gene encoding the variable light chain (VL) of the mAb. By performing RT-PCR using specific primers according to previous report (Meyeret. al, 2019), 1.2 % agarose gel electrophoresis was analyzed PCR product. The sesults revealed that VL chain of mAb clone CARA contains kappa (κ) light chain with the product size of 550 bp (Figure 3.2B).

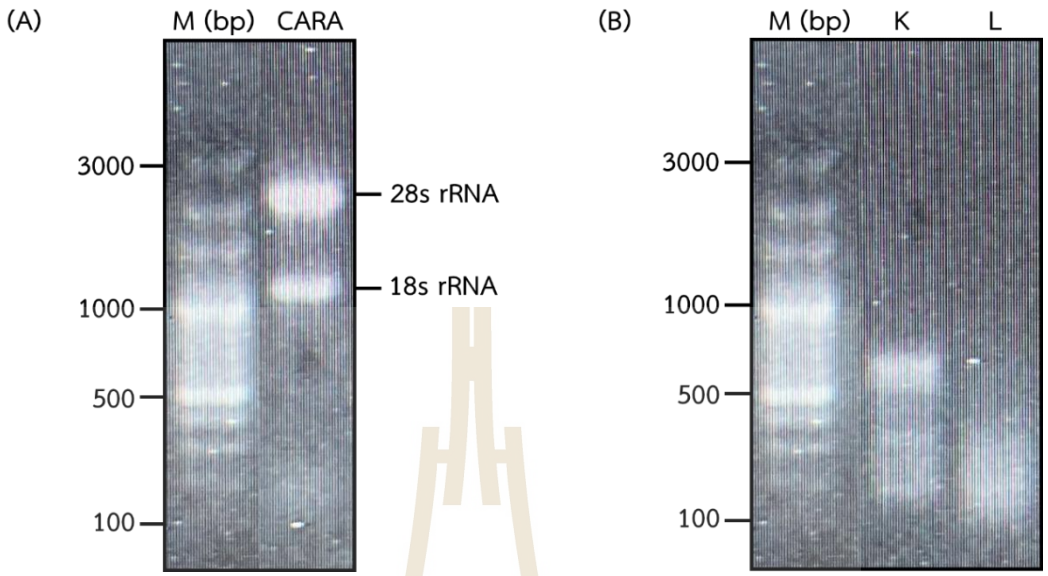

Figure 3.2 Agarose gel analysis of total extracted RNA and PCR products. (A) Total RNA was extracted from hybridoma cell producing mAb CARA ($2.5x10^6$ cells/ml) using GENzol™ reagent. The quality of the extracted RNA (2 µg/lane) was determined by resolving in 1.2% agarose gel electrophoresis containing SYBR-safe DNA gel stain in 0.5X Tris-acetate-EDTA (TAE) buffer. (B) RT-PCR; Synthesized cDNA was carried out using Viva cDNA synthesis kit (Vivantis, Malasia) and the reverse transcription primer was designed for the detection of mouse immunoglobulin kappa light chain (mIGk), or lambda light chain (mIGL).

3.2 Effect high-yield production of purified mAb CARA

To study the function of the mAb CARA on T cell biology, high yield of the purified mAb was required. The affinity chromatography using Hitrap® Protein L column was performed according to the specificity of protein L to the kappa light chain of mAb CARA. The production yields of each purification lot were summarized in Table 3.1. The purified mAb was examined by performing 10% SDS-PAGE under non-reducing or reducing conditions to indicate its purity. According to the results shown in Figure 3.3, two distinct bands corresponding to heavy and light chain were observed under reducing conditions at molecular weight of 55 and 25 kDa respectively. In contrast, at approximately 150 kDa was detected under non reducing condition. These results confirmed the high purity of the purified mAb.

Table 3.1 Production yield of the purified mAb clone CARA using affinity chromatography Hitrap® Protein L column.

Clone	Starting volume (ml)	Yield (µg)	Concentration (µg/ml)
CARA	48.6	249.12	859.03
	107	248.60	828.67
	104	352.00	1100.00
	200	271.27	602.82
	108	780.00	975.00
	140	609.56	609.56
	138	816.00	627.69
	145	813.11	813.11
	150	490.30	612.88
	148	626.6	696.22

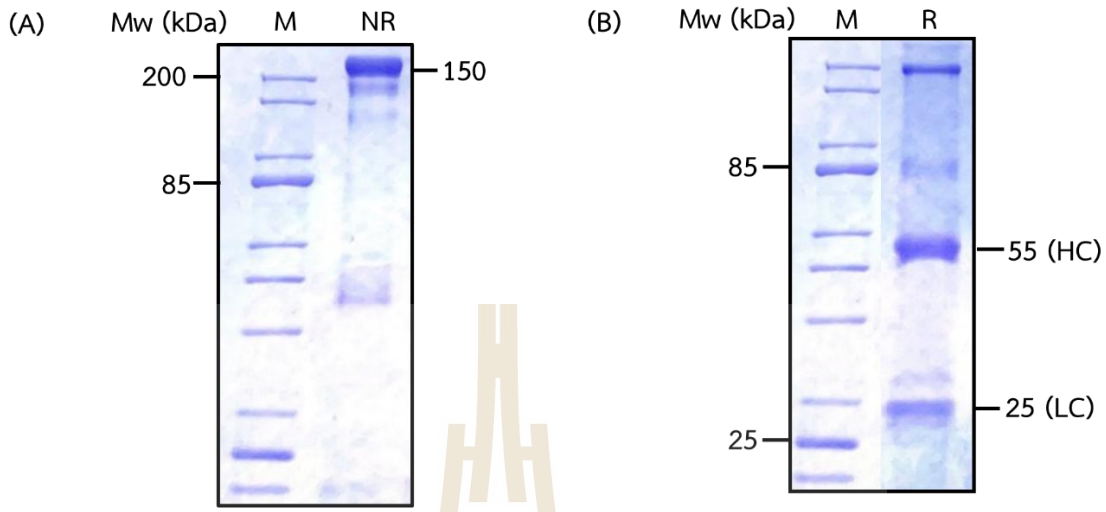

Figure 3.3 SDS-PAGE showing the purity of the purified mAb CARA. Five micrograms of purified mAb CARA were resolved in 10% SDS-PAGE under non-reducing (A) and reducing conditions (B). The separated proteins were visualized by staining the gel with Coomassie Brilliant Blue R250. The results shown represent one of each mAb CARA purification lots (HC: heavy chain; LC: light chain).

Activity the of purified mAb clone CARA was evaluated by indirect immunofluorescent staining of Molt4 cell surface and analyzed by flow cytometry according to the methods described in 2.2.5 Determination of antibody activity by indirect immunofluorescent staining and flow cytometry. Flow cytometry analysis showed positive reactivity of the purified mAb CARA to its recognizing molecule on Molt4 cell surface as shown in Figure 3.4. These results demonstrate that the purified mAb CARA retains its binding activity. Interestingly, the expression pattern of the mAb CARA recognizing molecule on the surface of Molt4 cells was further confirmed using laser scanning electron microscopy. Red fluorescence indicated the presence of the CARA recognizing molecule, while green fluorescence marked CD3 expression on T cells and DAPI indicated the nuclei in the cell. This result revealed a heterogeneous population of cells, which could be classified into $CARA^{neg}$, $CARA^{low+}$ and, $CARA^{high+}$ as shown in Figure 3.5.

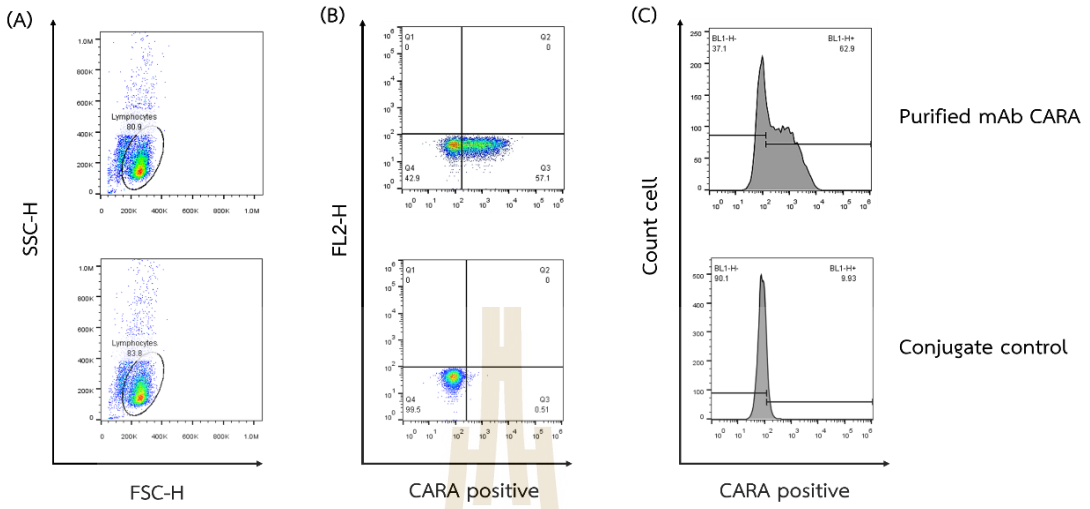

Figure 3.4 FACS profile representing binding activity of the purified mAb CARA. FACS profiles of (A-B) dot plot and (C) histogram plot. Molt4 cells ($1x10^7$ cell) were stained with purified mAb CARA (10 µg/ml). The binding of the target surface molecule on Molts was assessed using AF488-conjugated goat anti-mouse IgG at a dilution 1:1,000 and analyzed by a flow cytometer (Attune NxT, Invitrogen). Data shown are representative of one of ten independent experiments.

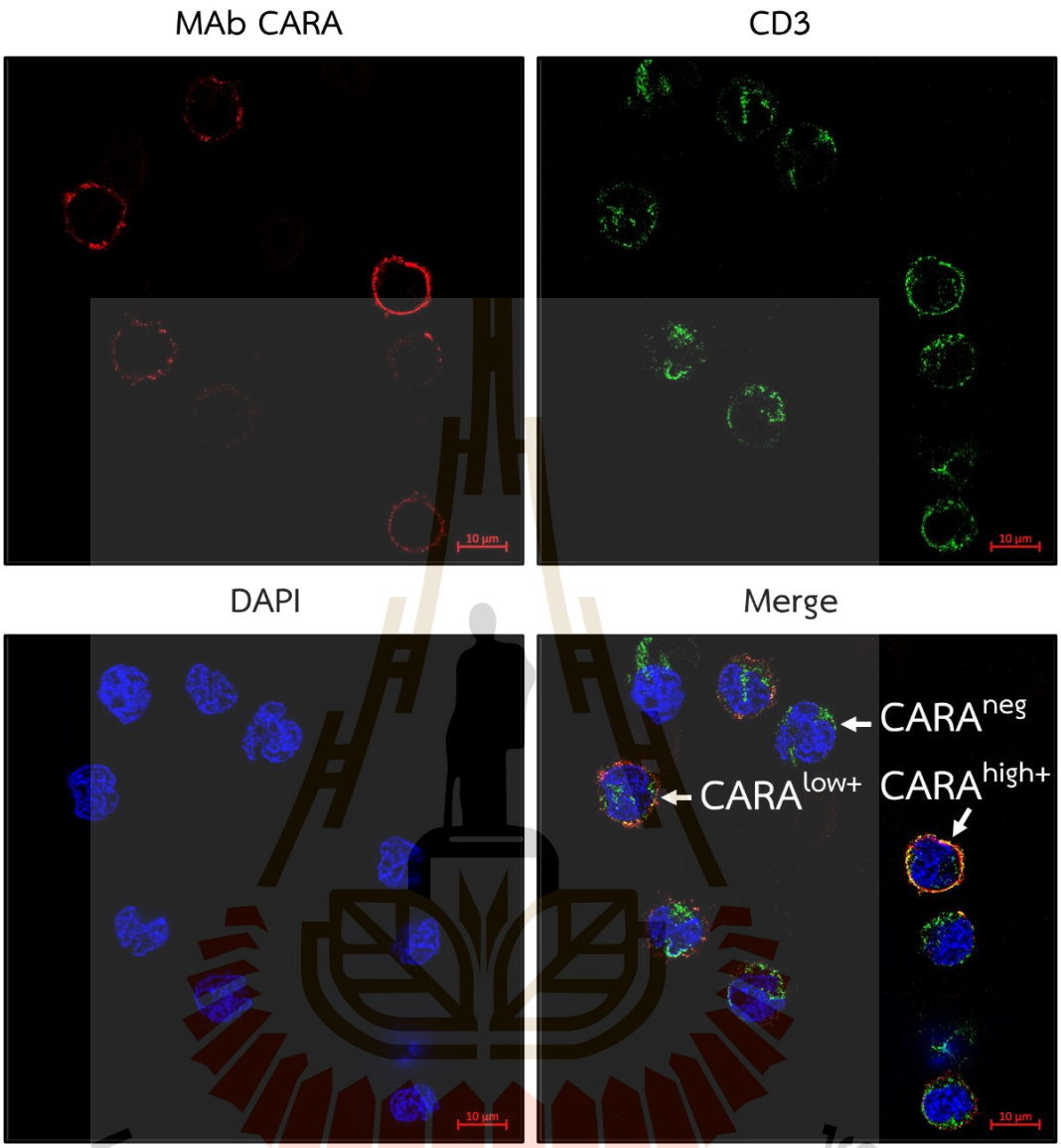

Figure 3.5 Cell surface expression of mAb CARA recognizing molecule on Molt4 cell lines**.** Immunofluorescence staining of CD3 and specific target molecule of mAb CARA on Molt4 cell lines. Cell nuclei were shown by DAPI. The expression was analysis using a field emission scanning electron microscope (Zeiss GeminiSEM 500, Zeiss, Germany) The result shown is representative of two independent experiments.

3.3 Investigation of the effect of mAb CARA on CD3-mediated T cell activation

T cell receives antigens through the TCR-CD3 complex by presenting antigen APCs, a mechanism of signalling will occur into the cell that results in various processes such as cell division and cytokine secretion. This study aims to investigated whether mAb CARA could modulated activation of CD3-mediated T cell. The immobilized anti-CD3 mAb clone OKT3 was utilized to induce T cell activation through CD3 triggering, stimulating signal 1 in the immune system. Cell proliferation was assessed using CFSE reduction-based analysis and monitored via flow cytometry. CFSE is a fluorescent dye used to track cell division. It passively diffuses into cells, where it covalently binds to intracellular proteins. When a labeled cell divides, the dye is equally distributed between daughter cells, leading to a progressive decrease in fluorescence intensity with each subsequent division, which can be monitored by flow cytometer. The results indicated that the mAb CARA could reduce cell proliferation in a dose dependent manners as shown by reduction of CFSE signal in the FACS profiles dot plot (Figure 3.6) and histogram plot (Figure 3.7). The fluorescence intensity of CFSE-labeled PBMCs gradually decreased following treatment with increasing concentration of mAb CARA, compared to the isotype-matched control, after 3 and 5 days of cocultivation with activated T cells. Statistical analysis revealed that the mAb CARA significantly suppressed the proliferation of CD3-mediated T cell in a dose dependent manner, relative to the isotype-matched control. Nonetheless, no effect was observed on unstimulated cells as shown in Figure 3.8 and Figure 3.9. Based on these results, a final concentration of 10 µg/ml of mAb CARA, the lowest concentration yielding the strongest suppressive effect on CD3-mediated T cell activation and it was selected for all subsequent experiments in this study.

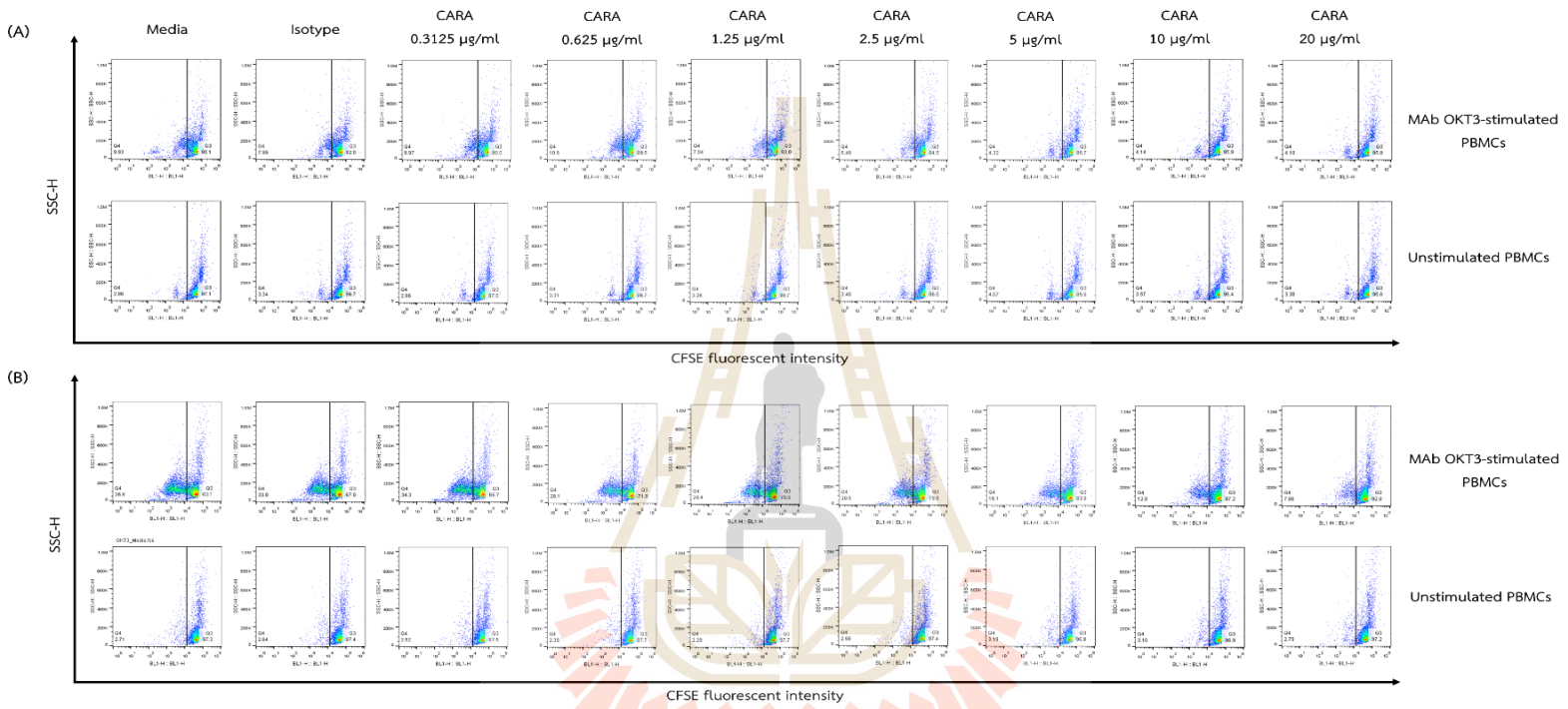

Figure 3.6 Dot plots of the FACS profiles show reduction of CFSE signal. The CFSE-labeled PBMCs ($1x10^5$ cells) were either stimulated with immobilized anti-CD3 mAb (OKT3 60 ng/ml) or unstimulated in the absence or presence of either mAb CARA at varying concentrations or mouse IgG3 isotype-matched control mAb. Cell proliferation was evaluated by monitering CFSE reduction following cultivation for (A) 3 days or (B) 5 days, using a flow cytometer (Attune NxT, Invitrogen). One of three independent experiments is shown as a representative result.

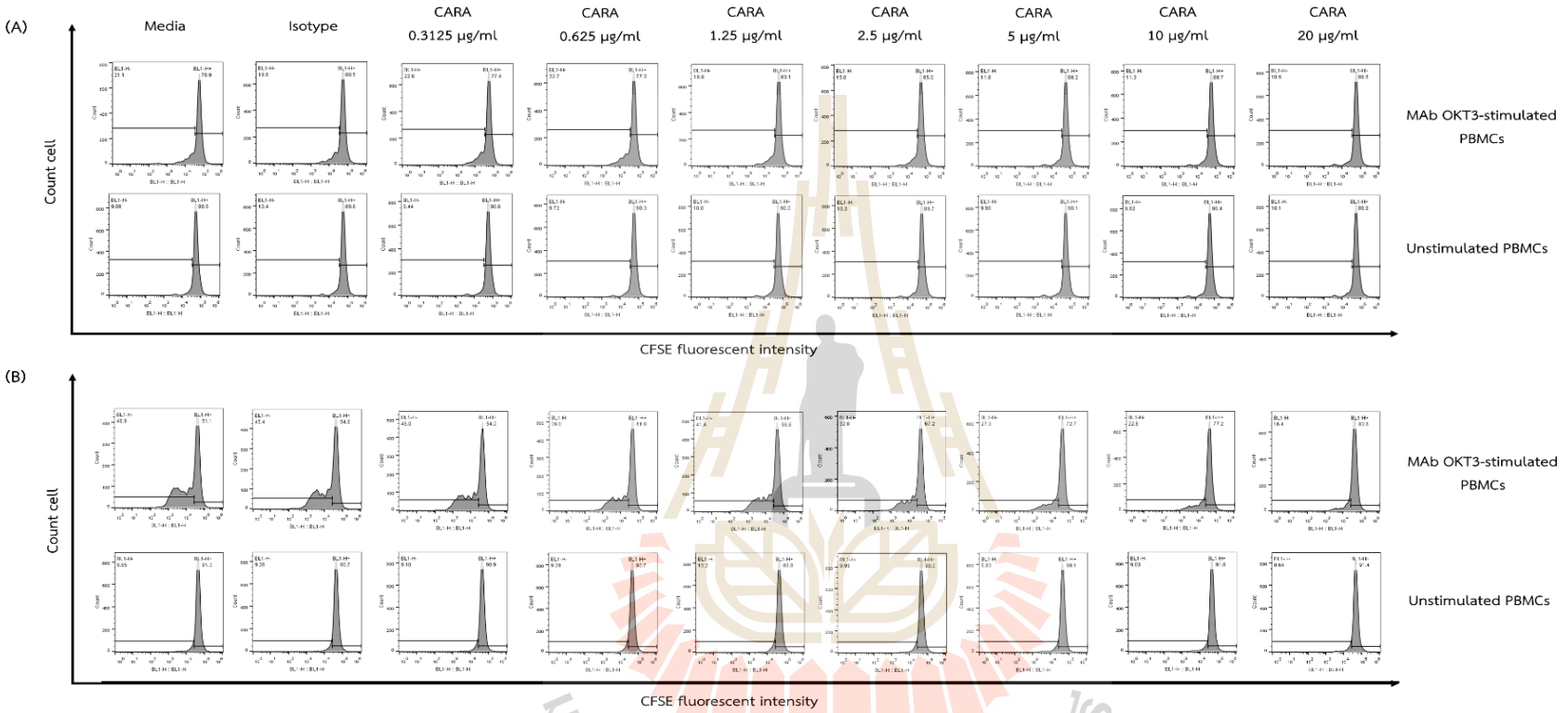

Figure 3.7 Histogram plots showing reduction of CFSE signal. The CFSE-labeled PBMCs ($1x10^5$ cells) were either stimulated with immobilized anti-CD3 mAb (OKT3 60 ng/ml) or unstimulated in the absence or presence of either mAb CARA at varying concentrations or mouse IgG3 isotype match control. Cell proliferation was evaluated by monitoring CFSE reduction following cultivation for (A) 3 days or (B) 5 days, using a flow cytometer (Attune NxT, Invitrogen). One of three independent experiments is shown as a representative result.

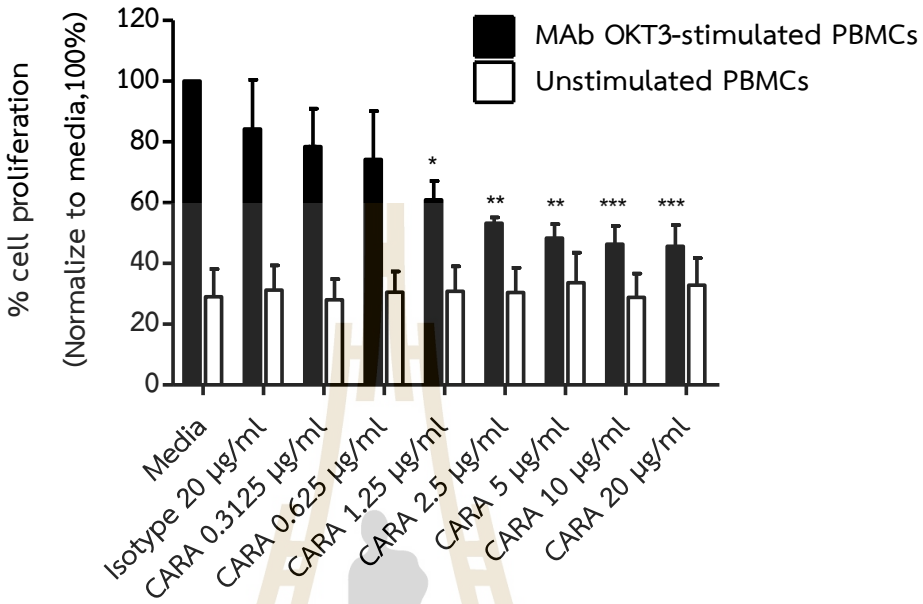

Figure 3.8 The bar graphs show percentage of cell proliferation after 3 days of cultivation. CFSE-labeled PBMCs ($1x10^5$ cells) under the defined conditions were normalized relative to cell stimulated with only mAb clone OKT3, set as 100%. The data represent the mean ± SD from three independent experiments, $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) indicate statistically significant differences compared to the isotype-matched control.

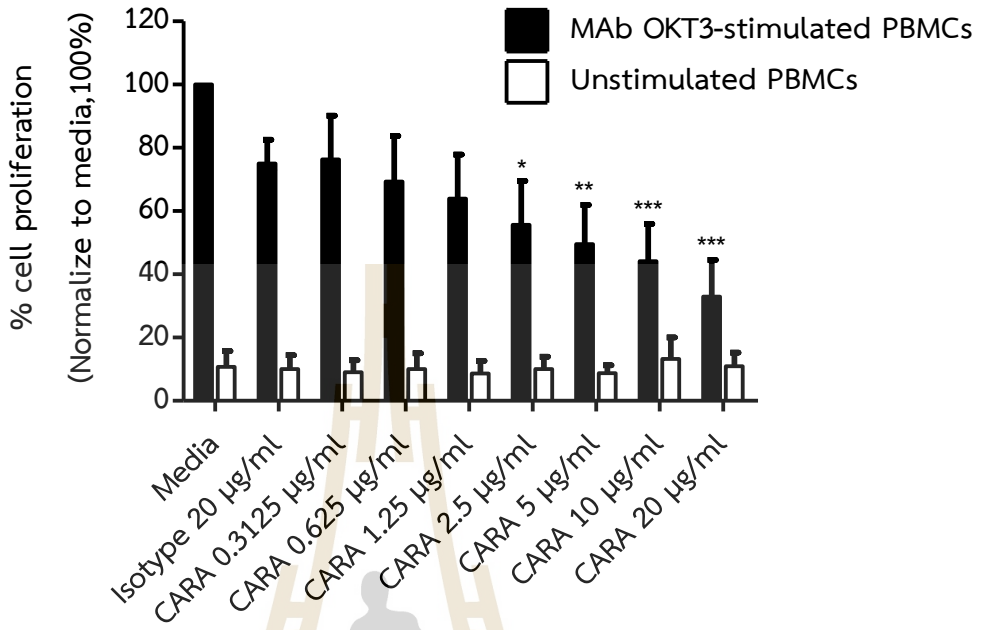

Figure 3.9 The bar graphs show the percentage of cell proliferation after 5 days of cultivation. CFSE-labeled PBMCs ($1x10^5$ cells) under the defined conditions were normalized relative to cell stimulated with only mAb clone OKT3 as 100%. The data represent the mean ± SD from three independent experiments, $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) indicate statistically significant differences compared to the isotype-matched control.

3.3.1 The mAb CARA reduced expression of T cell activation marker, CD69 and IL-2 receptor (CD25) during T cell activation

CD69 is an early activation marker that is rapidly upregulated upon T cell receptor (TCR) engagement. While, IL-2 receptor alpha chain (CD25), is considered as a late activation marker of T cells, which is essential for T cell proliferation and differentiation. These cells were used to evaluate the effect of mAb CARA on T cell activation status. The results revealed that mAb CARA significantly diminished cell surface CD69 expression on $CD3^+$ T cells compared to the isotype-matched control mAb (Figure 3.11A). The expression of CD25 tended to decrease upon presenting of mAb CARA, though the reduction was not statistically significant compared to isotype-matched control mAb (Figure 3.11B). These results indicated that mAb CARA significantly suppressed T cell activation and may play a role in modulating immune responses.

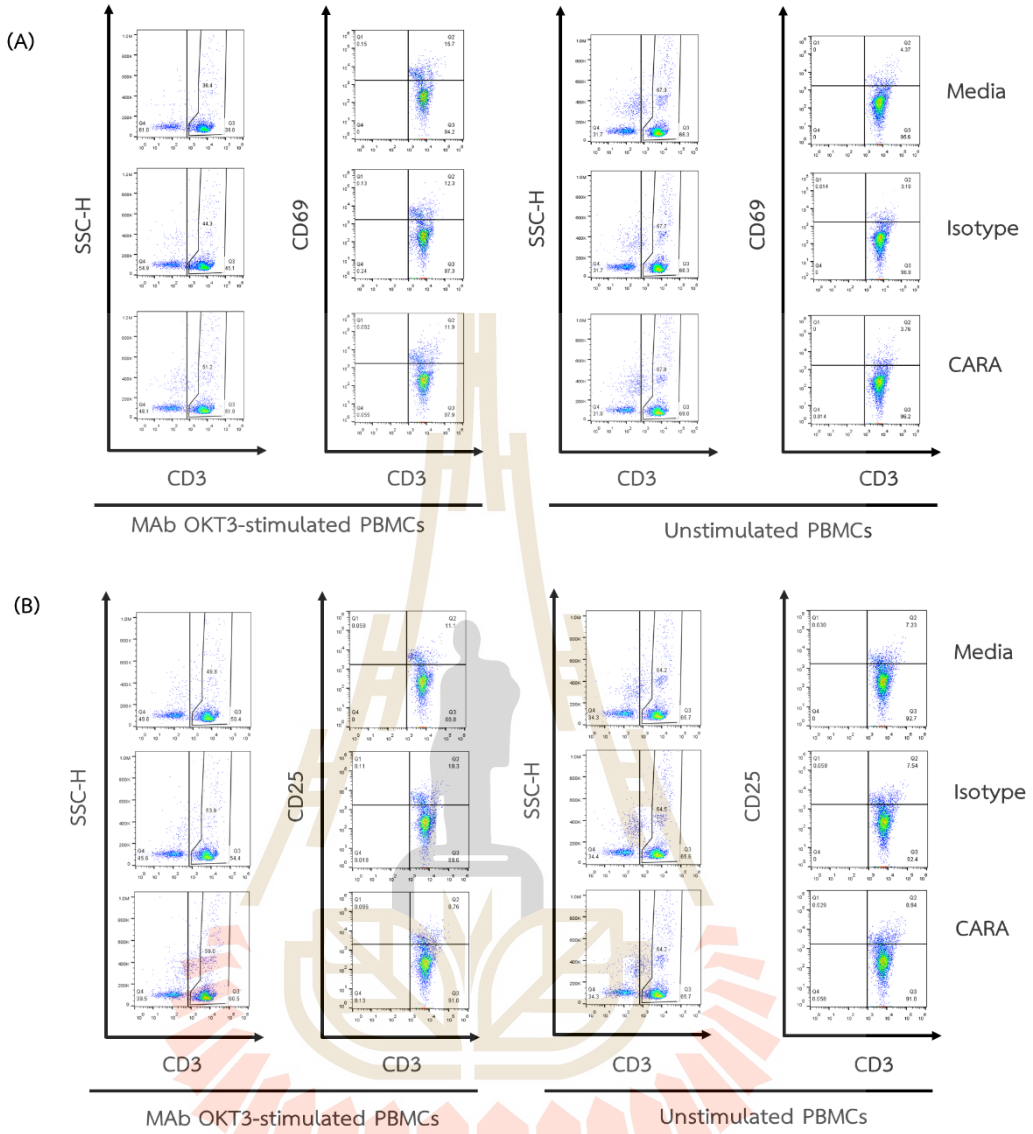

Figure 3.10 FACS profile of CD69 (A) or CD25 (B) expression on T cell. PBMCs ($5x10^5$ cells) were either stimulated with immobilized anti-CD3 mAb (OKT3 60 ng/ml) or unstimulated in 24-well plate in the absence or presence of mAb CARA or mouse IgG3 isotype-matched control mAb at final concentration 10 µg/ml at 37 °C in 5% CO_2 incubator for 24 h. The cultivated PBMCs ($1x10^7$ cells/ml) were double stained with FITC-conjugated-anti-human CD3 mAb and PE-conjugated-anti-human CD69 or CD25 mAb. Expression of CD69 or CD25 were determined using a flow cytometry. A representative result shows one of three independent experiments.

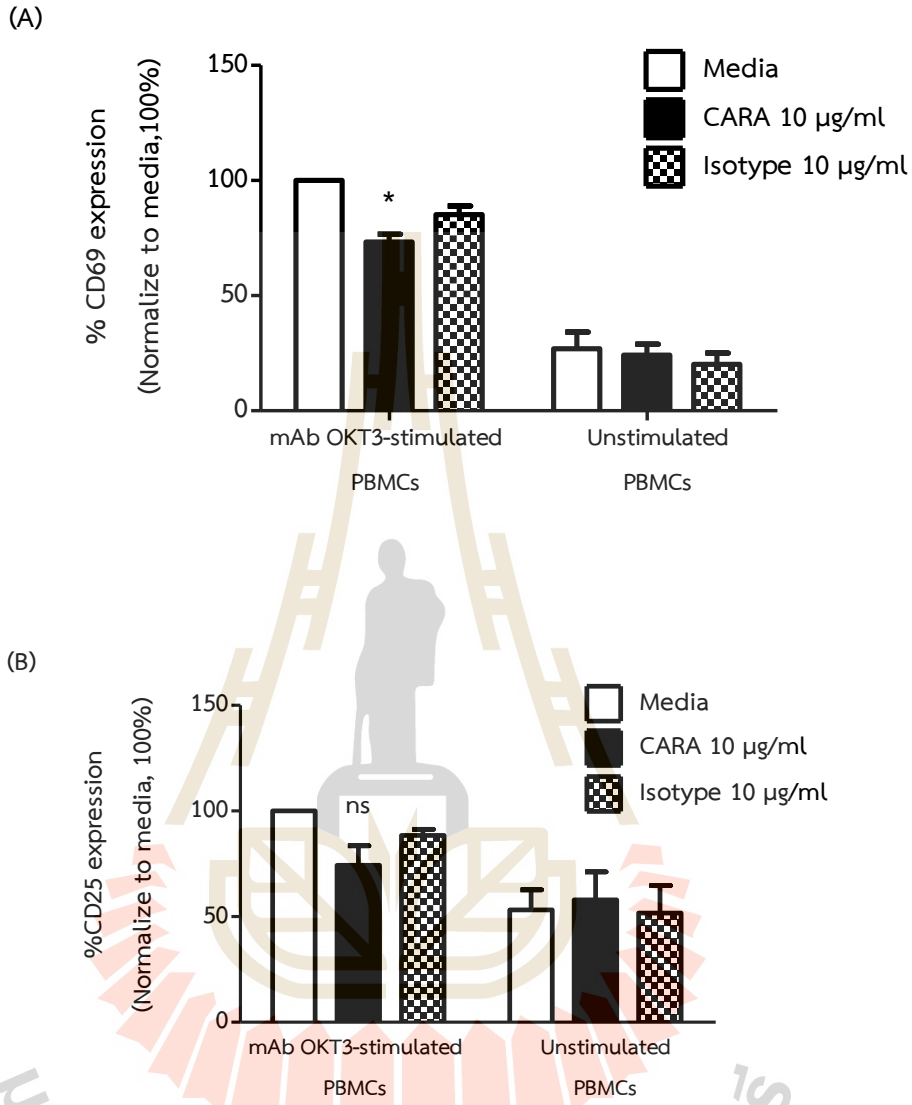

Figure 3.11 Bar graph shows the percentage of the cell surface CD69 and CD25 expression. The plot illustrates the percentage of the CD69-positive cells (A) and CD25-positive cells (B), calculated from dot plots of FACS profiles. Data represents the mean ± SD from three healthy donors, $p < 0.05$ (*) and $p < 0.05$ (ns) indicates not significant $p > 0.05$ when compared to OKT3-stimulated cells.

3.3.2 The expression of the mAb CARA recognizing molecule on PBMCs during T cell activation

As the results, indicating that mAb CARA effects cell proliferation and the expression of T cell activation marker. Consequenly, the expression of the mAb CARA-recognizing molecule on the surface of OKT3-stimulated PBMCs was analyzed at various time points using laser scanning electron microscopy. Red fluorescence signified the mAb CARA-recognizing molecule, while green fluorescence indicated CD3 expression on T cells. The analysis revealed consistent expression of the mAb CARA-recognizing molecule across all time points, with a heterogeneous distribution among the cell population. Specifically, both $CARA^{high+}$ and $CARA^{low+}$ were observed at each time point, reflecting variable expression levels of the target molecule in response to OKT3 stimulation.

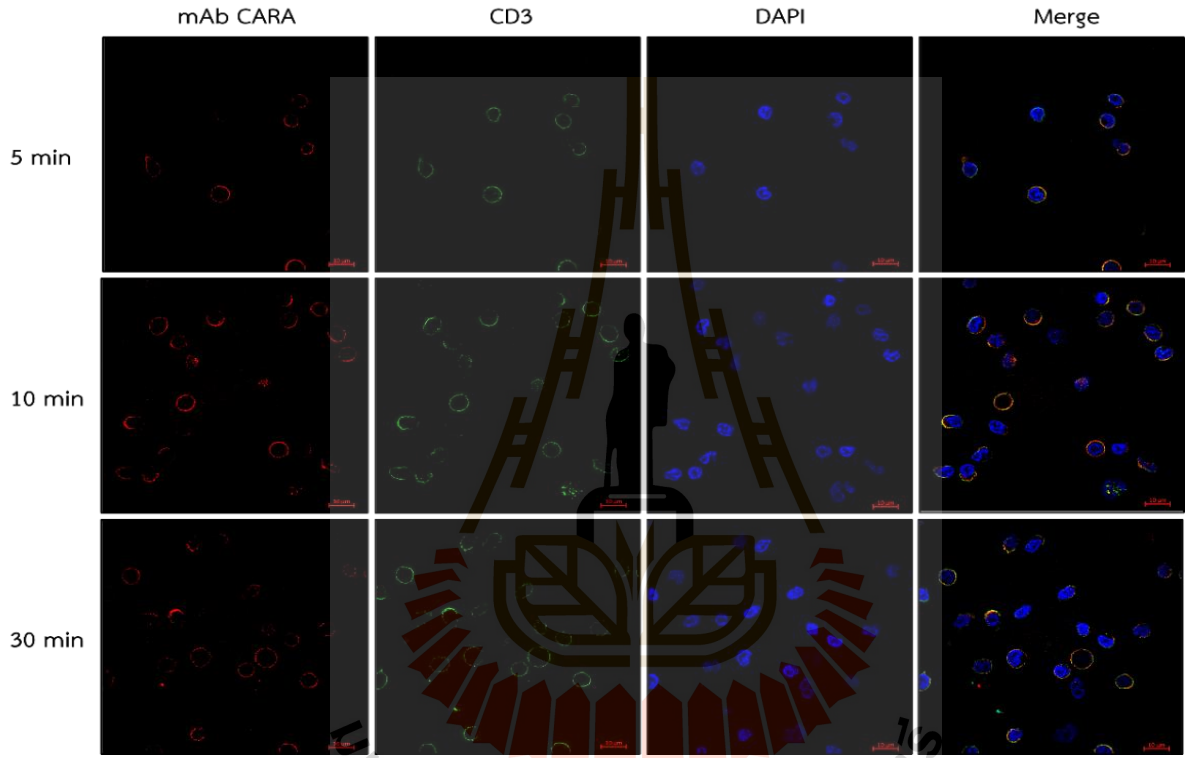

Figure 3.12 Localization of mAb CARA-recognizing molecule on OKT3-stimulated PBMCs. Immunofluorescence staining of CD3 and specific target molecule of mAb CARA on OKT3-stimulated PBMCs at various time points. Cell nuclei were shown by DAPI. The expression was analysis using a field emission scanning electron microscope (Zeiss GeminiSEM 500, Zeiss, Germany) A representative result show two independent experiment.

3.3.3 Mab CARA modulated cytokine secretion profile of CD3-medicated T cell activation

3.3.3.1 Total cytokine production

Upon T cell activation, several cytokines are secreted, playing a crucial role in immune modulation. The precise cytokine profile depends on the type of T cells and the nature of the stimulus, shaping the overall immune reaction. These include IL-2, which promotes T cell proliferation; IFN-γ, which enhances macrophage activation and antiviral defense; TNF-α mediating pro-inflammatory responses; IL-4 and IL-13 contributing to Th2 immunity; IL-10 exerting immunosuppressive effects, and IL-17A being associated with Th17-driven inflammation. Therefore, the effect of mAb CARA on cytokine secretion during CD3-mediated T cell activation was investigated. The total production of IL-2, IL-4, IL-10, IL-13, IL-17A, IFN-γ, and TNF-α in culture supernatant were quantified using sandwich ELISA kits after 24 h under each cultivation condition. The results demonstrated a significant reduction in production of all cytokines (Figure 3.13-Figure 3.17) following treatment with mAb CARA compared to the isotype-matched control mAb accepted for IFN-γ (Figure 3.18), and TNF-α (Figure 3.19). IL-2 exhibited the most substantial decrease, indicating a suppression of the T cell activation. Furthermore, the downregulation of IL-4, IL-10, IL-13, and IL-17A suggested that mAb CARA may have a board impact on immune responses, potentially inhibiting both regulatory and pro-inflammatory pathways. These findings suggested that mAb CARA reduces overall cytokine production and may help prevent excessive immune activation through its immunosuppressive effects.

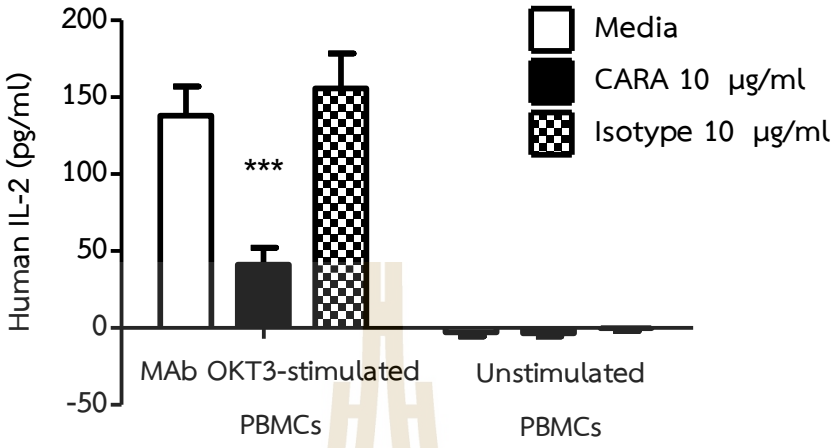

Figure 3.13 Effect of the mAb CARA on human IL-2 secretion after 24 h of CD3-mediated T cell activation. The PBMCs ($5x10^5$ cells) were either stimulated with immobilized anti-CD3 mAb (OKT3 60 ng/ml) or unstimulated in 24-well plate in presence or absence of either mAb CARA or mouse IgG3 isotype-matched control mAb at final concentration 10 µg/ml. The cells were cultured at 37 °C in 5% CO_2 incubator for 24 h. For each condition, the culture supernatant of PBMCs was harvested after cultivation for 24 hours. The concentration of IL-2 was determined using human IL-2 ELISA MAX™ Deluxe kits (BioLegend). Data shown represent the mean ± SD from three independent experiments, $p < 0.001$ (***) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

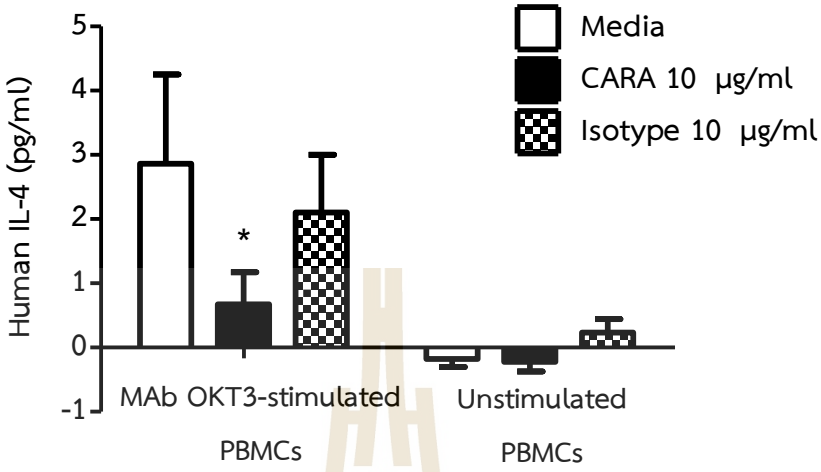

Figure 3.14 Effect of the mAb CARA on human IL-4 secretion after 24 h of CD3-mediated T cell activation. The PBMCs ($5x10^5$ cells) were either stimulated with immobilized anti-CD3 mAb (OKT3 60 ng/ml) or unstimulated in 24-well plate in presence or absence of either mAb CARA or mouse IgG3 isotype-matched control mAb at final concentration 10 µg/ml. The cells were cultured at 37 °C in 5% CO_2 incubator for 24 h. For each condition, the culture supernatant of PBMCs was harvested after cultivation for 24 hours. The concentration of IL-4 was determined using human IL-4 ELISA MAX™ Deluxe kits (BioLegend). Data shown represent the mean ± SD from three independent experiments, $p < 0.05$ (*) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

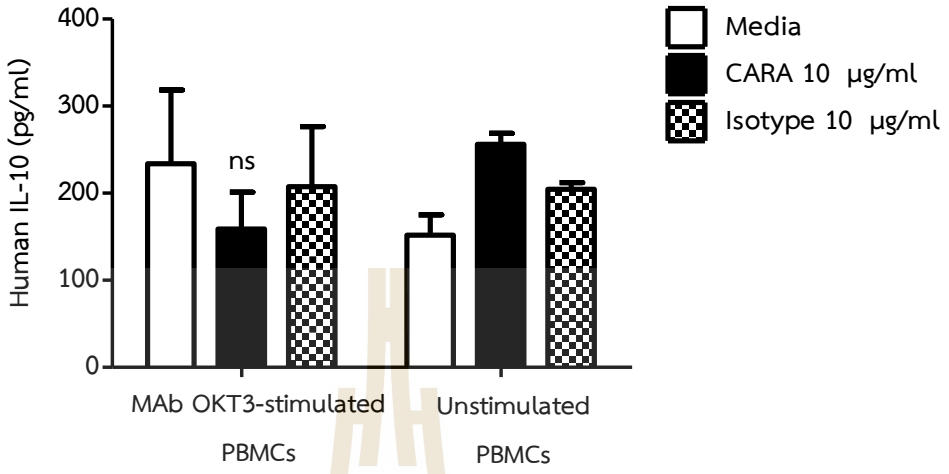

Figure 3.15 Effect of the mAb CARA on human IL-10 secretion after 24 h of CD3-mediated T cell activation. The PBMCs ($5x10^5$ cells) were either stimulated with immobilized anti-CD3 mAb (OKT3 60 ng/ml) or unstimulated in 24-well plate in presence or absence of either mAb CARA or mouse IgG3 isotype-matched control mAb at final concentration 10 µg/ml. The cells were cultured at 37 °C in 5% CO_2 incubator for 24 h. For each condition, the culture supernatant of PBMCs was harvested after cultivation for 24 hours. The concentration of IL-10 was determined using human IL-10 ELISA MAX™ Deluxe kits (BioLegend). Data shown represent the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

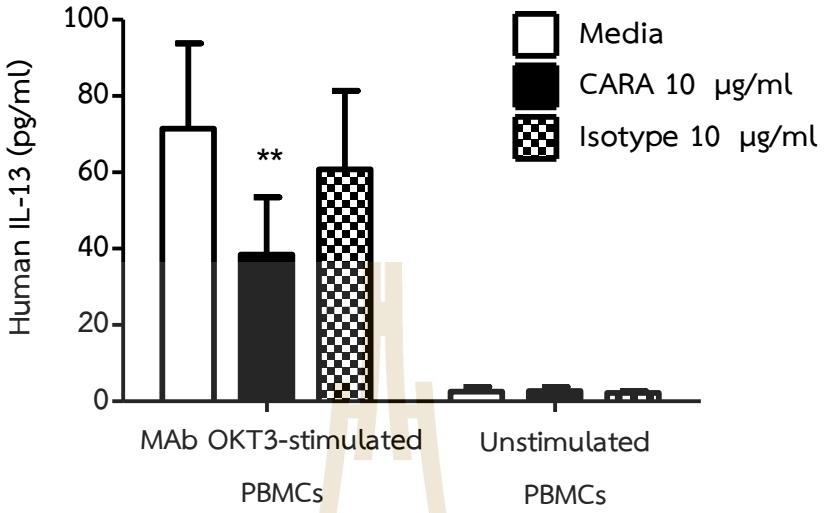

Figure 3.16 Effect of the mAb CARA on human IL-13 secretion after 24 h of CD3-mediated T cell activation. The PBMCs ($5x10^5$ cells) were either stimulated with immobilized anti-CD3 mAb (OKT3 60 ng/ml) or unstimulated in 24-well plate in presence or absence of either mAb CARA or mouse IgG3 isotype-matched control mAb at final concentration 10 µg/ml. The cells were cultured at 37 °C in 5% CO_2 incubator for 24 h. For each condition, the culture supernatant of PBMCs was harvested after cultivation for 24 hours. The concentration of IL-13 was determined using human IL-13 uncoated ELISA kit (Invitrogen). Data shown represent the mean ± SD from three independent experiments, $p < 0.01$ (**) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

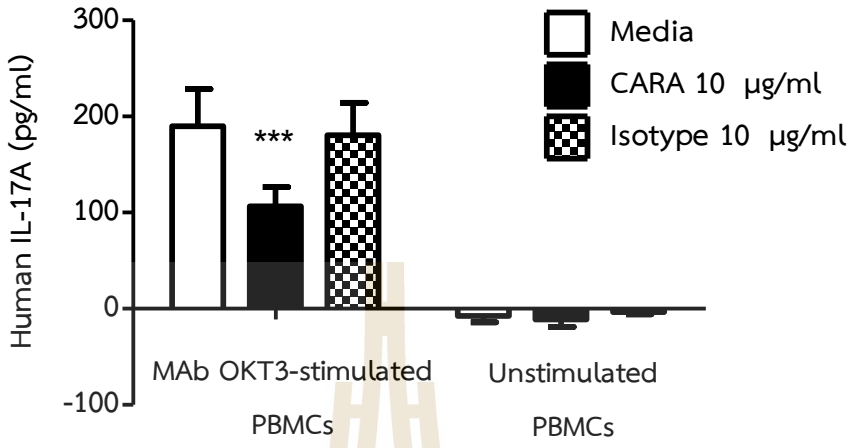

Figure 3.17 Effect of the mAb CARA on human IL-17 secretion after 24 h of CD3-mediated T cell activation. The PBMCs ($5x10^5$ cells) were either stimulated with immobilized anti-CD3 mAb (OKT3 60 ng/ml) or unstimulated in 24-well plate in presence or absence of either mAb CARA or mouse IgG3 isotype-matched control mAb at final concentration 10 µg/ml. The cells were cultured at 37 °C in 5% CO_2 incubator for 24 h. For each condition, the culture supernatant of PBMCs was harvested after cultivation for 24 hours. The concentration of IL-17 was determined using human IL-17 ELISA MAX™ Deluxe kits (BioLegend). Data shown represent the mean ± SD from three independent experiments, $p < 0.001$ (***) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

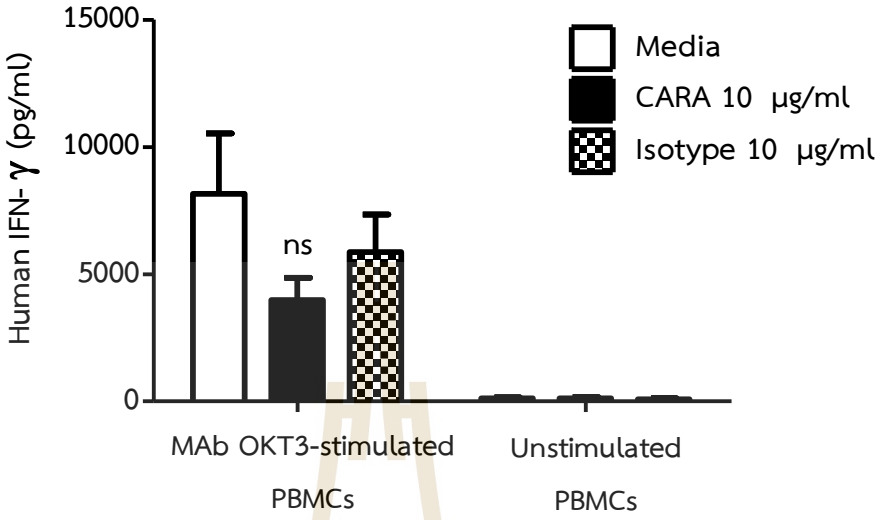

Figure 3.18 Effect of the mAb CARA on human IFN-γ secretion after 24 h of CD3-mediated T cell activation. The PBMCs ($5x10^5$ cells) were either stimulated with immobilized anti-CD3 mAb (OKT3 60 ng/ml) or unstimulated in 24-well plate in presence or absence of either mAb CARA or mouse IgG3 isotype-matched control mAb at final concentration 10 µg/ml. The cells were cultured at 37 °C in 5% CO_2 incubator for 24 h. For each condition, the culture supernatant of PBMCs was harvested after cultivation for 24 hours. The concentration of IFN-γ was determined using human IFN-γ ELISA MAX™ Deluxe kits (BioLegend). Data shown represent the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

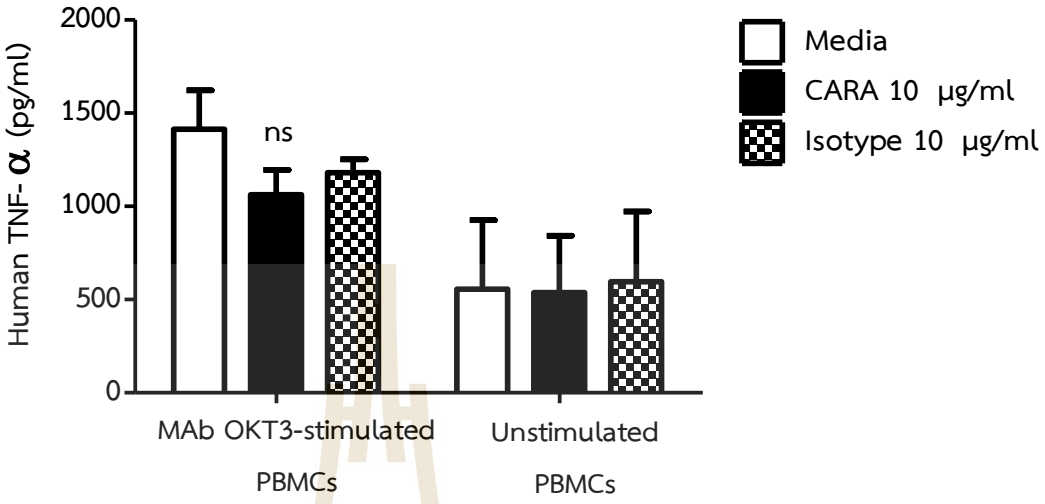

Figure 3.19 Effect of the mAb CARA on human TNF-α secretion after 24 h of CD3-mediated T cell activation. The PBMCs ($5x10^5$ cells) were either stimulated with immobilized anti-CD3 mAb (OKT3 60 ng/ml) or unstimulated in 24-well plate in presence or absence of either mAb CARA or mouse IgG3 isotype-matched control mAb at final concentration 10 µg/ml. The cells were cultured at 37 °C in 5% CO_2 incubator for 24 h. For each condition, the culture supernatant of PBMCs was harvested after cultivation for 24 hours. The concentration of TNF-α was determined using human TNF-α ELISA MAX™ Deluxe kits (BioLegend). Data shown represent the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

3.3.3.2 Intracellular Cytokine Staining (ICS)

Based on the total cytokine production, the mAb CARA appeared to decrease overall cytokine production. Since the molecule recognized by mAb CARA is exclusively expressed on T cells, an assessment was conducted to evaluate cytokine secretion by T cell. PBMCs were either stimulated or left unstimulated with immobilized anti-CD3 mAb clone OKT3 in the present or absence of mAb CARA or isotype-matched control mAb. The resulted in reduced IL-2 production (Figure 3.22) compared to the isotype-matched control on stimulated cells. This suggests that CARA may interfere with the pathways involved in activation of T cell and IL-2 expression.

However, increased and variable cytokine production was seen with other cytokines following mAb CARA treatment (Figure 3.23 - Figure 3.27). This indicates that mAb CARA may have a differential modulatory effect on T cell function, potentially enhancing certain signaling pathways. The variability in cytokine responses may reflect the effects of PBMCs cell populations. Therefore, this question remains unanswered.

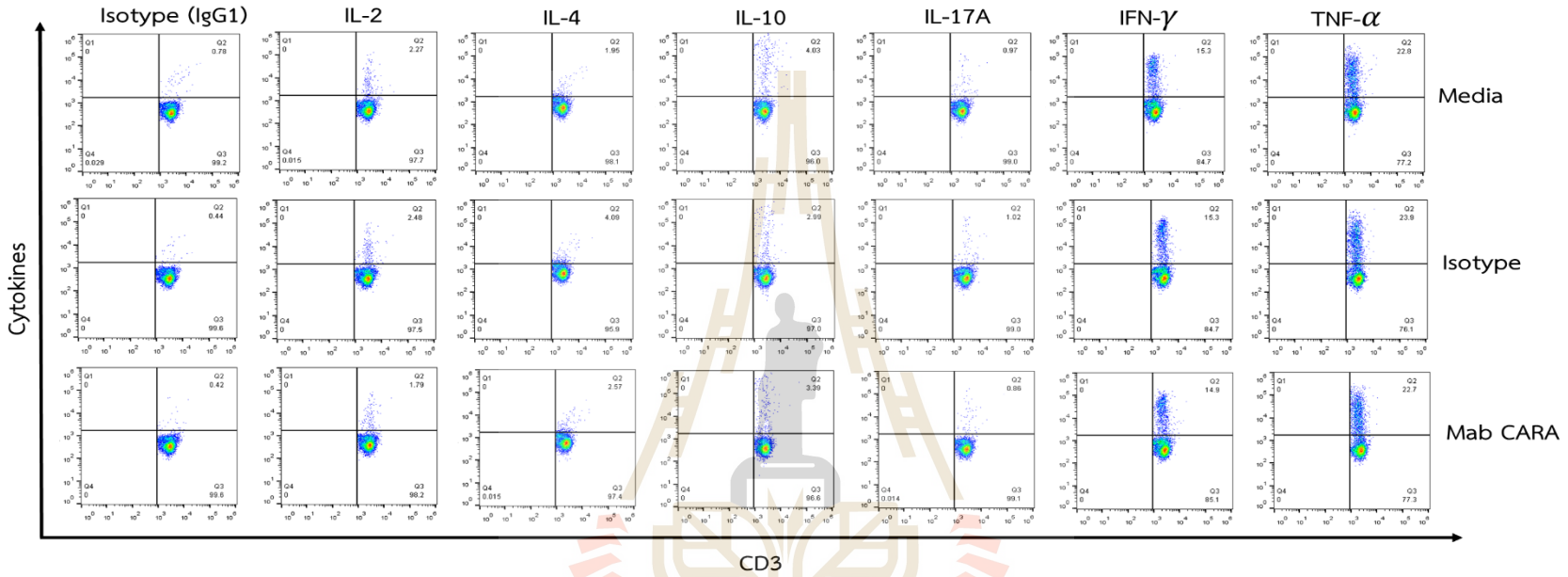

Figure 3.20 FACS profiles of dot plot show expression of cytokines production on CD3-mediated T cell activation. PBMCs ($5x10^5$ cells) were stimulated with immobilized anti-CD3 mAb clone OKT3 (100 ng/ml) in the absence or presence of soluble mAb CARA or mouse IgG3 isotype-matched control mAb at a final concentration of 10 µg/ml in 5% CO_2 incubator at 37 °C. After an hour of cultivation, brefeldin A (BioLegend) was added, and the cells were further cultivated for an addition 5 h. The cells were harvested and intracellularly stained with FITC-conjugated anti-CD3 mAb and PE-conjugated anti-human cytokine antibodies (IL-2, or IL-4, or IL-10, or IL-17A, or IFN-γ, or TNF-α) or PE-conjugated isotype-matched control mAb. The $CD3^+$ T cells were gated to determine cytokine-expressing cells. A representative result from one of three independent experiments is shown.

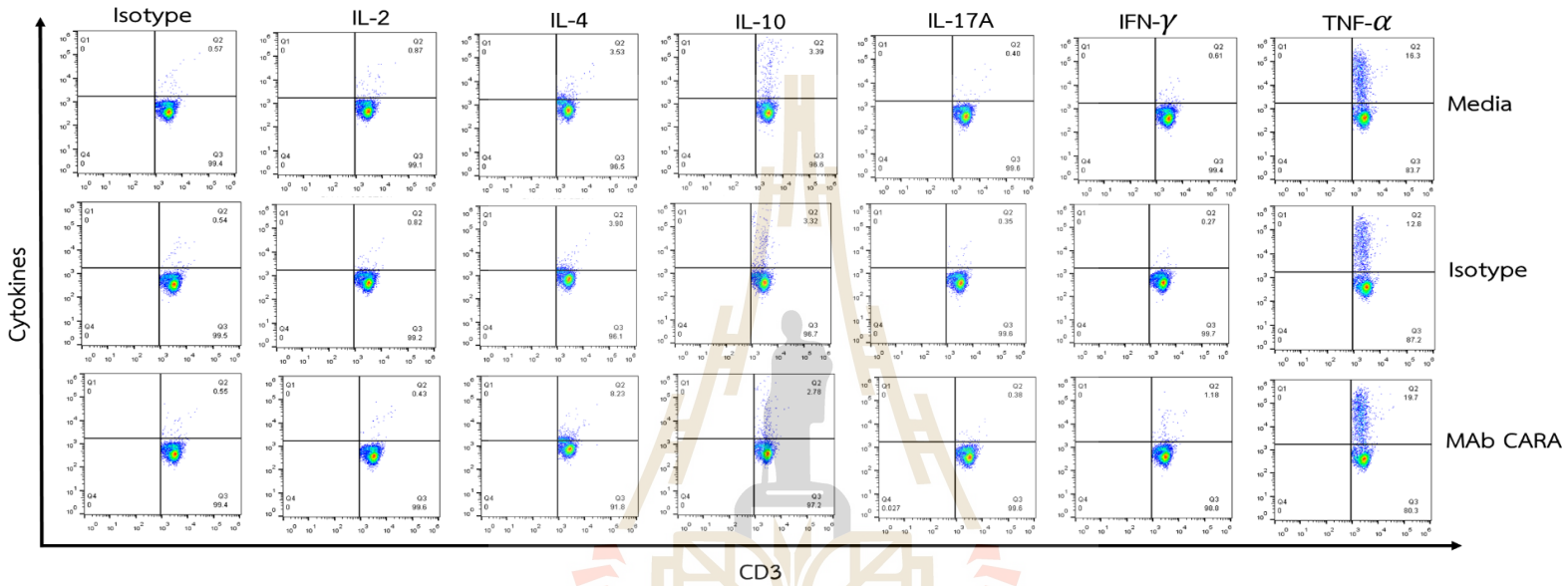

Figure 3.21 FACS profiles of dot plot show expression of cytokines production on unstimulated T cell. Unstimulated PBMCs ($5x10^5$ cells) were cultured in the absence or presence of either mAb CARA or mouse IgG3 isotype-matched control mAb at a final concentration of 10 µg/ml in 5% CO_2 incubator at 37 °C. After an hour of cultivation, brefeldin A (BioLegend) was added, and the cells were further cultivated for an addition 5 h. The cells were harvested and intracellularly stained with FITC-conjugated anti-CD3 mAb and PE-conjugated anti-human cytokine antibodies (IL-2, or IL-4, or IL-10, or IL-17A, or IFN-γ, or TNF-α) or PE-conjugated isotype-matched control mAb. The $CD3^+$ T cells were gated to determine cytokine-expressing cells. A representative result from one of three independent experiments is shown.

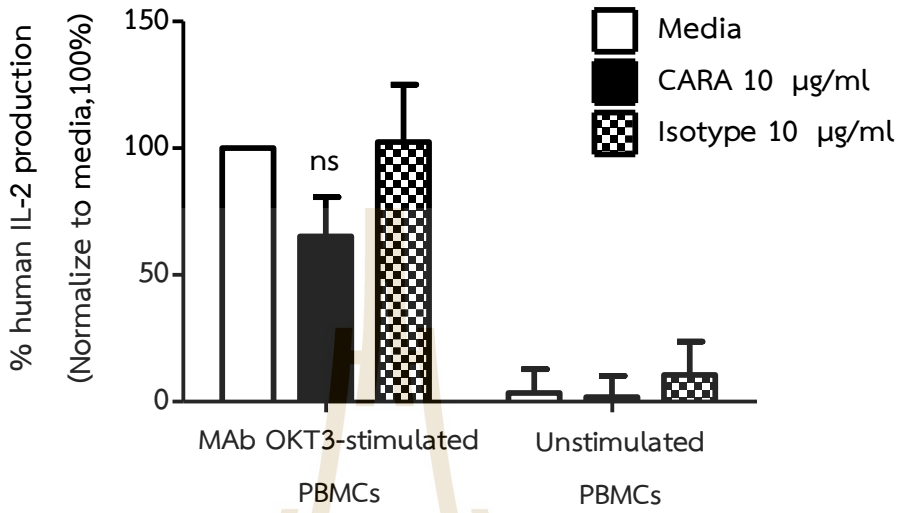

Figure 3.22 The bar graph illustrates the percentage of human IL-2 production after 6 h of cultivation. PBMCs ($1x10^5$ cells) under the conditions tested were normalized relative to cell stimulated with only mAb clone OKT3 as 100%. The data represents the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

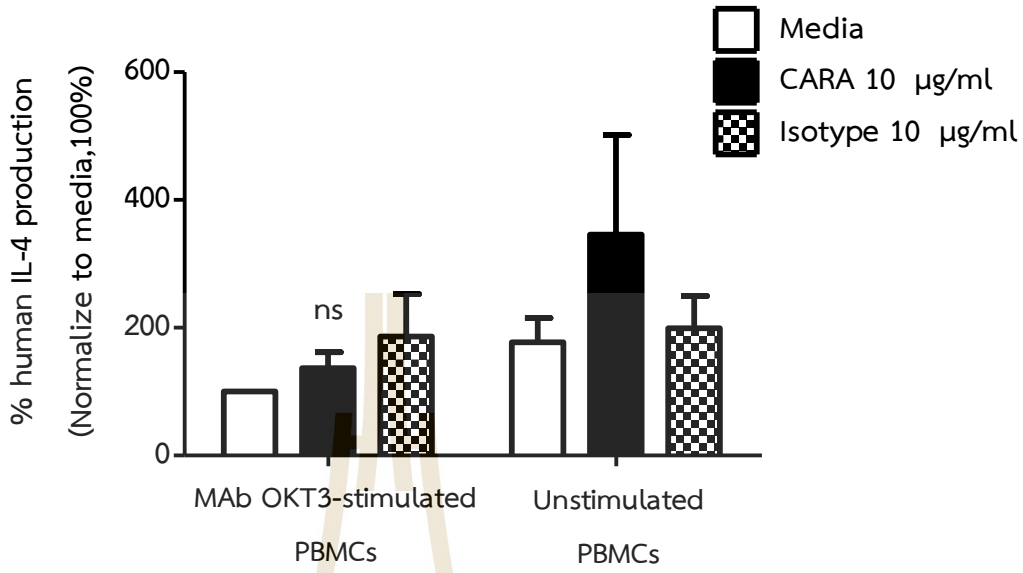

Figure 3.23 The bar graph illustrates the percentage of human IL-4 production after 6 h of cultivation. PBMCs ($1x10^5$ cells) under the conditions tested were normalized relative to cell stimulated with only mAb clone OKT3 as 100%. The data represents the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

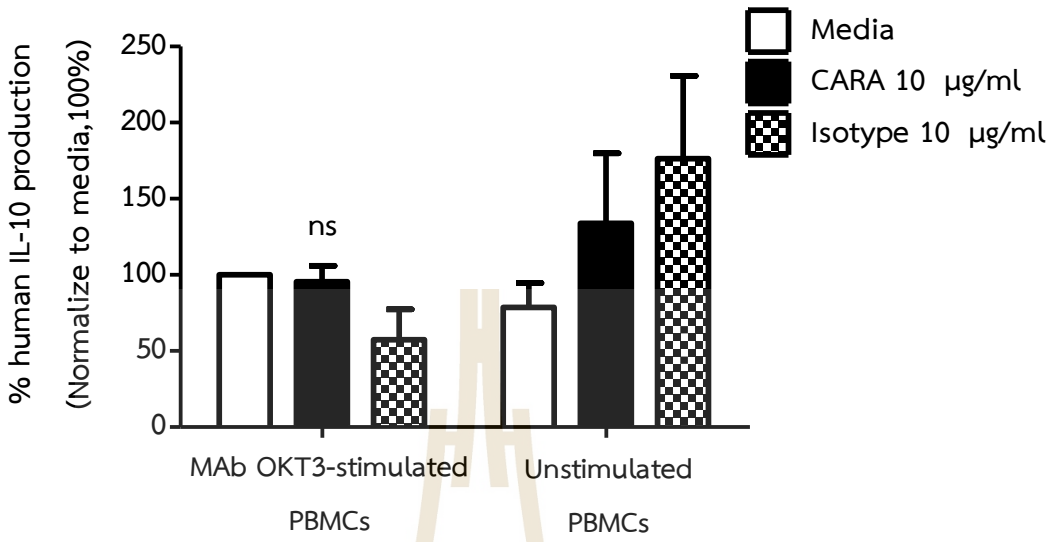

Figure 3.24 The bar graph illustrates the percentage of human IL-10 production after 6 h of cultivation. PBMCs ($1x10^5$ cells) under the conditions tested were normalized relative to cell stimulated with only mAb clone OKT3 as 100%. The data represents the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

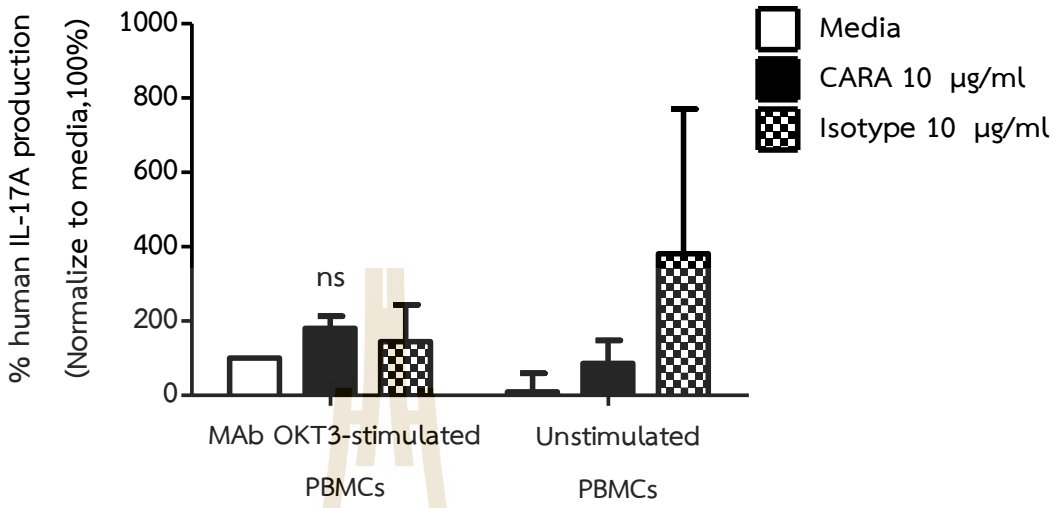

Figure 3.25 The bar graph illustrates the percentage of human IL-17A production after 6 h of cultivation. PBMCs ($1x10^5$ cells) under the conditions tested were normalized relative to cell stimulated with only mAb clone OKT3 as 100%. The data represents the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

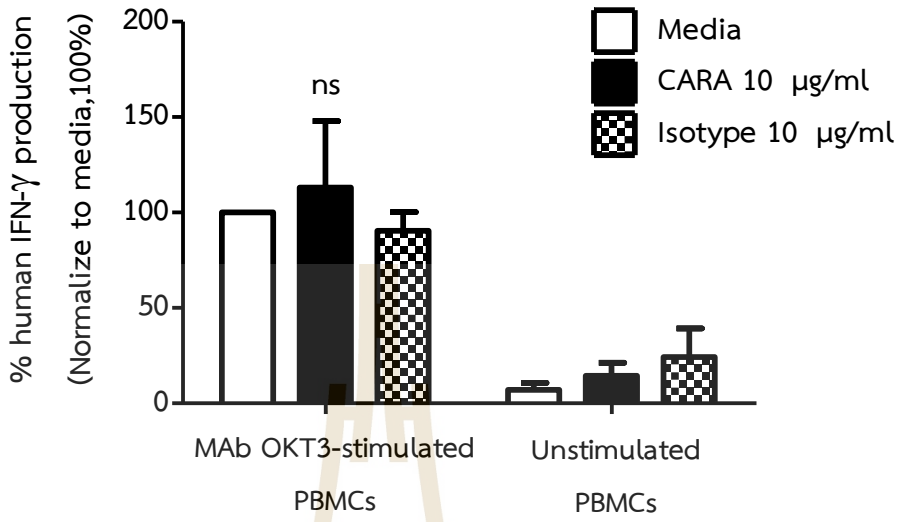

Figure 3.26 The bar graph illustrates the percentage of human IFN-γ production after 6 h of cultivation. PBMCs ($1x10^5$ cells) under the conditions tested were normalized relative to cell stimulated with only mAb clone OKT3 as 100%. The data represents the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

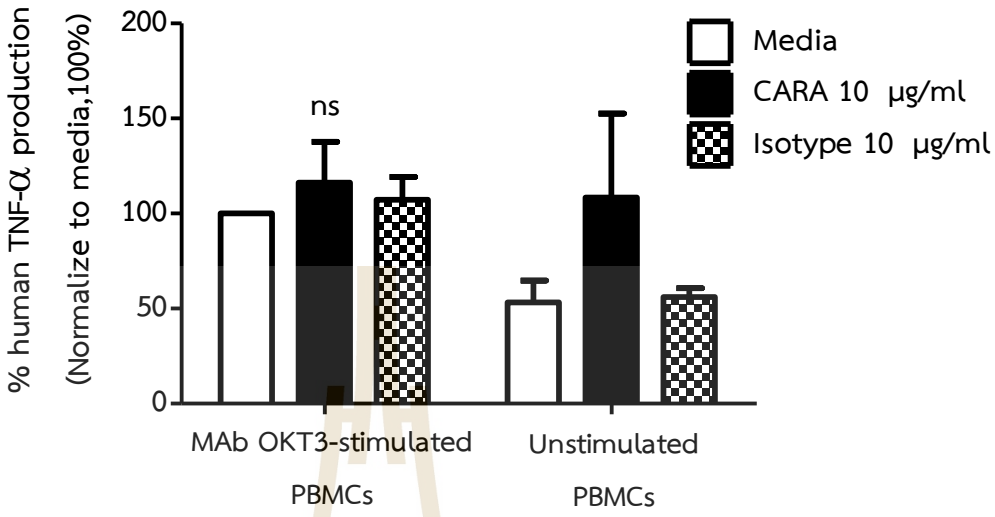

Figure 3.27 The bar graph illustrates the percentage of human TNF-α production after 6 h of cultivation. PBMCs ($1x10^5$ cells) under the conditions tested were normalized relative to cell stimulated with only mAb clone OKT3 as 100%. The data represents the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

3.4 Cell signaling using phosphotyrosine blot

Since mAb CARA was shown to influence cytokine production during T cell activation, it was important to determine whether it also affects intracellular signaling pathways involved in this process. Therefore, PBMCs were stimulated or unstimulated with OKT3 and treated with mAb CARA or isotype-matched control mAb at indication times. Phosphotyrosine blot revealed a marked increase in tyrosine phosphorylation of several proteins in a condition that cells were treated with mAb CARA compared to the isotype-matched control mAb as shown in Figure 3.28. Notably, enhanced phosphorylation was clearly observed at the protein band of approximately 63 and 65 kDa. As shown in Figure 3.29 and Figure 3.30, the bar graph represents the relative expression of phosphorylated proteins at ~63 kDa and ~65 kDa, normalized to the unstimulated control (0 min), which was set as 1. Statistical analysis confirmed that the phosphotyrosine signal of these two proteins did not significantly differ in phosphorylation between OKT3-stimulated PBMCs treated mAb CARA and those treated with isotype-matched control mAb.

Altogether, this study suggests that mAb CARA modulates CD3-mediated T cell signaling via phosphorylation of key signaling proteins involved in T cell activation. However, further investigation is required to identify the specific molecules involved.

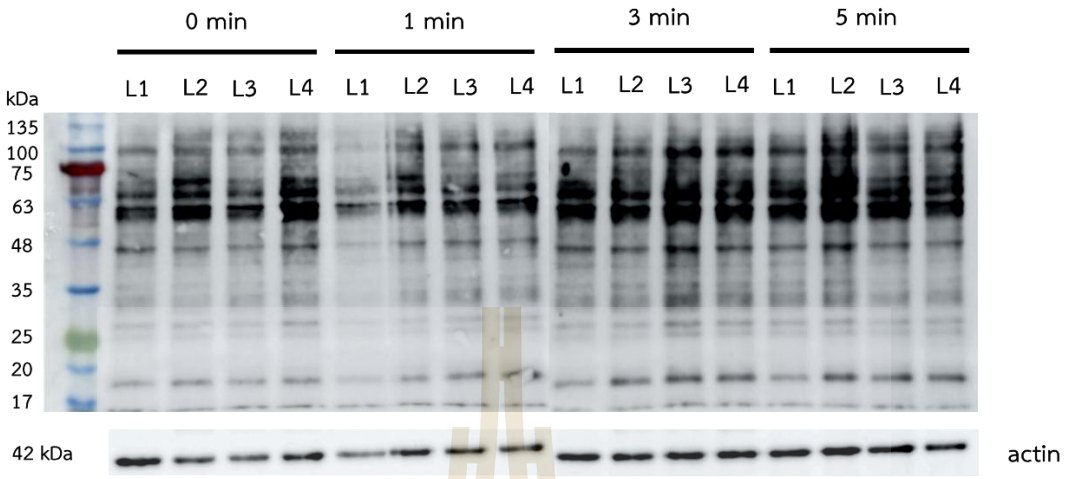

Figure 3.28 Phosphotyrosine blot shows change several phosphotyrisine proteins upon T cell activation. PBMCs ($1x10^7$ cells/ml) were stimulated with mAb OKT3 10 µg/ml or unstimulated in presence or absence of mAb CARA or isotype-matched control mAb at final concentration 10 µg/ml at indicated time points at 37 °C. The cells were lyzed and the cell lysates were resolved under reducing condition by 10% SDS-PAGE and subjected to Western blot using HRP-conjugated anti-phosphotyrosine antibody clone 4G10 at dilution of 1:5,000 in 2.5 % BSA in Tris-HCl buffer pH 7.5 as the detecting Ab. The protein bands were visualized using an enhanced chemiluminescent detection system and analyzed by Amersham Image Quant™ 500 CCD imaging system (Cytiva, USA). A representative result from one of three independent experiments is shown. (L1: Unstimulated PBMCs; L2: mAb OKT3-stimulated PBMCs with mAb CARA; L3: mAb OKT3-stimulated PBMCs and isotype-matched control mAb; L4: mAb OKT3-stimulated PBMCs)

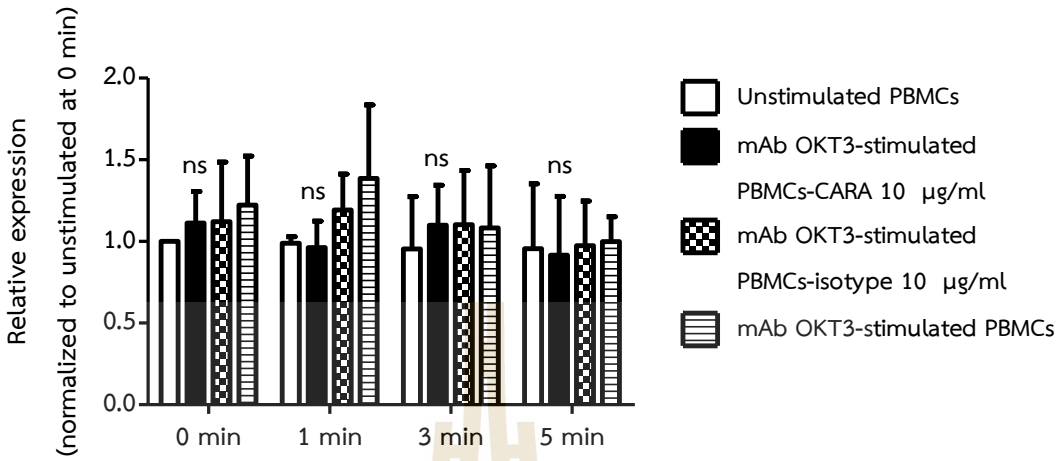

Figure 3.29 Bar graph shows relative expression of phosphorylated protein at molecular weight about 63 kDa. PBMCs ($1x10^7$ cells/ml) under the indicated conditions. The relative phosphotyrosine signal were normalized relative to unstimulated at 0 min of cultivation as 1. The data represents the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

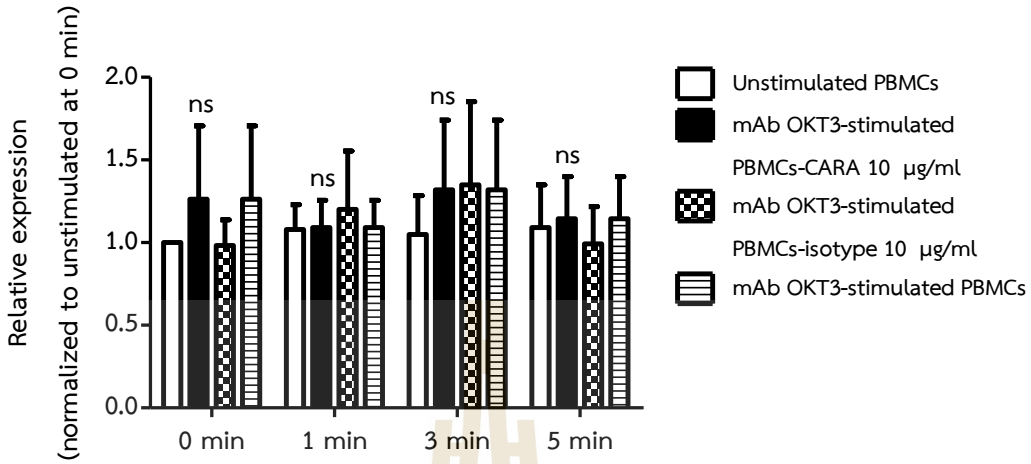

Figure 3.30 Bar graph shows relative expression of phosphorylated protein at molecular weight about 65 kDa. PBMCs ($1x10^7$ cells/ml) under the indicated conditions. The relative phosphotyrosine signal were normalized relative to unstimulated at 0 min of cultivation as 1. The data represents the mean ± SD from three independent experiments, $p > 0.05$ (ns) indicates statistically significant differences compared to each condition with OKT3-stimulated cells.

3.5 Identification of mAb CARA-recognizing molecule on human T cell surface

To identify molecules specifically recognized by a mAb CARA targeting a surface on T cells, immunoprecipitation was performed using Molt-4 cells. Protein L was used to pull down the antibody-bound proteins, followed by SDS-PAGE and Western blotting under reducing condition.

The results showed multiple protein bands, not limited to the expected size, suggesting non-specific binding Figure 3.31. In comparison, a previous attempt using Protein G Sepharose beads, preliminary results found a specific band at approximately 60–70 kDa, consistent with the positive control, but the result was not reproducible.

These results indicate that the current immunoprecipitation conditions using Protein L may not be optimal, as non-specific proteins were also precipitated. Further optimization is required to improve the specificity and reproducibility of target protein detection.

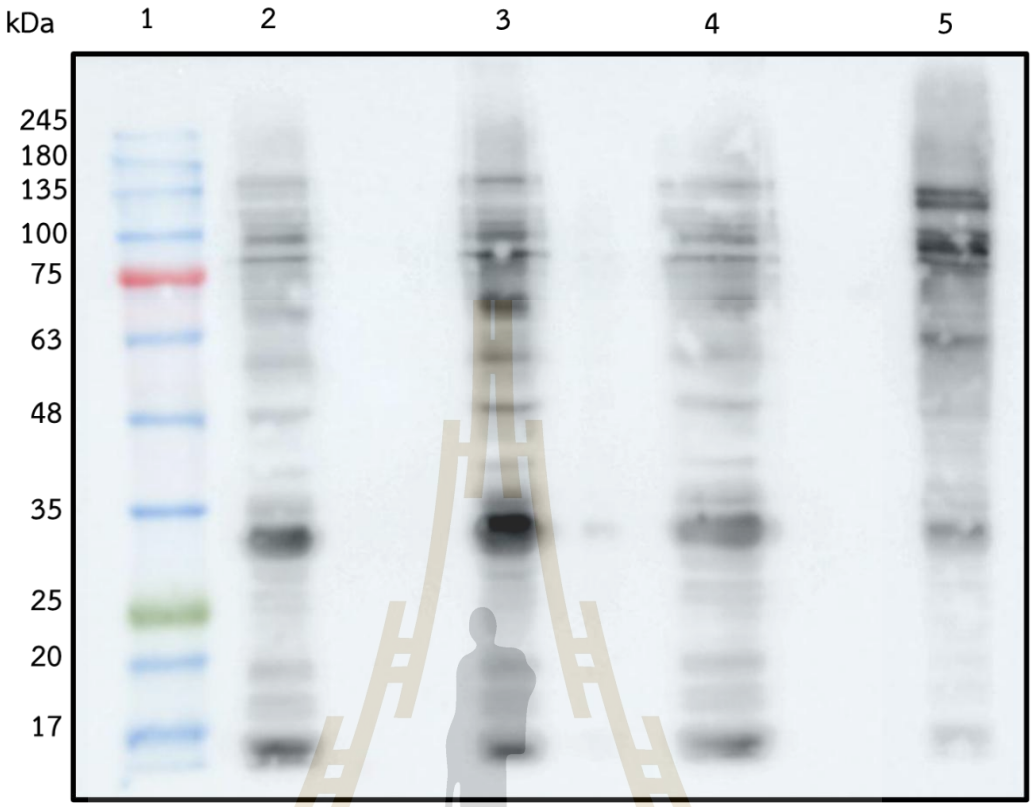

Figure 3.31 Biochemical identification of the mAb recognizing molecule by immunoprecipitation. Surface biotinylated Molt4 cell lysates were subjected to immunoprecipitation. The precipitated proteins under reducing conditions were then resolved by 10% SDS-PAGE and performed to Western blotting using HRP-conjugated streptavidin at a final dilution 1:5,000. The protein bands were detected and visualized using a chemiluminescent detection system and captured under Amersham Image Quant 500. One of three independent experiments is represented by this result. (L1: standard protein marker; L2: mAb M6-1B9; L3: mAb CARA; L4: negative control; L5: cell lysates)

CHAPTER IV
DISCUSSION AND CONCLUSION

T cell activation is a critical process in the adaptive immune response, requiring distinct signals for full activation. To investigate the biological functions and mechanisms underlying T cell responses, various monoclonal antibodies (mAbs) have been widely used as essential tools for exploration over the years. The in-house monoclonal antibody (mAb) CARA, targeting an unidentified molecule specifically expressed on an unidentified subset of T cells, proved to be a valuable tool for investigating its effects on T cell responses. Therefore, this study focused on exploring the effect of this mAb on T cell response.

Using the mAb as a tool for the study, high yield of the pure mAb CARA was required. According to isotypic determination, mAb CARA belongs to IgG3 isotype with kappa (κ) light chain. Affinity chromatography using protein G, a bacterial protein that binds to immunoglobulins, particularly the Fc region of IgG antibodies, was the first tried for mAb CARA purification. However, the system was not worked with mAb CARA, no purified mAb was obtained.

Thereby, the mAb CARA was purified from culture supernatant of hybridoma cell producing mAb CARA by affinity chromatography using HiTrap™ Protein L column. Protein L selectively binds subtype kappa light chains variable region (VL) of immunoglobulins (Igs) and also binds to single-chain variable fragments (scFvs) and antigen-binding fragments (Fabs). Unlike protein A and G, Protein L does not interfere with the Fc region while binding (Kittler et al., 2022). This purification system enriched a high yield of pure mAb CARA (Table 3.1) providing a sufficient quantity for the overall

study. The purity was confirmed by SDS-PAGE analysis, Under reducing conditions, clear separation of the heavy (55 kDa) and light (25 kDa) chains was observed and under non-reducing conditions, the intact antibody was shown at approximately 150 kDa, confirming high purity of the antibody and remain binding specificity to its recognition molecule based on immunofluorescent staining and flow cytometry analysis.

According to the results of immunofluorescent staining and flow cytometry analysis (Figure 3.4), the cellular expression profile of the mAb CARA-recognizing molecule on Molt4 T cell exhibits heterogeneous pattern, which could classify Molt4 cells into 3 subpopulations including $CARA^{neg}$, $CARA^{low+}$ and, $CARA^{high+}$. In parallel to flow cytometry analysis, microscopic study was used. As shown in Figure 3.5, the mAb CARA-recognizing molecule was detected via the red fluorescence signal of Alexa Fluor 633, while CD3 was visualized using a FITC-conjugated-CD3 mAb (green). Nuclear staining with DAPI (blue) enabled clear identification of individual cells. This result confirms the presence of three subpopulations based on red fluorescence intensity: $CARA^{high+}$ (strong), $CARA^{low+}$ (weak) and, $CARA^{neg}$ (undetectable). These findings suggest variability in surface presentation or availability of the epitope recognized by mAb CARA among the cell population. Notably, the specific molecule recognized by mAb CARA dose not colocalize with CD3 molecule on Molt4 cell surface, suggesting that its epitope is spatially distinct from the CD3 complex. Several possible explanations may account for this observation. First, the mAb CARA recognizing molecule may be part of a different membrane domain or microenvironment that is functionally or structurally segregated from the CD3/TCR complex. Such compartmentalization could reflect differences in protein-protein interactions (Douglass and Vale, 2005).

Moreover, the non-colocalization may indicate that mAb CARA targets a receptor or surface molecule that is upregulated or reorganized independently of T cell receptor activation. This spatial segregation could have functional implications, possibly influencing how signaling is coordinated or how the cell responds to its microenvironment. Further investigation, including co-immunoprecipitation or proximity labeling studies, would be valuable in identifying the molecular identity of the mAb CARA recognizing molecule and elucidating its relationship to CD3 and T cell function.

Therefore, the effect of mAb CARA was of particular interest for studying its immunological function on T cell biology. This study, PBMCs were used as cellular model and monoclonal antibody clone OKT3, which targets CD3 on the T cell receptor (TCR) complex was used to induce T cell activation. Upon binding to CD3, OKT3 mimics antigen recognition and triggers TCR signaling, initiating a cascade of intracellular events that lead to T cell activation (Van Wauwe, De Mey and Goossens, 1980)

Cell proliferation is a crucial event in T cell activation, serving as an essential process for expanding the immune response. Upon activation, T cells undergo rapid division, increasing their numbers to effectively combat infections or other immune challenges. This study, effect of the mAb CARA on CD3-mediated T cell proliferation using CFSE-based proliferation assay was performed and found that mAb CARA significantly suppressed CD3-mediated T cell proliferation in a dose dependent manner. Prominently, it did not affect unstimulated T cells, indicating that it specifically targets activated T cells, potentially offering a controlled mechanism for immune modulation. To ensure that the observed proliferation is due to live cells rather than dead cells, CFSE is a fluorescent dye commonly used to track cell proliferation. It passively diffuses into cells and covalently binds to intracellular proteins, allowing it to be retained over multiple cell divisions. However, CFSE initially stains both live and dead cells without discrimination. To determine whether a reduction in CFSE intensity results from cell division rather than cell death, the fluorescence histogram pattern is examined (Figure 3.7). In proliferating live cells, CFSE signal is diluted by half with each division, producing a series of stepwise peaks that shift progressively leftward on the histogram, each peak corresponding to a specific cell generation. This dilution pattern is characteristic and predictable. In contrast, dead or dying cells do not follow this pattern. Instead, CFSE signal reduction in non-viable cells tends to appear irregular or diffuse, lacking the distinct stepwise peaks seen in dividing live cells. To confirm accurate analysis, viability dyes such as propidium iodide, 7- Amino actinomycin D, or Zombie dyes should be used alongside CFSE to exclude dead cells during flow cytometry (Quah and Parish, 2010).

CD69 is recognized as an early activation marker of T cells, with its expression rapidly upregulated within hours following T cell receptor (TCR) engagement or stimulation by cytokines such as IL-2, IL-12, IL-4, IFN-α and IFN-γ cytokines through their receptors (Poloni et al., 2023). It plays an important role in regulating immune responses and is commonly used to identify recently activated T cells. The rapid upregulation of CD69 makes it a useful marker for detecting early-stage activation in response to stimuli. In contrast, CD25 is considered a late activation marker that is critical for T cell proliferation. While T cells become fully activated, they express higher levels of CD25, which enables them to respond to IL-2 signaling and sustain their expansion. The presence of CD25 can be used to monitor sustained activation and clonal expansion of T cells. Its expression is induced through TCR signaling, involving pathways such as nuclear factor-kappa B (NF-κB), and is maintained via IL-2-induced activation of activator of transcription 5 (STAT5) and signal transducer (Shatrova et al., 2016). According to suppression of T cell activation by mAb CARA, expression of CD69 and CD25 was also monitored by immunofluorescent staining and flow cytometry analysis. A significant reduction in the expression of the early activation marker CD69 was observed. CD25 expression level tended to reduce but not significantly observed. CD25 is typically upregulated within hours of T cell activation and reaches its peak expression around 3 to 4 days post-activation (Sheng, Vick, Collins, Yoon and Murphy, 2023). According to this study, CD25 expression was evaluated at 24 h after T cell activation, which may be a reason for low expression of CD25. These findings suggest that the mAb CARA may primarily interfere with early T cell activation events. This inhibition may trigger modulation of signaling pathways downstream of TCR engagement, potentially disrupting the activity of key transcription factors such as STAT5 or NF-κB .

As a result, activated T cells begin to produce a broad range of cytokines. The total cytokine output reflects the overall level of T cell activation and can be measured through techniques such as ELISA, while cell specific cytokine production can be determined by intracellular cytokine staining and flow cytometry analysis. Stimulation with OKT3 generally results in robust cytokine production, indicative of T cell activation. The profile and quantity of cytokines produced can vary depending on

additional co-stimulatory signals, the presence of accessory cells, or other modulatory factors such as cytokines or therapeutic antibodies like CARA.

Based on the investigation on the effect of mAb CARA on cytokine production, the results demonstrated that mAb CARA significantly reduced CD3-mediated T cell induction of total cytokine secretion, including IL-2, IL-4, IL-10, IL-13, and IL-17A. These cytokines are critical for T cell proliferation, differentiation, and the regulation of immune responses. The observed decrease suggests that mAb CARA may impair T cell activation and disrupt downstream signaling pathways involved in cytokine expression. However, no significant effect on IFN-γ and TNF-α production was observed.

This selective cytokine suppression suggests that mAb CARA may modulate specific immune pathways without inducing broad immunosuppression.

To investigate the effect of the mAb CARA on T cell specifically of cytokine production, intracellular cytokine staining was performed following stimulation. The results revealed that mAb CARA selective reduces IL-2 production, while other cytokines show variable expression patterns. This selective suppression of IL-2 suggests that mAb CARA may differentially modulate T cell activation pathways, potentially by interfering with signals required for IL-2 transcription or stability. These findings suggest a differential regulatory effect of mAb CARA on cytokine responses following T cell activation.

T cell activation involves the phosphorylation series of CD3-associated signaling molecules (such as Lck and ZAP-70), which subsequently activates multiple downstream signaling cascades involving MAPK, (NF-κB), and NFAT pathway. These pathways collectively promote transcription of genes involved in T cell proliferation, differentiation, and effector function (Paul and Schaefer, 2013).

To evaluate the effects of mAb CARA on T cell signaling, a phosphotyrosine Western blot was performed following OKT3 stimulation at various time points. The dynamics of phosphotyrosine proteins among indicated conditions was clearly observed. The most distinctly enhanced phosphotyrosine signal was observed in a

protein with a molecular weight of approximately 63–65 kDa in the presence of mAb CARA. The molecular size of these proteins closely corresponds to the known molecular weight of phosphorylated ZAP-70 (Chan, Iwashima, Turck and Weiss, 1992). This band was particularly prominent at 3 and 5 minutes of stimulation, indicating that mAb CARA enhances phosphorylation events associated with TCR signaling. Quantitative analysis further confirmed that mAb CARA treatment results in an increase in tyrosine-phosphorylated proteins in this molecular weight range compared to the isotype-matched control (Figure 3.29-Figure 3.30). T cell activation is initiated by TCR engagement and involves a cascade of tyrosine phosphorylation events that propagate intracellular signaling. One of the key mediators in this process is ZAP-70, a cytoplasmic tyrosine kinase that becomes rapidly phosphorylated upon TCR stimulation. ZAP-70 is recruited to phosphorylate ITAMs on the CD3ζ chain, where it plays a critical role in amplifying downstream signaling required for full T cell activation (Neumeister et al., 1995). Based on these findings, we propose that mAb CARA may enhance or prolong ZAP-70 activation, potentially amplifying early TCR signaling. To determine whether the mAb CARA disrupts CD3-mediated T cell activation via ZAP-70 phosphorylation modulation, further studies with specific identification are required.

Subsequently, this study has tried to identify the mAb CARA-recognizing molecule by immunoprecipitation technique. However, the difficulty of protein purification was quite challenged and still unable to identify.

In conclusion, the data collectively suggests that mAb CARA effectively suppresses T cell activation and cytokine production in response to CD3-mediated T cell stimulation, likely through intracellular signaling via tyrosine phosphorylation cascades. Although the specific molecular target of mAb CARA remains unidentified, its ability to selectively target activated T cells and reduce key cytokine production such as IL-2 highlights its potential use in modulating immune responses. These findings suggest possible therapeutic applications in conditions characterized by excessive T cell activation, such as autoimmune diseases or graft-versus-host disease (GVDH).

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APPENDICES

APPENDIX A

LIST OF CHEMICALS AND MATERIALS USED IN THIS STUDY

Chemicals/Materials	Source
Agarose gel	Norgen, Biotek Corp., Thorold, ON, Canada
Alexa Flour 488-conjugated goat anti-mouse IgG	Invitrogen, USA
Ammonium chloride (NH_4Cl)	Carlo erba, Milan, Italy
Ammonium (II) sulfate ($(NH_4)_2SO_4$)	Carlo erba, Milan, Italy
Amersham ECL Prime	Cytiva, USA
Bis-acrylamide	Acros Organics, New Jersey, USA
Brefeldin A solution, 1000X	Thermo Scientific, Rockford, USA
Bromophenol blue	Carlo erba, Milan, Italy
Bovine Serum Abumin (BSA) fraction V	Capricorn, Germany
Calcium chloride ($CaCl_2$)	Carlo erba, Milan, Italy
5(6)-Carboxylfluorescein diacetate-N-succinimidyl ester (CFSE) fluorescence, 25 mg Carboxyfluorescein succinimidly ester (CFSE)	Molecular probes-Invitrogen, Camarillo, CA, USA.
CD3 monoclonal antibody clone OKT3 (IgG2a isotype)	Provided by Prof. Dr. Watchara Kasinrerk
Chloroform	Carlo erba, Milan, Italy
Coomassie brilliant blue R250	AppliChem, Damstadt, Germany
Dimethylsulfoxide (DMSO)	Vivantis, Malaysia
Disodium hydrogen phosphate (Na_2HPO_4)	Carlo erba, Milan, Italy

Chemicals/Materials	Source
Ethanol	Carlo erba, Milan, Italy
Ethylenediaminetetraacetic acid-Disodium salt dyhydrate (EDTA)	Amersham Biosciences, Uppsala, Sweden
ELISA MAX Deluxe Set Human IL-2, IL-4, IL-10,-IL-17A, IFN-γ, TNF-α	Biolegend, San Diego, California, USA
EZ-LINKTM Sulfo-NHS-LC-Biotin-Sulfosuccinimidyl-S-(biotinamido) Hexanoate	Thermo Scientific, Rockford
Fetal Bovine Serum (FBS)	Gibco, gran Island, N.Y., USA
Fugizone (Amphotericin B)	Gibco, gran Island, N.Y., USA
FITC anti-human CD3 clone UCHT1 antibody	Biolegend, San Diego, California, USA
GENEzol reagent	Geneaid, Taiwan
Glycerol	Carlo erba, Milan, Italy
Glycine	Vivantis, Malaysai
Glacial acetic acid	Carlo erba, Milan, Italy
Hitrap^{TM} Protein L column	Cytiva, USA
Horseradish peroxidase conjugated-Rabbit anti-mouse immunoglubulins-Antibody	Dako, Glostrup, Denmark
Horeradish peroxidase conjugated-streptavidin	Genscript, USA
Human IL-13 uncoated ELISA kit	Invitrogen, USA
Isocove's Modified Dulbecco's-Medium (IMDM)	Gibco, Gran Islang, N.Y., USA
Isopropanol	Tedia, USA
Isotypic determination kit	Sigma, St. Louis, MO, USA
2-Mercaptoethanol	Acros Organics, New Jersey, USA
Methanol	Carlo erba, Milan, Italy

Chemicals/Materials	Source
Mouse IgG1 control PE-conjugated, clone PPV-06	ImmunoTools, Germany
PE anti-human CD25 clone BC96 antibody	Biolegend, San Diego, California, USA.
PE anti-human IL-2 clone MQ1-17H12 antibody	Biolegend, San Diego, California, USA
PE anti-human IL-4 clone MP4-25D2 antibody	Biolegend, San Diego, California, USA
PE anti-human IL-10 clone JES3-19F1 antibody	Biolegend, San Diego, California, USA
PE anti-human IL-17A clone BL168 antibody	Biolegend, San Diego, California, USA
PE anti-human IFN-γ clone B27 antibody	Biolegend, San Diego, California, USA
PE anti-human TNF-α clone Mab11 antibody	Biolegend, San Diego, California, USA
PerCP anti-human CD14 clone HCD14 antibody	Biolegend, San Diego, California, USA
Pierce™ BCA protein assay kit	Thermo Scientific, Rockford, USA
Pierce® Protein L Agarose	Cytiva, USA
Polyoxyethylenes orbitan monolaurate-(Tween 20)	Scharlau Chemie, S.A., Barcelona, Spain
Potassium chloride (KCl)	Carlo erba, Milan, Italy
Potassium hydrogen carbonate ($KHCO_3$)	Carlo erba, Milan, Italy
Potassium hydrogen phosphate (KH_2PO_4)	Carlo erba, Milan, Italy
Roswell Park Memorial Institute 1640-Medium (RPMI)	Gibco, Gran Islang, N.Y., USA
Skimmed milk	Himedia, Mumbai, India
Sodium carnbonate (Na_2CO_3)	Carlo erba, Milan, Italy

Chemicals/Materials	Source
Sodium chloride (NaCl)	Carlo erba, Milan, Italy
Sodium hydrogen carbonate ($NaHCO_3$)	Carlo erba, Milan, Italy
Sodiun hydrogen phospahte (NaH_2PO_4)	Carlo erba, Milan, Italy
Sodium hydroxide (NaOH)	Carlo erba, Milan, Italy
Sodium lauryl sulfate (SDS)	Carlo erba, Milan, Italy
SuperSignal™ West PicoPLUS-Chemiluminescent substrate kit	Thermo Scientific, USA
SYBR Safe DNA Gel Stain	Thermo Fisher Scientific, USA
Syringe filter (diameter 30 mm, 0.22 µm pore size sterile)	NES, China
Ultra-LEAFTM Purified Mouse IgG3, κ isotype Ctrl clone MG3-35 antibody	Biolegend, San Diego, California, USA
3, 3', 5, 5;-Tetramethylbenzidine substrate (TMB)	Merk, Darmstadt, Getmany
4G10® platinum anti-phosphotyrosine antibody	Merk, USA.
N, N, N', N'-Tetramethyl ethylenediamine (TEMED)	AppliChem, Damstadt, Germany
10 cm diameter tissue culture dish	SPL Lift Science, Gyeonggi-do, Korea
Tri-sodium citrate	Carlo erba, Milan, Italy
ViVa cDNA synthesis kit	Vivantis, Selangor Darul Ehsan, Malaysia
6-well tissue culture plate	SPL Lift Science, Gyeonggi-do, Korea
24-well tissue culture plate	Corning, USA
96-well tissue culture plate	Corning, USA
96-well ELISA plate	Nunc, Denmark
Whatman filter paper- (diameter 90 mm, 0.45 µm pore size)	GE Healthcare, Uppsala, Sweden

APPENDIX B

LIST OF INSTRUMENTS USED IN THIS STUDY

Instruments	Source
AKTA Start protein purification system	GE Healthcare Bio-Science AB, Uppsala, Sweden
Amersham Image Quant 500	Cytiva, UK
Attune NxT Flow Cytometer	Thermo Scientific, USA
Automatic high-pressure autoclave-Hirayama	Artisan Technology Group, USA
Autopipettes for tissue culture	Thermo Scienticis, USA
Autopipettes- Research plus	Eppendorf, Germany
Biohazard safety cabinet class II	ESCO, Singapore
Brushless microcentrifuge	Denvill Scientific, Canada
CO_2 incubator	Thermo Scientific, USA
Electrophoresis and electrotransfer Unit	Cosmo Bio, USA
Field emission scanning electron microscope	Zeiss GeminiSEM 500, Zeiss, Germany
Gene Amp PCR Systen-9700	Applied Biosystem, Singaopore
Himac compact refrigerated centrifuge-RXII series	Hitachi, Japan
Infinite 200 PRO microplate reader	Tecan, Switzerland
Lab culture horizontal laminar flow cabinet	ESCO, Thailand
Light microscope-CX21	Olympus, Japan
Microplate spectrophotometer	Tecan, Switzerland

Instruments	Source
MS major scieice (Semi-Dry mini blotter)	Major Science, Taiw
Multichannel pipette-ACURA855	Socorex, Switzerland
Multichannel pipette for tissue culture	Biohit, Finlan
Nanodrop spectrometer ND-1000	Thermo Scientific, USA
Rapid-Flow sterile disposable	Thermo Ficher Scientific, Rochester
Refrigerated microcentrifuge-5415R	Eppendorf, Germany
SDS-PAGE sets with power supply	Bio-Rad, USA
Trans-blot semi-dry transfer cel	Bio-Rad, USA
Ultra-sonicator	Crest, Pensylvania
Versatile refrigerated centrifuge-CT15RT	Techcomp, Kowloon, Hong Kong
pH meter	Satorius, Germany

APPENDIX C

REAGENTS AND BUFFER PREPARATION

1. Reagents for cell culture

1.1 Incomplete Isocove's modified dubecco's (IMDM) medium

IMDM	powder 1 pack
$NaHCO_3$	3.024 gm
Gentamycin (40 mg/ml)	1 ml

Dissolve in ddH_2O and adjust volume to 1,000 ml

Filtrate through 0.2 µm membrane filter

Add fungizone (250 µg/ml)	1 ml

Determine the sterility before use and store at 4 °C

1.2 Complete IMDM medium

Incomplete IMDM medium	180 ml
Heat-inactivared fetal bovine serum	20 ml

Determine the sterility before use and store at 4 °C

1.3 Roswell Park Memorial Institute (RPMI) 1640 medium

RPMI	powder 1 pack
$NaHCO_3$	3.024 gm
Gentamycin (40 mg/ml)	1 ml

Dissolve in ddH_2O and adjust volume to 1,000 ml

Filtrate through 0.2 µm membrane filter

Add fungizone (250 µg/ml)	1 ml

Determine the sterility before use and store at 4 °C

1.4 Complete RPMI medium

Incomplete RPMI medium	180 ml
Heat-inactivared fetal bovine serum	20 ml

Determine the sterility before use and store at 4 °C

2. Reagent for Enzyme-linked immunosorbent assay (ELISA)

2.1 Coating buffer (0.1 M carbonate-bicarbnate buffer pH 9.6)

Na_2CO_3 (MW.105.99)	1.06 gm
$NaHCO_3$ (MW. 84.01)	1.26 gm
ddH_2O	200 ml

Adjust pH to 9.6 with HCl

Adjust volume with ddH_2O to 250 ml

Store at RT

2.2 0.05% Tween-PBS

1X PBS	500 ml
Add Tween-20	0.25 ml

Mix well and store at RT

2.3 Blocking solution (2.5% skimmed milk-PBS)

Skimmed milk	1.25 gm
1xPBS	50 ml

Mix well

2.4 Stop reaction solution (1 N HCl)

ddH_2O	917.2 ml
Add con^{c} HCl (36 N)	82.8 ml

Store at RT

2.5 TMB substrate (for ELISA)

Commercial from Zymed (00-2023) 500 ml

Keep out of light and store at 2-8 °C

S. San Francisco, California, USA

2.6 10X Phosphate buffer (PBS) pH 7.2

NaCl (MW. 58.44) 80 gm

KCl (MW. 74.56) 2 gm

Na_2HPO_4 (141.96) 11.5 gm

KH_2PO_4 (MW. 136.09) 2 gm

ddH_2O 800 ml

Adjust pH to 7.2 with 1N NaOH/1N HCl

Adjust volume with ddH_2O to 1000 ml

2.7 1X PBS pH 7.2

10X PBS pH 7.2 100 ml

ddH_2O 900 ml

Mix well and store at RT

3. Reagent for Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis

3.1 1.5 M Tris-HCl pH 8.8 (4x Running gel buffer)

Tris-base (MW.121.1) 36.36 gm

ddH_2O 100 ml

Adjust pH to 8.8 with HCl

Adjust volume with ddH_2O to 200 ml

3.2 0.5 M Tris-HCl pH 6.8 (4x Stacking gel buffer)

Tris-base (MW.121.1)	12 gm
ddH_2O	100 ml

Adjust pH to 6.8 with HCl

Adjust volume with ddH_2O to 200 ml

3.3 10x Running buffer

Glycine	144.13 gm
Tris-base (MW.121.1)	30.28 gm
SDS	10 gm
ddH_2O	1,000 ml

3.4 0.5 M Iodoacetamide in ddH_2O

Iodoacetamide (Sigma: I-6125; MW.185)	0.555 gm
ddH_2O	6 ml

Mix well until dissolve and aliquot 100 µl/tube

Store at -20 °C before use

3.5 100 mM PMSF in acetone

PMSF (Sigma: P7626-250 mg)	0.0546 gm
Acetone	3.134 ml

Mix well until dissolve

Aliquot 100 µl/tube and store at -20 °C before use

3.6 Blotting buffer (Towbin buffer)

Glycine	14.14 gm
Tris-base (MW.121.1)	3.79 gm
SDS	1 gm
Methanol	250 ml

Adjust volume with ddH_2O to 1000 ml

Store at RT

3.7 30% Monomer (30.8%T, 2.7%Cbis): (200 ml)

Acrylamide (MW.71.08)	60 gm
Bis-acrylamide (MW. 154.2)	1.6 gm
ddH_2O to	200 ml

Filtrate with 0.45 mm filter paper and degas before use

Store at 4 °C (up to 3 months)

3.8 10% APS (Ammonium persulfate)

Ammonium persulfate (MW.228.2)	0.5 gm
ddH_2O	5 ml

Mix well, aliquot 100 µl/tube and store at -20 °C

3.9 10% SDS (Sodium dodecyl sulfate)

SDS (MW.288.38)	10 gm
ddH_2O	50 ml

Mix well and store at RT

3.10 5X reducing buffer: 10 ml

SDS	1 gm (10%)
0.5 M Tris-HCl pH 6.8	5 ml (250 mM)
Glycerol (100%)	2.5 ml (25%)
5% Bromophenol blue	0.5 ml (0.25%)
2-ME	0.5 ml (5%)
ddH_2O	1.5 ml

3.11 10X Non-reducing buffer: 10 ml

	Final con^{c}
SDS	2 gm (20%)
Glycerol (99% sigma)	5 ml (50%)
1% Bromophenol blue	1 ml (0.1%)
1.75 M Tris-base pH 6.8***	3.676 ml (0.625 M)

ddH_2O	0.324 ml
***1.75 M Tris-HCl pH 6.8	
Tris-base (MW.121.14)	21.192 gm
ddH_2O	50 ml
Adjust pH to 6.8 with HCl	
Adjust volume with ddH_2O to	100 ml
Store at RT	

3.17 0.025% Coomassie Brilliant blue R250

Coomassie brilliant blue R250 (Bio-rad)	0.125 gm
Methanol	200 ml
Acetic acid	35 ml
Add ddH_2O to	500 ml

3.18 Destaining gel solution I (40% Methanol, 7% Acetic acid)

Methanol	400 ml
Acetic acid	70 ml
Add ddH_2O to	1000 ml

3.19 Destaining gel solution II (5% Methanol, 7% Acetic acid)

Methanol	25 ml
Glacial acetic acid	35 ml
Add ddH_2O to	500 ml

4. Reagent for Phosphotyrosine blot

4.1 Tris-lysis buffer (150 mM NaCl, 20 mM Tris-base, 5 mM EDTA: for Phosphotyrosine blot and lipid raft)

Tris-base (MW.122.14)	2.423 gm
NaCl (MW.58.44)	8.766 gm
EDTA (MW. 372.24)	1.86 gm
ddH_2O	800 ml
Adjust pH to 7.5 with HCl	
Adjust volume with ddH_2O to	1000 ml
Filtrate with 0.45 mm filter paper	
Store at 4 °C	

4.2 Lysis buffer with protease inhibitors (1%Triton X-100): 10ml

10% Triton X-100	1 ml
Iodoacetamide (0.5 M in ddH_2O)	100 µl
Aprotinin (1 mg/ml)	100 µl
PMSF (100 mM in acetone)	100 µl
Leupeptin (0.5 M in ddH_2O)	100 µl
Pepstatin A (2 mM in ddH_2O)	10 µl
Tris-lysis buffer (pH7.5/pH 8.2)	8.590 ml
Aliquot 1 ml/ tube and store at -20 °C	

4.3 200 mM Sodium orthovanadate activation stock (MW.183.9)

In 20 ml lysis buffer add 100 ml of 200 mM stock of sodium orthovanadate activation and final concentration is 1 mM

Sodium orthovanadate activation stock (MW.183.9) 0.37 gm

ddH_2O 10 ml

Adjust pH to 10 (solution becomes yellow)

Boil solution until it turns colorless (~10 min)

Cool to RT

Readjust pH to 10

Repeat steps 3, 4 until solution remains colorless and pH stabilizes at pH 10

Store activated orthovanadate as aliquot at -20 °C (300 ml in each aliquot)

5. Reagents for Ab purification

5.1 1M Na_2HPO_4

Na_2HPO_4 2.84 gm

ddH_2O 20 ml

5.2 1M NaH_2PO_4

NaH_2PO_4 2.76 gm

ddH_2O 20 ml

5.3 Binding buffer (20 mM Sodium phosphate; pH 7.0)

1M Na_2HPO_4 5.8 ml

1M NaH_2PO_4 4.2 ml

ddH_2O 400 ml

Adjust pH to 7.0 with 1N HCl

Adjust volume with ddH_2O to 500 ml

Filtrate with 0.45 um filter paper and store at 4 °C

5.4 Elution buffer (0.1 M Glycine pH 2.7)

Glycine (MW.75.07)	3.753 gm
ddH_2O	350 ml

Adjust pH to 2.7 with 1 N HCl

Adjust volume with ddH_2O to 500 ml

Filtrate and store the solution at 4 °C

5.5 Neutralizing buffer (1 M Tris-HCl pH 9.0)

Tris-base (MW. 121.1)	12.114 gm
ddH_2O	60 ml

Adjust pH to 9.0 with concentrated HCl

Adjust volume with ddH_2O to 100 ml

Filtrate and store the solution at 4 °C

6. Reagents for cells staining

6.1 1%BSA-PBS-Azide: 1 Liter

NaN_3	0.05 gm
BSA	2.5 ml
Adjust volume with 1xPBS to	250 ml

Mix well until dissolve and store at 4 °C

6.2 4 % Paraformaldehyde in PBS (Fixative solution) 250 ml

Paraformaldehyde	4 gm
1X PBS	80 ml

Warm at 56°C until dissolve

Adjust volume with ddH_2O to 100 ml

Filtrate with 0.22 mm filter paper and store at 4 °C

6.3 10% NaN_3 in PBS

NaN_3	1 gm
1X PBS	8 ml

Mix well until dissolved with magnetic stirrer

Adjust volume to 10 ml

Store at RT

7. Reagents for intracellular staining

7.1 10% Saponin

Saponin	2.5 gm
1X PBS	20 ml

Mix well until dissolve (on heat)

Adjust volume to 25 ml

7.2 Permeabilizing buffer (0.1% saponin, 5% FBS, 0.02% NaN_3-PBS)

10% Saponin	1 ml
FBS	5 ml
10% NaN_3	0.2 ml
1X PBS	93.8 ml

Mix well and store at 4 °C

APPENDIX D

HUMAN RESEARCH ETHICS APPROVAL

COA no. 46/2567

Human Research Ethics Committee, Suranaree University of Technology

Certificate of Expedited Review Approval

Human Research Ethics Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand, has approved the following study which is to be carried out in compliance with the international guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline, International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Title of Project : Identification and functional study to T lymphocyte surface molecule recognizing by monoclonal antibody CARA

Project Code : EC-67-58

Principal Investigator : Miss Warapan Panchai

Department : Institute of Science

Review Method : Expedited Review

Continuing Report : At least once annually or submit the final report if finished.

Document Reviewed : Protocol, Information Sheet, Informed Consent (version 2.0, 18 April 2024) Curriculum Vitae, Certificate in ethics training

Signature.....Chairman
(Asst. Prof. Dr. Benjamart Chitsomboon)
Human Research Ethics Committee, Suranaree University of Technology

Date of Approval : 22 April 2024
Approval Expiry Date : 21 April 2025

Approval is granted subject to the following conditions: (see back of this Certificate)

สำนักการพิจารณาจาก
คณะกรรมการจริยธรรม
การวิจัยในมนุษย์
มหาวิทยาลัยเทคโนโลยีสุรนารี แล้ว
Approved by HREC-SUT

APPENDIX E

BIOSAFETY AND BIOSECURITY APPROVAL

เลขที่รับรอง SUT-IBC-005/2024

ใบรับรองการดำเนินงานด้านความปลอดภัยทางชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี

ชื่อโครงการ	: การบ่งชี้และการศึกษาหน้าที่ของโมเลกุลบนผิวเซลล์ทีลิมโฟไซต์ ที่ถูกจดจำโดยโมโนโคลนอลแอนติบอดี CARA
ชื่อโครงการภาษาอังกฤษ	: Identification and functional study of T lymphocyte surface molecule recognizing by monoclonal antibody CARA
รหัสโครงการ	: IBC-67-05
หัวหน้าโครงการ	: นางสาววราพรรณ พันธุ์ชัย
อาจารย์ที่ปรึกษา	: รองศาสตราจารย์ ดร.พนิดา ขันแก้วหล้า
สังกัด	: สำนักวิชาวิทยาศาสตร์
ประเภทงานวิจัย	: งานวิจัยประเภทที่ 2
ระดับห้องปฏิบัติการ	: Biosafety Level 1และ2 (BSL 1และ2)
รายงานความก้าวหน้า	: ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี

ข้อเสนอโครงการวิจัยและเอกสารประกอบของข้อเสนอโครงการวิจัยนี้ ได้รับการพิจารณาจากคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ มหาวิทยาลัยเทคโนโลยีสุรนารีแล้ว คณะกรรมการฯ ลงความเห็นว่า ข้อเสนอโครงการวิจัยที่จะดำเนินการมีความสอดคล้องกับแนวทางปฏิบัติเพื่อความปลอดภัยทางชีวภาพ และพระราชบัญญัติเชื้อโรคและพิษจากสัตว์ พ.ศ. 2558 จึงเห็นสมควรให้ดำเนินการวิจัยตามข้อเสนอการวิจัยนี้ได้

กรณีที่มีการปฏิบัติอย่างหนึ่งอย่างใดนอกเหนือจากที่กรอกไว้ในข้อมูลและที่เสนอไว้ในโครงการ คณะกรรมการฯ จะดำเนินการงดใบรับรองนี้ และแจ้งมายังคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ มทส. หรือหน่วยงานที่เกี่ยวข้องทราบ

ลงชื่อ.....
(รองศาสตราจารย์ ดร.ระพี อูทเคอ)
ประธานคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี

IBC SUT
ผ่านการพิจารณาจาก
คณะกรรมการควบคุม
ความปลอดภัยทางชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี แล้ว
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Development and Promotion of Science and Technology Talent project (DPST) student

PUBLICATION

Lowhalidanon K., **Panchai W.,** Khunkaewla, P. Effect of differential oxidation of LDL on foam cell formation and expression of MMP-9 and CD147 in PMA-derived macrophage foam cells. In preparation for submission.

PROCEEDING

Sritrakarn T., **Panchai W.**, Lowhalidanon K., Khunkaewla P. Reduction of foam cell formation by the novel generated monoclonal antibodies against human LDL. The 18th International Symposium of the Protein Society of Thailand (PST2024). At Convention Center, Chulabhorn Research Institute, Thailand during August 28-30, 2024.

POSTER PRESENTATION

Panchai W., Kasinrerk W., Khunlaewla P. Effect of a monoclonal antibody to human T cell surface molecule called CARA on CD3-mediated T cell activation. The 18th International Symposium of the Protein Society of Thailand (PST2024). At Convention center, Chulabhorn Research Institute, Bangkok, Thailand during August 28-30, 2024.