THE ROLE OF ADAPTOR PROTEIN NCK1 IN RELATION TO THE TYROSINE KINASE LCK FUNCTION IN T CELL RECEPTOR SIGNALING

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A Thesis Submitted to the Graduate School of Naresuan University

in Partial Fulfillment of the Requirements

for the Doctor of Philosophy Degree in Microbiology

May 2021

Thesis entitled "The role of adaptor protein Nck1 in relation to the tyrosine kinase Lck function in T cell receptor signaling"

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has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Microbiology of Naresuan University

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ACKNOWLEDGEMENT

Apart from my endeavors, the success of this study depended mainly on the encouragement and inspiration of many people. I would like to take this opportunity to express my gratitude to everyone who has been instrumental in the successful completion of my PhD life.

First of all, the author is grateful to thank her enthusiastic adviser, Associate Professor Dr. Sutatip Pongcharoen, for her continuous encouragement and for her guidance from the beginning to the end during my PhD study, and for providing me a great opportunity for further education in PhD degree by arranging her a Ph.D. grant according to the cooperation between The Royal Golden Jubilee Ph.D. Program (RGJ) of the Thailand Research Fund, jointly funded by Naresuan University for financial supports of my study, since 2012. Many thanks also go to my co-supervisor, Assistant Professor Dr. Jatuporn Ngoenkam, for his suggestions that allowed me to advance my research project.

My deep gratitude goes next to Professor Dr. Wolfgang Schamel, Freiburg University, Freiburg, Germany, who has given me the best experience about molecular science and research in immunological field for one year visiting. I am very grateful to many wonderful people that I have worked with in his lab for all of their help and friendship. In particular Anna Morath and Claudia Juraske, they have supported my daily life and research during my stay in Germany.

I would also like to especially thank to my laboratory colleagues in Pongcharoen's laboratory group for making nice atmosphere to work, especially, Assistant Professor Dr. Pussadee Paensuwan.

Finally, that is indispensable is my appreciation also extends to my family, Wipa for supporting me spiritually throughout my study and my life in general.

Piyamaporn Wipa

Title	THE ROLE OF ADAPTOR PROTEIN NCK1 IN RELATION TO THE TYROSINE KINASE LCK FUNCTION IN T CELL RECEPTOR SIGNALING
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Academic Paper	Ph.D. Dissertation in Microbiology, Naresuan University, 2020
Keywords	Nck, Lck, TCR-mediated T cell activation, T cell signaling

ABSTRACT

Following T cell antigen receptor (TCR) engagement, rearrangement of the actin cytoskeleton supports intracellular signal transduction and T cell activation. The non-catalytic region of tyrosine kinase (Nck) molecule is an adapter protein implicated in TCR-induced actin polymerization. Further, Nck is recruited to the CD3ε subunit of the TCR upon TCR triggering. In this study, the role of actin polymerization in the recruitment of Nck to the TCR was investigated. To this end, Nck binding to CD3ε was quantified in Jurkat cells using the proximity ligation assay (PLA). The result showed that inhibition of actin polymerization using Cytochalasin D delayed the recruitment of Nck1 to the TCR upon TCR triggering. Interestingly, CD3ε phosphorylation was also delayed. These findings suggest that actin polymerization promotes the recruitment of Nck to the TCR, enhancing downstream signaling, such as phosphorylation of CD3ε.

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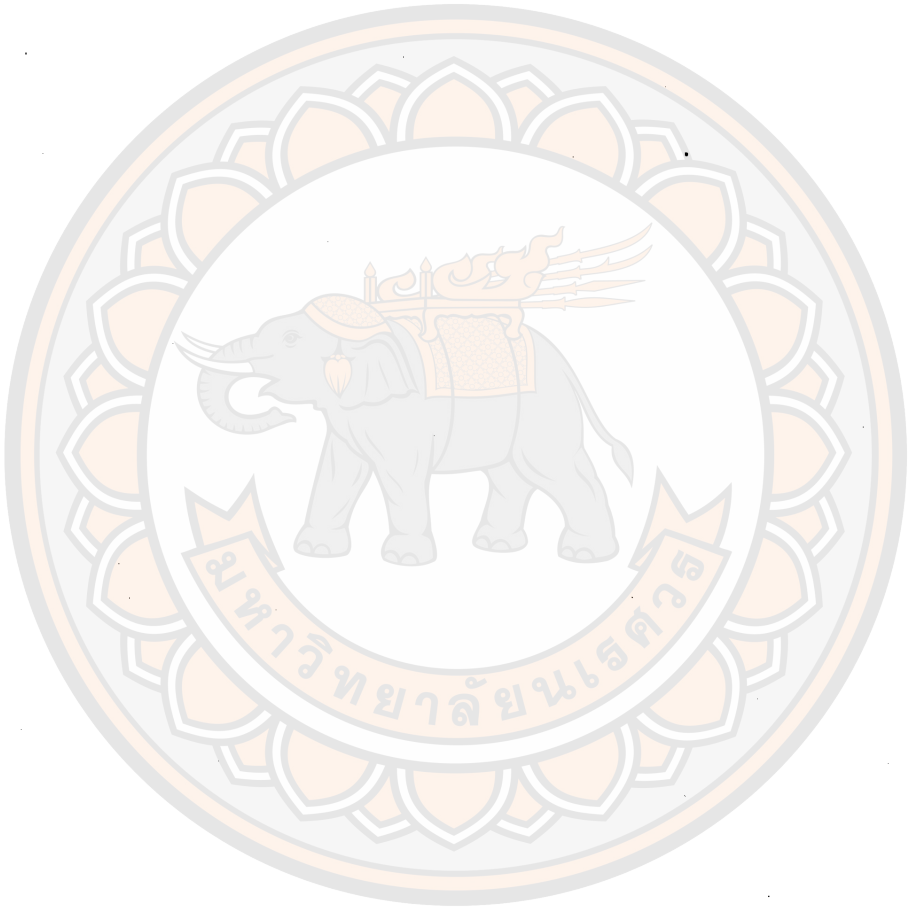

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ABBREVIATIONS

Ab	=	antibody
ADAP	=	adhesion- and degradation-promoting adaptor protein
AP-1	=	activation protein-1
APC	=	antigen presenting cell
Arp2/3	=	actin-related proteins 2 and 3
Cdc42	=	cell division cycle 42
CDRs	=	complementarity determining regions
CRAC	=	Ca^{2+} release-activated Ca^{2+}
CTL	=	cytotoxic T lymphocyte
DAG	=	diacylglycerol
Erk	=	extracellular signal regulated kinase
FBS	=	fetal bovine serum
FITC	=	fluorescein isothiocyanate
Gads	=	Grb2-related adaptor downstream of shc
GDP	=	guanosine diphosphate
Grb2	=	growth factor receptor-bound protein 2
IκB	=	inhibitor of κB
IKK	=	IκB kinase
IL-2	=	interleukin 2
IP_3	=	inositol 3,4,5-triphosphate
IS	=	immunological synapse
ITAM	=	immunoreceptor tyrosine-based activation motif
Itk	=	IL-2-inducible tyrosine kinase
JNK	=	c-Jun N-terminal kinase
LAT	=	linker for activation of T cells
Lck	=	lymphocyte protein -tyrosine kinase
mAb	=	monoclonal antibody
MAPK	=	mitogen-activated protein kinase
MEK	=	MAP kinase kinase

ABBREVIATIONS (CONT.)

MHC	=	major histocompatibility complex
Nck	=	non-catalytic region of tyrosine kinase
NFAT	=	nuclear factor of activated T cell
NF-κB	=	nuclear factor kappa-light-chain-enhancer of activated B cells
PBMC	=	peripheral blood mononuclear cell
PBS	=	phosphate buffered saline
PI-3K	=	phosphatidylinositol 3-kinase
PIP2	=	phosphatidylinositol 4,5-bisphosphate
PIP_3	=	phosphatidylinositol (3,4,5) trisphosphate
PKC	=	*protein kinase C*
PLCγ1	=	phospholipase Cγ1
pMHC	=	peptide-loaded major histocompatibility complex
PAK1	=	p21-activated serine-threonine kinase 1
PRS	=	proline-rich sequence
PTK	=	protein tyrosine kinase
pY/pTyr	=	phosphotyrosine
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	=	Src homology 2
SH3	=	Src homology 3
shRNA	=	short hairpin RNA
siRNA	=	small interfering RNA
SLP-76	=	SH-2-domain containing leukocyte protein of 76 kDa
SOS	=	son of sevenless
TCR	=	T cell antigen receptor
T_h cell	=	helper T cell
WASP	=	Wiskott-Aldrich syndrome protein
WAVE2	=	WASP-family verproline-homologous protein 2
WIP	=	WASP interacting protein
WT	=	wild type
ZAP-70	=	ζ-chain-associated protein 70 kDa

CHAPTER I

INTRODUCTION

Thesis rationale and statement of problems

T cells play an important role in adaptive immunity. T cell displays a structurally unique receptor with large and extreme diversity called T cell receptor (TCR). TCR is associated with CD3 complex molecules (TCR/CD3). A T cell generally recognizes an antigenic peptide presented by a major histocompatibility complex (MHC) molecule on the surface of an antigen presenting cell (APC). Apropriate binding affinity of TCR and peptide-MHC (pMHC) induces an enzyme called Lck (lymphocyte-specific protein tyrosine kinase) to phosphorylate the immune receptor tyrosine-based activation motifs (ITAMs) at the cytoplasmic tail of the TCR-CD3 complex. Then another enzyme known as ZAP-70 kinase is recruited to bind the phosphorylated ITAMs leading to phosphorylation and recruitment of other enzymes and adaptor proteins. These include, for instance, phospholipase Cγ (PLCγ), linker for activation of T cells (LAT), SH2-domain-containing leukocyte protein of 76 kDa (SLP-76), and non-catalytic region of tyrosine kinase (Nck) (Samelson, 2002) to form a signaling complex that is important for activation of many signaling pathways and T cell activation (Horejsi et al., 2004; Koretzky et al., 2006). Activation of T cells leads to T cell proliferation and differentiation as well as production of various cytokines and growth factors (Choudhuri et al., 2005; Smith-Garvin et al., 2009).

Nck is an adaptor protein composed of three SH3 domains at the amino-terminal and one SH2 domain at the carboxy-terminal (Buday et al., 2002; Lettau et al., 2009). In humans, there are 2 Nck isoforms that are Nck1 or Nckα and Nck2 or Nckβ (or another name is Grb4). The main function of Nck is to interact with other proteins to form a signaling complex. Studies in several cell types have shown that Nck can interact with more than 60 types of protein (Buday et al., 2002; Li et al., 2001). Nck binds to phosphorylated protein via its SH2 domain and binds to proline-rich sequence (PRS) via its SH3 domain. Thus, Nck functions to link a signal from the

upstream phosphotyrosine (pTyr) via SH2 domain to the downstream effector via SH3 domain (Lettau et al., 2009).

The lymphocyte-specific protein tyrosine kinase (Lck) is a kinase enzyme that plays a key role in T cell activation (Broman et al., 2004; Parsons, & Parsons, 2004). Upon TCR stimulation by binding with pMHC, the Lck molecules phosphorylate ITAMs of the TCR-CD3 complex to initiate intracellular signaling. Lck molecule is made up of lipids that undergo lipid modification including myristolylation and palmitoylation at the N-terminal. This structure is used to bind cell membranes. The N-terminal of Lck also contains 2 cysteine molecules for binding with CD4 or CD8 molecule. Lck has one SH3 and one SH2 domain, which can bind other proteins. Its SH3 domain binds to proline-rich sequence while its SH2 domain binds to phosphorylated protein. In addition, the amino acids located between lipid modification site and the SH3 domain are also proline-rich sequence that could bind other protein that carries SH3 domain. The C-terminal of Lck molecule is a catalytic tyrosine kinase domain (Turner et al., 1990; Kim et al., 2003).

Activation of Lck is induced by binding of TCR to pMHC. It is thought that the activation of Lck molecules are associated with binding of CD4 or CD8 co-receptor molecules to MHC class II or MHC class I molecules, respectively. This is because Lck molecules are found to be associated with the CD4 and CD8 molecules on the cell membranes (Rudd et al., 1998, Veillette et al., 1988). Moreover, activation of double positive thymocytes is involved in simultaneous stimulation of TCR and co-receptors (Wiest et al., 1996). However, deletion of CD4 or CD8 molecules does not affect T cell development and activation (Van Laethem et al., 2007). Recent studies have shown that there are 2 types of Lck molecules. One is membreane associated with CD4 and CD8 molecules and the other one is free in the cytoplasm. This may explain the dispensable role of CD4 and CD8 molecules in thymocyte development as some Lck molecules exist in the cytoplasm. It has also demonstrated that up to 40% of Lck molecules are in a stimulatory state even though the T cell is not activated and stimulation of TCR or co-receptors does not increase the activity of Lck (Turner JM, 1990; Nika et al., 2010). In many studies, for stimulation of primary T cells in vitro, anti-CD3 and anti-CD28 antibodies are generally used that can stimulate Lck, ITAMs and other proteins leading to T cell activation. Thus, Lck molecules may be activated

without any involvement of co-receptors CD4 and CD8 molecules. The distribution of activated Lck molecules are found to be clustered with phosphorylated TCR molecules. Therefore, stimulation of TCR is believed to recruit Lck to the TCR-CD3 complex (Rossy et al., 2013).

After TCR ligation, there is a conformational change at the CD3ε causing an exposure of the proline-rich sequence (PRS) of the cytoplasmic tail of the CD3ε. Then, Nck molecules are recruited to bind the CD3ε. The Nck uses its SH3.1 domain to bind the PRS of the CD3ε. This is thought to be important for stimulation of other proteins essential for phosphorylation of ITAMs leading to T cell activation (Gil et al., 2002). As aforementioned that Lck also contains the PRS. that can bind the Nck, it is possible that the recruitment of Lck to the TCR may relie on Nck molecules functioning as a linker. When TCR is activated, binding of Nck to the PRS of the CD3ε may facilitate the recruitment of Lck molecules to the CD3ε. Lck molecules then phosphorylate ITAMs of the TCR-CD3 complex, thus, initiating the intracellular signaling and activation of the T cell. Nevertherless, there has been no direct evidence that shows that stimulation of Lck is a direct result of TCR activation. It is also questioned that whether or not the Nck is directly involed in Lck stimulation. Preliminary experiments showed that when Jurkat T cells were stimulated with anti-TCR antibody, the Lck molecules were co-precipitated with CD3 molecules. In Nck1 and Nck2 knock-down Jurkat cells, there was a decline in co-precipitation of Lck and CD3 molecules. This finding demonstrates that upon T cell activation, Lck molecules are recruited to the TCR-CD3 complex and the Nck1 and Nck2 may function as a linker. Beside that finding, Lck was also co-precipitated with Nck1 and Nck2. From these data, it was hypothesized that activation of the TCR may result in a recruitment of cytoplasmic Lck to the TCR-CD3 complex and this would be involved function of Nck adaptor protein. This event would be very important for the initiation of T cell activation.

Morover, the TCR-peptide-MHC binding results in intracellular signaling that involves a dynamic rearrangement of the actin cytoskeleton. The latter event is important for T cell cytokine production, proliferation, survival, metabolism and differentiation.

Filamentous actin (F-actin) is enriched at the interface of the conjugation between the T cell and APC, the so called immunological synapse. Prevention of actin reorganization by using actin polymerization inhibitors such as Cytochalasin D (CytD) can result in impaired synapse formation, intracellular signaling and thus T cell activation (Bunnel et al., 2001; Kumari et al., 2014).

Understanding the molecular mechanisms that regulate actin rearrangement has expanded in the past years. Various signaling molecules have been described that are involved in intracellular signaling from the TCR to actin rearrangement. Engagement of the TCR by pMHC- or anti-TCR/CD3 antibodies stabilizes the TCR in an active CD3 conformation that allows the Lck to access the tyrosines in the cytoplasmic tails of the signaling subunits of the TCR, namely CD3ζ, CD3ε, CD3δ and CD3γ (Swamy et al., 2016; Schamel et al., 2017). This results in the phosphorylation of CD3 (Lin et al., 2001) and of multiple effector molecules, including the zeta chain-associated protein kinase of 70 kDa (ZAP70), the linker of activated T cells (LAT) and the Src homology (SH)-2 domain containing leukocyte protein of 76 kDa (SLP-76) (Courtney et al., 2018; Guy et al., 2009; Wunderlich et al., 1999). Nck and its associated molecule the Wiskott-Aldrich syndrome protein (WASp) are then recruited to phosphorylated SLP-76 in the vicinity of the activated TCR (Ramesh et al., 1999; Griffiths et al., 2002). Here, WASp is activated by the Rho family GTPase Cdc42 and subsequently leads to the initiation of actin filament formation (Ramesh et al., 1999). Previous studies have confirmed the involvement of Nck recruitment associated with LAT, SLP-76, and WASp in the process of actin polymerization induced by TCR engagement (Barda-Saad et al., 2005; Paensuwan et al., 2015).

Interestingly, Nck bound to the TCR might bind simultaneously to WASp and thereby recruit WASp to the TCR (Paensuwan et al., 2015), regulating actin polymerization. Indeed, inhibition of the Nck-TCR interaction reduces actin polymerization (Borroto et al., 2016) showing that the Nck pool at the TCR significantly contributes to actin rearrangements. However, the vice-versa scenario, i.e. whether actin polymerization affects Nck recruitment to the TCR, has not been investigated so far. Recently, it has demonstrated that Nck1, rather than Nck2, plays a major role in TCR signaling in human T cells (Ngoenkam et al., 2014). Hence, this

work was focused on Nck1 by examining the involvement of actin polymerization in Nck recruitment to the TCR. It was found that inhibition of actin polymerization is associated with a delayed recruitment of Nck1 to the TCR upon TCR triggering. This might be explained by a delayed phosphorylation of CD3ε by Lck.

Objectives of the study

1. To examine the involvement of actin polymerization in Nck recruitment to the TCR
2. To characterize the role of Nck1 in TCR signaling in αβ T cells
3. To study the involvement of Nck1 in relation to the tyrosine kinase Lck function in signal transduction

Hypotheses of the study

The role of adaptor protein Nck1 may be an important mediator of Lck recruitment to the TCR/CD3 signaling complex upon T cell activation.

Scope of the study

The human Jurkat T leukemia cell line (clone E6-1) was used in this study. The plasmid expressing CRISPR Nuclease/Cas9, a guide sgRNA oligonucleotide sequence againt the Nck1 region was used and electroporated into the cell line to produce a stable Nck1-deficient (N1KO) T cells. These Nck1 knockout cells were then stimulated with anti-CD3 antibody with/without inhibition of actin polymerization to study the effects of actin polymerization in Nck recruitment to the TCR, including the influence of Nck1 knockout on TCR-mediated signal transduction, the activation of intracellular signaling molecules in T cells. Furthermore, these N1KO cells were evaluated in relation to the Lck function in T cell receptor signaling.

Key words

Nck, CD3ε, T cell receptor, Lck, actin polymerization, T cell signaling

Expected outputs of the study

Actin polymerization associated with Nck1 recruitmet to the TCR is related to CD3 phosphorylation by Lck in TCR signal trasduction. This also work provided the methods and new knowledge for preparation of the stable Nck1-deficient Jurkat T cells using CRISPR/Cas9 technique.

CHAPTER II

LITERATURE REVIEW

This chapter begins with the overview of general concepts of immune response. Subsequently, the importance of T cells and the composition of TCR-CD3 complex that is required for antigen recognition and signing transduction is described. Then, the mechanisms of T cell activation in depth is explained. Finally, Nck is described in T cell function. The role of Nck in term of the effect on Lck fuction via TCR-mediated signaling transduction is highlighted.

The main point of this study was also focused on an enzyme kinase Lck that is important for initiation of intracellular signaling in T cell after TCR binding to pMHC. Lck molecules are activated causing stimulation of other signaling proteins. However, there are several mechanisms that have been proposed for Lck activation. Some researchers have proposed that Lck is bound to the C-terminal of co-receptors CD4 and CD8 and activation of the Lck occurs when TCR binds pMHC molecule and the CD4 or CD8 co-receptor binds the same MHC molecule. However, there is no direct evidence showing that binding of CD4/CD8 to MHC molecule stimulates Lck. This idea has also been disputed when it has been demonstrated that a deletion of CD4 or CD8 molecule does not affect T cell development and activation (Van et al., 2007). Recently, studies have shown that in the absence of T cell stimulation, up to 40% of Lck molecules are in an activated state (Nika et al, 2010). Moreover, stimulation of TCR or co-receptors does not increase Lck activity (Turner et al., 1990; Nika et al., 2010). Thus, Lck activation can occur without the involvement of the co-receptors. A study of the distribution of activated Lck molecules has shown that the activated Lck molecules are clustered with phosphorylated TCR molecules. Therefore, stimulation of TCR is believed to recruit Lck to the TCR-CD3 complex (Rossy et al., 2013), although there has been no direct evidence to prove this. As Lck has the PRS in its molecule that can bind Nck and Nck can be recruited to CD3ε, I hypothesized that activation of TCR results in the recruitment of Lck to the TCR-CD3 complex with the Nck molecules as the linker or mediator.

Overview of the immune response

The immune system has a primary function to prevent the host from pathogen invasion and toxic substances. It uses a complex of protective mechanisms to control and eliminate the pathogen and toxins. It can produce the memory cells, which respond effectively when they encounter their specific antigens a second time. The collective and coordinated response of the immune sytem to foreign antigens is termed "the immune response". The human immune system consists of the innate and adaptive immune responses that are different in terms of their mechanisms of action. Innate immunity (also called natural or native immunity) is the first line of defense against pathogens and acts rapidly after encounter with invading pathogens.

The innate immune system relies on germ line-encoded pattern recognition receptors (PRRs) to recognize molecular patterns shared in many microbes, but not present in host cells or tissues (Chaplin, 2006). The response of innate immunity remains unchanged even though antigens are encountered many times because it generally lacks immunological memory. Cells that play a central role in innate immunity are phagocytic cells (neutrophils, monocytes, and macrophages), inflammatory cells (basophils, mast cells, and eosinophils) and natural killer (NK) cells (Figure 1). The molecular components generated by innate immunity for controlling the pathogens are complements, acute-phase proteins, and cytokines (Delves et al., 2000).

Unlike the the early response of innate immunity, the adaptive immune system responds later. The term adaptive immunity is defined according to the fact that it can develop and adapt a response to infection. Importantly, it provides long-lasting memory to specific pathogen and can respond more rapidly and effectively to the second and subsequent exposure to the same antigens. The principal cells of the adaptive immune system are antigen specific T and B cells (Figure 1). These cells express the highly antigen-specific receptors to individual unique pathogens by a somatic cell DNA rearrangement. The antigen-binding molecules of B cells are membrane-bound immunoglobulins (Ig) or referred to as the B cell receptor (BCR), whereas those of T cells are TCRs. Unlike B cells that can recognize and bind antigen directly, T cells recognizes a processed short pathogenic peptide fragment bound on MHC molecules that are expressed on the surface of specialized cells, called antigen

presenting cells (APCs) such as dendritic cells, macrophages, and B cells (Madden, 1995). T cells can eliminate the intracellular pathogens by activating macrophages, thereby resulting in killing of intracellularly-infected cells (Abbas et al., 2017). These T cells are called cytotoxic T lymphocytes (CTLs) or $CD8^+$ T cells. In contrast to T cells, B cells can secrete antigen-specific membrane-bound Igs (antibody) to eradicate the relevant extracellular microorganisms. In most cases, interaction of the B cell with T cell is required for providing full activation of the B cell and, therefore, enhancing antibody production following antigen binding. T cells that interact with B cells and enhance antibody production are called T helper (T_h) cells or $CD4^+$ T cells (Abbas et al., 2017). T_h cells, in addition to the enhancement of antibody production by B cells, also provide signals for activating and directing other immune cells that are particularly important for effective immune response.

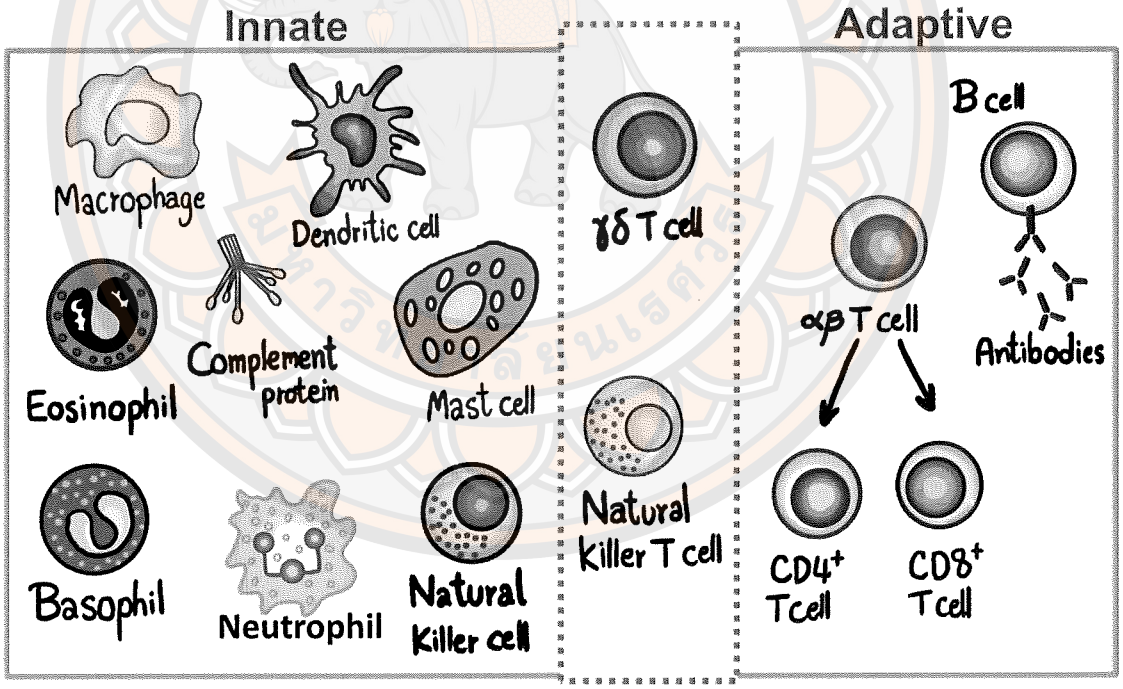


Figure 1 Cells of the immune systems

Cells that play a central role in innate immunity are phagocytic cells (neutrophils, monocytes, and macrophages), inflammatory cells (basophils, mast cells, and eosinophils) and natural killer (NK) cells and antigen specific T and B cells in adaptive immune system.

T cells

Pathogenic microbes and malignant cells are neutralized by the immune system to prevent humans from diseases. T cells are essential for this function in (Courtney et al., 2018) that they play an important role in adaptive immunity. T cells can be divided into αβ and γδ T cells, depending on the type of a structurally unique T cell antigen receptor (TCR), expressed on cell surface for binding to antigens. Whereas most αβ TCRs recognize peptides presented by MHC molecules, γδ TCRs recognize stress-induced self-antigens (Groh et al., 1998; Uldrich et al., 2013), lipids or pyrophosphates that are secreted by microbes or overproduced in tumor cells (Vantourout et al., 2013; Chien et al., 2007; Bonneville et al., 2010; Zou et al., 2017; Chitadze et al., 2017). In contrast to γδ T cells, which accounts about 1-10% of all T cells in human blood, the main subset of T cells expressing TCRαβ chains comprise about 90% of circulating T cells in the peripheral blood of healthy adults (Juraske et al., 2018).

T cells play a central role in defense against foreign antigens. One major type of T cells functions by producing soluble mediators to stimulate other cells or to help them perform better functions. These T cells are called helper T cells or CD4+ T cells. The cells that obtain T cell help are particularly B cells. The stimulated B cells secrete specific antibodies to neutralize pathogens or to activate other phagocyte cells to effectively ingest microorganisms. Another major type of T cells can secrete effector molecules to directly destroy infected cells. These T cells are known as cytotoxic T cells or CD8+ T cells. Nevertheless, to be able to perform the above functions, T cells have to interact with APCs that present antigens associated with their MHC molecules on the cell surface. The APCs that are dendritic cells, macrophages, and B cells can ingest pathogens and process and then present the pathogen derived peptides associated with their MHC molecules to T cells. The so called activated T cells then recognize and respond to this antigen presentation (Janeway et al., 2007).

T cell activation plays a central role in immune response. The full mechanisms underlying T cell activation remain unclear and are continually being investigated. There are various signaling molecules involved in transmitting the signal from TCR to the nucleus that leads to T cell activation.

Structure of T cell receptor (TCR-CD3) complex

T cell antigen receptors consist of a clonotypic TCRαβ or TCRγδ heterodimer, and the CD3δε, CD3γε, and CD3ζζ dimers. TCRαβ and TCRγδ bind to the antigen and the CD3 chains transduce the signal of antigen binding into the cell by phosphorylation of the tyrosines in their cytoplasmic tails by Lck kinase. Consequently, the tyrosine kinase ZAP70 can bind to phosphorylated CD3 and the signal of ligand binding is transmitted further to intracellular signaling cascades, such as Ca2+ influx and the Ras/Erk pathway, ultimately resulting in the activation of the T cell. This includes the execution of the cytotoxic activity to kill infected or tumor cells, up-regulation of CD69 and CD25, as well as secretion of cytokines. However, how antigen binding to the TCR is communicated to the cytosolic tails of CD3 is not well understood. The αβ TCR is in equilibrium between two reversible conformations: the antigen-stabilized active CD3 conformation and the resting conformation adopted by non-engaged TCRs (Gil et al., 2002; Swamy et al., 2016; Schamel et al., 2017).

The principal functions of T cells are defense against extracellular microorganisms and activation of other cells, such as B cells and macrophages. The responses of T cells result in T cell proliferation, differentiation and secretion of cytokines and growth factors. The initiation of T cell responses requires its specific recognition to antigen, stable contact between T cell and APC, and the signal transduction from the cell surface to the nucleus of T cells. Each of these events is mediated through different sets of molecules of the T cells; a highly variable TCR, adhesion molecules (e.g. integrins), and invariant signaling proteins (CD3 and ζchains), respectively (Dustin et al., 2000; Friedl et al., 2005; Smith-Garvin et al., 2009). Each T cell expresses a structurally unique receptor (~30,000 TCRs per cell) with large and extreme diversity (Saizawa et al., 1987). Normally, T cells scan the surface of APCs to detect foreign peptides that are derived from invading pathogens. In the absent of pathogens, it is believed that APCs present only self peptides on MHC

that can bind to TCR complex with low affinity, thereby failing to activate the T cells. In contrast, when the foreign antigens are present, these foreign peptides in the context of pMHCs can bind TCR with higher affinity and consequently leads to the activation of T cells (Davis et al., 2007).

T cells exhibit different responses when they are stimulated with a variety of agonist peptide antigens that differ in quality and quantity. Additionally, T cells can enter to an unresponsive state when they are exposed to an antagonist (Madrenas, 1999). In most cases, APCs generally express higher concentration of self-pMHC than antigenic pMHCs. The affinity of self-pMHC binding to TCR is ten-fold lower than that of the antigenic pMHCs binding to TCR (1-50 μM), but self-pMHC cannot elicit T cell response (Davis et al., 1998; Davis et al., 2007). Interstingly, T cells can respond to higher amounts of ligand in a range of 10^5-fold to 10^6-fold (Bachmann et al., 1998; Cocharan et al., 2000; Irvine et al., 2002). It is not completely understood how T cells show these different response, how T cells are activated only to antigenic-peptide but not for self-peptide, and how T cells display an unsaturated response to a range of pMHC concentration. The molecular composition of TCR-CD3 complex is postulated to allow a flexible T cell response (Alarcon et al., 2003; Alarcón et al., 2006; Molnar et al., 2010).

TCR is a multisubunit complex composed of antigen-binding moiety, formed by a disulfide-bonded T-cell receptor α (TCRα) and β (TCRβ). TCRαβ heterodimer noncovalently associates with signaling transduction subunits CD3 and ζ dimers (CD3εγ and CD3εδ heterodimers and ζζ (CD247) homodimers) to form a TCR-CD3 complex (Malissen, 2003). The majority of T cells express TCRαβ, while a small subset of T cells expresses TCRγδ. These γδ T cells bind directly to pathogen-derived glycoproteins or nonclassical MHC molecules (Chien et al., 1996; Adams et al., 2005). TCRαβ is noncovalently coupled to the conserved signaling transduction subunits (CD3 and ζ chain) (Kane et al., 2000; Werlen et al., 2002; Malissen, 2003). CD3γε and CD3δε heterodimer have an extracellular Ig domain in each chain. In contrast to CD3 heterodimer, ζζ homodimers have no extracellular Ig domain because they consists of only nine amino acid residues, which cannot form an Ig domain. The regions next to extracellular part are a stalk region, negatively charged transmembrane segment, and long intracellular tails containing ITAMs, respectively (Molnar et al., 2010). There is

an additional sequence in CD3ε molecule that is a proline-rich sequence (PRS) located upstream of ITAMs (Gil et al., 2002; Szymczak et al., 2005; Tailor et al., 2008).

The transmembrane domains of TCRαβ, CD3 and ζ chains contain totally nine basic/acidic amino acid residues. Three basic residues are found at TCRαβ (one residue within β chain and two within α chain), whereas each of the three signaling dimers contains a pair of acidic residues in the centre of their transmembrane (Clevers et al., 1988). It has been proposed that pairwise ionic interactions between these basic and acidic residues mediate the assembly of TCR-CD3 complex (Call et al., 2002; Call et al., 2010). Moreover, the extracellular parts of the receptor and signaling molecules are also important for mediating the association of CD3 and ζ chains to TCRαβ (Kuhns et al., 2007). However, the information of the organization and structure of the complete TCR-CD3 complex are not completely clear.

T cell receptor signaling

The fully activation of T cell requires signaling transduction though the TCR-CD3 complex but the TCR-CD3 complex has no intrinsic catalytic activity on its own. However, the long cytoplasmic tail of each CD3 and ζ chains contains totally ten copies of ITAMS, a single copy on the CD3γ, δ, and ε chain, and as a triplicate repeat on the ζ chain (Reth, 1989). These ITAMs contain the conserved sequence of amino acids of YXX(L/I)X_{6-8}YXX(L/I) (where x is any amino acid), in which both tyrosines (underline) serve as a substrate for the Src protein tyrosine kinases (PTK). Two tyrosine residues within each ITAM are phosphorylated following TCR engagement resulting in their transformation into high-affinity ligand for the Syk kinase family member ZAP70 (Iwashima et al., 1994). Recruitment of ZAP70 to phosphorylated ITAMs initiates a cascade of multiple signaling proteins activation of diverse pathways, eventually leading to alterations in gene expression, T cell proliferation, and secretion of various cytokines.

Upon TCR ligation, the earliest detectable intracellular signal after TCR ligation is the phosphorylation of the ITAMs (CD3 and ζ) by Src (Lck and Fyn) kinases, which is associated either with the TCR (Samelson et al., 1990) or with the CD4 and CD8 coreceptor (Veillette et al., 1988; Barber et al., 1989). Tyrosine phosphorylation of the ITAMs serves as docking sites for interaction with ZAP70 via

its tandem SH2 domains. It is important to note that not all the ITAMs are phosphorylated on every occasion following TCR engagement. Instead, the stoichiometry of ITAM phosphorylation and the number of ZAP70 recruited are determined by the binding affinity of the TCR and its peptide ligands (Sloan-Lancaster et al., 1994; Madrenas et al., 1995).

ZAP70 molecule contains two tandemly arranged SH2 domains, which recognize specifically and can bind to the phosphorylated ITAM motifs with high affinity (Hatada et al., 1995). The kinase activity of ZAP70 is mediated by its binding to doubly phosphorylated ITAMs, which leads to the release of ZAP70 from its autoinhibited conformation. This event results in the exposure of the regulatory phosphorylation sites (Tyr315 and Tyr319) for Lck-mediated phosphorylation, which may prevent reversion to inactive conformation even after ITAM disengagement (Leonard et al., 2007). In addition, tyrosines in the activation loop of the ZAP70 kinase domain (Tyr492 and Tyr 493) are then phosphorylated by Lck or by ZAP70 itself in *trans* to further promote its catalytic activity (Deindl et al., 2007). Once activated, ZAP70 phosphorylates a number of signaling proteins, including the transmembrane adaptor protein linker for LAT and the cytosolic adaptor protein SLP-76 (Horejsi et al., 2004). These two adaptors form the backbone to organize signaling complexes in the correct spatiotemporal manner to allow activation of multiple signaling pathways (Horejsi et al., 2004; Koretzky et al., 2006).

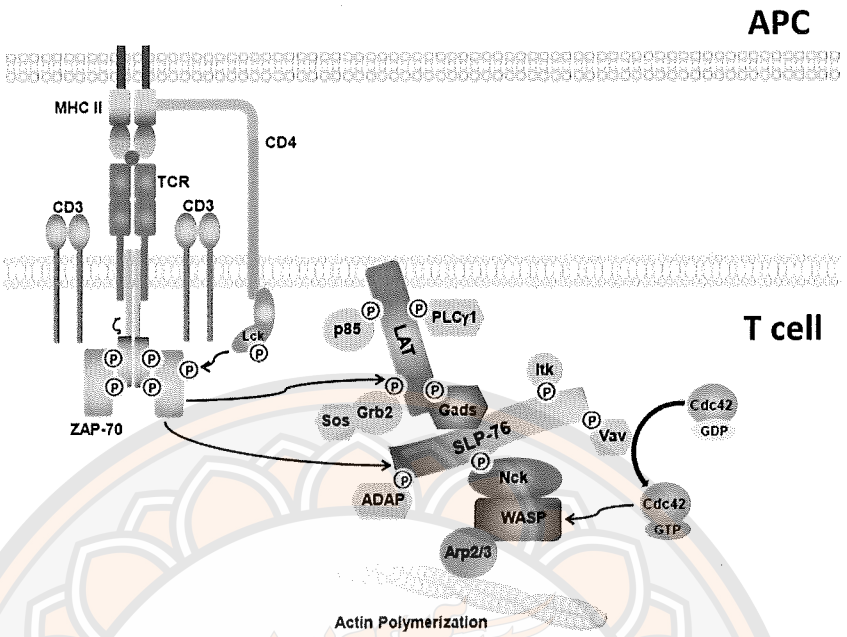


Figure 2 The schematic diagram of molecular signaling cascades that link the TCR signaling to cytoskeleton rearrangement

Source: Ngoenkam, 2013

TCR ligate with its ligand, antigenic peptide bound on the MHC molecule that is expressesed on the antigen presenting cells (APCs) surfaces. This leads to the phosphorylation of ITAMs located at the cytoplasmic tails of CD3 and ζ molecules by tyrosine kinase Lck that is associated with the cytoplasmic tail of CD4 molecule. ZAP-70 is then recruited to the phosphorylated ITAMs, where it is activated. The activated ZAP-70 in turn phosphorylates LAT and SLP-76. The phosphorylated LAT serves as docking sites for adaptor proteins such as Grb2 and Gads. Subsequently, SLP-76 is recruited to LAT-nucleated complex via Gads. Like LAT, SLP-76 is an adaptor protein that contains the sites for interaction with various proteins such as Nck, Vav, Itk and ADAP. Nck then recruits the adapter protein WASP. The GEF Vav promotes the exchange of Cdc42-GDP to Cdc42-GTP that is essential for WASP activation. WASP activates the Arp2/3 complex, which then induces the formation of branced actin filament networks.

Adaptor protein Nck and its struture

Nck is an adaptor protein of 47 kDa. Nck is composed of one SH2 domain at the carboxy-terminal and 3 SH3 domains at the amino-terminal (Figure 3) (Lettau et al., 2009; Buday et al., 2002). In humans, there are 2 types of the Nck protein: Nck1 or Nckα and Nck2 or Nckβ (the latter is also called Grb4). The main function of the Nck protein is to interact with other proteins to form an intracellular signaling complex. Thus, the Nck protein is characterized as an adapter protein that links a signal from upstream phosphotyrosine (pTyr) via the SH2 domain to downstream effector via the SH3 domain (Figure 3) (Lettau et al., 2009). Studies in many cell types have shown that Nck can interact with more than 60 different proteins (Buday et al., 2002; Li et al., 2001). The gene encoding the Nck1 is located on the chromosome 3q21, while the gene encoding the Nck2 is on chromosome 2q12. The two Nck isoforms share about 68% similarity of the amino acids and the major difference is found in the linker regions between each domain (Figure 3). The SH2 (src homology 2) domain of the Nck is localized to the carboxy-terminal. The SH2 domain of the two Nck isoforms share about 82% similarity of the amino acids. The SH2 function is to bind phosphorylated tyrosine, pTyr or pY). Besides binding to pY, the SH2 domain can also recognize the surrounding amino acids. The general amino acid sequence that the Nck1 and Nck2 protein recognize is pYDxV (when x is any amino acid). For T cells, after TCR ligation the SH2 domain of the Nck binds pY at the Y113 and Y128 of the SLP-76 molecule (Frese et al., 2006).

Each of the SH3 domains of the Nck is composed of about 50-60 amino acids. This sequence interacts with the PRS that is PXXP. It has been reported that the SH3 domains of the Nck1 and Nck2 can interact with more than 30 different proteins, the majority being involved in regulation of actin cytoskeleton, such as, N-WASp (neuronal-Wiskott-Aldrich Syndrome protein) and WIP (WASP interaction protein) (Buday et al., 2002; Li et al., 2001). All the three SH3 domains of the Nck1 and Nck2 are related but have different properties. When comparing the amino acids in each pair of the domain, for instance SH3.1 of the Nck1 and Nck2; SH3.2 of the Nck1 and Nck2; SH3.3 of the Nck1 and Nck2, each pair has a similar amino acid sequence of about 80%. However, when the three SH3 domains of the same Nck molecule are compared, the amino acid sequence is similar in only about 30%. For interaction with

other proteins, only one domain of the SH3 domains is required (Buday et al., 2002; Li et al., 2001). Nevertheless, some studies have found that more than one of the SH3 domains may be used to bind other proteins (Wunderlich et al., 1999).

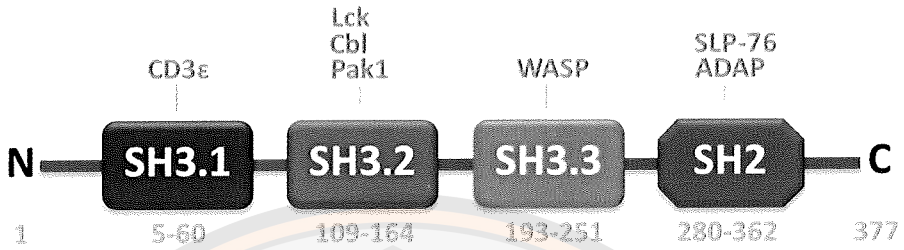


Figure 3 Structure of the Nck family

The Nck1 and Nck2 are composed of similar domains that are three domains of the src homology 3 (SH3) domain at the N-terminal and one domain of the SH2 domain at the C-terminal. Each domain of the Nck1 and Nck2 shares some similarities. The number at the C-terminal of the Nck1 and Nck2 is the total number of amino acids of the Nck proteins. The Nck acts as an adapter protein for binding of other proteins as a link between tyrosine phosphorylation and downstream effector. The SH2 domain binds to phosphorylated protein that have pY-D-x-V motif, whereas the SH3 domains bind to effector molecule that have P-x-x-P motif. Examples of proteins and effector molecules are shown.

Function of Nck in T cells

The Nck protein has been reported to be involved in actin polymerization. After TCR ligation and tyrosine phosphorylation of SLP-76 molecules, proteins with SH2 domain are recruited including the guanine nucleotide exchange factor VAV and Nck. Then the WASp is recruited to bind the SH3.3 domain of the Nck. In this case, the SLP-76 molecules function as a scaffold to bring the Nck and WASp into close proximity with VAV and Cdc-42-GTP (Barda-Saad et al., 2005). The VAV stimulates Rho family GTPases, Cdc42 and RAC (Rho family GTPases comprise Rac, Cdc42, and Rho). Activation of Cdc42 and RAC facilitates aggregation of the actin filaments using the nucleation promoting factor (NPFs) such as actin-related proteins 2/3

(Arp2/3) and proteins in the WASp family (WASp, N-WASp, WAVE1, WAVE2, and WAVE3) (Billadeau et al., 2007; Smith-Garvin et al., 2009; Reicher et al., 2010).

In addition to interacting with SLP-76 molecules in a control of actin polymerization, Nck can also interact directly with the PRS of the CD3ε (Gil et al., 2002). This event occurs after the TCR binds to its ligand resulting in a conformational change of the CD3ε and a subsequent exposure of its PRS leading to recruitment of the Nck to bind it via the SH3.1 domain of the Nck. This event occur prior to and independent of tyrosine phosphorylation. This finding of CD3ε-Nck interaction is important for T cell activation and actin polymerization in T cell (Gil et al., 2002) as well as activation of ZAP70 (Borroto et al., 2013). Recruitment of Nck to CD3ε may promote the recruitment of Lck to the TCR-CD3 complex. This is because Lck contains the PRS that can bind to the SH3 domain of the Nck (Borroto et al., 2013). However, until now there has been no report about binding of Nck and Lck and its impact on T cell activation. Because of the similarity of the amino acid sequences, Nck1 and Nck2 have overlapping function. Mice lacking of either Nck1 or Nck2 protein survive. However, mice lacking of both Nck1 and Nck2 die in utero at day 9.5 (Bladt et al., 2003). It has been reported that Nck1 and Nck2 are not redundant in terms of activation of human T cells (Ngoenkam et al., 2014). Jurkat T cells with reduced expression of the Nck1 protein decrease phosphorylation of Erk and MEK and have a decrease in the recruitment of Lck and ZAP-70 to the TCR as well as in the activation of a transcription factor AP-1. These result in impaired expression of IL-2 and CD69. In contrast, activation of the TCR in the cells that lack Nck2 protein results in normal IL-2 and CD69 expression (Ngoenkam et al., 2014). Thus, beside the important role of Nck in regulation of actin polymerization, Nck also plays a pivotal role in T cell intracellular signaling.

Lck and its function in TCR signaling

The lymphocyte-specific protein tyrosine kinase (Lck) is categorized to be in the Src kinase enzyme family. Lck plays a critical role in intracellular signaling for cellular responses that include cell growth, differentiation, movement, and protein production. Lck has a molecular weight of 56 kDa. In T cells, Lck primarily functions in increasing and regulating signals receiving from the TCR after the TCR is

stimulated by pMHC. Activation of Lck molecules leads to phosphorylation of tyrosine in the ITAMs of the TCR-CD3 complex (Van et al., 1996). This event results in a recruitment of ZAP-70 to the phosphorylated ITAMs and the ZAP-70 molecules themselves are also phosphorylated by the activity of the Lck enzyme (Iwashima et al., 1994). The phosphorylated activated ZAP-70 molecules then phosphorylate LAT molecules. The phosphorylated LAT molecules then become a docking site for several other enzymes and adaptor protein, all of which being associated with activation of signaling pathways such as MAPK signaling pathway, calcium influx, and PKC signaling pathway (Smith-Garvin et al., 2009). Activation of these pathways has an endpoint in the nucleus that involves activation and translocation of the transcription factors NF-AT, NF-κB, and AP1 molecules into the nucleus. These transcription factors then bind several gene promoters including IL-2 promotor leading to IL-2 protein expression (Horejsi et al., 2004; Koretzky et al., 2006).

Structure of Lck molecule

The N-terminal of the Lck molecule has lipids that have undergone lipid modification including myristoylation and palmitoylation in order that it can bind the cell membranes (Figure 4A). The next region is a unique domain that contains around 60 amino acids, of which there are 2 cysteine molecules that can bind cytoplasmic tail of the co-receptor CD4 or CD8 molecule (Rudd et al., 1988; Shaw et al., 1989; Veillette et al., 1988). Lck molecule also contains Src-homology 3 (SH3) domain and SH2 domain. The former binds to other proteins that have a PRS sequence, while the latter binds to other proteins that have phosphorylated tyrosine. Next from the SH2 domain of the Lck is a short linker region followed by a catalytic kinase domain carring tyrosine at the 394 residue (Tyr394). The Tyr394 is the site to be phosphorylated by a process called autophosphorylation, which makes the Lck be in an active form (open conformation). Thus, the Tyr394 is an activation loop of the Lck molecule. Next from the kinase domain of the Lck is a short C-terminal domain that is critical in regulation of the Lck activity. In this domain, the tyrosine at the 505 residue (Tyr505) when phosphorylated causes intramolecular interaction between Tyr505 and SH2 domain of the Lck molecule itself. This results in inhibition of Lck kinase activity

and the molecule becomes an inactive form (close conformation). Therefore, the Tyr505 is said to be an inhibition loop of the Lck molecule (Boggon, & Eck, 2004).

Control of Lck activation

The kinase activity of the Lck molecule is controlled by phosphorylation and dephosphorylation in a reversible process. The phosphorylation and dephophorylation can occur on two tyrosine residues, particularly, the positive regulatory tyrosine 964 on the kinase domain and the negative regulatory tyrosine 505 on the C-terminal segment. An enzyme that phosphorylates Lck molecule is the Csk (C-terminal Src kinase). An enzyme that dephosphorylates Lck molecule is the phosphatase CD45. Phosphorylation of Tyr505 by Csk causes binding of the Tyr505 to the SH2 domain of Lck itself. Molecular arrangement within the Lck molecule prevents the kinase domain and maintains the Lck in an inactive form or closed conformation (Filipp *et al.*, 2012). Moreover, the closed conformation can be stabilized by the binding of the SH3 domain and the PRS in the linker region between the SH2 domain and the kinase domain (Figure 4B). On the contrary, dephosphorylation of Tyr505 by CD45 tyrosine causes the detachment of the SH2 domain from the Tyr505 and the Lck becomes primed conformation (the tyrosine 394 and 505 are not phosphorylated). This is the basal state of the kinase activity (Figure 4B), in which Tyr394 can be phosphorylated by Lck in a process called trans/autophosphorylation that make the Lck active (it is now in opened conformation). Dephosphorylation of Tyr394 is involved in the activity of SH2 domain-containg phosphatase-1 (SHP-1) (Stefanova et al., 2003) and of CD45 (Saunders, & Johnson, 2010).

CD45 is a negative regulator of Lck. Dephosphorylation of Tyr394 considerably requires CD45 whereas that of Tyr505 does not significantly require CD45 activity. Thus, it has been proposed that in resting T cells, Lck is in an inactive form as there is high expression of CD45 molecules. It is also interesting that Lck can be found phosphorylated at both Tyr394 and Tyr505 simultaneously. This is called DPho active that can be found in about 20% of Lck molecules in T cell (Nika et al., 2010). There are 2 steps in DPho active process. The first step involves ligand binding of SH2 and/or SH3 domains leading to an inhibition of closed conformation. For the second step, there are two possible mechanisms. The first one may involve

phosphorylation of Tyr394 by Lck activity. The second one may be that Csk phosphorylates Tyr505 of Lck that has already a prior phosphorylation of the Tyr394. It is also thought that membrane compartmentalization is essential for CD45 to regulate Lck activity in T cell. After TCR stimulation, Csk molecules are released from the plasma membrane, while the CD45 molecules having a large ecto domain are separated from the Lck. Thus, translocation of CD45 close to or away from Lck is believed to be a result of membrane compartmentalization (Horejsi, 2003). However, it has been reported that only small amount of CD45 molecules are present with Lck on the plasma membrane. Thus, the mechanism underlying the involvement of CD45 in dephosphorylation of pTyr505 of Lck after TCR activation is not fully understood.

Most Lck molecules are linked to the co-receptor CD4 or CD8 molecules. It is believed that binding of CD4 or CD8 to MHC class II or class I upon antigen presentation can stimulate Lck (Rudd et al., 1998, Veillette at al., 1988). On the other hand, CD4 or CD8 molecules are involved in locating Lck to the TCR-pMCH complex rather than sending a signal as there is evidence of TCR signaling in the absence of co-receptors CD4 or CD8 (Van et al., 2007; Artyomov et al., 2010). Moreover, previous studies have demonstrated that there are two types of Lck molecules. One type of Lck is associated with CD4 and CD8 molecules and the other type is free in the cytoplasm. Thus, whether or not the CD4 or CD8 molecules are presence does not affect the activation of thymocytes because there are free Lck molecules in the cytoplasm that can function normally (Turner et al., 1990). In resting T cells, up to 40% of Lck molecules are active and TCR stimulation does not increase Lck activity. This is evident from the finding that the ratio of phosphorylation and conformation state of Lck is not changed (Nika et al., 2010) and it is hypothesized that location of Lck may be critical for its function in TCR signaling. The conformational state of Lck molecules may also affect their clustering and distribution (Rossy et al., 2013). When they are made to be in a 'stable open conformation' by changing the tyrosine (Y) at the residue 505 to phenylalanine (F), there is no phosphorylation at this residue and the SH2 domain can not bind the Lck molecules. Thus, they are in open conformation. Using J.CaM1.6 cells that are Lck deficient T cell line, stimulation of these cells that has been reconstituted with 505F causes a more Lck clustering compared with wild-type Lck. In contrast, changing of tyrosine at 394 into

phenylalanine, resulting in inhibition of phosphorylation at this site while there is still heavy phosphorylation of Tyr505, the SH2 domain can bind the pTyr505 making the Lck molecule remains in close conformation. In this case, stimulation of the cells leads to less Lck clustering. Co-mutation of tyrosine 505 and lysine 273 to phenylalanine and arginine, respectively, which makes the Lck molecues remain in open conformation and inhibits their kinase activity, does not affect Lck clustering. This suggests that Lck clustering is not associated with kinase activity but is associated with the conformational state.

The lipid raft may also be involved in Lck clustering. Lck can be associated with plasma membrane via its N-terminal related with myristoylation and palmitoylation (Otahal et al., 2010; Ballek et al., 2012). Movement of Lck to the lipid raft is found to be important to Lck activity and regulation of TCR signaling (Rodgers et al., 2005). Nevertheless, inhibition of lipid-raft accumulation does not influence Lck clustering, indicating that only association of Lck to the plasma membranes is not enough to cause Lck clustering either before or after T cell activation. Thus, Lck clustering may rather be a result of the ability of Lck to bind other proteins. Mutations of the SH2 (R154K) and SH3 (W97A) domains that inhibit Lck from binding to other proteins do not affect Lck clustering. The explanation could be that Lck clustering is controlled by binding of Lck molecules themselves, or the Lck may use other domains, such as the PRS, to bind other proteins in order to form clusters. In addition, in J.CaM1.6 cells reconstituted with wild-type Lck, before cell stimulation there are only small amounts of CD45 and Lck being together and these associated molecules significantly decline after cell stimulation. However, Lck molecules are mostly with phosphorylated TCRζ, suggesting that stimulation of T cell leads to distribution of Lck to the TCR, which may be involved in TCR signaling.

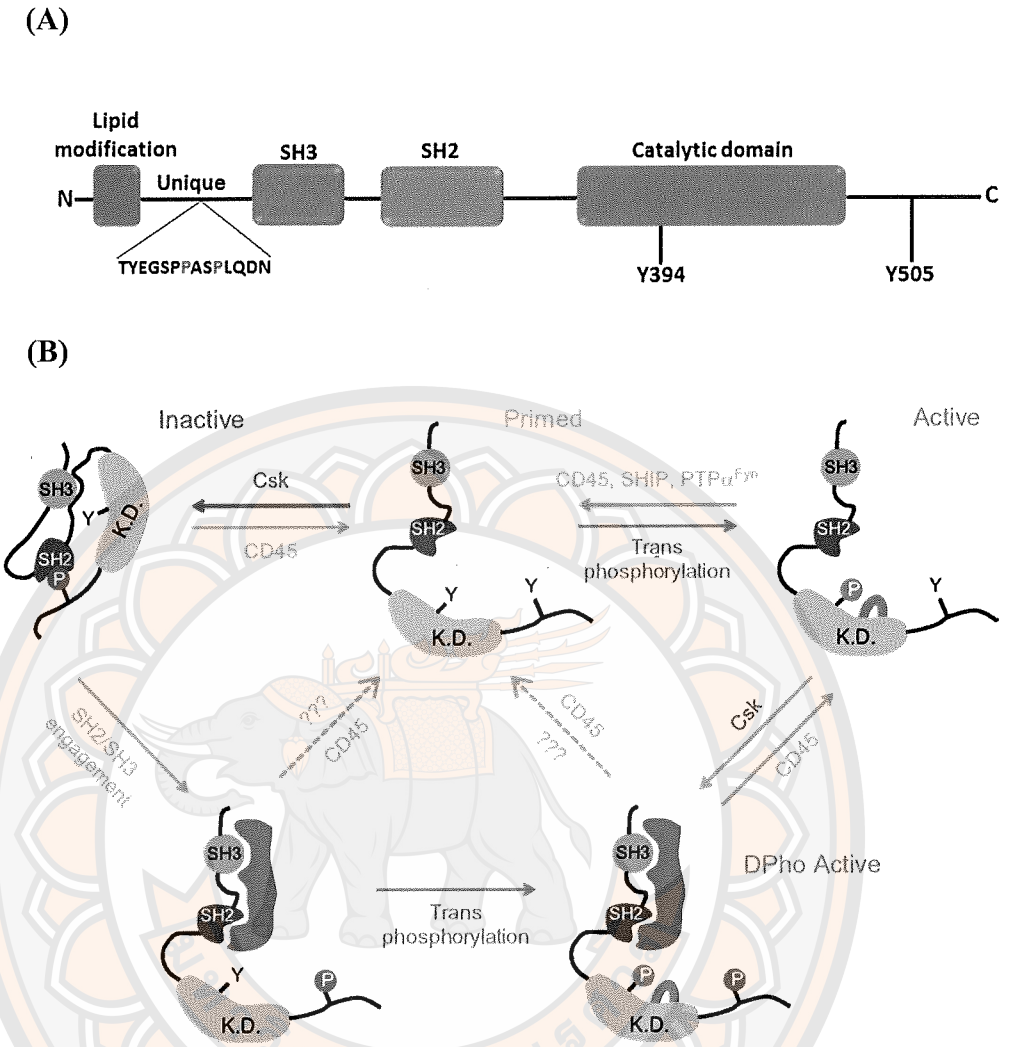


Figure 4 Structure of Lck and a control of Lck stimulation

(A) A linear structure of Lck that comprises several domains. The unique domain has a proline rich region (PxxP) and (B) A model of Lck conformation associated with phosphorylation of the 2 tyrosine residues: the positive regulatory tyrosine 394 at the kinase domain (K.D.) and the negative regulatory tyrosine 505 at the C-terminus (Filipp et al., 2012).

CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodologies of this research including materials, apparatus and methods. The detail of each topic is described below.

Equipments

1. Antibodies

·Goat anti-mouse IgG-HRP conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

Goat anti-rabbit IgG-HRP conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

Mouse monoclonal anti-human β-actin antibody (Cell Signaling Technology, Danvers, MA, USA)

Mouse monoclonal anti-human CD3 (clone UCHT1) antibody (Upstate Biotechnology, Lake Placid, NY, USA)

Mouse monoclonal anti-human CD3 (clone OKT3) antibody (eBioscience, San Diego, CA, USA)

Mouse monoclonal anti-human CD3ε (LE-CD3) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

Mouse monoclonal anti-human CD3ζ (E-3) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

Mouse monoclonal anti-human Nck2 antibody (clone AF3F11) (Abnova Corp., Taipei, Taiwan)

Mouse monoclonal anti-human Nck2 antibody (clone 8.8) (Sigma Aldrich, MO, USA)

Mouse monoclonal anti-T Cell Receptor (clone C305) antibody (Upstate Biotechnology, Lake Placid, NY, USA)

Mouse monoclonal cocktail IgG2b anti-Phosphotyrosine (4G10) (Upstate Biotechnology, Lake Placid, NY, USA)

Mouse monoclonal anti-ZAP-70 antibody (BD BioSciences, Qume Drive San Jose, CA, USA)

Mouse monoclonal anti-phospho-ZAP-70 (pY319) antibody (BD BioSciences, Qume Drive San Jose, CA, USA)

PE-conjugated anti-CD69 antibody (eBioscience, San Diego, CA USA)

Rabbit monoclonal anti-human β-actin antibody (Cell Signaling Technology, Danvers, MA, USA)

Rabbit monoclonal anti-phospho-Epsilon (Tyr1) (obtained from Schamel's lab, Freiburg University, Germany)

Rabbit monoclonal anti-phospho-Epsilon (Tyr2) (obtained from Schamel's lab, Freiburg University, Germany)

Rabbit monoclonal anti-phospho-ERK1/2 (clone AW39R) antibody (Upstate Biotechnology, Lake Placid, NY, USA)

Rabbit monoclonal anti-human Nck1 antibody (Cell Signaling Technology, Danvers, MA, USA)

Rabbit monoclonal anti-phospho-MEK1/2 antibody (Cell Signaling Technology, Danvers, MA, USA)

Rabbit monoclonal anti-phospho-p38 antibody (Cell Signaling Technology, Danvers, MA, USA)

Rabbit monoclonal anti-phospho-Src family (Tyr416) (D49G4) antibody (Cell Signaling Technology, Danvers, MA, USA)

2. Cell lines

Jurkat T cell (clone E6-1; ATCC, Rockville, MD, USA)

Lck-deficient derived Jurkat T cell (J.CaM1.6; ATCC, Rockville, MD, USA)

3. Chemicals

Acrylamide (Invitrogen, Carlsbad, CA, USA)

Agarose (AMRESCO, Solon, OH, USA)

Ammonium persulfate (APS) (Sigma Aldrich, St. Louis, MO, USA)

Ampicillin (Sigma Aldrich, St. Louis, MO, USA)

Aprotinin Bovine recombinant (Sigma Aldrich, St. Louis, MO, USA)

Cytochalasin D (CytD) (Sigma Aldrich, St. Louis, MO, USA)

Dulbecco's Modified Eagle Medium (DMEM), High Glucose (GIBCO, Gaithersburg, MD, USA)

Fetal bovine serum (GIBCO, Gaithersburg, MD, USA)

Hepes Buffer Solution (Sigma-Aldrich, St. Louis, MO, USA)

HIYIELD GEL/PCR DNA Fragments extraction kit (RBC Bioscience Corp, Taipei County Taiwan)

HIYIELD Plasmid mini kit (RBC Bioscience Corp, Taipei County Taiwan)

IL-2 ELISA Kit (R&D Systems, Minneapolis, MN, USA)

Iscove's Modified Dulbecco's Media (IMDM) (GIBCO, Gaithersburg, MD, USA)

LB broth (AMRESCO, Solon, OH, USA)

pLVX-shRNA1 vector (Clonetech, Mountain View, CA, USA)

Leupeptin (AMRESCO, Solon, OH, USA)

L-glutamine (JRH Biosciences, Woodland, CA, USA)

M-PER mammalian protein extraction reagent (Pierce, Rockford, Il, USA)

Mouse serum (Sigma-Aldrich, St. Louis, MO, USA)

N, N', methylenebisacrylamide ((Invitrogen, Carlsbad, CA, USA)

NF-AT luciferase reporter plasmid (Addgene plasmid 17870, Cambridge, MA, USA)

NF-κB and AP-1 luciferase reporter plasmid vectors (kindly provided by Assist. Prof. Dr. Prateep Warnnissorn (Naresuan University)

NucleoBond Xtre Maxi EF (Macherey-Nagel, Easton, PA, USA)

Penicillin/streptomycin (JRH Biosciences, Australia) (JRH Biosciences, Woodland, CA, USA)

Phenylmethanesulfonylfluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA)

Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA)

Polyoxyethylene 10 Oleyl Ether (Brij97) (Sigma-Aldrich, St. Louis, MO, USA)

Polyoxyethylene 20 (Tween 20) (Bio Basic Inc, Markham, Canada)

Precision Plus Protein Western C Standard (Bio-Rad, Hercules, CA, USA)

Precision StrepTactin-HRP conjugate (Bio-Rad, Hercules, CA, USA)

Primer Base (scale 50 nmole) (Invitrogen, Carlsbad, CA, USA)

Protein G Mag Sepharose Beads (GE Healthcare, Waukesha, WI, USA)

Puromycin (Clontech, Mountain View, CA, USA)

QIAprep Miniprep Kit (Qiagen, Valencia, CA, USA)

QuikChange® II XL Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA)

Restriction enzymes: *Bam*HI, *Eco*RI and *Mlu*I (Takara Shuzo, Tokyo, Japan)

Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA)

RPMI 1640 medium (GIBCO, Gaithersburg, MD, USA)

Sodium Dodecyl Sulfate (SDS) (AMRESCO, Solon, OH, USA)

Sodium Fluoride (NaF) (UNIVAR, Redmond, WA, USA)

Sodium Orthovanadate (Na_3VO_4) (Sigma-Aldrich, St. Louis, MO, USA)

Supersignal Wet Pico Chemoluminescent Substrate (for HRP) (Thermo Fisher Scientific, Waltham, MA, USA)

Synthesis of cDNA cloning and mutagenesis primers (Invitrogen, Carlsbad, CA, USA)

Synthesis oligonucleotides (Invitrogen, Carlsbad, CA, USA)

T4 DNA ligase enzymes (Takara Shuzo, Tokyo, Japan)

TBE (Tris-Borate-EDTA) Buffer powder (AMRESCO, Solon, OH, USA)

TEMED (Invitrogen, Carlsbad, CA, USA)

Triton X-100 (AMRESCO, Solon, OH, USA)

Tris (AMRESCO, Solon, OH, USA)

TrisHCl (Invitrogen, Carlsbad, CA, USA)

4. Labwares

15- and 50-ml tube (Corning Incorporated, Corning, NY, USA)

24- and 96-well plate (Nunc A/S, Rosklide, Denmark)

25-cm^2 cultured flasks (Nunc A/S, Rosklide, Denmark)

Acrodisc 25 mm Syring Filter S-450, 0.45 mm (PALL Corporation, Port Washington, NY, USA)

Syring (NIPRO Corporation, Osaka, Japan)

5. Apparatus

CCD camera (GE Healthcare, Little Chalfont, UK)

CO_2 incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA)

FACScalibur (Becton-Dickinson, San Jose, CA, USA)

Gel Doc (Bio-Rad, Hercules, CA, USA)

Luminometer (Promega, Madison, WI, USA)

Microplate reader (PerkinElmer Life Sciences, Downers Grove, Il, USA)

Real-Time PCR (Corbett Research, Sydney, NSW, Australia)

High Speed Refrigerated Centrifuge (TOMY Seiko Co., Ltd., Tokyo, Japan)

Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA)

Shaker (Heidolph Instruments GmbH & Co. KG, Schwabach / Germany)

Thermo Cycler (Bio-Rad, Hercules, CA, USA)

Tube rotator (Biosan Ltd, Riga, Latvia)

Water bath (JULABO Labortechnik GmbH, Seelbach, Germany)

Research methodologies

1. T cell culture

The human Jurkat T cell line (E6-1 clone) obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and the N1KO Jurkat cells produced in our laboratory were used for studying TCR signaling and T cell activation. Both cell types were cultured in RPMI 1640 medium (Gibco ThermoFisher Scientific, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine and 100 U/ml penicillin G and 100 µg/ml streptomycin (HyClone, Fisher Scientic, Loughborough, UK) in a humidified incubator with 5% CO_2 at 37°C.

2. Cell stimulation

The cells were harvested and starved in RPMI 1640 medium without FBS for 1 hr at 37°C. Subsequently they were either stimulated with the C305 antibody diluted 1:50 for the indicated time points or left untreated as a control. This antibody dilution corresponded to ~0.12 mg/ml of C305.

3. Generation of Nck1-deficient Jurkat T cells

3.1 Principles of CRISPR/Cas9 technology

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR- associated (Cas) system is the genome editing technique that is quite efficient solution. The core principle is derived from components of a simple bacterial immune system, the CRISPR-Cas9 system permits targeted gene cleavage and gene editing in a variety of eukaryotic cells, and because the endonuclease cleavage specificity in CRISPR-Cas9 system is guided by RNA sequences, editing can be directed to virtually any genomic locus by engineering a short noncoding guide RNA (gRNA) sequence that function in guiding the Cas9 endonuclease protein to a specific genomic locus via base pairing between the crRNA sequence and the target sequence. The CRISPR-Cas9 system has great promise in broad applications such as stem cell engineering, gene therapy.

In bacteria CRISPR loci are composed of a series of repeats separated by segments of exogenous DNA, called spacers (~30 bp in length). The repeat-spacer array is transcribed as a long precursor and processed within repeat sequences to generate small crRNAs that specify the target sequences cleaved by the endonuclease Cas9 protein. CRISPR spacers are then used to recognize and silence exogenous genetic elements at the DNA level. Essential component for cleavage is a three-nucleotide sequence motif (NGG) immediately downstream on the 3' end of the target region, known as the protospacer-adjacent motif (PAM). The PAM is present in the target DNA, but not the crRNA that targets it (Figure 5) (Liang et al., 2016)

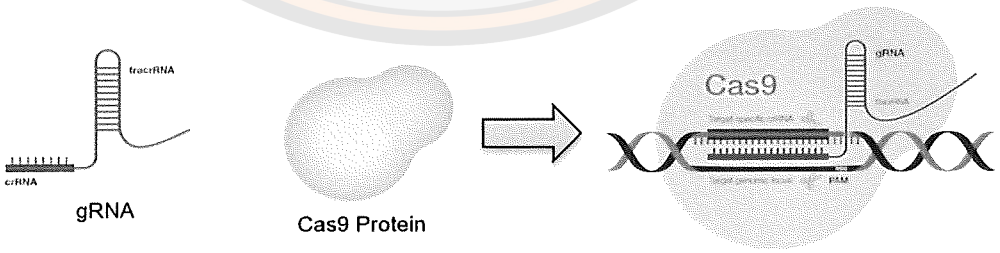


Figure 5 Schematic representation of the CRISPR gRNA and Cas9 protein in The CRISPR/Cas9 System

Source: Modified from Thermo Fisher Scientific

The two main compotents of the CRISPR/Cas9 system; gRNA and Cas9 nucleas protein that is essencial for the specific gene editing in target cells are shown.

Ater the binging to the target sequence, the Cas9 protein induces a specific double-strand break. When the DNA is cleavaged, after that, the break is repaired by cellular repair machinery through non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms. Together with target specificity defined by a very short RNA-coding region, the CRISPR-Cas9 system greatly simplifies genome editing (Figure 6).

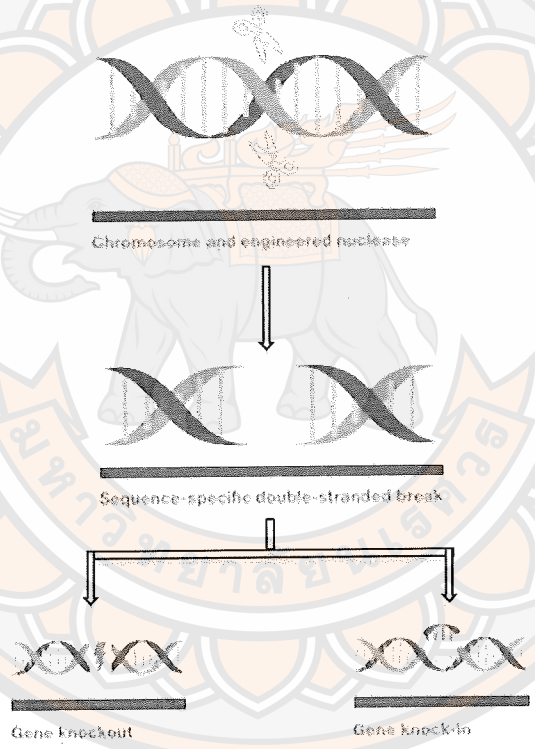


Figure 6 Schematic representation of A CRISPR-Cas9 targeted double-strand break. Cleavage occurs on both strands

Source: Modified from Thermo Fisher Scientific

Two different modifications of target cells including gene knock-out or gene knock-in of gene targets using the CRISPR-Cas9 technique.

3.2 Construction of Nck1 specific CRISPR-Cas9 plasmid anf transfection into Jurkat T cell

The Jurkat variant N1KO (deficient in Nck1) was generated by transiently expressing Cas9, a guide sgRNA againt the Nck1 region. The Nck1 sgRNA oligonucleotide sequence used was 5'-GTCGTCAATAACCTAAATAC-3'(Table 1 and Figure 7). This guide sgRNAs were cloned into the GeneArt® CRISPR Nuclease (OPF) vector [Invitrogen, #A21174] (Figure 8) that was then electroporated into Jurkat T cell using Amaxa Nucleofector II using the manufacturer's protocol X-005. The stable Nck1-deficient single cells were obtained from these lines by limiting dilution. Successful positive knock-out individual clones were selected by fluorescence analysis and screening with immunoblot probed for anti-Nck1 antibody. The selected clones had 100% downregulation of endogenous Nck1 and TCR expression was evaluated by flow cytometry. Clones with similar expression levels of TCR were selected for further studies that was named N1KO.

Table 1 The oligonucleotides for construction of Nck1 gRNA in CRISPR-Cas9 vector

Target Name	Sequence (5' to 3')
Nck1	5'-GTCGTCAATAACCTAAATAC-3'

Figure 7 Schematic representation of A CRISPR-Cas9 targeted gene in Nck1 struture

The location of a target oligonucleotides in Nck1 protein structure for design the specific gRNA sequence in CRISPR-Cas9 vector for Nck1-deficient Jurkat T cells generation.

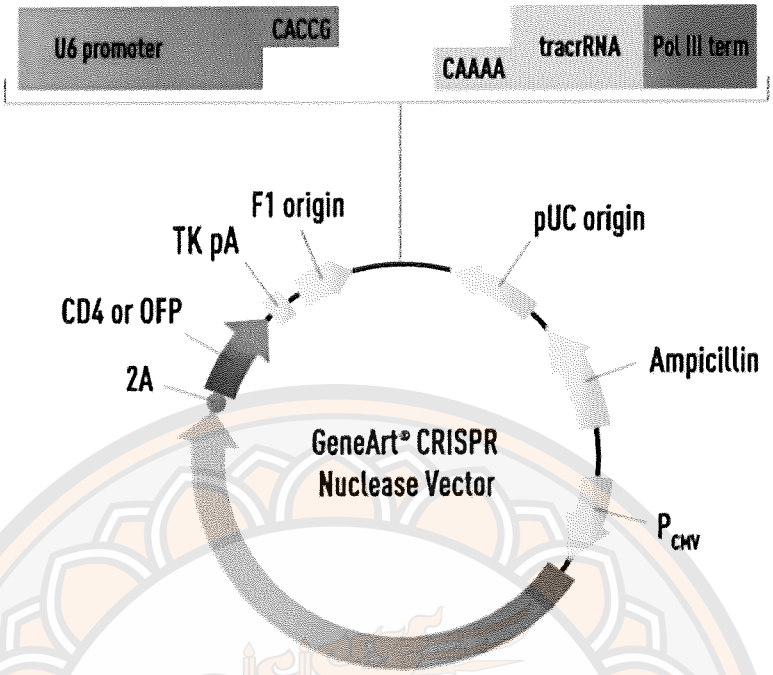


Figure 8 Schematic diagram of CRISPR-Cas9 expression vector

Source: Modified from Thermo Fisher Scientific

The vector is designed to express a guide RNA (gRNA) that compremented with endogenous Nck1 gene in cells. The gRNA is insertd at the downstream of human U6 promoter. The expression of inserted gRNA oligos is driven by the RNA Pol III-dependent human U6 promoter. The expression of orange fluorescent protein (OFP) gene is contained in this vector for selection of stable transfectant. In addition, a pUC origin of replication and *E.* coli ampicillin resistance gene are also contained in this vector for using in bacterial propagation and selection. CRISPR Nuclease Vectors were inserted with oligos of gene of interst and then transfectd to target cells for stable knockout the gene of interest.

4. Inhibitor treatment and fluorescence microscopic visualization

To determine the optimal inhibitory concentration to inhibit actin polymerization, Jurkat cells were treated with CytD for 30 min at concentrations of 5 μM and 10 μM at 37°C before cell stimulation. The investigation of F-actin formation inhibition was performed as previously known for application in fixed cells.[28] Briefly, chambered coverglasses (LabTek, Thermo Fisher Scientific) pretreated with 0.01% w/v poly-L-lysine solution (Merk KGaA) were coated with anti-CD3 antibody (1 μg/ml) and incubated at 4°C overnight. After washing with phosphate-buffered saline (PBS), $2x10^5$ cells in 200 μl medium containing 10% FCS and 10 mM HEPES were seeded into the chambers and incubated for 5 min. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After washing, the samples were blocked for 1 hr with 10% FCS and 0.02% sodium azide in PBS. Subsequently, the chambers were incubated with rhodamine-phalloidin staining (diluted in PBS at 1:200) and they were observed for red fluorescence signals under a fluorescence microscope (Nikon Eclipse Ti) using NIS-Elements D software with 2560x1920 record pixels. Three to five high power fields in each slide were always included for visualization in order to avoid slide-to-slide variation.

5. Multiplex TCR signaling assay

Ten million Jurkat T cells were pretreated with 5 μM CytD for 30 min at 37°C before stimulated with C305 antibody (1:50) at 37°C for the indicated time points (30 sec, 1 min, 5 min, 10 min, and 30 min) or left untreated as a control. After stimulation, cell pellets were lysed in 1 ml of MILLIPLEX® MAP Lysis Buffer (Merck KGaA) containing freshly prepared protease inhibitors. The working concentration of protein for the assay was 7.5 μg of total protein/well (25 μl/well at 300 μg/ml). Subsequently, the levels of phosphorylation of each protein were determined using T-Cell Receptor Magnetic Bead Kit 96-well Plate Assay, Milliplex Map Kit (Merck KGaA), following the manufacturer's instructions and the median fluorescence intensity (MFI) was measured with the MAGPIX, Luminex® system (Austin, USA).

6. In situ proximity ligation assay

Jurkat T cells were grown on diagnostic microscopic slides (Thermo Scientific). Cells were pretreated with 5 μM CytD for 30 min at 37°C. Then, cells were stimulated with the anti-TCR antibody C305 (1:50) at 37°C for 5 and 10 min or left untreated as a control. Subsequently, cells were fixed with paraformaldehyde, permeabilized with 0.5% saponin, and blocked with blocking solution. Then, cells were co-incubated with goat anti-CD3ε M20 antibody (Santa Cruz Biotechnology) and rabbit anti-Nck1 antibody (Cell Signaling Technology). A proximity ligation assay (PLA) between the CD3ε and Nck1 molecules was performed using the Duolink kit (Olink Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. The PLA signals appeared as red fluorescence dots. Cell nuclei were stained with DAPI. A fluorescence microscope (Nikon Eclipse Ti) was used for imaging and analysis. The number of the PLA signal dots was scored with the Blob-Finder program (Uppsala University).

7. Immunoprecipitation and Western blotting

Jurkat T cells that were either pretreated or not with CytD for 30 min were stimulated with anti-TCR antibody (C305, 1:50) at 37°C for 3, 10 and 30 min or left unstimulated. Cells were then lysed in 100 μl lysis buffer (20mM Tris-HCl pH 8.0, 137 mM NaCl, 2 mM EDTA, 10% glycerol, protease inhibitor mixture (Sigma-Aldrich), 1 mM PMSF, 5mM iodoacetamide, 0.5 mM sodium orthovanadate, 1 mM NaF, and 0.3% Brij96V) for 30 min on ice. TCRs from the total cellular lysates were then immunoprecipitated with 1 mg anti-CD3ε antibody (OKT3)-coupled protein-G Sepharose beads (Amersham Pharmacia Biotech). The SDS-PAGE and immunoblotting were performed with the antibodies indicated, and visualization was done using a CCD camera (ImageQuant LAS4000; GE Healthcare Life Sciences). Band intensity was assessed by the ImageJ software.

8. Statistical analysis

Data were analyzed using SPSS software and presented as means ± SEM. Differences between two experimental groups were analyzed with Student's T-test. Significant differences were considered when p values were less than 0.05.

CHAPTER IV

RESULTS

Analysis of F-actin distribution in Jurkat T cells treated with CytD

In order to study the function of actin polymerization in the recruitment of Nck1 to the TCR, CytD was used to disturb actin polymerization. To confirm that the the use of CytD has the expeted inhibitory effect on actin polymerization, Jurkat T cells were treated with 5 μM and 10 μM CytD for 30 min before TCR stimulation or left unstimulated as a control. Subsequently, the TCR was stimulated with the anti-idiotypic monoclonal antibody C305 that binds the variable regions of the endogenous TCR expressed by Jurkat cells (Weiss et al., 1984). Then the amount of polymerized actin (F-actin) was probed with rhodamine-conjugated phalloidin and was assessed by fluorescence microscopy (Figure 9a,b). As anticipated, TCR stimulation led to actin polymerization showing an apical ring of F-actin. CytD treatment prevented actin polymerization as compared to the staining pattern observed in C305 stimulated cells without CytD. The result suggested that 5 μM of CytD was sufficient to inhibit actin polymerization under our experimental system and this concentration was used in all following experiments.

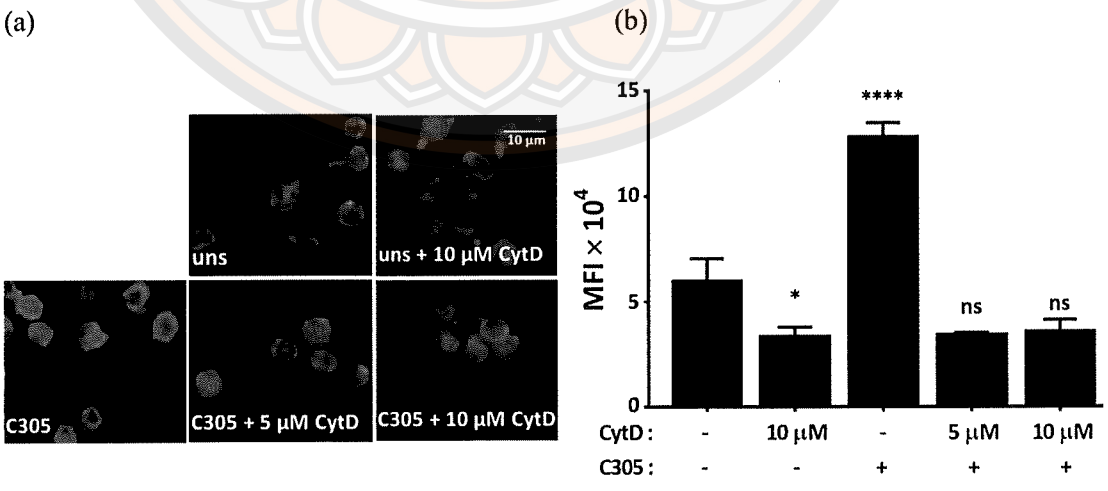


Figure 9 Inhibition of actin polymerization by cytochalasin D (CytD)

Jurkat T cells were treated without or with 5 or 10 μM of CytD for 30 min at 37°. Subsequently, the cells were stimulated for 5 min at 37° with the anti-T-cell receptor (TCR) antibody C305 (1 : 50) or left untreated (uns) (a). Cells were stained with rhodamine-coupled phalloidin to visualize F-actin formation using fluorescence microscopy. Original magnification is 600x. The fluorescence intensities of cells were quantified by IMAGEJ and the mean fluorescence intensity is displayed. Significant differences were determined by unpaired, two-tailed Student's t-test. Results were the average of two independent experiments (ns, non-significant, *P < 0.05, ****P < 0.0001) (b).

Requirement of actin polymerization for an efficient TCR-induced Nck1-CD3ε interaction

A possible involvement of actin polymerization in the recruitment of Nck to CD3ε has not been studied so far. To determine this, we used the in situ proximity ligation assay (PLA), which is a technique that allows visualisation of the close proximity between endogenous proteins in fixed cells by red fluorescent dot detection (Weiss et al., 1984). Recently, we established the PLA to quantify the proximity of Nck with the cytoplasmic tail of CD3ε in T cells using anti-Nck1 and anti-CD3ε antibodies (Paensuwan et al., 2016) (Figure 10a). Here, we left Jurkat T cells untreated as a control or treated them with CytD to prevent actin polymerization. Subsequently, the cells were stimulated with the anti-TCR antibody C305 for 5 and 10 min at 37°C or left unstimulated. PLA was performed with anti-Nck1 and anti-CD3ε antibodies and analyzed by fluorescence microscopy. The red fluorescent dots, which indicate the close proximity between Nck and the TCR were counted (Figure 10b). Without CytD treatment and as reported before (Paensuwan et al., 2016), we detected an increase in the number of red dots when the TCR was stimulated for 5 min compared to unstimulated cells, showing that endogenous Nck was recruited to CD3ε upon TCR stimulation. At 10 min of stimulation Nck recruitment to the TCR ceased, demonstrating that this is a very transient event following TCR triggering (Figure 10c).

In the presence of CytD, the anti-TCR induced proximity between Nck and CD3ε was delayed, so that Nck recruitment was hardly detectable after 5 min, but

prominent after 10 min of stimulation (Figure 10b, c). Thus, actin cytoskeletal rearrangement is necessary for a fast recruitment of Nck1 to the TCR, including a fast shut off of the signal. To test whether the induced Nck-TCR proximity was caused by Nck binding to the TCR, Jurkat cells were stimulated under the same conditions as in Figure 10b and subjected to immunoprecipitation with anti-CD3 antibody. Consistent with the data from the PLA, Nck binding to the TCR was increased upon TCR triggering (Figure 10d). Importantly, lower amounts of Nck were co-immunoprecipitated with the TCR from anti-TCR C305 antibody-stimulated and CytD-pretreated cells as compared with C305-stimulated cells alone (Figure 10d, e). Collectively, these data indicate that actin polymerization is required for an efficient recruitment of Nck to the TCR.

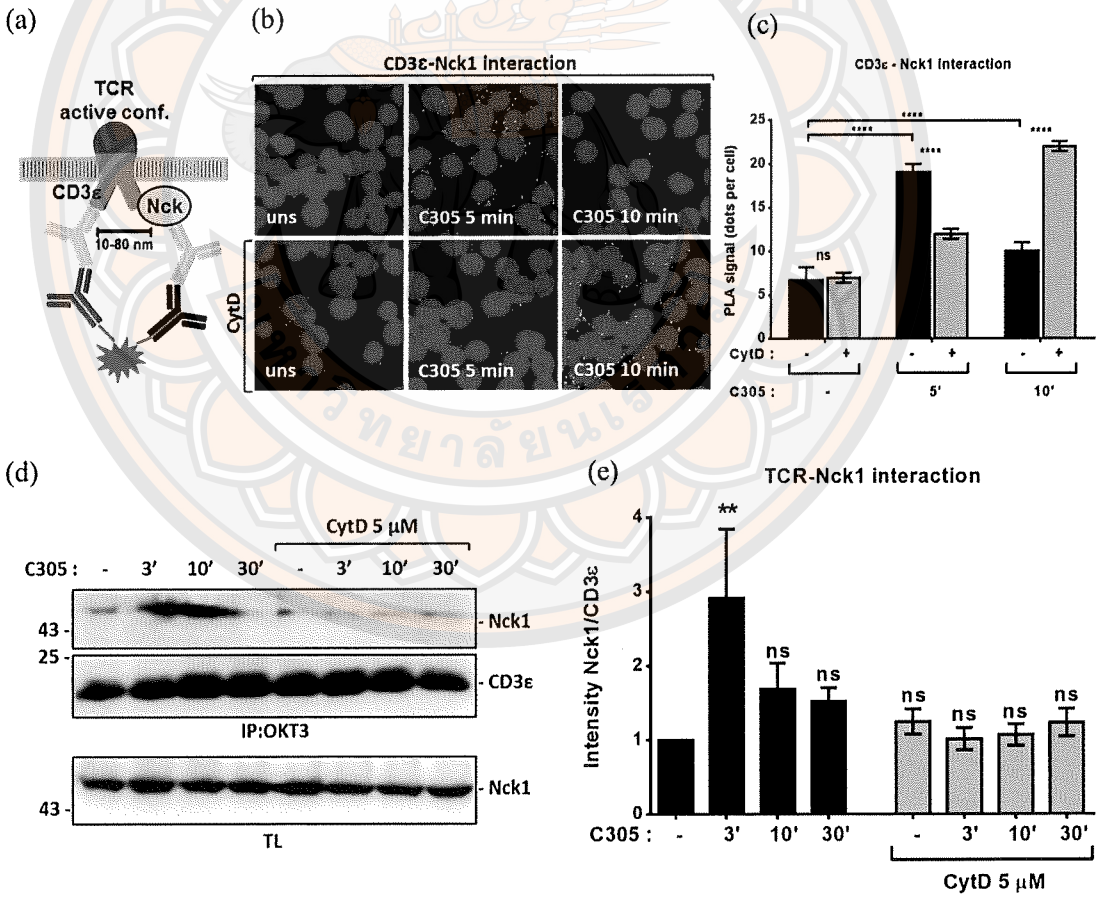


Figure 10 Involvement of actin polymerization in tyrosine phosphorylation of CD3ε and the recruitment of non-catalytic region of tyrosine kinase 1 (Nck1) to the T-cell receptor (TCR)

Schematic of the in situ proximity ligation assay (PLA) using anti-CD3ε and anti-Nck1 antibodies (a). The proximity between Nck1 and the TCR was detected by in situ PLA in intact fixed Jurkat cells. Jurkat T cells were either left untreated or treated with 5 μM cytochalasin D (CytD) for 30 min at 37°. Subsequently, cells were stimulated with the anti-TCR antibody C305 (1 : 50) for 5 and 10 min or left unstimulated. After fixation and permeabilization, PLA was performed using goat anti-CD3ε (M20ε) and rabbit anti-Nck1 primary antibodies, followed by secondary antibodies. Nuclei were stained with DAPI. Cells were imaged using a Nikon Eclipse Ti-U fluorescence microscope. Original magnification is 600x (b). The corresponding quantification of the red PLA dots from (b) and the mean SEM is displayed; statistical analysis was done by two-tailed Student's t-test. For each condition an average of 500 cells were analyzed. Three independent experiments were performed (c). Jurkat cells were pretreated with 5 μM CytD for 30 min at 37°C and stimulated as in (b). (d) Cell lysates were subjected to immunoprecipitation with the anti-CD3 antibody (OKT3). After sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the Western blot was developed with anti-Nck1 and anti-CD3ε antibody. The corresponding lysates were developed with anti-Nck1 antibody. Data are representative of four independent experiments. The intensity of the Nck1 and CD3ε bands in the immunoprecipitation was quantified using IMAGEJ software and is presented as a ratio of Nck1 to CD3ε normalized to the unstimulated/untreated cells. The data represent the mean SEM (ns, non-significant, $^{**}P < 0.01$, $^{****}P < 0.0001$) (e).

Antibody-mediated CD3ε phosphorylation was delayed under CytD treatment

Nck recruitment to the TCR requires both an interaction of Nck with the exposed PRS of CD3ε and with a phosphorylated tyrosine in CD3 (Paensuwan et al., 2016). Since the exposure of the CD3ε PRS occurs concomitantly with ligand-binding to the TCR (Gil et al., 2002; Swamy et al., 2016), we hypothesized that CD3ε phosphorylation might decrease under CytD treatment. To explore this possibility, we tested for CD3ε phosphorylation using C305 as a TCR stimulus and immunoprecipitation of the TCR (Figure 11a). In the absence of CytD, CD3ε phosphorylation was maximal at 3 and 10 min of stimulation and it was decreased at 30 min. In contrast, in the presence of CytD, there were lower amounts of tyrosine phosphorylation at CD3ε at all stimulation time points (Figure 11a). Hence, CytD prevented tyrosine phosphorylation of CD3ε after TCR mediated engagement.

To substantiate this finding, CD3ε tyrosine phosphorylation in total cell lysates was measured using the Luminex system and western blotting. To this end, Jurkat T cells were stimulated with the antibody C305 for the indicated time points in the absence or presence of CytD and the levels of tyrosine phosphorylated CD3ε were quantified (Figure 12a, b, respectively). Consistent with the immunoprecipitation data, the phosphorylation of CD3ε was a transient event peaking at around 5 min. In sharp contrast, CytD treatment resulted in a delay of phosphorylation of CD3ε with the peak occurring at 10 min (Figure 12c). Together this suggested that inhibition of actin polymerization was associated with a delayed and reduced tyrosine phosphorylation at CD3ε upon TCR triggering.

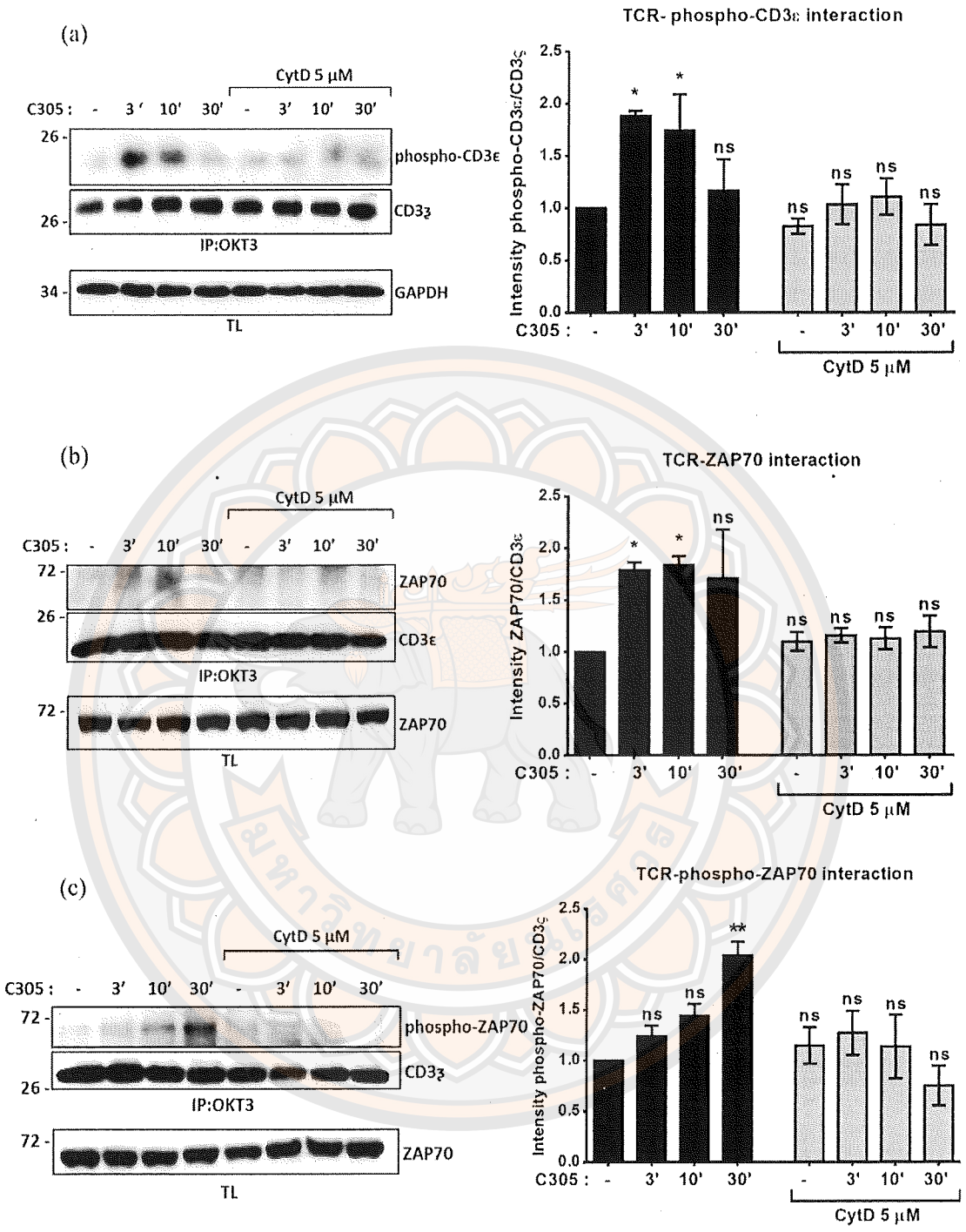


Figure 11 Inhibition of the actin polymerization impairs tyrosine phosphorylation at CD3e and recruitment of f chain-associated protein kinase of 70 000 MW (ZAP70) to the T-cell receptor (TCR)

Jurkat cells were left untreated or pretreated with 5 μM cytochalasin D (CytD) for 30 min at 37° before stimulation with the anti-TCR antibody C305 (1 : 50) at 37°C for the indicated time-points or left unstimulated (uns) as in Figure 10(d). After stimulation, cell lysates were subjected to immunoprecipitation with the anti-CD3 antibody OKT3. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) the Western blot was developed with anti-phospho-CD3ε and anti-CD3ζ antibodies. The lysates were developed with anti-GAPDH antibody (a). Jurkat cells were stimulated as in (a). Cell lysates were subjected to immunoprecipitation with the anti-CD3 antibody OKT3. After SDS–PAGE the Western blot was detected with anti-ZAP70 and anti-CD3ε antibodies (b), and anti-phospho-ZAP70 and anti-CD3ζ antibodies (c). The corresponding lysates were developed with an anti-ZAP70 antibody. The intensity of the phospho-CD3ε and CD3ζ bands (a), the ZAP70 and CD3ε bands (b), and the phospho-ZAP70 and CD3ζ bands (c) in the immunoprecipitation was quantified using IMAGEJ software and is presented as a ratio of phospho-CD3ε to CD3ζ (a), of ZAP70 to CD3ε (b), and of phospho-ZAP70 to CD3ζ (c) normalized to the unstimulated/untreated cells. Data are representative of four experiments, and the statistical analysis was performed as in Figure 10(e) (ns, non-significant, $*P < 0.1$, $**P < 0.01$).

Actin polymerization is required for the stimulation-induced interaction between ZAP70 and the TCR

To test whether the interaction between TCR and ZAP70 was altered by inhibiting actin polymerization, we performed a time course experiment of ZAP70 co-immunoprecipitation with the TCR using the anti-TCR C305 antibody as a stimulus in the presence or absence of CytD (Figure 11b). Binding of ZAP70 to TCR was decreased upon TCR triggering in the presence of CytD compared with C305 stimulated cells alone (Figure 11b). This was correlated with a decrease of phosphorylated ZAP70 which was co-immunoprecipitated with TCR in the presence of CytD (Figure 11c). These data indicate that actin polymerization is required for an efficient recruitment of ZAP70 to the antibody stimulated TCR.

Defective actin filament formation correlates with impaired TCR-induced phosphorylation of intracellular signaling proteins

Following the proximal TCR phosphorylation data of Figure 11, we hypothesized that upon TCR triggering actin polymerization would also be involved in more downstream signaling. To investigate this, Jurkat T cells were stimulated with the anti-TCR antibody C305 at 37°C for the indicated time points in the absence or presence of CytD. Then, the phosphorylation of ZAP70, Extracellular-signal Regulated Kinase (Erk) and the transcription factor CREB in total cell lysates were measured by the Luminex system (Figure 12c, 12e, 12g, respectively) and by Western blotting (Figure 12d, 12f, 12h, respectively). As predicted, the impaired actin polymerization was associated with decreased and delayed phospho-ZAP70, decreased phospho-Erk, and decreased phospho-CREB. Therefore, actin polymerization promoted the phosphorylation of ZAP70, Erk and CREB upon TCR triggering.

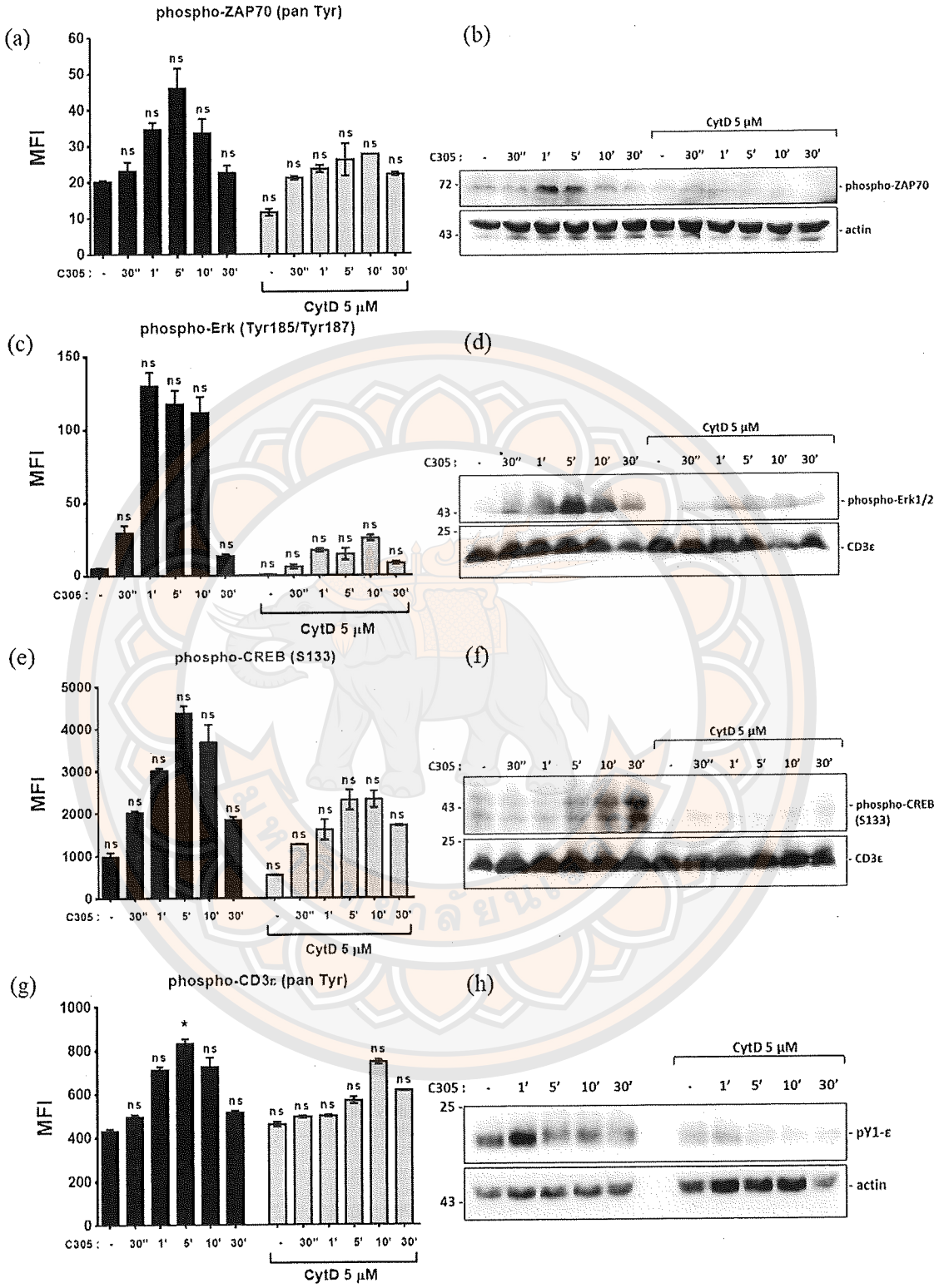


Figure 12 Down-regulation of the proximal and downstream signaling proteins upon disturbance of polymerized actin

Phosphorylation of CD3ε (pan-phospho-Tyr CD3ε) (a), ζ chain-associated protein kinase of 70 000 MW (ZAP70; pan-phospho-Tyr ZAP70) (c), Erk (phospho-Tyr185/ Tyr187) (e) and CREB (phospho-Ser133) (g) was measured with the Luminex system. Jurkat T cells that were either left untreated or pretreated with 5 μM cytochalasin D (CytD) for 30 min at 37°C before stimulation with anti-T-cell receptor (TCR) antibody C305 (1 : 50) at 37° for the indicated time-points or left unstimulated. Cells were then lysed and the mean fluorescence intensity (MFI) was measured using the MAGPIX, Luminexsystem. The graph represents the MFI of duplicate wells from two independent experiments. Jurkat cells were stimulated and lysed as in the Luminex experiments (a,c,e,g). Total cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot. Membranes were detected with anti-phospho-CD3ε (b), anti-phospho-ZAP70 (d), anti-phospho Erk1/2 (f) and anti-phospho-CREB (h) antibodies. The corresponding lysates were detected with an anti-actin or anti-CD3ε antibody. Data are representative of three independent experiments. Significant differences were determined by ordinary one-way analysis of variance (ns, non-significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Phosphorylation of CD3ε depends on the presence of Nck

Next, we tested whether CD3ε phosphorylation was caused by the recruitment of Nck to the TCR. To this end, we stimulated Nck1-CRISPR/Cas9 knock out (N1KO) and wild type Jurkat cells with the anti-TCR antibody C305 or left cells unstimulated. All cells expressed similar amount of TCR on their cell surface as shown by flow cytometry (Figure 13a). We showed that an efficient phosphorylation of CD3ε required the presence of Nck1, since this phosphorylation was reduced in N1KO cells after TCR stimulation compared to the control Jurkat cells containing Nck1. These data indicate that Nck recruitment to the TCR is required for an efficient phosphorylation of CD3ε (Figure 13b). Therefore, this finding places the recruitment of Nck to the TCR upstream of CD3ε phosphorylation.

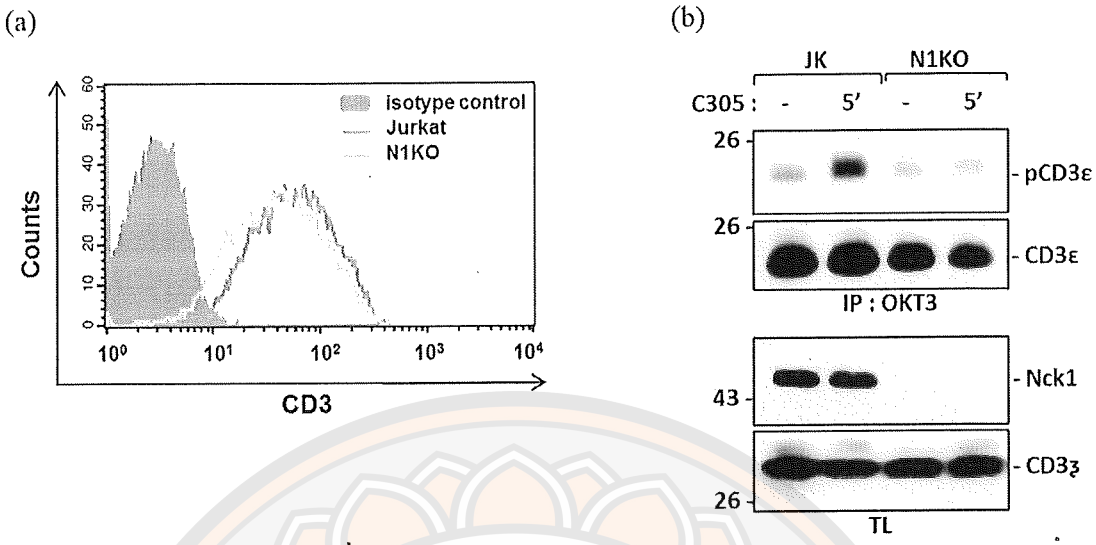


Figure 13 Non-catalytic region of tyrosine kinase 1 (Nck1) is required for the phosphorylation of CD3ε

Jurkat wild-type and Nck1-CRISPR/Cas9 knockout (N1KO) Jurkat cells were stained with allophycocyanin-conjugated anti-CD3ε antibody UCHT1. The expression levels of surface CD3 molecule of the cell clones were evaluated by flow cytometry. The clones with similar expression of T-cell receptor (TCR) were selected for further studies (a). The cells were either stimulated with C305 or left unstimulated for 5 min at 37°C. Cell lysates were collected and TCRs were immunoprecipitated with the anti-CD3 antibody OKT3. After sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the Western blot was developed with anti-phospho-CD3εY1 (pCD3ε) and anti-CD3ε antibodies. The corresponding lysates were developed with anti-Nck1 and anti-ζ antibodies (b).

CHAPTER V

DISCUSSION

This study demonstrated the role of actin polymerization in proximal TCR signaling involving the recruitment of the adaptor protein Nck to the TCR upon TCR triggering. Disruption of actin polymerization resulted in a delayed recruitment of Nck1 to the TCR. This was correlated with a delay in tyrosine phosphorylation of the CD3ε. In the present finding, Nck was recruited as early as 5 min after TCR ligation. A previous work has demonstrated that a mechanotransduction caused by TCR engagement at the immune synapse leads to actin polymerization (Comrie et al., 2016). Together with this present finding, Nck is required for CD3ε phosphorylation by Lck, in which F-actin acts to link Nck and Lck to initiate this phosphorylation. Nck and F-actin, therefore, could function to promote each other at proximal TCR signaling.

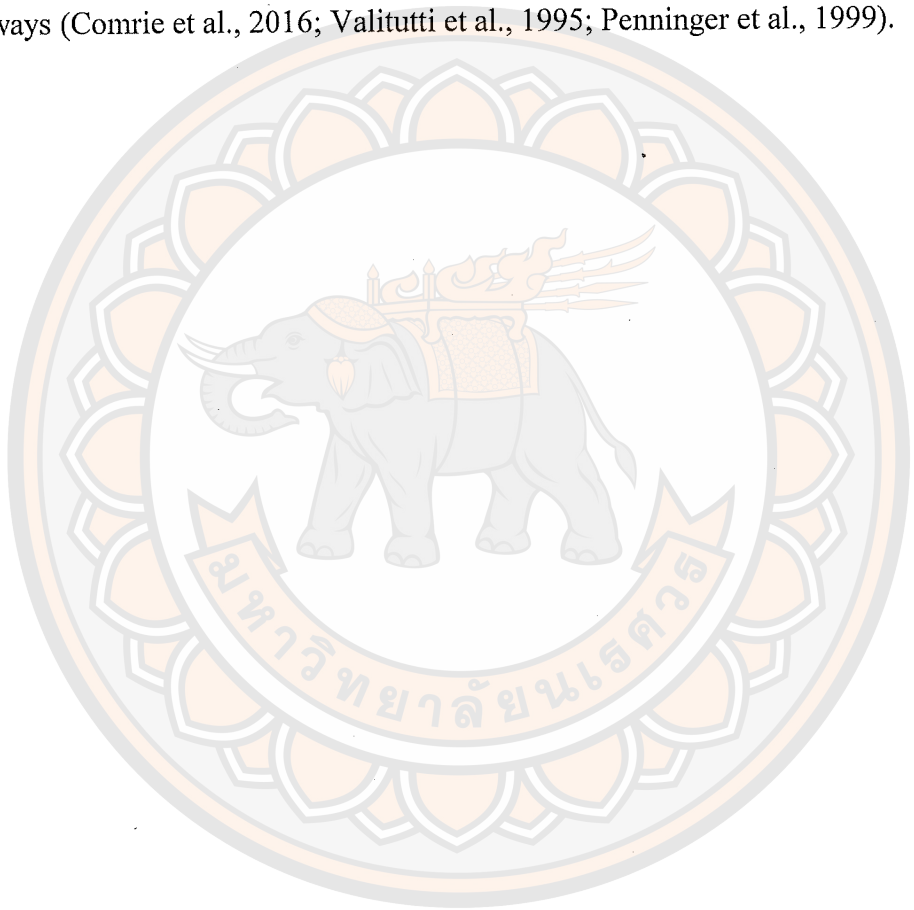
Inhibition of actin polymerization might delay CD3ε phosphorylation by the following mechanisms: i) Upon TCR triggering, Nck interacts with a proline motif of the unique domain of the lymphocyte specific protein tyrosine kinase Lck (vazquez et al., 2007), the interaction being enhanced by a specific T cell adaptor protein TSAd (Hem et al., 2015). Therefore, it could be that disruption of actin polymerization impairs recruitment of Nck-TSAd-Lck to the TCR, leading to delayed CD3 phosphorylation. ii) Proximal TCR signaling involves very early and simultaneous binding between Nck, CD3ε, and Lck. This signaling complex formation might require actin polymerization in stabilizing the recruitment of either of these molecules. Thus, inhibition of actin polymerization then causes a delayed formation of the signaling complex and as a consequence the delayed CD3ε phosphorylation. iii) Actin polymerization was shown to be important for the directed movement of TCRs upon TCR triggering. There has been a study using CytD to inhibit actin retrograde flow. It was demonstrated that actin retrograde flow is required for centripetal TCR microcluster transport and drives the receptor cluster dynamics at the immunological synapse in Jurkat T cells (Yi et al., 2012). Importantly, TCR clustering enhances phosphorylation of CD3 (Minguet et al.,

2007; Taylor et al., 2017). Thus, without actin polymerization TCR clustering might be delayed resulting in less CD3 phosphorylation.

TCR microcluster formation by transgenic TCR T cells on synthetic lipid bilayers with MHC-peptide agonist and ICAM-1 depends upon F-actin (Campi et al., 2005). Another report using latrunculin-A to inhibit F-actin formation showed that TCR microclusters stopped translocating immediately after latrunculin-A treatment. This inhibitory effect was primarily on disorganization of the pSMAC, but very little on the cSMAC, showing that the stability of the cSMAC might not depend on F-actin. In addition, the newly formed TCR-microclusters could be inhibited by F-actin inhibitor, whereas those already generated were resistant to F-actin inhibitor (Varma et al., 2006). These findings are in accordance with another report suggesting that the TCR microcluster formation is resistant to inhibition by Src family kinase inhibitor PP2, but can be inhibited by latrunculin-A (Campi et al., 2005). In our present study, phosphorylation of CD3ε was reduced when F-actin was inhibited, thus, it is likely that F-actin supports Src kinase-independent formation of TCR microclusters in response to MHC-peptide agonist for TCR signal amplification.

Actin drives the process of cell polarization and maintains the cell-cell contact; its polymerization also likely provides a scaffold for clustering, translocation and spatial segregation of proteins, these are key steps to amplify and maintain T cell signaling (Barda-Saad et al., 2005; Billadeau et al., 2007; Dustin et al., 2007; Gomez et al., 2008; Beemiller et al., 2010). These data were consistent with our study showing that actin polymerization is required for an efficient recruitment of ZAP70 and Nck1 to the antibody stimulated TCR. Therefore, the actin polymerization is essential for the translocation of signaling proteins, especially for the recruitment of proximal signaling molecules to TCR to sustain initial T cell signaling. The present finding shows that the presence of Nck is required for an efficient phosphorylation of CD3ε. However, F-actin has been demonstrated to be essential for micro-adhesion rings, LAT and SLP76 clusters, but not for TCR microclusters and ZAP70 clusters (Hashimoto et al., 2016). F-actin might support phospho-PLC-γ as actin foci similar to the F-actin localized at the core of the synapse-like structure that supports SLP76 clusters and the micro-adhesion ring (Hashimoto et al., 2016; Kumari et al., 2014). This is in agreement with the present study demonstrated that the inhibition of actin

filament formation resulted in the impaired TCR-induced phosphorylation of intracellular signaling proteins including ZAP70, Erk and CREB. Previous studies have also shown the involvement of F-actin in the function of calcium signaling molecules and calcium influx (Varma et al., 2006; Huppa et al., 2003; Valitutti et al., 1995). It has also been suggested that actin polymerization acts very early at immune synapse when TCR is ligated and it also serves as a scaffold to which signaling molecules would bind and thus be protected from degradation by proteasomal pathways (Comrie et al., 2016; Valitutti et al., 1995; Penninger et al., 1999).



CHAPTER VI

CONCLUSIONS

The present study reveals that the TCR engagement leads to actin polymerization, this might be caused by fast and very low level downstream signaling. The polymerized actin stabilizes the exposure of the PRS of CD3ε and allows the early binding of the SH3.1-Nck to the PRS. This transient and weak interaction promotes CD3ε phosphorylation, possibly because Nck can interact with Lck. Then, Nck can bind to TCR stably using its SH3 and SH2 domains, resulting in a subsequent induction of the downstream signaling cascade (Figure 14). Collectively, our present report identified the important mechanism of actin polymerization in the recruitment of the endogenous Nck1 molecule to phosphorylated CD3ε contributing to T cell activation.

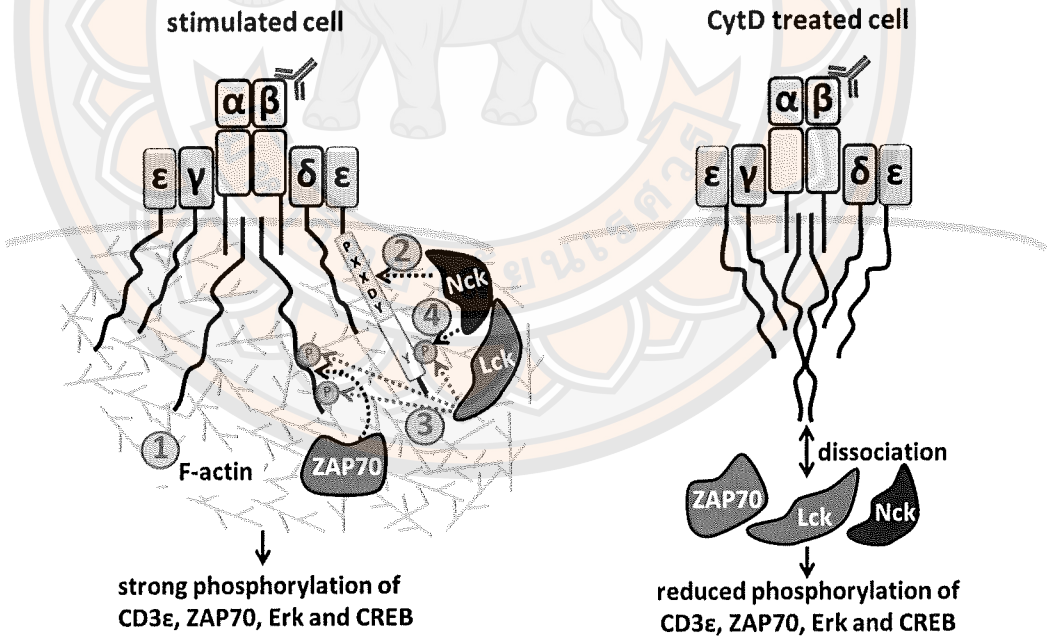


Figure 14 Model of the role of actin polymerization in promoting non-catalytic region of tyrosine kinase (Nck) -binding to the ligand-/antibody-engaged T-cell receptor (TCR)

Source: Wipa et al., 2019

Engagement of the TCR first leads to actin remodeling (1); this might be caused by fast and very-low-level downstream signaling. The polymerized actin promotes binding of the SH31(Nck) domain to the proline-rich sequence (light blue box) in the CD3ε (2). This transient and weak interaction promotes the phosphorylation of CD3, possibly due to the interaction of Nck with Lck (3). If the second immunoreceptor-tyrosine based activation motif (ITAM) tyrosine of CD3ε is phosphorylated, Nck can bind using its SH31 and SH2 domains in a cooperative manner (4). This binding mode is strong and together with f chain-associated protein kinase of 70 000 MW (ZAP70) binding to doubly phosphorylated ITAMs leads to T-cell activation (left panel). If actin polymerization is blocked, the following downstream events do not occur in an efficient manner (right panel).

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APPENDIX

APPENDIX A PREPARATION OF REAGENTS

1. PBS (phosphate-buffered saline), pH 7.2 to 7.4

NaH_2PO_4 (anhydrous; 1.9 mM)	0.23	g
Na_2HPO_4 (anhydrous; 8.1 mM)	1.15	g
NaCl (154 mM)	9.00	g

Add H_2O to 1 liter

2. 50% (v/v) protein G–Sepharose bead slurry

protein G–Sepharose bead (Sigma, Amersham Pharmacia Biotech) slurry in Non-denaturing lysis buffer

1. Take 150 µl of stock protein G bead and add with 300 µl lysis buffer.
2. Leave on the rotating wheel for 15 min at 4°C.
3. Spin at 2000 rpm for 30 sec and resuspend with the lysis buffer.
4. Repeat step 3 for 2-3 times.
5. Prepare 50% slurry by adding equal volume of lysis buffer to the beads.

3. 2X EMBO buffer

Tris-HCl, pH 8.0	40	mM
NaCl	274	mM
EDTA, pH8.0	4	mM
Glycerol	20 %	(w/v)

Adjust pH of buffer to 7.4 with HCl. Store up to 6 months at 4°C

4. Protease and Phosphatase inhibitors

Immediately before use add:

PMSF	1	mM

(store 100 mM stock in 100% ethanol up to 6 months at –20°C)

Leupeptin	10	µg/ml

(store 10 mg/ml stock in dH_2O up to 6 months at –20°C)

Aprotinin	10	µg/ml

(store 10 mg/ml stock in dH_2O up to 6 months at –20°C)

Sodium orthovanadate	0.5	mM

(Prepare a 50 mM solution in dH_2O. Store at –20°C)

Sodium fluoride	1	mM

(Prepare a 100 mM solution in dH_2O. Store at –20°C)

5. Non-denaturing lysis buffer, 1 ml

2X EMBO buffer	500	µl
50 mM Na_2VO_3	10	µl
1 mM NaF	10	µl
5 mM IAA	10	µl
1 mM PMSF	10	µl
100X protease inhibitor	50	µl
5% Brij 96	60	µl
dH_2O	400	µl

6. Wash buffer for immunoprecipitation, 100 ml

2X EMBO buffer	50	ml
5% Brij 96	10	ml
dH_20	40	ml

7. Separating gel solution 15 ml (12% gel)

30% acrylamide/0.8% bisacrylamide	6	ml
Tris-HCl, pH 8.8	3.75	ml
10% SDS	0.150	ml
dH_2O	5.1	ml
10% ammonium persulfate	7.5	µl
TEMED	75	µl

8. 30% acrylamide/0.8% bisacrylamide, 100 ml

Acrylamide	29.2	g
Bisacrylamide	0.8	g

Adjust vol. to 100 ml. filter the solution through a 0.45 µm filter.

Store at 4 °C in the dark.

Discard after 30 days.

9. Tris.Cl, pH 8.8 (1.5 M Tris.Cl , 100 ml

Tris base	18.15	g
dH_2O	60	ml

Adjust to pH 8.8 with 1 N HCl. Add H_2O to 100 ml total volume.

Filter the solution through a 0.45 µm filter.

Store at 4 °C.

10. 10% SDS

SDS	10	g
dH_2O	100	ml

store at RT

11. 10% ammonium persulfate (APS)

APS	0.1	g
dH_2O	1	ml

Make fresh before use.

12. Stacking gel solution 5 ml (4% gel)

30% acrylamide/0.8% bisacrylamide	0.65	ml
Tris-HCl/SDS, pH 6.8	1.25	ml
10% SDS	0.05	ml
dH_2O	3.05	ml
10% ammonium persulfate	5	µl
TEMED	25	µl

13. Tris.Cl/SDS, pH 6.8 (0.5 M Tris.Cl) 100 ml

Tris base	6	g
dH_2O	60	ml

Adjust to pH 6.8 with 1 N HCl.

Add H_2O to 100 ml total volume.

Filter the solution through a 0.45 μm filter, store at 4 °C.

14. 5X SDS - reducing sample buffer

0.5 M Tris-HCl pH 6.8	5	ml
100% glycerol	8	ml
10% SDS	0.150	ml
β-mercaptoethanol	2	ml
0.5% bromophenol Blue	2	ml
dH_2O	5	ml

Aliqout and store -20°C

15. 5X SDS - nonreducing sample buffer

0.5 M Tris-HCl pH 6.8	1.25	ml
100% glycerol	2	ml
10% SDS	2	ml
0.5% bromophenol Blue	0.5	ml
dH_2O	4.25	ml

Aliqout and store -20°C

16. 10X SDS- running buffer, 5 L

Tris-base	150	g
Glycine	720	g
SDS	50	g

Add H_2O to 5 L in total volume.

17. 10X semidry transfer buffer, 2 L

Tris-base	116.2	g
Glycine	68.6	g
10% SDS	75	ml
10% NaN_3	20	ml

Adjust to pH 8.3

Add H_2O to 2 L in total volume.

18. Working 1X semidry transfer buffer, 1L

1X semidry transfer buffer	800	ml
Methanol	200	ml

19. 4% paraformaldehyde in PBS, 50 ml

Paraformaldehyde (PFA)	2	g
PBS	50	ml

Let the solution in 56 °C in water bath until PFA powder dissolved completely (under hood)

Filter the solution through a 0.2 µm filter, store at -20 °C.

20. Wash buffer for immunoblotting, 0.1% Tween20 in PBS, 1000 ml

1X PBS	990	ml
10% Tween20	10	ml

21. 10X TBE buffer, 1 liter

890 mM Tris base	108	g
890 mM boric acid	55	g
0.5 M EDTA, pH 8.0	40	ml

22. Pervanadate (PV)

50 mM sodium orthovanadate	50	µl
dH_2O	57	µl
Mix		
30% H_2O_2 Mix	15	µl

Incubate for 5 min at RT and use within the next 30 min

The solution becomes a little bit yellowish/brownish.

23. RPMI 1640 10% FBS with everything:

RPMI 1640	500	mL
Inactivated FBS (fetal bovine serum)	50	mL
(100X) non-essential amino acids (L-glutamine)	5	mL
Streptomycin/penicillin solution (10000:10000)	5	mL

APPENDIX B PREPARATION AND TRANFORMATION OF COMPETENT *E. coli* USING CALCIUM CHLORIDE

1. Preparation of competent *E.coli*

Bacteria from glycerol stock are streaked on LB agar plate and incubated at 37°C for 24 hours. A single bacterial colony is inoculated into 3 ml of LB broth and further incubated at 37°C for 24 hours with agitation at 200 rpm. One millilitre of the culture broth is transferred to 100 ml LB broth in a 250 ml-flask and further cultivated for 3 hours with shaking at 200 rpm. Fifty millitters of bacterial culture are then transferred to a sterile ice-cold 50-ml polypropylene tube and stored on ice for 10 minutes. Cells are harvested by centrifugation at 2700 x *g* for 10 minutes at 4°C. The supernatant is discarded and the cell pellet is resuspended thoroughly by pipetting up and down in 1 ml of ice-cold $MgCl_2$-$CaCl_2$ solution followed by adding of 29 ml ice-cold $MgCl_2$-$CaCl_2$. Cells are stored on ice for 20 minutes. After centrifugation, the cell pellet is resuspended by gently swirling in 2 ml ice-cold 0.1 M $CaCl_2$. Cells are now ready for transformation.

2. Preparation of bacterial transformation

A total volume of 200 µl of $CaCl_2$-treated bacterial cells are transferred into sterile eppendorf tube. The plasmids of interest at a final amount of plasmid DNA of 100 ng are added to the cells that were then gently swirled. The sample is stored on ice for 30 min, and then incubated in water bath pre-set at 42°C for exactly 90 seconds without shaking. The sample is rapidly chilled on an ice bath for 1-2 minutes. 800 µl steriled LB medium are added to the sample and this was further incubated for 45 minutes at 37°C. 50-150 µl are placed on the LB agar containing 100 µg/ml of ampicillin using spread plate technique. The sample is incubated at 37°C for 12-16 hours.