CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Signaling transduction allows cells to response to changes in the environment outside them. Signaling pathways usually start with receptor proteins that alter intracellular molecules creating various signal transduction cascades. Several signaling molecules, which regulate the formation of multimeric protein complexes, can be divided into three groups: (1) enzymes, such as Src family tyrosine kinases, phospholipase Cγ and Ras GTPase-activating protein (RasGAP), (2) regulator molecules, such as the Vav family of Rho guanine nucleotide exchange proteins, Cbl and STAT proteins, and (3) adapter proteins without any known enzymatic capabilities (Buday, Wunderlich, and Tamas, 2002, pp. 723-732). Adaptor proteins usually contain several domains within their structure (e.g., Src homology 2 (SH2) and SH3 domains) which allow specific interactions with several other specific proteins. These proteins mediate specific protein–protein interactions that drive the formation of protein complexes for the activation of signal transduction cascades inside the cell. To date, the identified SH2/SH3 domain-containing adaptor proteins are Crk, Grb2, the p85 subunit of phosphatidylinositol-3' (PI-3') kinase, and Nck (McCarty, 1998, pp. 913-921).

The Nck Family of Adaptor Proteins

Nck is a cytosolic adaptor molecule of 47-kDa expressed in a wide variety of cell types and tissues. The human Nck cDNA was originally cloned by Lehmann, Riethmuller, and Johnson (Lehmann, Riethmuller, and Johnson, 1990, pp. 1048). Using monoclonal antibodies recognizing the melanoma specific MUC18 antigen, Nck was identified as a false positive during the screening of a melanoma cDNA expression library. Nck has been cloned for the second time by virtue of its ability to bind the phosphorylated C-terminal tail of the EGF receptor in the CORT screening technique (Skolnik, et al., 1991, pp. 83-90). The murine homolog of Nck, termed Grb4 was later cloned in attempting to identify novel SH2 domain containing proteins (Margolis, et al., 1992, pp. 8894-8898). In human cells, the Nck family comprises two

members (Nck1/Nckα and Nck2/Nckβ, also termed Grb4). Nck1 and Nck2 display 68% identity at the amino acid level. While the human *nck-1* gene has been localized to the 3q21 locus of chromosome 3, the *nck-2* gene can be found on chromosome 2 at the 2q12 locus (Huebner, et al., 1994, pp. 281-287; Vorobieva, et al., 1995, pp. 91-94). Notably, the largest differences are mainly located in the linker regions between the interaction modules (Figure 2). However, hardly any Nck1- or Nck2-specific downstream target has been identified so far. In fact, in many instances the interactions have not been clearly attributed to Nck1 or Nck2. Therefore, Nck1 and Nck2 are generally termed Nck in the published data. Nck acts as a link between extracellular and intracellular signaling molecules and cytoskeleton (Lehmann, Riethmuller and Johnson, 1990, pp. 1048; Park, 1997, pp. 231-236; Ullrich and Schlessinger, 1990, pp. 203-212). This molecule consists of a single C-terminal SH2 domain, which binds to phosphotyrosine residues, and three N-terminal SH3 domains, which bind to PRS within specific peptide sequence contexts of proteins. It has been reported that Nck is capable of associating with approximately 50 different proteins, suggesting that it is used by a wide variety of intracellular signaling pathways (Buday, Wunderlich and Tamas, 2002, pp. 723-731). Nck is capable of associating with a number of phosphotyrosine proteins, such as activated epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptor tyrosine kinases (Chou, et al., 1992, pp. 5834-5842; Li, et al., 1992. pp. 5824-5833; Meisenhelder and Hunter, 1992, pp. 5843-5856), downstream of tyrosine kinases (Dok) (Noguchi, et al., 1999, pp. 1748-1760), and SLP-76 (Wunderlich, et al., 1999, pp. 1068-1075) via its SH2 domain. Various biochemical analyses have identified several proteins which interact with one or more of the Nck SH3 domains, such as son of sevenless (Sos) (Hu, Milfay, Williams, 1995, pp. 1169-1174; Okada and Pessin, 1996, pp. 25533-25538), Vav (Ramos Morales, Druker and Fischer, 1994, pp. 1917-1923), Wiskott-Aldrich syndrome protein (WASP) (Rivero-Lezcano, et al., 1995, pp. 5725-5731), Nck-associated p21-activated kinase 1 (PAK1) (Bokoch, et al., 1996, pp. 25746-25749) and CD3ε (Gil, et al., 2002, pp. 901-912). Moreover, several Nck ligands bind to more than one SH3 domain of Nck suggests that a cooperative interaction is necessary for tight complex formation (Wunderlich, Goher and Farago, 1999, pp. 253-262). Nck utilizes the specificity of its individual SH3 domains to facilitate multiple interactions

with different associated proteins. In this way, Nck can recruit proline-rich proteins to the plasma membrane or multiple protein complexes found either in the cytoplasm or in association with the actin cytoskeleton. Many aspects of tissue development and homeostasis, activation and effector function of immune cells but also malignant transformation and invasiveness of tumor cells depend on regulated changes of cell polarity, morphology and migration that are often regulated through protein complexes formed by Nck (Buday, Wunderlich and Tamas, 2002, pp. 723-731; Lettau, Pieper and Janssen, 2009, pp. 1-13). In T cells, Nck plays a pivotal role in the T cell receptor (TCR)-induced reorganisation of the actin cytoskeleton and the formation of the immunological synapse. However two different mechanisms and adapter complexes are discussed below. In the first pathway, Nck is recruited to the TCR complex via phosphorylated SH-2-domain containing leukocyte protein of 76 kDa (SLP-76) (Wunderlich, et al., 1999, pp. 1068-1075), another central constituent of the membrane proximal activation complex. In the second pathway, dependent on an activation-induced conformational change in the CD3ε subunits, a direct binding of Nck to components of the TCR/CD3 complex was shown (Gil, et al., 2002, pp. 901-912). In this regard, activation-dependent association between Nck and CD3ε PRS occurs prior to ITAM phosphorylation. Thus, it is possible that the recruitment of Nck to CD3ε interferes with subsequent phosphorylation of the CD3εITAM by Src kinases. However, the relationship between ITAM and non-ITAM-requiring interactions in T cell signaling remains to be investigated.

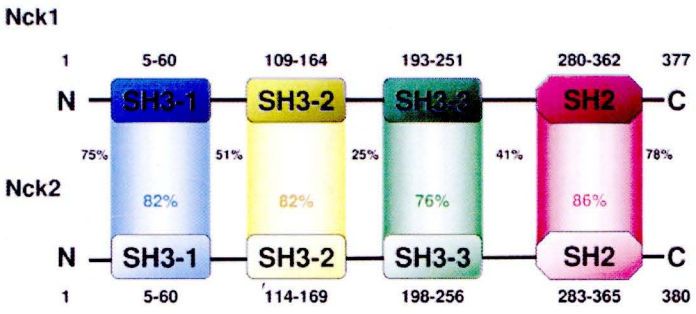

Figure 2 Modular composition of Nck adaptor protein.

Note: Nck adapters are proteins of 47 kDa consisting of three SH3 domains and a C-terminal SH2 domain linked by small spacer regions. Nck1 displays 68% amino acid identity to Nck2. As indicated in the figure, the differences between Nck1 and Nck2 are mainly located in the linker regions between the interaction modules, whereas the individual SH2 and SH3 domains show a high degree of homology.

Source: Lettau, Pieper and Janssen, 2009, pp. 1-13

Nck and T Cell Effector Function

Actin Reorganization in T Cell Activation

T lymphocytes play central roles in the adaptive immunity, both as modulators of other cells and as effector cells acting directly against infected cells. The activation of T lymphocytes is mediated by the interaction of T cell antigen receptors (TCRs) with their ligands, major histocompatibility molecule-peptide (MHC-peptide) complexes (Unanue, 1984, pp.395-428). The biochemical signals that are triggered in T cells by antigen recognition are transduced not by the TCR itself but by invariant proteins called CD3 and ζζ within the TCR complex. The TCR complex is a multi molecular complex composed of at least eight polypeptide subunits, a TCRα/β (or TCRγ/δ) heterodimer noncovalently bound to homodimers CD3εδ, CD3εγ, and ζζ. The CD3 and ζ chains function as the signaling components through their immunoreceptor tyrosine-based activation motifs (ITAMs) located in their cytoplasmic domains. The TCR:CD3 surface expression is essential for T cell development and function and is therefore a key element in the initiation of the adaptive immune response (Love, et. al., 1993, pp. 918-921; Dave, et. al., 1997, pp. 1360-1370; Haks, et. al.,1998, pp. 1871-1882; Wang, et. al.,1999, pp. 88-94).

TCR activation triggers actin dynamics for controlling cellular shape and polarity. This process regulates vital T cell responses, such as T cell adhesion, motility, and proliferation. These depend on the recruitment of Nck and WASP to the site of TCR activation. Upon optimal TCR engagement, ITAMs are phosphorylated, resulting in activation of Zap70. Activated Zap70 then phasphorylates linker for activation of T cells (LAT) and SLP-76. Nck can interact with phosphorylated SLP-76

through its SH2 domain and associate with PAK, Vav1, and WASP through its SH3 domains (Bubeck, et al., 1998, pp. 607-616; Bunnell, et al., 2002, pp.1263-1275; Fischer, et al., 1998, pp. 554-562; Galisteo, et al., 1996, pp. 20997-21000; Rivero-Lezcano, et al., 1995, pp. 5725-5731; Wunderlich, et al., 1999, pp. 1068-1075) (Figure 3). The main function of the multidomain adapter protein WASP is the activation of the actin-related proteins 2 and 3 (Arp2/3) complex that finally leads to the formation of branched actin filament networks. WASP is controlled by autoinhibition and is activated by binding to cell division cycle 42 (Cdc42) via its GTPase-binding domain. Activity can be further enhanced by phosphoinositides binding to a basic region of the WASP molecule. Interactions with proteins like WIP, intersectin or Grb2 and also phosphorylation by Src kinases have also been reported to affect WASP activity. However, in most cases, the precise molecular mechanisms are only poorly understood. Nck not only passively interacts with WASP and could thus recruit WASP to molecular activation clusters (Rivero-Lezcano, et al., 1995, pp. 5725-5731), but also modulates WASP activity (Rohatgi, et al., 2001, pp. 26448-26452). Therefore, this adapter protein also plays an essential role in the regulated activation-dependent reorganization of TCR-associated signaling complexes and platforms.

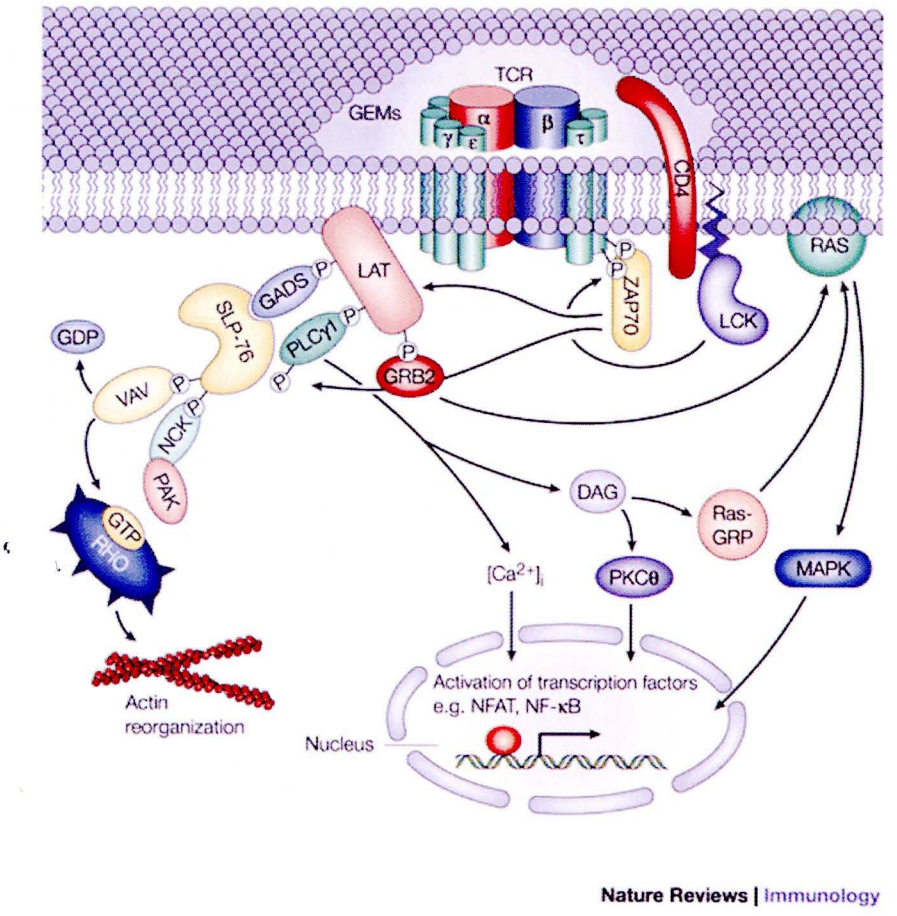

Figure 3 A model for TCR signaling.

Note: Following TCR engagement, SRC-family protein tyrosine kinases (PTKs; e.g. lymphocyte protein-tyrosine kinase; Lck) are activated, giving rise to phosphorylation of CD3 molecules of the TCR complex and activation of SYK-family PTKs (e.g. ζ-chain-associated protein 70 kDa; ZAP70). Activated ZAP70 phosphorylates linker for activation of T cells (LAT) and SLP76. Tyrosine-phosphorylated LAT then recruits several Src-homology 2 (SH2) domain-containing proteins, including growth factor receptor-bound protein 2 (GRB2), Grb2-related adaptor downstream of shc (GADS) and phospholipase Cγ1 (PLCγ1) to lipid rafts. Through its constitutive association with GADS, SLP76 is also recruited to LAT following TCR stimulation. SLP76 also constitutively associates with the SH3 domain of PLCγ1. The formation of a multimolecular complex between LAT, GADS, SLP76 and PLCγ1 is required

for optimal PLCγ1 activation. Activation of PLCγ1 results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 3,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 production leads to increases of cytosolic free Ca^{2+} $[Ca2^+]_i$, whereas DAG can activate both protein kinase C (PKC) θ and Ras guanyl nucleotide-releasing protein (RasGRP). Phosphorylated LAT also recruits the SH2 domain of GRB2 to lipid rafts, and therefore, the GRB2-associated Ras guanine nucleotide exchange factor (RasGEF), SOS, thereby providing an additional possible mechanism of Ras activation through LAT. Tyrosine-phosphorylated SLP76 also associates with the RHO-family GEF, VAV, and the adaptor protein, Nck. A trimolecular complex between SLP76, VAV and PAK1 has been proposed as a potential mechanism for SLP76 regulation of actin cytoskeletal rearrangements following TCR stimulation.

Source: Koretzky and Myung, 2001, pp. 95-107

Nck and TCR Signaling

The major issue in T cell activation is to understand how the ligand-binding components communicate to the signaling chains on the cytoplasmic side of the membrane upon appropriate interaction with MHC-peptide complex. Many models have been proposed for how TCR signaling is initiated, including TCR complex clustering and conformational change. The clustering model has stipulated that multimeric MHC-peptide brings individual TCR/CD3 into close proximity, thereby enabling transphosphorylation of the receptors by associated tyrosine kinases. This model has received much support based on the finding that soluble monomeric MHC-peptide, unlike dimeric or oligomeric MHC-peptide, is unable to elicit TCR-CD3 activation (Abastado, et al., 1995, pp. 439-447; Boniface, et al., 1998, pp. 459-466; Cochran, et al., 2000, pp. 241-250; Stone and Stern, 2006, pp. 1498-1505). Furthermore, T cell activation can be induced by intact anti-CD3 or anti- TCRα/β, but not by the corresponding Fab fragments (Kaye and Janeway, 1984, pp. 1397-1412). However, it has been shown that a small number of peptides, even one MHC-peptide complex molecule, can initiate signaling events (Irvine, et. al., 2002, pp. 845-849)

suggesting that clustering model does not account for the great sensitivity of the TCR complex.

Alternatively, conformational change in the TCR complex upon antibody binding has been proposed to explain studies of T cell signaling where receptor multimerization or the avidity of antibodies for the receptor do not sufficiently explain the biochemical results (Janeway, 1995, pp. 223-225). In general, crystallographic studies have argued against the idea of a large conformational change within the TCRαβ Ig ectodomains upon ligand engagement (Hennecke and Wiley, 2001, pp. 1-4; Rudolph and Wilson, 2002, pp. 52-65). Despite the lack of available structural data regarding the conformational change within the αβ heterodimer, there is increasing evidence that CD3 subunits undergo a ligand-induced conformational change. This idea is more attractive because the ITAMs in the cytoplasmic domains of these components are the proximal targets of tyrosine phosphorylation. Tyrosine residues within these motifs, once phosphorylated by the Src family tyrosine kinase (SFK), Lck and Fyn, become binding sites for different SH2-containing proteins, including the tyrosine kinase ZAP70 and then initiate a cascade of downstream signaling events that result in nuclear transcriptional changes as well as cellular morphological changes (Lin and Weiss, 2001, pp. 243-244). Several studies indicate that activated Zap70 promotes recruitment and phosphorylation of downstream signaling molecules including SLP-76, Vav1, a Rho family exchange factor, and Nck (Alberola-Ila, et al., 1997, pp. 125-154; Bubeck, et al., pp. 607-616; Galisteo, et al., pp. 20997-21000; Lin and Weiss, 2001, pp. 243-244). The assembly of this multiprotein complex regulates target effectors that interact through Nck, including Pak1. Consistent with this model, TCR-dependent activation of Pak1 and inducible association of Pak1 with Nck have been reported. Furthermore, dominant negative Pak1 and Nck inhibited TCR-mediated activation of nuclear factor of activated T cell (NFAT) transcription factor (Yablonski, et al., 1998, pp. 5647-5657).

Another current model for TCR signaling is a non-ITAM-requiring mechanism. In this regard, CD3ε cytoplasmic tail undergoes a conformational change that exposes the PRS for Nck SH3.1 binding upon multivalent ligands-mediated TCR triggering (Gil, et al., 2002, pp. 901-912; Gil, et al., 2005, pp. 517-522). These studies have shown that the exposure of TCR transgenic mouse T cells to antigen-pulsed

presenting cells promotes Nck binding to TCR/CD3 complex, which helps to allay concerns about the artificiality of the antibody induction. Nck recruitment to CD3ε occurs earlier than phosphorylation of the CD3ε ITAM and is independent of tyrosine kinase activity and disruption of the Nck/CD3ε interaction inhibits immunological synapse maturing and T cell activation. This mechanism might display an alternative means to more directly link T cell activation to the cytoskeleton.The protease-sensitivity assay has revealed that the cytoplasmic tails of CD3ε subunit adopt a compact structure in the triggering and conformational change is transmitted to the tails of CD3ε subunits. In non-stimulated cells, The CD3 subunits are in a closed conformation, marking the PRS and preventing its interaction with Nck (Risueno, Schamel and Alarcon, 2008, p. 1747). These would account for the ability of Nck to bind to CD3ε from resting T cells. However, because Nck and some of its binding proteins, including SLP76, are tyrosine phosphorylated (Buday, 1999, pp. 187-204; Lin and Weiss, 2001, pp. 243-244), it is proposed that the conformational change promoting Nck recruitment and the activation of phosphotyrosine kinases as a consequence of TCR-CD3 crosslinking soon come together into a common activation pathway (Gil, et al., 2005, pp. 517-522) (Figure 4). Nck-defective murine T cells failed to proliferate and to produce IL-2 upon stimulation with anti-CD3ε antibody but not with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Roy et al., 2010, pp. 15529-15534). Nck deletion also impaired TCR-mediated calcium mobilization and ERK phosphorylation in activated T cells. Conflicting data indicate that interaction between the CD3ε PRS and Nck is not required for T cell development and function (Szymczak, et al., 2005, pp. 270-275). In mice lacking CD3ε PRS motif, there is no difference observed in the number and percentage of T cell subsets in the thymus and spleen, and there is also no apparent defect in positive or negative selection. Furthermore, the proliferative response of mutant T cells to staphylococcal enterotoxin B (SEB) and anti-CD3 is normal. Biochemical and structure studies have demonstrated that the Nck-CD3ε is also capable of downregulating T cell activation by inhibiting of CD3ε ITAM phosphorylation and subsequent ITAM-dependent recruitment of downstream signaling molecules by Fyn and Lck kinases *in vitro* and/or reducing TCR cell surface expression upon physiological stimulation in mouse primary lymph node cells (Takeuchi, et al., 2008, pp. 704-716). In this context, Nck

binds to a noncanonical PxxDY region juxtaposed to the PRS in CD3ε chain. Accordingly, the PxxDy motif encompasses a putative internalization motif, YxxI/L, although this sequence has been described to behave only as a weak internalization signal of TCR/CD3ε endocytosis (Borroto, et al., 1999, pp. 25-31). Moreover, another experiment suggests that the capacity of CD3ε to bind Nck may serve to amplify TCR signaling and to promote ITAM phosphorylation in response to weak TCR stimuli (Tailor, et al., 2008, pp. 243-255). In this scenario, strong MHC agonists triggered cytokine release in both naïve and differentiated murine CD8+ T cell types whereas weak agonists, only affected differentiated cells. The responsiveness correlated with the ability of the agonist to elicit a CD3ε conformational change as measured by the ability of CD3ε to bind Nck. The mutation of the CD3ε proline motif and the ITAM significantly impaired the response to weak antigens in differentiated but not naïve cells. Therefore, the importance of Nck-mediate T cell activation is still a matter of debate.

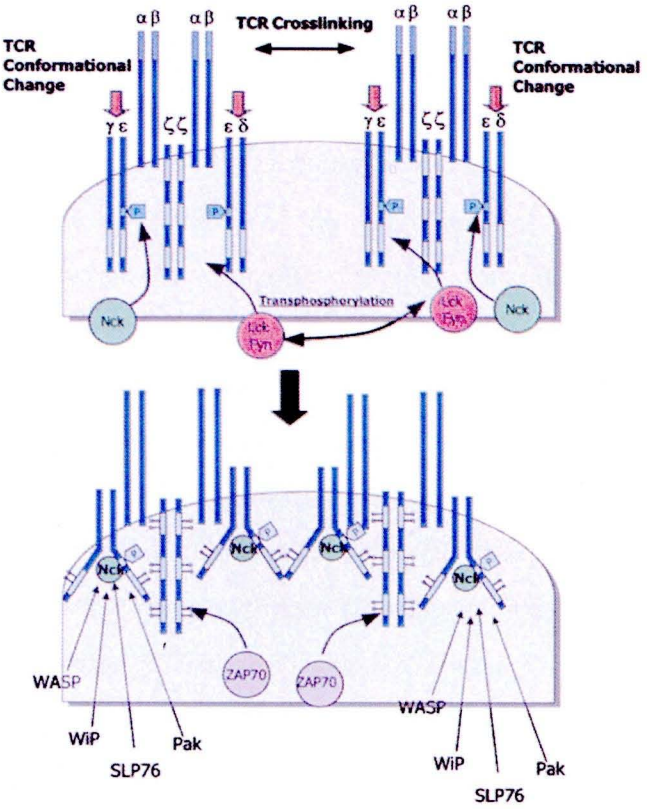

Figure 4 Model of T Cell Activation by TCR-CD3.

Note: Antigen/MHC binding to the variable regions of the TCRα/β heterodimer induces both TCR-CD3 clustering and a conformational change in the TCR. TCR-CD3 clustering is necessary for the activation of TCR-CD3-associated src kinases Lck and Fyn, which subsequently phosphorylate the ITAMs (indicated as pinned boxes). Independent from PTK activation and crosslinking, a conformational change in the cytoplasmic tails of the CD3 subunits is produced leading to an exposure of the PRS in CD3ε (indicated as a boxed P) and Nck recruitment. A second layer of intervention would be recruitment of the PTK, ZAP70 and other signaling proteins to phosphorylated ITAMs, and the recruitment of WASP, WAS-interacting protein (WIP), SLP76, and Pak to Nck.

Source: Gil, et al., 2002, p. 906

Nck Small Interfering RNA (siRNA) Transfection

The small RNA interference (RNAi) technique has emerged as a convenient and effective tool to manipulate the expression of specific gene for study a protein function. RNAi was first discovered in *Caenorhabditis elegans,* when it was noted that injection of a double-stranded RNA (dsRNA) that was homologous to a specific gene resulted in the efficient sequence-specific gene silencing (Fire, et al., 1998, pp. 806-811). siRNA, also known as short interfering RNA or silencing RNA, is 20-25 nucleotide-long double strand RNA molecule which can inhibit the expression of a particular gene, which shares a homologous sequence with siRNA. RNAi was first discovered in plant (Hamilon and Baulcombe, 1999, pp. 950-952) and in 2001, the synthetic siRNA can induce RNAi in mammalian cells (Elbashir, et al., 2001, pp. 494-498). siRNA is a part of post-transcriptional gene silencing process that requires active participation of cellular machinery. The process of RNAi consists of two main steps, an initiator step and an effector step. The first step in the RNAi pathway involves the processing of large dsRNAs into small, 21–23 nucleotide long siRNA molecules (Zamore, et al., 2000, pp. 25-33; Elbashir, et al., 2001, pp. 494-498). A specific RNAase III enzyme was then found to be responsible for cleaving the dsRNAs and

was named Dicer (Bernstein, et al., 2000, pp. 363-366). The second important stage of mRNA degradation is mediated by a multiprotein complex with nuclease activity known as RNA-induced silencing complex (RISC). The siRNAs are in turn unwound and incorporated into RISC, generating a sequence-specific nuclease that guides the cleavage of specific complementary mRNAs (Hammond, et al., 2000, pp. 293-296) (Figure 5). This guide role of siRNA was proposed after the observation that dsRNA would only lead to the degradation of an mRNA with a homologous sequence, leaving the rest of the RNA in the cell unaffected.

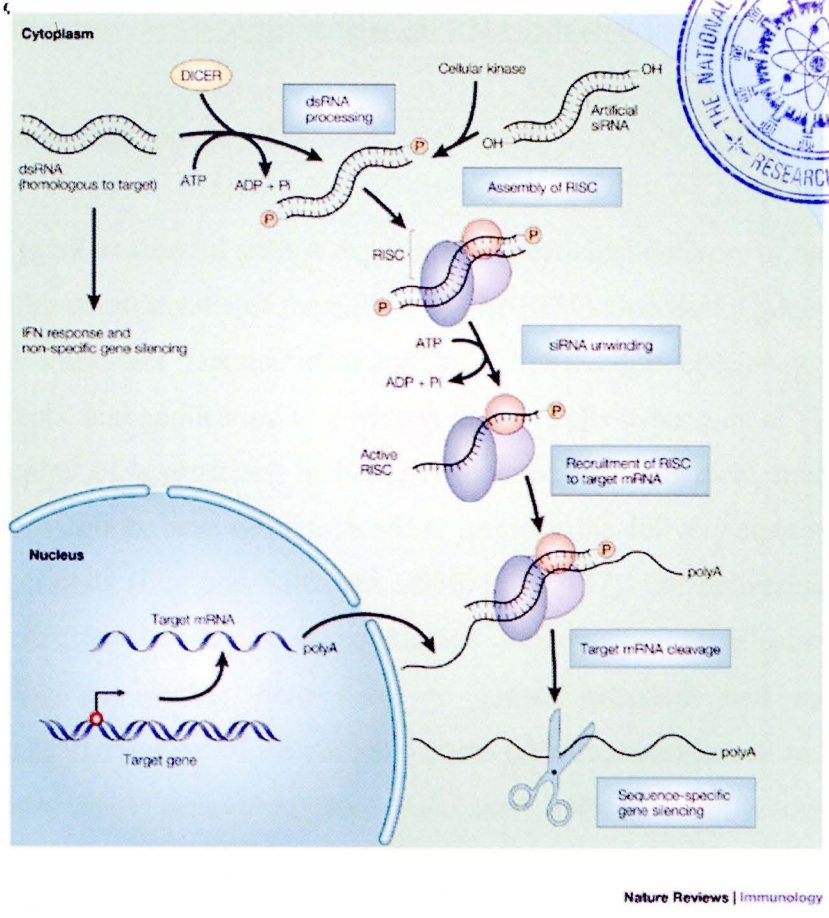

Figure 5 Gene silencing by RNA interference.

Note: Long double-stranded (dsRNA) is cleaved by DICER - a conserved RNase III protein - into RNA duplexes of 21–23 nucleotides that contain two unpaired

nucleotides at the 3' end of each strand. This reaction has been documented mainly in lower eukaryotic systems. In higher eukaryotes, dsRNA promotes non-specific inhibition of gene expression through the activation of the interferon (IFN) response. Artificial siRNAs are converted into functional siRNAs that have 5' phosphate groups by a cellular kinase. These artificial siRNAs do not need to be processed from long dsRNA. This allows activation of RNAi without concomitant activation of the IFN response. siRNAs assemble with cellular proteins to form RISC, which contains a helicase that unwinds the duplex siRNA and a ribonuclease that cleaves target sequences. The RISC is directed by the antisense strand of siRNA, through sequence complementarity, to the target messenger RNA, which is subsequently cleaved.

Source: Stevenson, 2003, pp. 851-858

A key to all successful siRNA experiments is efficient delivery of the siRNA into cells and subsequent uptake of the siRNA by the RISC. Successful gene delivery requires several important factors, including high transfection efficiency, lack of harmful side effects, and application to a wide variety of cells (Chang, et al., 1991, pp. 153-160). A number of approaches, including virus-mediated, chemical, mechanical, and electrical transfection, have been proposed to improve the delivery and expression of genes in target cells (Luo and Saltzman, 2000, pp. 33-37). For delivering siRNA into T-cell line, an effective and cost-saving method is electroporation. Electroporation involves applying an electric field pulse to induce transient cell membrane permeability via the formation of microscopic pores (electropores) in cell membrane. These electropores allow molecules, ions, water, and nucleic acids to traverse the membrane. If the electric field pulse has the proper characteristics, the electroporatd cells recover and continue to function normally. However, several problems exist with transfection by electroporation method. First, it has a relatively low cell viability and transfection rate than any other transfection method. Second, there is a need for optimization of the cell-specific protocol in order to increase transfection efficiency. Third, it involves a sensitive and complicated experimental process (Chang, et al., 1991, pp. 153-160). Therefore, a novel electroporation device using a capillary tip and

a pipette that was effective on a wide range of mammalian cells, including cell lines and primary cells has been developed. The capillary electroporation system considerably reduced cell death during electroporation because of its wire-type electrode, which has a small surface area. The capillary electroporation minimized the effective surface area of the electrode so that the transfection rate was greatly enhanced compared to that of a conventional cuvette. The three key parameters that influence eletroporation efficiency are voltage, pulse-length, and number of pulses. A common strategy to detect siRNA-mediated gene silencing has been semiquantitative or quantitative reverse transcription-polymerase chain reaction (RT-PCR). A disadvantage of this approach is that down-regulation at the mRNA transcript level may not extend to the protein level. To directly measure knockdown protein levels by siRNA, Western blot methodology using specific antibodies is the most efficient way (Burns, et al., 2003, pp. 5556-5571; Leirdal and Sioud, 2002, pp. 744-748).

siRNA-mediated gene silencing in the Jurkat T cell line is a transient phenomenon because there was a modest increase in the targeted protein levels after 72 hours compared to 48 hours post-transfection (Vang, et al., 2004, pp. 2191-2199). A partial knock-down of Nck by electroporated siRNA transfection yielded 50-60% reduction of Nck protein level in PHA-stimulated T cell blasts at 72 hours post-transfection (Lettau, et al., 2006, pp. 5911-5916). siRNA against Nck1 and Nck2 can also down-regulate the endogenous Nck1 and Nck2 in pheochromocytoma cell line PC12 and cortical neuron from rat (Guan, et al., 2007, pp. 6001-6011). Nck protein level has been shown to be reduced to <10% of the control level after Nck1 siRNA treatment in immunoblotting from rat mammary adenocarcinoma cells, MTLn3 (Yamaguchi, et al., 2006, pp. 441-452).

Relationship of Nck with Apoptosis

Apoptosis, or programmed cell death, is a form of cell death characterized by distinct morphological changes and energy-dependent biochemical mechanisms. Various morphological features occur during apoptotic process such as membrane blubbing, cell shrinkage with preservation of an intact plasma membrane, chromosomal condensation, and DNA fragmentation. Apoptotic cells exhibit several biochemical modifications such as protein cleavage, protein cross-linking, DNA

breakdown, and phagocytic recognition. Apoptosis occur normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissue. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001, pp. 367-401). Several methods have been developed for the quantitative detection of apoptotic cells, including technique based on the labeling of apoptotic cells by a Ca^{2+}-dependent phosphatidylserine-binding protein termed Annexin-V. In the early stages of apoptosis, phosphatidylserine is translocated from the inner to the outer layer of the cytoplasmic membrane, resulting in the exposure of this phospholipids at the external cell surface (Vermes, et al., 1995, pp. 39-51). Externalization of phosphatidylserine residues on the outer plasma membrane of apoptotic cells allows detection of Annexin V in tissues, embryos, or cultured cells. A fluorochrome, such as Propidium iodide (PI) is commonly used for identifying late apoptotic or necrotic cells by assessing plasma membrane integrity. Disruption of plasma membrane allows uptake of PI, while intact plasma membranes exclude it. The ability of PI to bind and label oligonucleosomal fragments makes it possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometry (Nicoletti, et al., 1991, pp. 271–279).

Nck translocates to the nucleus upon cellular stress (Kremer, Adang and Macara, 2007, pp. 837-850). In this scenario, the Nck-interacting protein, suppressor of cytokine signal-7 (SOCS7) (Martens, et al., 2004, pp. 239-248; Matuoka, et al., 1997, pp. 488-492), regulates the nucleocytoplasmic distribution of Nck. Septins in turn bind to SOCS7 and this interaction retains both SOCS7 and Nck in the cytoplasm. Following DNA damage, both proteins accumulate in the nucleus. This accumulation is essential both for morphological changes (e.g. the disintegration of stress fibers and loss of cell-polarity) and for the activation of downstream members of the DNA damage cascade and cell-cycle arrest. Thus, Nck somehow links a DNA damage checkpoint to the actin cytoskeleton (Figure 6). Interestingly, the set of nuclear Nck-interacting proteins significantly differ from cytosolic interactions (Lawe, Hahn and Wong, 1997, pp. 223-231). However, only few nuclear Nck-interacting proteins have been identified so far. Therefore, the depletion of Nck from the cytosol presumably accounts for the observed effects rather than a specific action within the nucleus. Nck has also been implicated in cellular responses to endoplasmic reticulum stress, which

leads to the inhibition of translation and downregulation of protein synthesis (Latreille and Larose, 2006, pp. 26633-26644). In this context, nuclear Nck might recruit additional effectors/regulators of the actin machinery to the site of gene transcription by its interaction with the SFPQ/NONO complex. A nuclear localization of Nck in 1–5% of the individual human T cells and a colocalization with nuclear proteins SFPQ and NONO were observed (Lettau, et al., 2010, pp. 658-669). Nevertheless, the physiological trigger for the nuclear translocation of Nck as well as the precise function of nuclear Nck in T cells remain to be investigated.

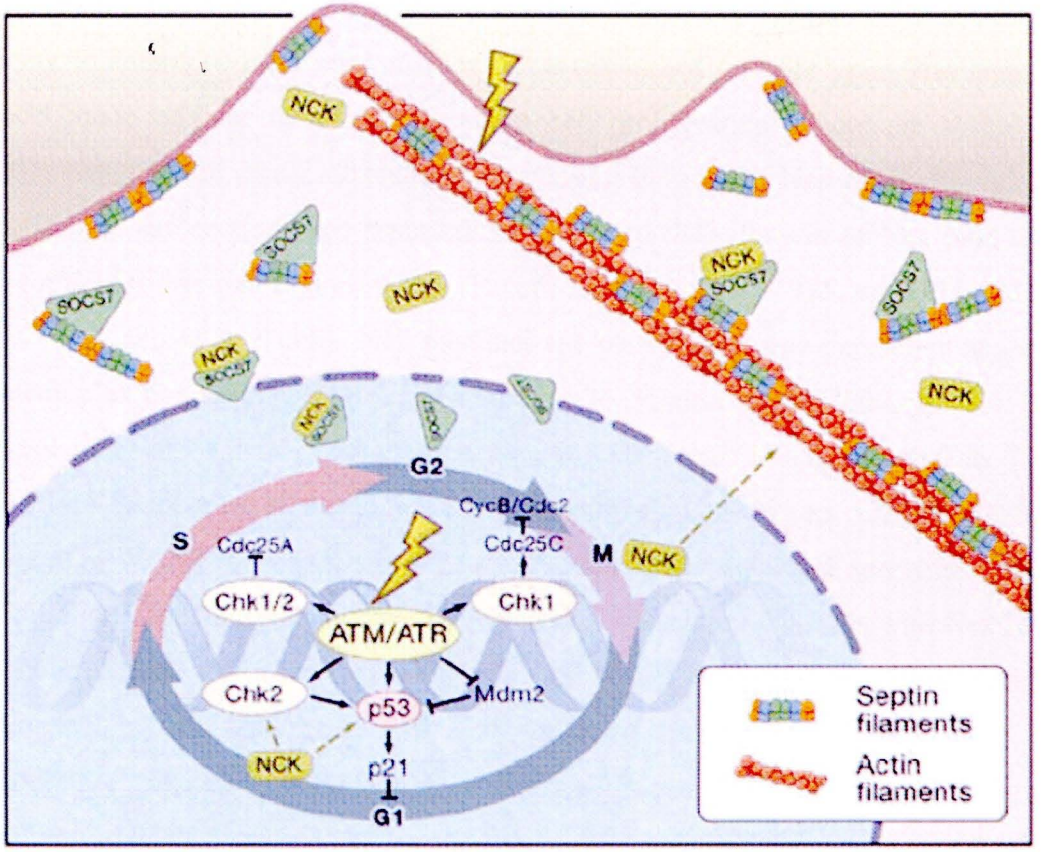

Figure 6 Septins and DNA damage checkpoint.

Note: Septin heteropolymers serve as cytiplasmic sinks for the adaptor protein SOCS7. SOCS7 autonomous transfers to nucleus, accompany Nck, another multifunctional adaptor protein involved in actin polymerization. Perturbation of septin filaments, as well as genotoxic stress such as UV irradiation, induces

nuclear translocation of Nck, which disorganizes actin stress fibers and modifies the cell-cycle/DNA damage checkpoint machinery.

Source: http://140.116.60.1/lin5612/microimm/96-semi/cchang2.htm

Relationship of Nck with T Cell Proliferation

Proliferative expansion of T cells is critical for effectiveness of the adaptive immune system. Several reports have been shown that Nck interacts with Grb2 and SOS GTP exchange factor (Hu, Milfay and Williams, 1995, pp. 1169-1174; Okada and Pessin, 1996, pp.25533-25538; Wunderlich, Farago and Buday, 1999, pp. 25-29), leading to enhanced transcription from a Ras-dependent reporter gene. Ras signaling affects many cellular functions, including cell proliferation. There are a few past studies that provide detail information concerning the role of Nck on proliferation of T cells. The earlier study has reported that the third SH3 domain of Nck bind to the C-terminal proline-rich region WASP (Rivero-Lezcano, et al., 1995, pp. 5725-5731).

In activated T cells, Nck mediates the phosphotyrosine-dependent assembly of a complex containing WASP, Nck and SLP-76 (Krause, et al., 2000, pp. 181-194). Several lines of evidence suggest that Nck/WASP interaction is essential in T cell activation (Snapper, et al., 1998, pp. 81-91; Zhang, et al., 1999, pp. 1329-1342). Mice deficient in WASP show specific defects in F-actin production, IL-2 secretion, and cell proliferation. Conflicting data have indicated that interaction of CD3ε with Nck might not be essential for T cell development and T cell response to strong antigens (Szymczak, et al., 2005, pp. 270-275). In mice lacking CD3ε PRS motif, the proliferative response of mutant T cells to SEB and anti-CD3 mAb is normal. Proliferation of PRS-mutant naïve mice T cells does not differ from wild-type CD3ε in response to strong agonists (Tailor, et al., 2008, pp. 243-255). A recent study employing a highly sophisticated mouse model has shown that Nck-defective T cells fail to proliferate upon stimulation with anti-CD3ε Ab but not with the DAG mimetic PMA and ionomycin (Roy, et al., 2010, pp. 15529-15534). The unmodified response of Nck-deficient T cells to PMA and ionomycin stimulation is due to signaling pathway that bypasses the TCR signaling apparatus (Figure 7). PMA induces a PKCs-mediated oxidative signal and RasGRP activation, whereas ionomycin induces

intracellular calcium mobilization. These results suggest the involvement of Nck in proximal TCR signaling.

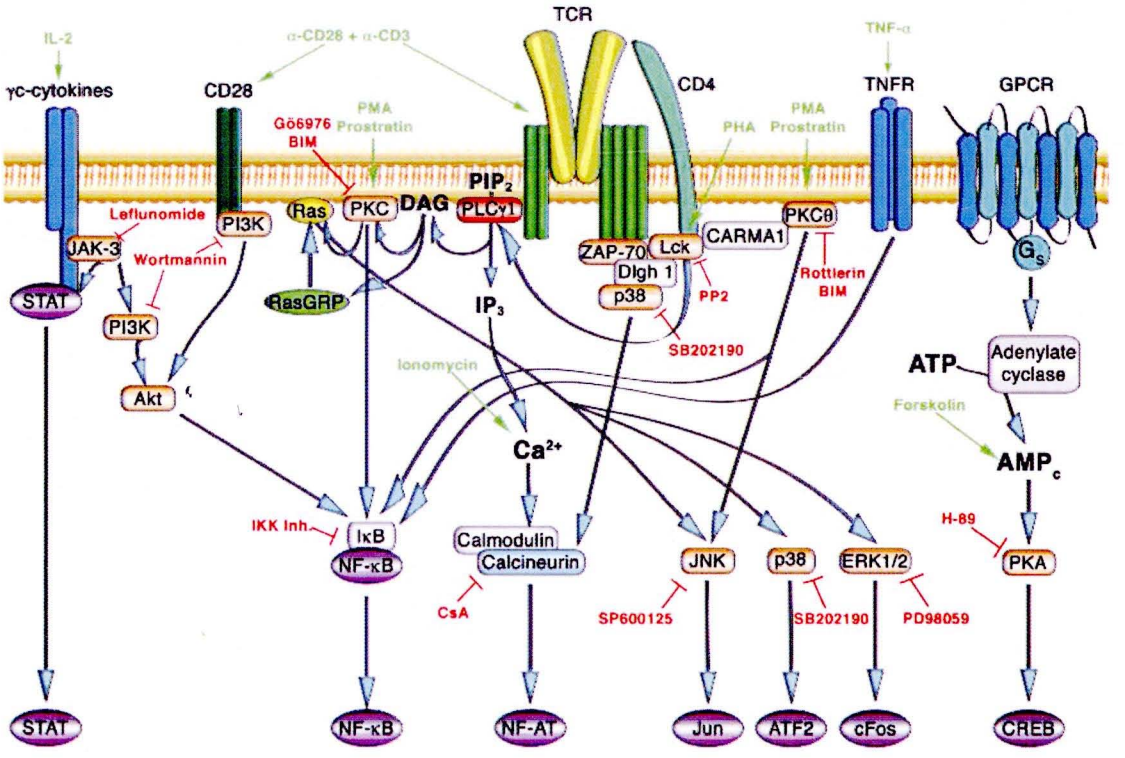

Figure 7 T cell-signaling pathways.

Note: PMA and ionomycin induce PKC activation and intracellular calcium mobilization which bypassing the TCR signaling machinery.

Source: Bosque and Planelles, 2009, pp. 58-65

The Activation Antigen CD 69

The CD69 molecule, also designated as activation inducer molecule (AIM), early activation Ag-1, leu-23, and MLR-3 Ag, is a member of the NK cell gene complex family of C-type lectin-like signal-transducing receptors. The human CD69 gene is located on the chromosome 12p13, a region that contains susceptibility loci for several autoimmune diseases (Lopez-Cabrera, et al., 1995, pp. 21545-21551), syntenic to the Cia3 trait loci on rat chromosome 4 and mouse chromosome 6 (Ziegler, et al.,

1993, pp. 1643-1648). Sequence analysis of the 5'-flanking region of the CD69 gene revealed the presence of a potential TATA element 30 base pairs upstream of the major transcription initiation site and several putative binding sequences for inducible transcription factors (NF-κB, Egr-1, AP-1), which might mediate the inducible expression of this gene (Cebrián, et al., 1988, pp. 1621-1637; Santis, et al., 1995, pp. 2142-2146; Testi, Phillips and Lanier, 1989, pp. 1854-1860). CD69 is a type II transmembrane glycoprotein with C-type lectin, disulfide-linked dimer composed of two glycosylated polypeptide chains of 28 and 32 kDa (Cosulich, et al., 1987, pp. 4205–4209; Hara, et al., 1986, pp. 1988-2005). Both chains have the same 24-kDa protein backbone, but appear to be differently glycosylated by N-linked oligosaccharides in their extracellular domains (Bjorndahl, et al., 1988, pp. 4094-4100; Lanier, et al., 1988, pp. 1572-1585). The three molecular forms exist by randomly association of 28-28, 28-32 and 32-32 kD chains present on the surface of the same cell (Figure 8). Both dimer chains are constitutively phosphorylated on serine residues (Cosulich, et al., 1987, pp. 4205-4209; Gerosa, et al., 1991, pp. 159-168; Hara, et al., 1986, pp. 1988-2005). Human CD69 consists of an intracellular domain of 40 residues, a transmembrane domain of 21 residues, and an extracellular domain of 138 residues and contains only a single consensus sequence for N-linked glycosylation site (Hamann, Fiebig and Strauss, 1993, pp. 4920-4927; Lanier, et al., 1988, pp. 1572-1585; Ziegler, et al., 1993, pp. 1643-1648).

CD69 is not present on the surface of resting hematopoietically derived leukocytes, but is rapidly expressed upon activation. Thus, CD69 is persistently expressed in vivo by T cells under certain conditions characterized by chronic inflammation (Laffo´n, et al., 1991, pp. 546-552) and in vitro on constant stimulation with proinflammatory cytokines or through certain adhesion receptors (McInnes, et al., 1997, pp. 189-195; Sancho, et al., 1999, pp. 886-896). A specific ligand for CD69 has not been identified but has been postulated to involve carbohydrate moieties (Bajorath, et al., 1994, pp. 4094-4100; Bezouska, et al., 1995, pp. 68-74). The CD69 molecule is expressed on the surface of T cells upon *in vitro* activation with a wide variety of agents, including anti-CD3/T cell receptor and anti-CD2 mAbs, activators of PKC, and PHA (Cebrián, et al., 1988, pp. 1621-1637; Hara, et al., 1986, pp. 1988-2005). CD69 cross-linking transduces intracellular signals that generate a variety of cellular

responses, suggesting that CD69 is a multipurpose cell-surface trigger molecule important in the development and activation of many different hematopoietic cell types, including bone marrow cells, monocytes, platelets, T and B lymphocytes, and natural killer cells (Moretta, et al., 1991, pp. 1393-1398; Testi, et al., 1994, pp. 479-483; Testi, Phillips and Lanier, 1989, pp. 1123-1128 ; Ziegler, Ramsdell and Alderson, 1994, pp. 456-465). Several reports have suggested that CD69 involved in both cell migration and cytokine secretion upon leukocyte activation (Esplugues, et al., 2003, pp. 1093-1106; Feng, et al., 2002, pp. 535-544; Nakayama, et al., 2002, pp. 87-94; Shiow, et al., 2006, pp. 540-544). However, CD69 engagement also triggers apoptosis in different cell types, such as monocytes or eosinophils (Ramirez, et al., 1996, pp. 192-199; Walsh, et al., 1996, pp. 2815–2821) and might mediate inhibitory signals on IL-1 receptor (IL-1R)- or CD3-mediated T-cell proliferation (Cosulich, et al., 1987, pp. 4205-4209).

In T cells, CD69 is detectable after stimulation of the TCR/CD3 complex or after the T cells are stimulated by phorbol esters. Induction of CD69 mRNA in activated murine thymocytes and T cells is very rapid, peaking between 30 and 60 min poststimulation, and transient, dropping to nearly resting levels by 8 h (Ziegler, et. al., 199, pp. 1228-1236). After human T cell stimulation, transcripts are rapidly induced as early as 30 minutes and CD69 membrane molecule is observed 3 hours later (Lopez-Cabrera, et al., 1993, pp. 537-547). CD69 acts as a costimulatory molecule for activation of PKC (Bjorndahl, et al., 1988, pp. 4094-4100; Cosulich, et al., 1987, pp. 4205-4209). Anti-CD69 mAb induced a prolonged elevation of intracellular [Ca2+] but this signal alone is unable to effectively activate PKC. When PKC is simultaneously activated by PMA, stimulation of CD69 induces expression of IL-2 and IFN-gamma genes, enhancement of CD25 expression, and ultimately resulting in IL-2/CD25 dependent T cell proliferation (Testi, Phillips, and Lanier, 1989, pp. 1854-1860). The intracellular signals through CD69 result in increase intracellular calcium concentration and enhanced binding activity of the transcription factor AP-1, which is considered to play an important role in the early events of cytokine and cytokine receptor synthesis in T cells (Castellanos, et al., 1997, pp. 5463-5473; Lopez-Cabrera, et al., 1995, pp. 21545-21551). Furthermore, rapid degradation of CD69 mRNA contributes to the regulation of CD69 expression on the cell surface (Santis, et al.,

1995, pp. 2142-2146). CD69 possibly contributes to the deletion of autoreactive lymphocytes by inducing apoptosis and, thus, abnormal expression of CD69 could be involved in the pathogenesis of autoimmunity (Croft, 1994, pp. 431-437; Green and Scott, 1994, pp. 476-487). Many clones of Jurkat cells, including E6-1 have been reported to express CD69 upon stimulation with anti-CD3 Ab (Münch, et al., 2005, pp. 10547-10560), PMA plus PHA (Brocardo, et al., 2001, pp. 297-305), PHA (Münch, et al., 2005, pp. 10547-10560), or PMA plus ionomycin (Roose, et al., 2005, pp. 4426-4441; Taylor-Fishwick and Siegel, 2005, pp. 3215-3221). It has been shown that TCR ligation is associated with a rapid conformational change in the CD3ε that exposes an Nck-binding PRS (Gil et al., 2002, pp. 901-912). In this study, Nck-CD3ε interaction-inducing (OKT3) Ab can induce both IL-2 release and CD69 expression in Jurkat cells. The authors proposed that the recruitment of Nck to the TCR:CD3 complex plays an important role in TCR signaling. In contrast, a functional analysis has shown that there are no differences observed in CD69 expression after SEB stimulation in T cells from mice expressing wild-type or CD3ε PRS mutation (Szymczak et al., 2005, pp. 270-275). These data suggest that CD3ε:Nck interaction is not required for T cell activation and function.

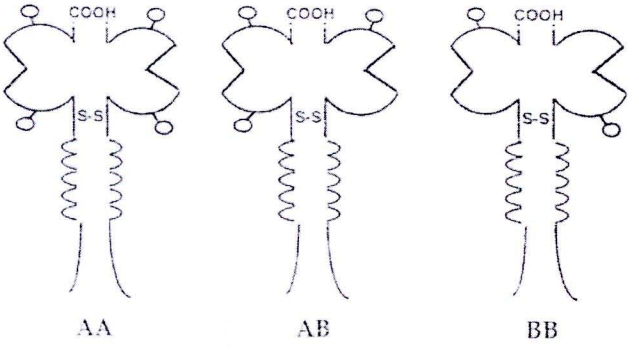

Figure 8 Multiple dimeric forms of human CD69 molecules result from Variable modification of typical and atypical glycosylation motifs.

Note: A schematic diagram shows the various forms of CD69 proteins expressed at the cell surface, including CD69 AA homodimers, AB heterodimers, and BB homodimers.

Source: Vance, et al., 1997, pp. 23117-23122

Interleukin 2 (IL-2)

A soluble factor mitogenic for lymphocytes was first found in 1965 in the culture media of mixed leukocytes and named Blastogenic Factor (BF) (Gordon and Maclean, 1965, pp. 795-796; Kasakura and Lowenstein, 1965, pp. 794-795). Later, Gillis and Smith established a quantitative assay for T cell Growth Factor (TCGF) in culture fluids of activated T cells that promote the long-term proliferation of T cells (Gillis and Smith, 1977, pp. 54-56). TCGF was assigned the more generic nomenclature of IL-2, as lymphokines typically mediate diverse biological functions in immunity.

IL-2 is a member of the four-helix bundle family of cytokines with a molecular weight of 15.5 kDa. IL-2 is mainly produced by activated $CD4^+$ and $CD8^+$T cells, to a lesser extent by activated dendritic cells, natural killer cells, and natural killer T cells. IL-2 is rapidly and transiently produced upon engaging the TCR and costimulatory molecules such as CD28 on naive T cells. TCR signaling induces AP-1, increases the levels of active nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65/rel, and causes calcineurinmediated dephosphorylation of nuclear factor of activated T cells (NFAT), promoting its translocation into the nucleus. These transcription factors, in conjunction with constitutive factors bind to specific sites in a cooperative fashion in the promoter domain, contributing to IL-2 gene transcription. Based primarily upon in vitro studies, IL-2 has been considered a potent immunoregulatory cytokine that promotes the proliferation of activated T cells (Figure 9). However, the principle function of this cytokine in vivo has been challenged. Several pieces of evidence have shown that mice deficient in IL-2, or IL-2 receptor α (CD25) and a patient with a CD25 mutation develop the IL-2 deficiency syndrome characterized by the accumulation of activated $CD4^+$ T cells and production of autoantibodies (Sharfe, et al., 1997, pp. 3168-3171; Suzuki, et al., 1995, pp. 1472-

1476; Willerford, et al., 1995, pp. 521-530). These observations indicate that IL-2 is involved in maintaining self-tolerance in vivo. A number of more recent studies have raised the possibility that IL-2 is essential in the development of efficient memory response. IL-$2^{-/-}$ TCR transgenic $CD4^{+}$ T cells readily proliferate and contract after stimulation with peptide-pulsed DCs, but these IL-$2^{-/-}$ T cells poorly survive and yield a low number of memory cells (Dooms, et al., 2004, pp. 5973-5979; Dooms, et al., 2007, pp. 547-557). While the role of IL-2 in vivo is controversial, IL-2 plays an essential role in triggering proliferation, differentiation, and survival of mature T cells in vitro (Lotze, Strausser and Rosenberg, 1980, pp. 1007-1008; Morgan, Ruscett and Gallo, 1976, pp. 1007-1008; Strausser and Rosenberg, 1978, pp. 1491-1495). Thus, IL-2 is a multifaceted cytokine with immunostimulatory and immunosuppressive properties. After mitogen or antigen stimulation, T cells are induced to secrete IL-2 and enhance expression of specific membrane receptors for this cytokine (Robb, Munck and Smith, 1981, pp. 1455–1474; Smith, 1980, pp. 337-357). Its biologic responses are induced after binding to a high-affinity IL-2 receptor (IL-2R).

Primary IL-2 R signaling is through the JAK/Stat pathway and MAPKs which promote specific target gene transcription (Beadling, et al., 1994, pp. 5605-5615). Several clones of Jurkat cells, including E6-1 have been shown to produce large quantities of IL-2 in the cell culture supernatant when stimulated with anti-CD3 plus anti-CD28 Abs (Fang, et al., 2005, pp. 4966-4971), PMA plus ionomycin (Fang, et al., 2005, pp. 4966-4971; Schrager and Marsh, 1999, pp. 8167-8172), or PHA plus PMA (Sasagawa, et al., 2006, pp. 1214-1221; Schrager and Marsh, 1999, pp. 8167-8172).

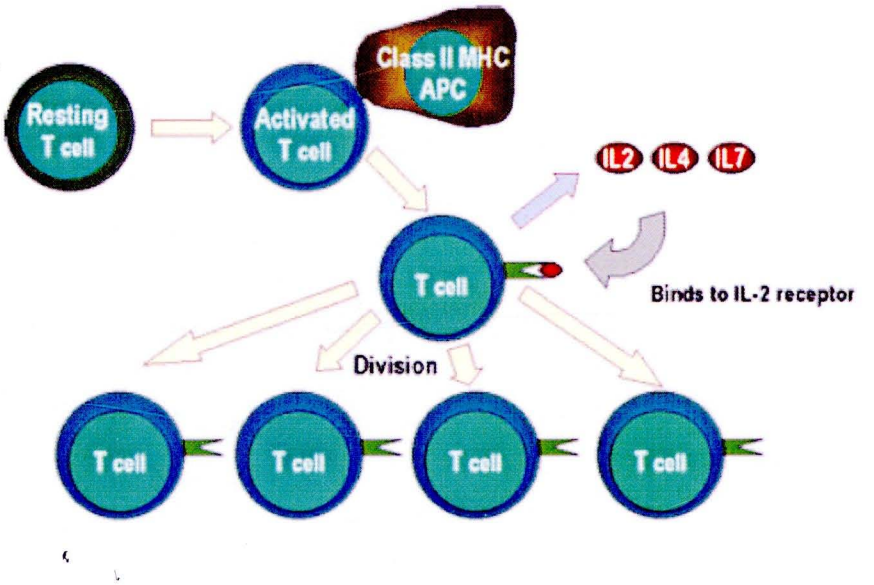

Figure 9 T cell proliferation and cytokines.

Note: Activation of T cells results in the formation of high affinity IL-2 receptors and induction of the synthesis and secretion of IL-2 and IL-4. These bind to their receptors and the cells proliferate.

Source: http://www.pathmicro.med.sc.edu