CHAPTER V

DISCUSSION AND CONCLUSION

Nck1 is an important regulator of the cytoskeletal reorganization and of the formation of the immune synapse between T cells and antigen presenting cells (APCs). Two different models for the association of Nck with TCR/CD3 complex have been proposed. In an ITAM-requiring pathway, Nck is recruited to the membrane proximal site via interaction with SLP-76, forming a trimolecular complex of SLP-76/Vav1/Nck that is involved in WASP-dependent actin cytoskeletal rearrangement (Barda-Saad, et al., 2005 pp. 80-89; Rivero-Lezcano, et al., 1995, pp. 5725-5731) and Pak1-dependent T cell activation (Yablonski, et al., 1998, pp. 5647-5657). In a non-ITAM-requiring pathway, the N-terminal SH3 domain of Nck directly binds to a PRS in CD3ε that exposes due to a conformational change in the TCR complex after TCR ligation (Gil, et al., 2002, pp. 901-912). The authers proposed that Nck recruits associated regulatory proteins such as Slp 76, WASP, or Pak1 to CD3ε and this event displays an alternative means to link T cell activation to the actin cytoskeleton.

In this present report, Nck1 siRNA was transfected into Jurkat T cells, and it was used as a cell model to knockdown Nck1 protein in order to investigate the role of Nck1 in T cell activation and function. siRNA, 20- to 25- nucleotide double- stranded RNA, mediates sequence-specific elimination of complementary mRNA (Elbashir, et al., 2001, pp. 494-498; Hammond, et al., 2000, pp. 293-296; Zamore, et al., 2000, pp. 25-33). In order to deliver siRNA into the T-cell line, an effective and cost-saving route is the by the way of capillary electroporation system (Kim, et al., 2008, pp. 1353-1360). A partial knockdown of Nck by electroporated siRNA transfection yielded a 50-60% reduction of Nck protein level in PHA-stimulated human T cell blasts (Lettau, et al., 2006, pp. 5911-5916). The Nck1 protein expression was inhibited after transfection with Nck1-specific siRNA, suggesting that the transfection was successful. The selective reduction in IL-2 secretion by Nck1 knock-downed Jurkat T cells stimulated with anti-CD3ε/anti-CD28 antibodies was accompanied by a lower expression of CD69. This is not unexpected because surface expression of CD69 is a marker of Ras activation, which is critical for proliferation and transcription of the IL-

2 gene (Finco, et al., 1998, pp. 617-626). Nck is known to interact with Grb2 and SOS GTP exchange factor (Buday, et al., 2002, pp. 723-731; Hu, et al., 1995, pp. 1169-1174; Okada and Pessin, 1996, pp. 25533-25538), leading to enhanced transcription from a Ras-dependent reporter gene. Ras signaling affects many cellular functions, including cell proliferation. This reduced IL-2 secretion did not result from cell apoptosis as no difference in the percentage of apoptotic cells was found between the transfected and control groups in both resting and stimulated condition. These findings suggest that the decreased expression of Nck1 by transfection of Nck1 siRNA could not induce Jurkat T cell apoptosis. Therefore, it is possible that the role of Nck1 has no relationship with cell apoptosis or other Nck. It is also possible that adaptor molecules compensated for the function of Nck1 when the Nck1 expression was silenced by Nck1 siRNA.

An earlier study have indicated that recruitment of Nck to the highly conserved PRS of CD3ε, is essential for optimal T cell activation and synapse formation (Gil, et al., 2002, pp. 901-912). The PRS within CD3ε only gets available due to conformational change upon TCR engagement and directly bind to SH3.1 domain of Nck. Recruitment of Nck to CD3ε occurs prior to phosphorylation of the CD3ε ITAM, suggesting that it regulates the earliest stages of TCR signaling. It has been shown that Nck/CD3ε interaction-inducing Abs was a better inducer for both IL-2 release and CD69 expression than noninducing Abs for Jurkat T cells. Also in this study, the stable overexpression of Nck SH3.1 decreased cell spreading, IL-2 release and synapse formation by preventing the interaction of endogenous Nck with CD3ε. However, the overexpressed SH3.1 domain may also block the association of Nck with other binding partners besides CD3ε. A recent study employing a highly sophisticated mouse model showed that Nck-defective T cells failed to proliferate upon stimulation with anti-CD3ε Ab but not with PMA and ionomycin (Roy, et al., 2010, pp. 15529-15534). Furthermore, culture supernatants of T cells from Nck-deficient mice exhibited a reduction in IL-2 content compared to untreated mice after CD3ε/CD28 stimulation. They suggested that the unmodified response of Nck-deficient T cells to PMA and ionomycin stimulation was due to a signaling pathway that bypassed the TCR signaling apparatus, which indicated that involvement of Nck in proximal TCR signaling. This present study is consistent with those results, since

CD3ε/CD28 stimulation was unable to induce CD69 expression and IL-2 secretion in Nck1 knock-downed cells. However, the proliferation and CD69 expression of Nck1 knock-downed cells stimulated with PHA and PMA/ionomycin or PHA/PMA, respectively, were not changed. Because stimulation with PHA and PMA or PMA and ionomycin bypassed TCR signals by directly triggering Lck and PKC activity and inducing intracellular calcium mobilization (Smith-Garvin, et al., 2009, pp. 591-619), it is reasonable to suspect that Nck1 impairs TCR/CD3-mediated CD69 expression and IL-2 production.

One functional analysis observed no differences in proliferation and CD69 expression after long treatment with strong stimulators in T cells from mice expressing wild-type or CD3ε PRS mutation (Szymczak, et al., 2005, pp. 270-275). Furthermore, the defects in T cell development and function of CD3ε-deficient mice could be rescued using a mutant CD3ε lacking the highly conserved proline residues. This data suggests that interaction of CD3ε PRS with Nck might not be essential for T cell development and function. However, in addition to CD3ε, interaction of Nck with SLP-76 or LAT (Barda-Saad, et al., 2005, pp. 80-88; Rivero-Lezcano, et al., 1995, pp. 5725-5731) induces WASP-mediated actin cytoskeleton rearrangement and T cell activation (Badour, et al., 2004, pp. 395-407; Snapper, et al., 1998, pp. 81-91; Zhang, et al., 1999, pp. 1329-1342). Thus, it seems to be the case that Nck1 may cause recruitment of these regulatory molecules to the plasma membrane in order to enhance proximal TCR signal potency and T cell functions. An alternative possibility is that there are other Nck-binding residues in CD3ε instead of the PRS that are responsible for CD3ε/Nck interaction because the mutant CD3ε PRS still retains weak interaction to Nck SH3.1 (Kesti, et al., 2007, pp. 878-885). This weak interaction might not be identified by immunoprecipitation but it could be physiologically significant, such as for the promotion of ITAM phosphorylation and synapse formation and subsequently signal transduction. Besides, the apparent lack of defect in T cells expressing CD3ε with a mutated PRS might be due to the redundancy among the CD3 subunits in the TCR complex that all support ZAP70 binding. In this regard, each of the CD3 subunits contains one or more ITAMs, whereas only CD3ε has an Nck binding motif. Thus, the functional modification provided by the CD3ε/Nck interaction might be masked by the other CD3 subunits. This experiment do not reveal the role of individual CD3 subunits

in T cell signaling and a fully conclusive to claim the dispensability of the Nck binding site of CD3ε is unclear. In another experiment using differentiated murine T cells, it was found that abrogation of the putative interaction between Nck and the CD3ε PRS affected the number and cytokine production in response to weak agonists but not strong antigens (Tailor, et al., 2008, pp. 243-255). On the basic of these observations, it has been suggested that the CD3ε PRS enhances weak TCR signals by promoting synapse formation and CD3ε phosphorylation. Considering previous report that Lck contains a binding site for Nck (Mingueneau, et al., 2008 pp. 522-532), Nck might play a role in recruiting Lck to the unphosphorylated TCR complex and Lck could confer higher signaling competence to weak antigens. The authors suggest that Nck/CD3ε interaction is essential to enhance mature T cell reaction to TCR-mediated stimulation.

Conflicting data from biochemical studies have shown that the interaction of Nck with CD3ε is also capable of down-regulating T cell activation by inhibiting CD3ε ITAM phosphorylation and subsequent ITAM-dependent recruitment of downstream signaling molecules and/or reducing TCR cell surface expression via a non-ITAM-requiring mechanism. (Takeuchi, et al., 2008, pp. 704-716). In this context, the SH3.1 domain of Nck2 binding to an uncommon PxxDY motif of CD3ε blocked the tyrosine phosphorylation of the ITAM of CD3ε, and it affected the internalization signal of TCR. However, this was concluded from the structure of the Nck2 SH3.1/CD3ε complex, and it experimentally demonstrated the ability to inhibit tyrosine phosphorylation of CD3ε peptide by recombinant Fyn and Lck in an *in vitro* phosphorylation assay. The discrepancy from data presented here may be due to differences in the isoforms of the Nck being tested. Perhaps Nck1 could bind to PxxDY motif at a lesser degree and a subsequent ITAM phosphorylation may not be affected.

In summary, Nck1-specific siRNA transfection did not induce Jurkat T cell apoptosis. Nck1 appears to be important in TCR/CD3-mediated activation, as evidenced by the inhibition of T cell function when its expression is down-regulated. Nck1 could bridge TCR activation to downstream signaling mechanisms, leading to functional events, particularly to the T cell proliferation, expression of CD69 and production of IL-2. In this scenario, formation of Nck-mediated signaling complex

may result in the propagation of proximal TCR signals. The reduced Nck1 expression in this present study led to an impairment CD69 expression and IL-2 production by T cells. Taken together, these data suggest that the Nck1 adaptor protein is a crucial regulator of TCR/CD3-mediated activation and function. Further investigation is required to define the exact function of interaction of Nck with the other binding partners such as Sos, HIP55 (HPK1-interacting protein of 55 kDa), or FYB (Fyn-binding protein) during T cell activation and effector function.