CHAPTER IV

RESULTS

Nck1 siRNA Transfection in Jurkat T Cells Inhibited Nck1 Protein Expression

siRNA has been used successfully for targeting Nck by electroporation in human T cell blasts (Lettau, et al., 2006, pp. 5911-5916). Delivery of siRNA against Nck1 to Jurkat T cells by way of capillary electroporation system was investigated. In order to investigate the siRNA import into the cell and optimal electrical parameters, the pEGFP entrance after electric pulse delivery was first analyzed by flow cytometry. The percentage of cells expression EGFP (EGFP positive cells) was approximately 45-55% in the first three most efficient conditions (Figure 12). To determine the proper condition for transfection of Jurkat T cells, three electroporation parameters, which were voltage, pulse width and pulse number (1700 V, 20 msec, 1; 1600V, 10 msec, 3; or 1400V, 20 msec, 2) and two concentrations (100 pmol or 50 pmol) of siRNA against Nck1 and non-specific siRNA negative control were used and reduction of protein expression was monitored by Western blotting. As shown in Figure 13, the Nck1 protein expression was reduced in Nck1 siRNA-transfected groups at 48 hours after transfection. The greatest reduction of Nck1 protein level appeared when Jurkat T cells were electroporated at 1600V, 10 msec, and 3 with 50 pmol of Nck1 siRNA compared with the blank control and non-specific siRNA negative control. The Nck1 siRNA knockdown was specific because it did not affect expression of unrelated protein β-actin. These parameters led to 40-60% cell viability as determine by visualization of trypan blue negative cells.

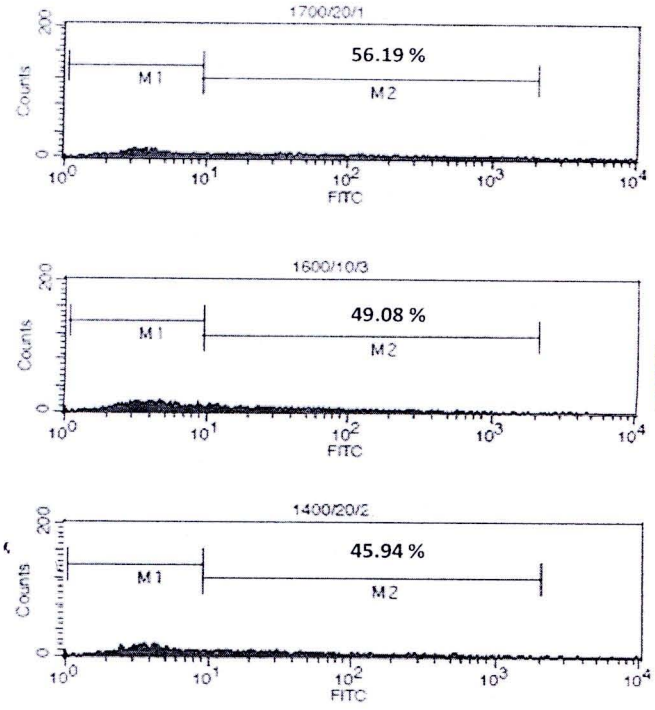


Figure 12 Expression EGFP of electrotransfer Jurkat cells.

E6-1 Jurkat T cells were transfected with pEGFP in various electric parameters; (A) 1700 V, 20 msec, 1, (B) 1600V, 10 msec, 3, or (C) 1400V, 20 msec, 2.

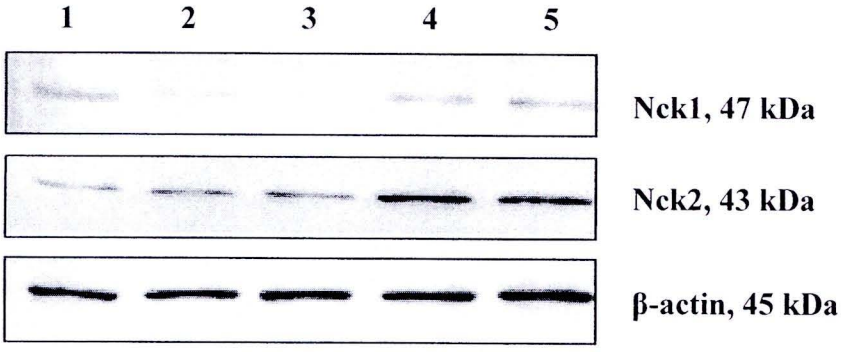


Figure 13 Expression of Nck1 protein was reduced after Nck1-specific short interfering (si)RNA transfection.

E6-1 Jurkat T cells were transfected with Nck1-specific or negative control siRNA for 48 hours. Cell extracts were subjected to Western blot analysis with anti-Nck1 mAb and anti-β-actin mAb. Lane 1, untransfection; lane 2, transfection with 100 pmol Nck1 siRNA; lane 3, transfection with 50 pmol Nck1 siRNA; lane 4, transfection with 100 pmol non-specific siRNA negative control; lane 5, transfection with 50 pmol non-specific siRNA negative control.

Loss of Nck1 Did Not Induce Apoptosis in Jurkat T Cells

It has been shown that Nck translocates to the nucleus upon DNA damage. This accumulation is essential for the activation of downstream regulators of the DNA damage cascade and cell-cycle arrest (Kremer, et al., 2007, pp. 837-850). A well-known early hallmark of apoptosis is externalization of the normal inward-facing phosphatidylserine of the cell's lipid bilayer to the outer cell membrane (Elmore, S. 2007, pp. 495-516). Annexin-V, a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues, can be used to detect apoptotic cells by flow cytometry analysis. To test whether or not the loss of Nck1 was associated with apoptosis, annexin V-FITC and PI staining were performed using flow cytometry. In the figure of the biparameter flow cytometry analysis (Figure 14), the lower left zone showed the viable cells (annexinV-/PI-), the upper left zone represented necrosis cells (annexinV-/PI+), the lower right zone showed early apoptosis (annexinV+/PI-), and the upper right zone showed late apoptosis (annexinV+/PI+). The combination of the upper and lower right zones represented total apoptotic cells.

The percentage of apoptotic cells in the Nck1 siRNA-transfected group was 11.80% ± 0.76%, which did not differ from that of the untransfected control group (9.81% ± 0.69%) and the non-specific siRNA negative control group (10.72% ± 0.63%) (Figure 14 A-C). Similar, the percentage of apoptotic cells in transfected Jurkat T cells stimulated with anti-TCR antibody was 14.75% ± 1.54%, which also did not differ from the untransfected control (13.08% ± 1.81%) and the negative siRNA control (14.00% ± 1.71%) groups (Figure 14 D-F). This finding indicated that an apoptotic effect of Nck1 siRNA treatment was minimal and Jurkat T cell survival was independent of Nck1 expression in both normal and stimulated condition.

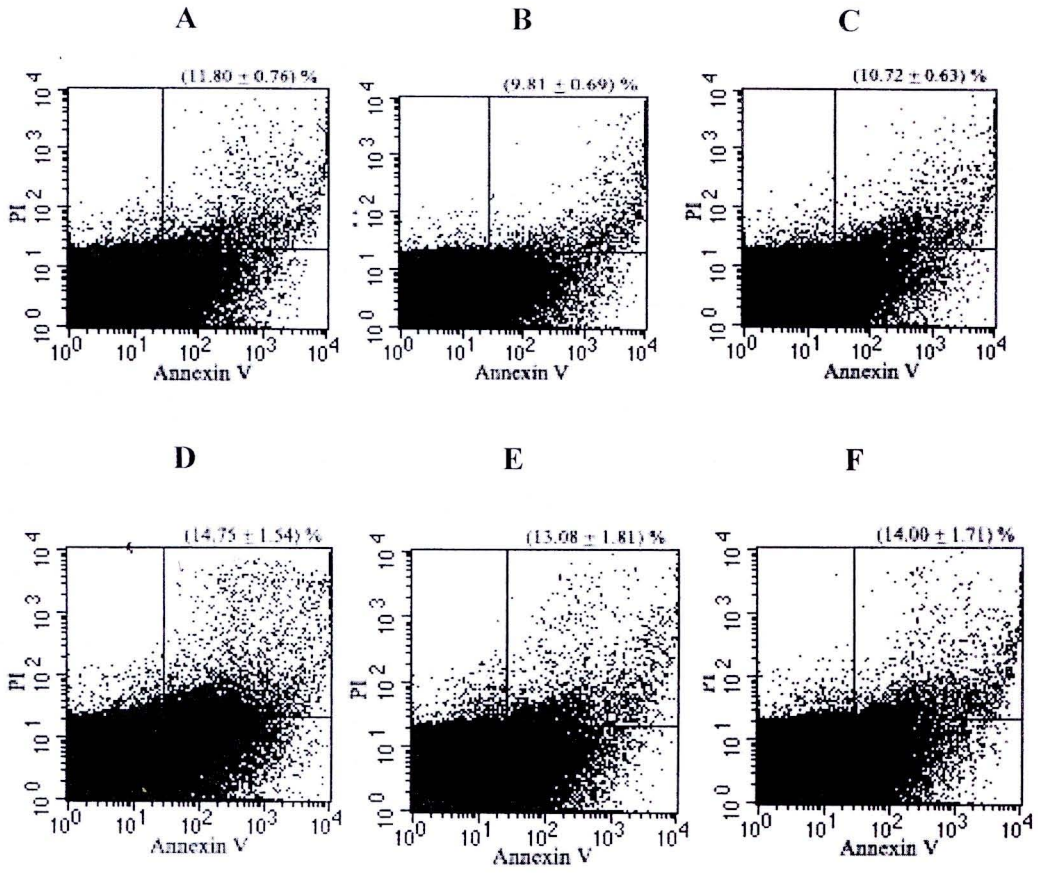


Figure 14 Nck1 knockdown did not induce apoptosis in Jurkat T cells.

E6-1 Jurkat T cells were transfected with Nck1-specific siRNA (A), untransfected (B) or transfected with non-specific siRNA negative control (C). Jurkat T cells were also stimulated with anti-T cell receptor (TCR) monoclonal antiboby (mAb) and there was no difference in % apoptosis of TCR-stimulated Jurkat T cells transfected with Nck1-specific siRNA (D), untransfected (E) or transfected with non-specific siRNA negative control (F). Shown are mean percentage ± SD of apoptotic cells from three experiments.

Loss of Nck1 Did Not Impair Jurket T Cell Proliferation

T-cell proliferation is an important in vitro parameter of the in vivo immune function. Several reports have shown that Nck interacts with Grb2 and SOS GTP exchange factors (Hu, et al., 1995, pp. 1169-1174; Okada and Pessin, 1996, pp. 25533-25538; Wunderlich, et al., 1999, pp. 25-29), leading to enhanced transcription from a

Ras-dependent reporter gene. Ras signaling affects many cellular functions, including cell proliferation. T-cell proliferation was measured by an enzyme-linked immunosorbent assay (ELISA) based on the incorporation of bromo-2'-deoxyuridine (BrdU) into the newly synthesized DNA of proliferating cells. To determine whether Nck1 was involved in proliferation of T cells, normal and transfected Jurkat T cells were cultured for 24 hours in the presence of PHA, anti-TCR antibody and IL-2. The proliferative response in the Nck1 siRNA-transfected cells did not differ significantly from that in untransfected cells and non-specific siRNA-transfected cells (Figure 15). The results suggested that Nck1 was not required for the proliferation of Jurkat T cells stimulated with PHA-, anti-TCR antibody- or IL-2.

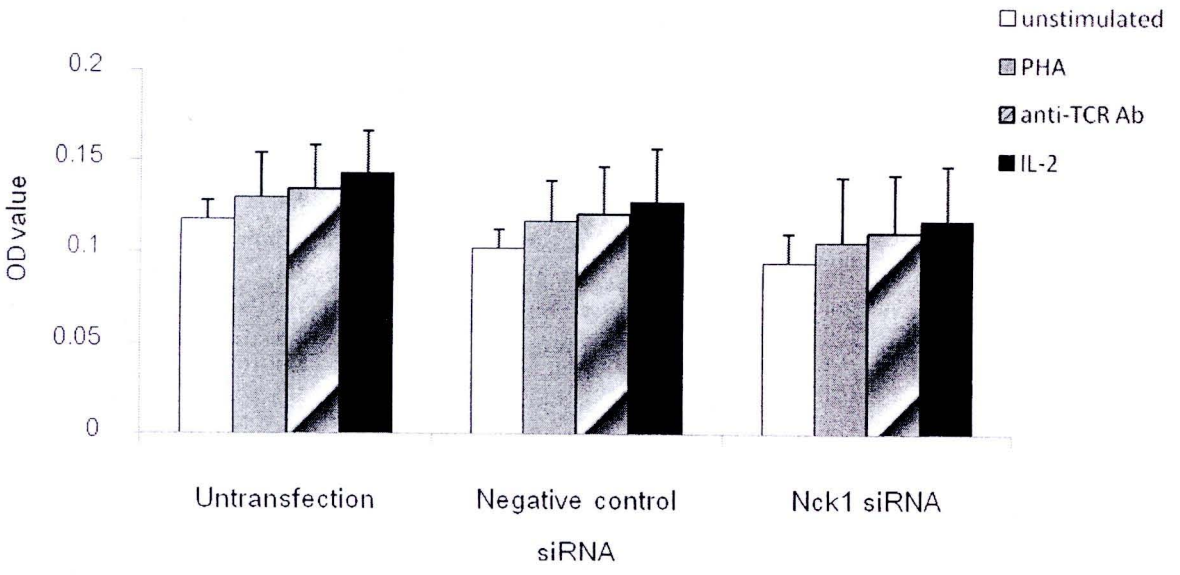


Figure 15 Nck1 was not required for PHA-, anti-TCR mAb and IL-2 induced Jurkat T cells proliferation.

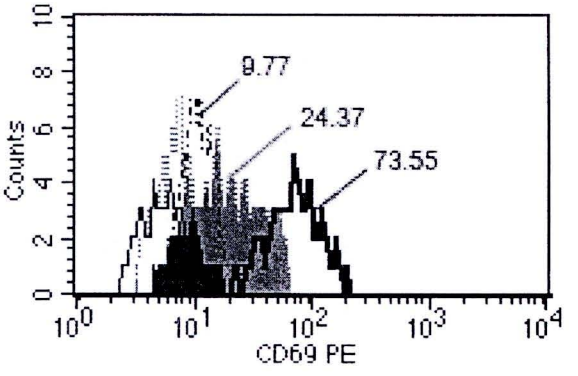
Untransfected, negative control siRNA-transfected or Nck1 siRNA-transfected Jurkat T cells were unstimulated (□), stimulated with PHA (□), anti-TCR Ab (▨), or IL-2 (■) for 24 hour. The proliferation was measured by using BrdU-incorporation assay. Values are presented as mean ± SD from three experiments. OD, optical density read at 450 nm.

Nck1 is Required for TCR/CD3-Mediated Activation

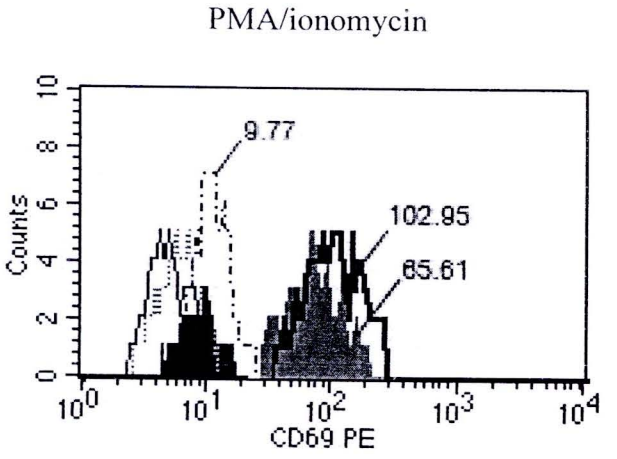
CD69 expression represents one of the earliest available markers for quantifying T cell activation. It is a lymphoid activation antigen that acts as a potent signal-transmitting receptor on lymphocytes. It is involved in cytokine gene regulation and cell migration upon lymphocyte activation (Shiow, et al., 2006, pp. 540-544). The flow cytometric detection of CD69 expression by activated T cells was used in this study. To assess the influence of Nck1 down-regulation on the expression levels of the CD69, negative control and Nck1 knock-down Jurkat T cells were stimulated with anti-CD3ε/anti-CD28 antibodies, PMA/ionomycin or PHA/PMA. In the Nck1 siRNA-transfected group, CD69 was detectable by all three types of stimulation. After PHA/PMA or PMA/ionomycin stimulation, Nck1 siRNA-transfected cells had similar CD69 expression compared with the negative control siRNA group. However, after anti-CD3ε/anti-CD28 antibody stimulation, Nck1 siRNA-transfected cells had significantly lower CD69 expression compared with the negative control siRNA group (Figure 16). Previous studies have shown that the proximal TCR signaling machinery could be bypassed via PMA/ionomycin or PHA/PMA stimulation (Bosque and Planelles, 2009, Suppl. 3). These results indicated clearly that CD69 expression upon TCR/CD3-mediated stimulation was affected by Nck1 down-regulation.

A

CD3/CD28



B



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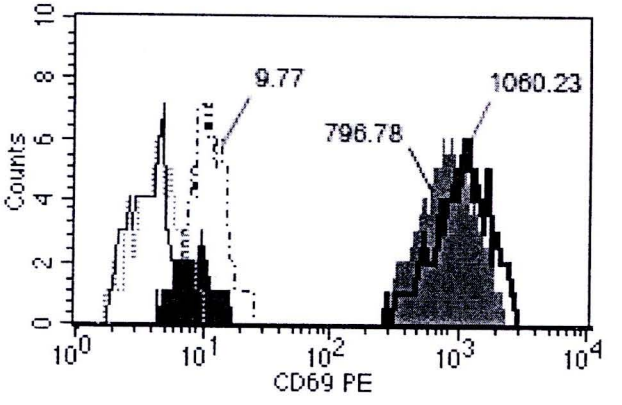

Figure 16 Nck1 regulated expression of CD69 in TCR/CD3-mediated activation.

Jurkat T cells were stimulated with anti-CD3ε/anti-CD28 antibodies (A), PMA/Ionomycin (B) or PHA/PMA (C) for 24 hours. Histograms show CD69 expression of untransfected Jurkat T cells without any stimulation (black dotted line), negative control siRNA-transfected (thick solid line) or Nck1 siRNA-transfected Jurkat T cells (gray histograms). Each cell population was stained with anti-CD69 PE and isotype control antibodies (untransfected: black histogram, negative control siRNA-transfected: gray dotted line or Nck1 siRNA-transfected Jurkat T cells: thin solid line) and was analyzed by flow cytometry for CD69 expression. Shown are mean fluorescence intensity of cells positive for CD69 expression.

Knockdown of Nck1 Resulted in the Reduction of IL-2 Production

Stimulated T cells are the main producers of IL-2. The expression of IL-2 is also used as a marker for T cell activation. IL-2 is a critical autocrine growth factor up-regulated upon T cell activation, which is required for the clonal expansion of T cells (Gillis and Watson, 1980, pp. 1709-1719). Previous reports suggest that CD69 expression on Jurkat T cell surface is an endogenous marker of Ras activation, which is required for the transcription of the IL-2 gene (Finco, et al., 1998, pp. 617-626). The impact of the Nck1 knock-down on IL-2 production induced by PHA/PMA or anti-CD3ε/anti-CD28 mAb was examined by using ELISA. Consistent with the CD69 results, Jurkat T cells expressing reduced levels of Nck1 failed to respond to anti-CD3ε/anti-CD28 antibody stimulation by producing IL-2 when compared with untransfected cells or cells transfected with the negative control siRNA (Figure 17). In contrast, IL-2 production upon PHA/PMA stimulation from Nck1 siRNA-transfected cells was comparable to that from negative control siRNA-transfected cells. Because stimulation with PHA/PMA bypasses the TCR signaling machinery, these results suggest that Nck1 reduction specifically induced an impairment of TCR/CD3-mediated T cell activation and function.

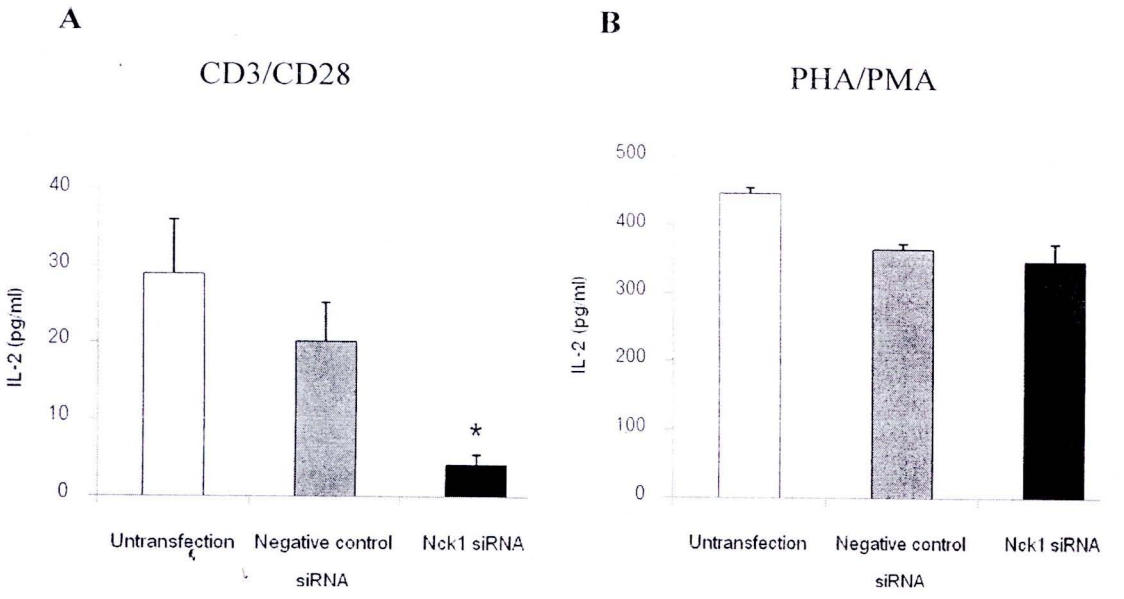

Figure 17 Nck1 was required for IL-2 production by Jurkat T cells in response to CD3/CD28 stimulation.

Untransfected (□), negative control siRNA-transfected (□) or Nck1 siRNA-transfected (■) Jurkat T cells were stimulated with anti-CD3ε/anti-CD28 antibodies (A) or PHA/PMA (B) for 24 hours. Then, supernatants were harvested and assessed for IL-2 production by ELISA. Results represent mean ± SD of three experiments. * $P < 0.05$.