CHAPTER III

METHODOLOGY

Materials and Mathods

This research project is divided into 7 parts:

1. Cell culture
2. Nck siRNA transfection of Jurkat T cells
3. Western blot analysis
4. Analysis of apoptosis by flow cytometry
5. Cell proliferation assays
6. Detection of CD69 upregulation
7. IL-2 ELISA assays

Cell Culture

Jurkat cells (clone E6-1; American Type Culture Collection, Rockville, MD, USA) were grown in the RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated foetal bovine serum (Gibco), 100 IU/ml penicillin/streptomycin (JRH Biosciences, Victoria, Australia), and 2mM L-glutamine (JRH Biosciences) at 37°C in a humidified atmosphere with 5% carbon dioxide.

Nck1 siRNA Transfection of T Cells

In order to knock-down Nck, Jurkat T cells were transiently transfected with control siRNA, or siRNA specific, for Nck1 mRNA (Invitrogen, Carlsbad, CA, USA) by electroporation. Approximately 2×10^5 cells per electroporation were collected, washed with RPMI-1640, and resuspended in solution R plus stealth RNAi™ siRNA duplexes specific for Nck1 (No. 983; Invitrogen). Negative controls were performed by using negative stealth siRNA low GC content (Invitrogen). The siRNA was imported into Jurkat Tcell, and optimal electrical parameters were investigated by using a green fluorescent protein reporter plasmid (pEGFP) (Invitrogen) 10 µl according to the manufacture's instruction. The EGFP positive cells were quantified flow cytometry. To determine the concentration of siRNA, the first three most

efficient conditions were used with the 100 pmol or 50 pmol of Nck1 stealth siRNA and with the non-specific siRNA negative control (a scrambled version of this sequence). Cells were electroporated in a microporator pipette at various voltages (V), pulse widths (msec), and pulse numbers by using the MicroPorator (Digital Bio Technology, Seoul, Korea). The transfected Jurkat cells were subsequently cultured in 500 µl of RPMI-1640 plus FBS for 48 hours. The delivery of siRNA into cells was determined by measuring the green fluorescence mean of each sample with FACScaliber flow cytometry and Cell Quest software (Becton Dickinson, San Jose, CA, USA). The trypan blue exclusion test was used to determine the number of viable knockdown T cells.

To determine the concentration of siRNA, the first three best efficiency conditions were used with 100 pmol, 50 pmol, 25 pmol, or 12.5 pmol of Nck1 stealth siRNA (No. 983) and 100 pmol or 50 pmol of negative contol (negative stealth siRNA low GC content). After microporation, Jurkat cells were harvested and expression of Nck1 protein was analysed by Western blotting of whole-cell lysates with anti-Nck1 mAb (Cell Signaling Technology, MA, USA).

Western blot analysis

Normal and transfected Jurkat cells were lysed and cytoplasmic protein was extracted using an M-PER mammalian protein extraction reagent (Pierce Biotechnology, IL, USA). The cell suspension of each sample was washed in PBS and pelleted by centrifugation at 2,500 x g for 10 minutes. The supernatant was discarded. Sixty microlitters of M-PER reagent was added and resuspended in each tube. The cell suspension was shaken gently for 10 minutes. Cell debris was removed by centrifugation at 14,000 x g for 15 minutes. Supernatant was transferred to a new tube for protein analysis. Protein concentration was then determined by using a commercially available BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology). BCA working reagent (WR) was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. 0.1 ml of each sample replicate was pipetted into a test tube and 2.0 ml of the WR was added to each tube, mixed well, and incubated at 60°C for 30 minutes. After cooling to room temperature (RT), all of the

samples were measured for absorbance at 540 nm with a spectrophotometer (PerkinElmer Life Sciences, IL, USA.)

Samples (5 μg) were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to an Immmobilon-P membrane (Millipore). After washing three times with Tris buffered saline (TBS) and 0.1% Tween 20, the membrane was blocked with 25 ml of blocking solution (TBS, 1% bovine serum albumin (BSA), 10% FBS, and 0.1% Tween 20) for 1 hour. The membrane was incubated with 10 μl rabbit monoclonal antibodies raised against Nck1 or β-actin (Cell Signaling Technology). Then, horse horseradish peroxidase-conjugated goat anti-rabbit antibody diluted 1:1,000 was added for 1 hour. Protein–antibody interaction was then visualized by chromogenic detection using a Western breeze chromogenic immunodetection system (Invitrogen). Molecular weights were determined by comparing with SeeBlueR Plus2 Pre-stained Standard (Invitrogen) and an equal loading was determined by the presence of β-actin.

Apoptosis Assay

Annexin-V-FITC and PI stainings were performed to determine the level of apoptosis (Figure 10) with FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA). Six experimental groups were designed as follow: untransfected cells, negative control siRNA-transfected cells, Nck1 siRNA-transfected cells, untransfected TCR-stimulated cells, negative control siRNA-transfected TCR-stimulated cells, and siRNA-transfected TCR-stimulated cells. Normal and transfected Jurkat T cells were either not stimulated or stimulated with 300 ng/ml anti-TCR antibody (clone C305; Millipore, Temecula, CA, USA) for 24 hours. The cells were washed twice with cold PBS and resuspended in a binding buffer (10 nM Hepes–NaOH, 140 nM NaCl, 2.5 nM CaCl2, pH 7.4) at a concentration of $1x10^6$ cells/ml. 100 μl ($1x10^5$ cells) of the solution was transferred to a tube for FACSCaliburs, and 2 μg of FITC-labeled annexin V and/or 2 μg of PI were added. The tube was gently vortexed and incubated for 15 minutes at RT in the dark. At the end of the incubation period, 200 μl of binding buffer was added to each tube and cells were examined immediately by the FACScaliber flow cytometer (Becton Dickinson) using CellQuestPro software. Flow cytometry was performed on Jurkat cells gated on the

basis of their forward and side light scatters with any cell debris excluded from the analysis. Apoptotic cells were defined as $FITC^+/PI^-$ cells and $FITC^+/PI^+$ cells. The gated Jurkat cells were then plotted for Annexin V-FITC and PI in a 2-way dot plot to assess the percentage of apoptotic Jurkat T cells.

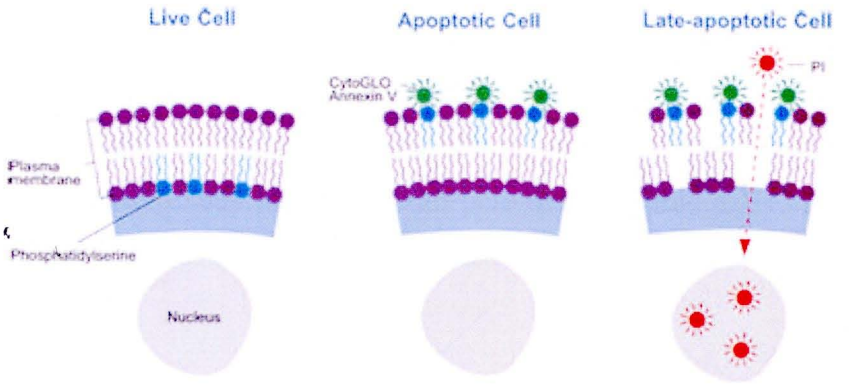

Figure 10 Annexin V detects cell membrane exposure of PS.

Note: During early apoptosis, the plasma membrane loses asymmetry causing PS to be translocated from the cytoplasmic face of the plasma membrane to the external face which can be detected using Annexin V. As apoptosis progresses and the plasma membrane becomes compromised, PI can be used as an additional marker of apoptosis.

Source: http://www.biomol.de

Cell proliferation assays

A colorimetric BrdU (5-bromo-2′-deoxyuridine) ELISA kit (Roche Diagnostics, Mannheim, Germany) was used to measure cell proliferation (Figure 11). Jurkat cells were transfected with the siRNA targeting Nck or negative control siRNA and cultured for 48 hours at 37 °C. Normal and Tranfected Jurkat T cells (2×10^4 cells/well) were cultured in 96-well microculture plates in the presence or absence of 5 μg/ml PMA, 100 U/ml IL-2 or 300 ng/ml anti-TCR antibody (Millipore) for 24 hours. 20 μl/well of BrdU labeling reagent (final concentration, 10 μM) was added and cells

were incubated for 24 hours. The cells were harvested by centrifugation at 300 × *g* for 10 minutes and were fixed in 200 µl/well of Fix Denat solution for 30 minutes. Then, the Fix Denat solution was removed by flicking off and tapping before incubation with peroxidase-labeled anti-BrdU for 90 minutes. Then antibody conjugate was removed by flicking off and the plate was rinsed three times with 200 µl/well of washing solution. 100 µl/well of substrate solution tetramethylbenzidine (TMB) was added for 15 minutes in dark at RT. The reaction was terminated by the addition of 25 µl of stop solution (1 M H_2SO_4). Absorbance was measured at 450 nm on a microplate reader (PerkinElmer Life Sciences). All proliferation assays were performed in triplicates. Culture medium alone and cells incubated with peroxidase-labeled anti-BrdU in the absence of BrdU were used as controls for non-specific binding.

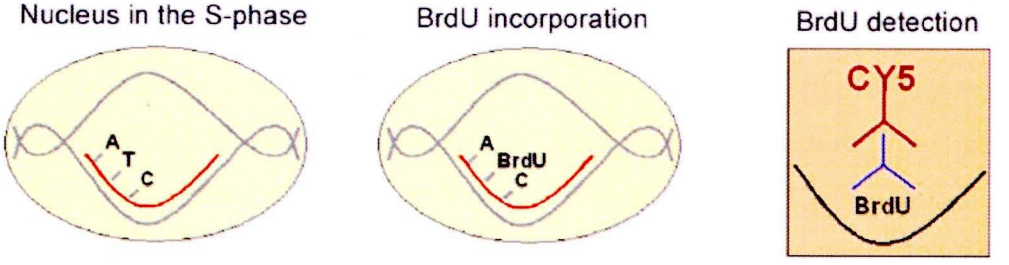

Figure 11 BrdU incorporated into the genomic DNA of proliferating cells.

Note: BrdU is an analog of the DNA precursor thymidine. If BrdU is added to the cell culture, proliferating cells will incorporate it into their DNA. The amount of BrdU in the newly synthesized cellular DNA can be detected with specific anti-BrdU. The immune complexes are detected by the subsequent substrate reaction.

Source: http://www.smp-cell.org/smp-cell/cell.org

Detection of CD69 Upregulation

Normal and transfected Jurkat cells were stimulated with 1 µg/ml PHA plus 10 ng/ml PMA or 25 ng/ml PMA plus 1 µM ionomycin or with 10 µg/ml anti-CD3ε

mAb (clone OKT3; e-Bioscience, San Diago, CA, USA) plus 10 μg/ml anti-CD28 mAb (e-Bioscience) for 24 hours at 37 °C (Brocardo, et al., 2001, pp. 297-305) before treatment with 20 mM of ethylenediamine tetraacetic acid (EDTA; BD Biosciences, San Jose, CA, USA) for 15 minutes at RT. The cells were centrifuged and washed twice with phosphate-buffered saline (PBS) containing 0.5% FBS, fixed in 1% paraformaldehyde, washed with phosphate-buffered saline (PBS) containing 2.0% FBS and then incubated with phycoerythrin (PE)-conjugated mouse anti-human CD69 mAb (e-Bioscience) or isotype control mAb (BD Pharmingen) for 30 minutes at 4°C protected from light. Finally, cells were washed, resuspended in a staining buffer (PBS containing 0.5% BSA) and CD69 expression analyzed by FACScalibur (Becton Dickinson). Jurkat T cells were gated according to side scatter (SSC) and forward scatter (FSC). The positive cells were $CD69^+$. A total of 15,000 cells were acquired.

IL-2 ELISA Assays

IL-2 levels were determined using a Human IL-2 Quantikine Immunoassay Kit (D2050; R&D Systems, MN, USA) following the manufacturer's instructions. Normal and transfected Jurkat T cells (1×10^5 cells/ml) were incubated with 6 μg/ml PHA and 1 ng/ml PMA (Sasagawa, et al., 2006, pp. 1214-1221) or with 10 μg/ml anti-CD3ε mAb plus 10 μg/ml anti-CD28 mAb (e-Bioscience) at 37 °C for 24 hours. 100 μl of cell-culture supernatants were collected, centrifuged and stored at –80°C until assayed.

100 μl of a buffered protein base was added into each well of an anti-IL-2 mAb coated 96-well microplate provided in the kit. 100 μl of standard, sample, or control were added to each well and incubated for 2 hours at RT. The plate was then washed with wash buffer three times. Subsequently, the plate was incubated with 200 μl per well of anti-IL-2 detection antibodies conjugated with horseradish peroxidase for 2 hours. After washing with wash buffer again, 200 μl of substrate solution containing a 1/1 mixture of H_2O_2 and tetramethylbenzidine was added and the solution is incubated for 20 minutes in the dark. The reaction was terminated by the addition of 50 μl of stop solution (2 M H_2SO_4). The optical density of each well at 450 nm was determined using a microplate reader (PerkinElmer Life Sciences). The amount of IL-2 was calculated as the relative percentage to 1200 pg/μl (i.e., 1200 pg/μl of IL-2 is

defined as 100%). All measurements were performed in duplicate and were repeated at two different occasions. Mean values of the two measurements were used for statistical analysis.

Statistical Analysis

All experiments were performed in duplicate, as stated otherwise, and were repeated on three different occasions. Statistical analyses were performed using SPSS software. All data were expressed as mean ± standard deviation (SD). Differences between experimental groups were analysed with analysis of variance (ANOVA) followed by Dunnett's comparison test. The differences were considered to be significant when $P<0.05$.

Table 1 Project Time Line

Activity Description	Month (Year 2010-2011)											
	Sep 10	Oct 10	Nov 10	Dec 10	Jan 11	Feb 11	Mar 11	Apr 11	May 11	Jun 11	Jul 11	Aug 11
1. Materials and equipments preperation	←	→										
2. Sample preparation		←	→									
3. Experimental operation				←	→							
4. Data collection and Analysis								←	→			
5. Conclusion and Final report									←	→		
6. Publication											←	→

Table 2 Financial Analysis

Budget List	Cost
1. Personnel budget	-
2. Operating budget	**350,000**
2.1 Salaries and Wages	-
Wages	-
2.2 Expense	**30,000**
Travel and Hotel fees	4,000
Scientific equipments fees	20,000
Publication and Dissemination	6,000
2.3 Materials and Equipments	**320,000**
Laboratory Materials:	270,000
- RPMI-1640, trypsin, FBS, penicillin-streptomycin, DMSO, glucanase, Stealth RNAi™ siRNA Duplexes specific for Nck1, negative control low GC content, FITC Annexin V Apoptosis Detection Kit I, BrdU reagent, PE-conjugated mouse anti-human CD69 mAb, isotype control mAb, ELISA assay reagents, BCA assay kit, etc.	
Laboratory Equipments:	48,000
- 75 cm^3 culture flasks, 96 well plates (flat- and U-bottom), serological pipette, pipette tips, 1.5, 5, 15, 50 ml steriled centrifuge tubes, microporator tips, etc.	
Office Materials and Equipments:	2,000
3. Investment budget	-
Total	**350,000**