CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and equipments

Chemicals and equipments used in this study are listed in Appendix A. The list of restriction enzymes are detailed in Appendix D. The recipes for reagent preparations are shown in Appendix E.

2.2 Cell culture

Human cervical cancer cell line (HeLa), a generous gift from Prof. Dr. Andre Lieber, (University of Washington, Seattle, WA, USA), and human embryonic kidney cell line (293A) (Invitrogen, Green Island, NY) were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 2 mM L-glutamine (Gibco, Grand Island, NY), penicillin (100 Units/ml) and streptomycin (100 µg/ml) (Gibco, Grand Island, NY). SupT1, the human T-cell lymphoblastic lymphoma cell line, kindly provided by Prof. Dr. Watchara Kasinrerk (Chiang Mai University, Thailand) was grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY). The medium was supplemented with 10% FBS, penicillin (100 Units/ml) and streptomycin (100 µg/ml) (Gibco, Grand Island, NY). All cell lines were cultured in a humidified 5% CO_2 atmosphere at 37˚C.

2.3 *E. coli* strains and vectors

E. coli strain XL-1 Blue and BL21 (DE3) were purchased from Stratagene (La Jolla, CA). The pCDM8 containing human CD4 gene and *E. coli* strain MC1061/P3 were generous gifts from Prof. Dr. Watchara Kasinrerk. *E. coli* strain BJ5183, modified

pAdTrack and pAdEasy vectors were kindly provided by Prof. Dr. Carlos F. Barbas (the Scripps Research Institute, La Jolla, CA, USA).

2.4 CD4-specific DARPin 23.2

2.4.1 Expression of CD4-specific DARPin 23.2

E. coli strain BL21 (DE3) carrying along pTriEx-CD4 specific DARPin 23.2 (GenBank: AM997265.1) were constructed in Prof. Dr. Chatchai Tayapiwatana's laboratory. The isolated bacterial clone was initially cultured in 3 ml of LB broth supplemented with 100 µg/ml penicillin in a 37°C shaking incubator at 200 rpm for overnight. Five hundred microliters of overnight culture were added to 50 ml Terrific (TR) broth supplemented with 100 µg/ml penicillin and 1% (w/v) glucose in a shaking incubator at 37°C until $OD_{600\ nm}$ reach 0.5. For large scale bacterial culture, 50 ml of pre-culture bacteria was added to TR broth supplemented with 100 µg/ml penicillin and 1% (w/v) glucose at total volume of 500 ml. The cells was grown in 37°C shaking incubator until $OD_{600\ nm}$ reach 0.8. The expression of CD4-specific DARPin 23.2 was induced by adding 0.1 mM IPTG and continued shaking at 30°C for 4 hours. The cells were harvested, spinned down at 1,000 g for 10 min at 4°C. The cells were wash once with PBS. The cells were resuspended in PBS including Protease Inhibitor Cocktail Set VII (Calbiochem, San Diego, CA) then go through 3 cycles of freezing/thawing and sonication prior to purification step.

2.4.2 Purification of CD4-specific DARPin 23.2

All the buffers used in the purification step were filtered and degased in advance of the process. After clarifying the bacterial cell lysate by centrifugation at 15,000 g for 30 min at 4°C, the CD4-specific DARPin will be purified using HiTrap HP affinity column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The bacterial lysate was mixed with Binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH7.4) at 1:1 ratio. The mixture was filtered with 0.45 µm filter to remove additional debris. The column was washed with distilled water to remove 20% ethanol for 5 – 10 column volumes (CV). Binding buffer was applied to equilibrate the column for another 5 – 10 CV. The filtered sample was introduced to the column. The Washing buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH7.4) was assigned to the

column until the absorbance reached the baseline. The gradient elution step was performed using Elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH7.4). The eluted protein was collected in 1-ml fractions.

The purity of purified CD4-specific DARPin was analyzed by SDS-PAGE. The purified fractions of CD4-specific DARPin were pooled according to purity and dialyzed in PBS. Before keeping DARPin at -70°C, glycerol was added to the protein at final concentration of 10%. The concentration of the protein was investigated using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Western immunoblotting determined the expression of CD4-specific DARPin. Two micrograms of purified CD4-specific DARPin was separated on 15% SDS-PAGE. The electrophoresis condition was performed at constant voltage of 100 volts using running buffer. The separated protein was blotted onto PVDF membrane at 58 mA for 90 min. The membrane was blocked with 5% skim milk in PBS for 1 hour at room temperature. The immunoblotting detection was performed by adding mouse anti-His tag (0.3 µg/ml) (Abm, Richmond, BC, Canada) diluted in blocking solution at 4°C for overnight. The membrane was washed four times with 0.05% Tween-20 in PBS. The blots was incubated with HRP conjugated goat anti-mouse immunoglobulins (1:3000) (KPL, Gaithersburg, MD) at room temperature for 1 hour. After washing step, the reaction was visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and exposed on Kodak Medical X-ray Film General Purpose Blue (Carestream Health, Inc, Rochester, NY).

2.4.3 CD4-specific DARPin 23.2 titration on SupT1 cells

To determine the proper concentration of CD4-specific DARPin 23.2 to be used in the experiment, binding of CD4-specific DARPin 23.2 to CD4 molecules on SupT1 cells was demonstrated using flow cytometry. SupT1 cells at 5×10^5 cells per reaction, were washed twice with PBS before the Fc receptors being blocked with 10% AB serum in 1% FBS-PBS-0.02% NaN_3 on ice for 30 min. CD4-specific DARPin 23.2 was added to the cells at final concentration of 10 and 1 µg/ml and 100, 10 and 1 ng/ml. The reactions were incubated on ice for 30 min and followed by washing step once with 1% FBS-PBS-0.02% NaN_3. The binding of CD4-specific DARPin 23.2 to CD4 molecules were investigated by adding mouse anti-His mAb (1 µg/ml) as the protein had histidine (6×His)

tag at the N-terminus. After incubation for 30 min on ice and washing step, rabbit anti-mouse immunoglobulins conjugated with RPE (5 µg/ml) (Dako, Glostrup, Denmark) was included to the reaction and incubated on ice for 30 minutes. The cells were washed three times with 1% FBS-PBS-0.02% NaN_3 in advance of being resuspended in 1% paraformaldehyde in PBS. The reactions were analyzed by FACSort (Becton Dickinson, Franklin Lakes, NJ) and Flowing Software version 2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland).

2.4.4 Binding of CD4-specific DARPin 23.2 on PBMC

To validate the ability to bind to CD4 molecules on primary cells of CD4-specific DARPin 23.2, PBMC was isolated from peripheral blood donor using density gradient centrifugation. Ten milliliters of EDTA blood was mixed with 10 ml of PBS. The blood mixture was gently overlayered over 5 ml of Isoprep (Robbins Scientific Corporation, Sunnyvale, CA) and centrifuge at 400 g for 30 min at room temperature. The buffy coat layer which contained high concentration of PBMC was collected. The PBMC was resuspended in PBS and centrifuged for 5 min at room temperature. The washing step with PBS was repeated again.

The PBMC at 5×10^5 cells per reaction was blocked with 10% AB serum in 1% FBS-PBS-0.02% NaN_3 on ice for 30 min. One hundred nanograms per milliliter of purified CD4-specific DARPin 23.2 was added to the reaction. The incubation was done on ice for 30 min. The washing step was performed with 1% FBS-PBS-0.02% NaN_3. Mouse anti-His mAb (1 µg/ml) was added to the reaction and incubated for 30 min on ice. CD4 molecules were stained separately with mouse monoclonal antibody against CD4 (MT4; generously provided by Prof. Dr. Watchara Kasinrerk, Chiangmai University, Chiang Mai, Thailand)) at final concentration of 10 µg/ml and incubated on ice for 30 min. After washing step, RPE conjugated rabbit anti-mouse immunoglobulins (5 µg/ml) was added to the reaction. The cells were incubated on ice for 30 min and followed by washing step. Ten microliters of IOTest® CD3-FITC (Immunotech, Marseille, France) was added to the PBMC. After 30 min incubation on ice, three times of washing steps with 1% FBS-PBS-0.02% NaN_3 was performed prior to resuspension the PBMC pellet in 1% paraformaldehyde in PBS. The cells were further analyzed by flow cytometry.

2.5 Production of CD4-specific DARPin (F100R) and CD4-specific DARPin (F100G)

2.5.1 Generation of CD4-specific DARPin (F100R) and CD4-specific DARPin (F100G)

To generate CD4-specific DARPin with mutation at phenylalanine position 100, the site-directed mutagenesis was performed. The original vector, pTriEx-CD4-specific DARPin 23.2 was mutated at position 100 into arginine (F100R) or glycine (F100G) using QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA) as protocol directed. The position of F100 to be mutated was shown in Figure 2.1. The overview of the protocol was illustrated in Figure 2.2. The primers used in the experiment were shown in Table 2.1.

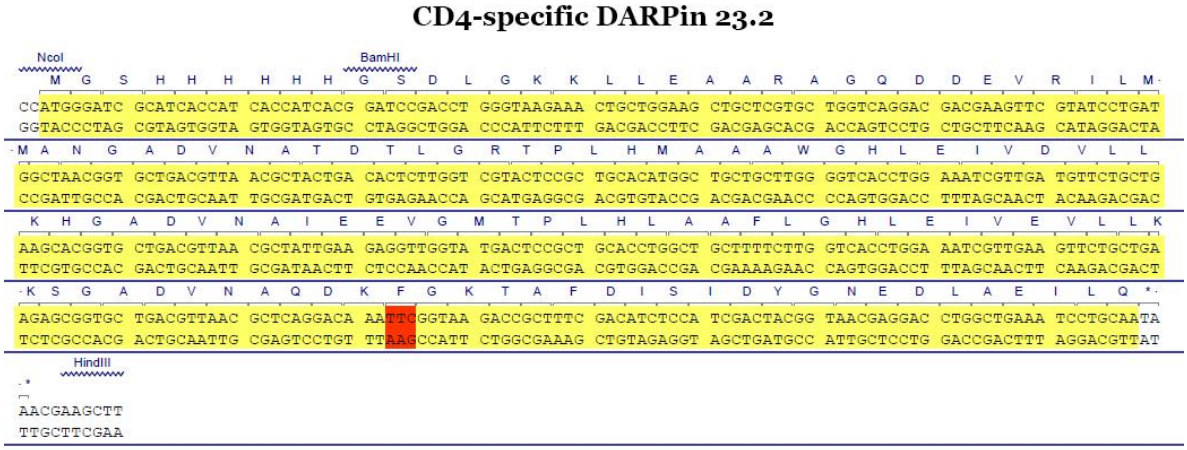

Figure 2.1 Nucleotide and amino acid sequences of CD4-specific DARPin 23.2. Deep orange highlight represented phenylalanine at position 100 (F100) to be mutated.

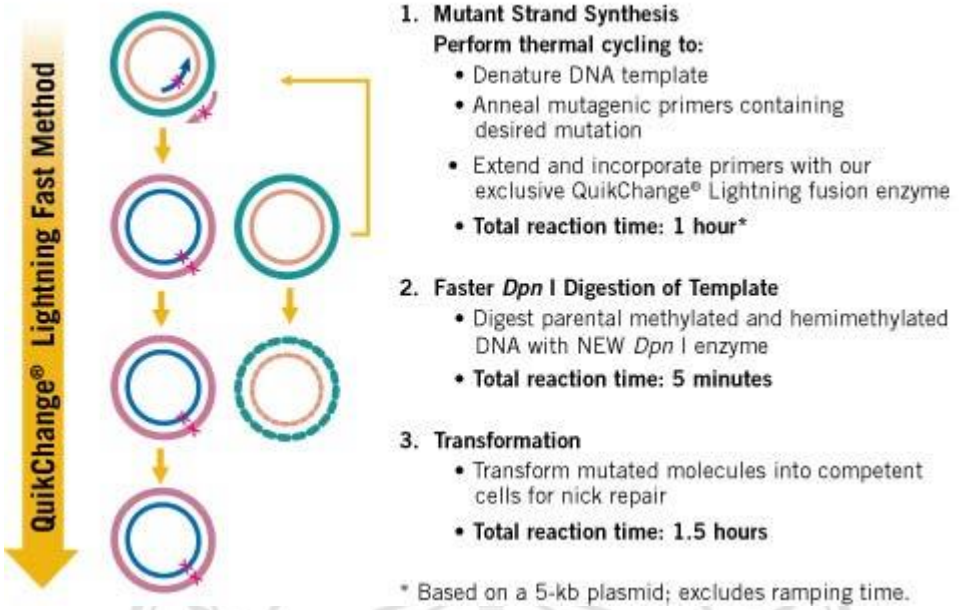

Figure 2.2 Schematic protocol in mutagenesis method using two primers to induce mutation at specific site. Treatment with *Dpn*I will eliminate the original vector, allowing only mutated vector to be transformed into competent cells for nick repair. (http://www.genomics.agilent.com/article.jsp?pageId=380&_requestid=809015)

Table 2.1 Primers used to generate CD4-specific DARPin (F100R) and CD4-specific DARPin (F100G)

Name	Sequences
F100R Forward	5’-AAC GCT CAG GAC AAA CGC GGT AAG ACC GCT TTC-3
F100R Reverse	5’- GAA AGC GGT CTT ACC GCG TTT GTC CTG AGC GTT-3’
F100G Forward	5’-AAC GCT CAG GAC AAA GGC GGT AAG ACC GCT TTC-3’
F100G Reverse	5’-GAA AGC GGT CTT ACC GCC TTT GTC CTG AGC GTT-3’

The mixture of 25 ng vector, 125 ng of each primer, dNTP mix, QuikSolution reagent, reaction buffer and QuikChange Lightning Enzyme in the total volume of 51 µl went through the initial denaturation step at 95°C for 2 min, 18 cycles of 95°C for 20 sec, 60°C for 10 sec, and 68°C for 3 min, then final extension at 68°C for 5 min. The mutated vector was later treated with *Dpn*I at 37°C for 5 min. Ten microliters of *Dpn*I-treated vector mixtures were transformed into competent XL-1 Blue. The clones were grown on LB agar supplemented with ampicillin (100 µg/ml) and tetracycline (10 µg/ml). Plasmid miniprep was performed to extract plasmids from the selected clones. The plasmids were screened for the gene by restriction enzyme analysis, *Hind*III and *Xho*I. The correct mutant clones were confirmed by sequencing.

2.5.2 Production of CD4-specific DARPin (F100R) and CD4-specific DARPin (F100G)

To produce soluble mutated DARPin, vectors encoded CD4-specific DARPin (F100R) and CD4-specific DARPin (F100G) were transformed into *E. coli* strain BL21 (DE3). The isolated clones carrying transformed vectors, pTriEx- CD4-specific DARPin (F100R) or pTriEx-CD4-specific DARPin (F100G) were picked on LB agar supplemented with ampicillin (100 µg/ml). For large scale production, the clones were cultured as described in the production of CD4-specific DARPin 23.2 (WT). The DARPins were purified using HiTrap HP affinity column. The purity of the DARPin fractions were determined by SDS-PAGE analysis. The purified proteins were dialyzed in PBS for overnight prior to addition of glycerol at final concentration of 10%. Protein concentration were considered by using Pierce™ BCA Protein Assay Kit. For Western immunoblotting, the purified DARPins were probed with mouse anti-His tag followed by goat anti-mouse immunoglobulins conjugated with HRP. The reaction was visualized by exposing the chemiluminescent reaction on to X-ray film.

2.6 Binding affinity of CD4-specific DARPin (F100R) and CD4-specific DARPin (F100G) to CD4 molecules

To investigate the binding affinity of CD-specific DARPin (F100R) and CD4-specific DARPin (F100G) comparing to CD4-specific DARPin 23.2 (WT), the experiment was performed by flow cytometry. SupT1 cells at 5×10^5 cells per reaction

were washed twice with PBS. The cells were incubated with 10% AB serum in 1% FBS-PBS-0.02% NaN_3 for 30 min on ice. Purified CD4-specific DARPin (F100R) and CD4-specific DARPin (F100G) were added to the cells at final concentration of 100 ng/ml. For wild type DARPin, the purified protein was added at 100 ng/ml. The reactions were incubated on ice for 30 min. The cells were washed once with 1% FBS-PBS-0.02% NaN_3. The binding of DARPin to CD4 molecules was followed by introducing mouse anti-His mAb (1 µg/ml) and incubate for 30 min on ice before being washed with 1% FBS-PBS-0.02% NaN_3. RPE conjugated rabbit anti-mouse immunoglobulins (5 µg/ml) was introduced to the reactions and incubated with the cells for 30 min on ice. The reactions were washed three times with 1% FBS-PBS-0.02% NaN_3 and finally resuspended in 1% paraformaldehyde. The cells were analyzed by flow cytometry.

2.7 Adenovirus receptors and CD4 expression profile of HeLa cell line

HeLa cell line was determined for adenovirus receptors, CAR and αv integrin on the cell surface, along with the expression of CD4 using flow cytometry. HeLa cells at 5×10^5 cells were used per reaction. The cells were washed three times with PBS. Ten percent human AB serum in 1% FBS-PBS-0.02% NaN_3 was used as blocking solution. The cells were incubated with blocking solution for 30 minutes on ice. The specific antibody against CAR (clone RmcB [48]) (1:100), αv integrin (clone L230) (1:100) (provided by Prof. Dr. Andre Lieber, University of Washington, Seattle, WA, USA) and CD4 (MT4) (10 µg/ml) were introduced to the cells and incubated on ice for 30 minutes. The cells were wash once with 1% FBS-PBS-0.02% NaN_3. The antibodies were tracked using rabbit anti-mouse immunoglobulins conjugated with RPE (5 µg/ml) and incubated on ice for 30 minutes. The cells were washed three times before reconstituted with 1% paraformaldehyde in PBS. The expression of these molecules were investigated using flow cytometry. The FACS data was analyzed using Flowing Software.

2.8 Generation of Ad5-CD4 (WT), Ad5-CD4 (K35A) and Ad5-CD4 (K35AQ40A)

2.8.1 Generation of CD4 (K35A) and CD4 (K35AQ40A)

The original vector pCDM8-CD4 were mutated to generate p+CDM8-CD4 (K35A) and pCDM8-CD4 (K35AQ40A) using the QuikChange® Lightning Site-

Directed Mutagenesis Kit as protocol directed. The amino acid sequence will be mutated from lysine to alanine at position 35 for CD4 (K35A). For CD4 (K35AQ40A) lysine at position 35 and glutamine at position 40 were both mutated into alanine. The sequences to be mutated are shown in Figure 2.3. The primers used in mutation were shown in Table 2.2.

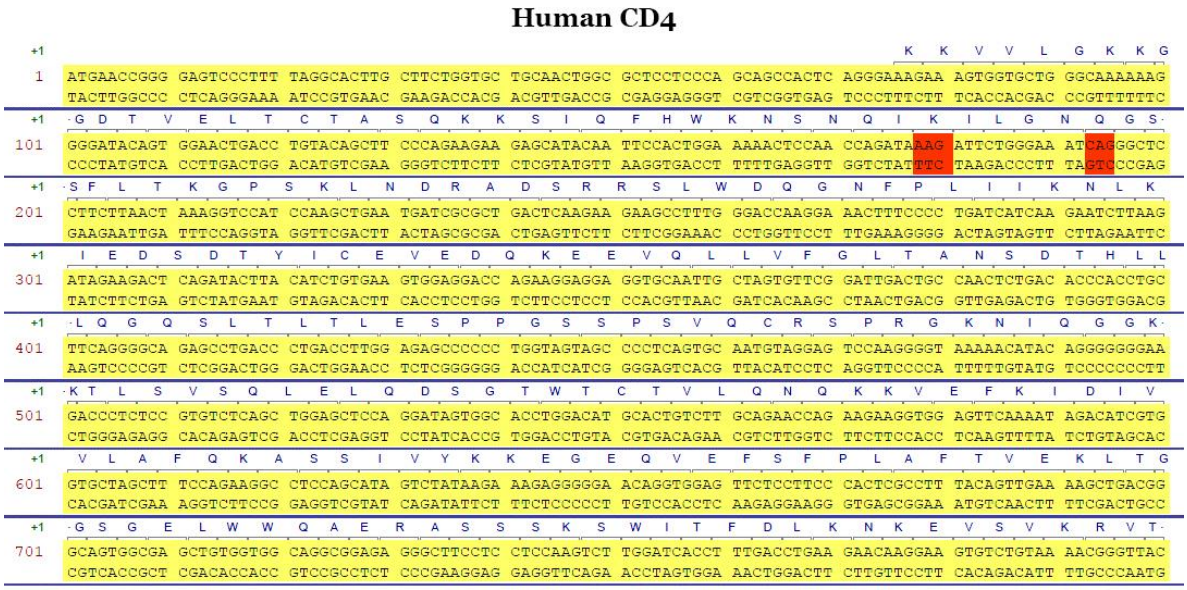

Figure 2.3 Part of extracellular domain of human CD4 gene, displaying D1 and D2, exhibited the position of K35 and Q40 to be mutated (highlight in deep orange).

Table 2.2 Primers used to generate CD4 (K35A) and CD4 (K35AQ40A)

Name	Sequences
CD4K35A Fwd	5'-AAC TCC AAC CAG ATA GCC ATT CTG GGA AAT CAG-3'
CD4K35A Rev	5'-CTG ATT TCC CAG AAT GGC TAT CTG GTT GGA GTT-3'
CD4K35AQ40A Fwd	5'-GCC ATT CTG GGA AAT GCC GGC TCC TTC TTA ACT-3'
CD4K35AQ40A Rev	5'-AGT TAA GAA GGA GCC GGC ATT TCC CAG AAT GGC-3'

The mixture of 25 ng vector, 125 ng of each CD4K35A primer, dNTP mix, QuikSolution reagent, reaction buffer and QuikChange Lightning Enzyme in the total volume of 51 µl went through the initial denaturation step at 95°C for 2 min, 18 cycles of 95°C for 20 sec, 60°C for 10 sec, and 68°C for 5 min, then final extension at 68°C for 5 min. The mutated vector was later treated with *Dpn*I at 37°C for 5 min. Ten microliters of *Dpn*I-treated pCDM8-CD4 (K35A) was transformed into *E. coli* MC1061/P3 and spread on LB agar plate containing ampicillin (50 µg/ml), kanamycin (40 µg/ml) and tetracycline (10 µg/ml). The single colonies were screened for CD4 (K35A) and CD4 (K35AQ40A) by PCR using a pair of primers, FwdCD4SfiI (5'-GAG GAG GAG GTG GCC CAG GCG GCC AAG AAA GTG GTG CTG GGC-3') and RevCD4SfiI (5'-GAG GAG GAG CTG GCC GGC CTG GCC AAT GGG GCT ACA TGT CTT C-3'). The recognition sequences of *Sfi*I were underlined. Plasmid minipreparation was performed to extract the mutated plasmid from the PCR-positive clones. The plasmids were screened again using *Nhe*I (Thermo Scientific, Waltham, MA) restriction enzyme. The mutated sequence was confirmed by sequencing.

To generate CD4 (K35AQ40A), the confirmed pCDM8-CD4 (K35A) went through the second site-directed mutagenesis process using the pair of CD4K35AQ40A primers shown in Table 2.2. The *Dpn*I-treated pCDM8-CD4 (K35AQ40A) was

transformed into *E. coli* MC1061/P3. The clones were screened and confirmed with the same method stated above.

2.8.2 Construction of adenovirus vector carrying CD4 (WT), CD4 (K35A) and CD4 (K35AQ40A) gene

The vector are based on adenovirus serotype 5 using AdEasy™ Adenoviral Vector System. The Ad5-CD4 (WT) was cloned and proved from Prof. Dr. Chatchai Tayapiwatana's lab. CD4 (K35A) and CD4 (K35AQ40A) fragments were amplified from pCDM8-CD4 (K35A) and CD4 (K35AQ40A) by Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, Lithuania) with FwdCD4SfiI and RevCD4SfiI using standard PCR protocol. The purified fragments of CD4 (K35A) and CD4 (K35AQ40A) were treated with *Sfi*I (Thermo Scientific, Waltham, MA) to expose the recognition sites. The *Sfi*I-treated CD4 mutants were ligated with *Sfi*I-excised modified pAdTrackCMV using T4 DNA ligase (Thermo Scientific, Waltham, MA) at 4°C for 16 hours. The ligated pAdTrackCMV-CD4 were separately transformed into *E. coli* strain XL1-BLUE. The clones were screened and confirmed using PCR, restriction enzyme analysis and sequencing.

The pAdTrackCMV-CD4 (K35A) and pAdTrackCMV-CD4 (K35AQ40A) were digested with *Mss*I (*Pme*I) (Thermo Scientific, Waltham, MA) before being transformed into pre-transformed-pAdEasy *E. coli* clone BJ5183. Inside the bacterial cell, the homologous recombination formed through left and right homology arms. The single colony grew on LB agar plate containing kanamycin (70 µg/ml) reflected the recombined adenovirus vector carrying CD4 (K35A) and CD4 (K35AQ40A) gene. The clones were screened using standard PCR protocol and restriction enzyme analysis. The recombined adenoviral vector were purified in large scale using HiSpeed® Plasmid Midi Kit (Qiagen, Hilden, Germany).

2.8.3 Production of Ad5-CD4 (K35A) and Ad5- CD4 (K35AQ40A)

The adenovirus vector carrying CD4 (K35A) and CD4 (K35AQ40A) genes were linearized by *Pac*I (Thermo Scientific, Waltham, MA) before being transfected into 293A cells using Lipofectamine® (Invitrogen, Carlsbad, CA) and Plus™ Reagent (Invitrogen,

Carlsbad, CA) as manufacturer's protocol directed. Twenty four hours before transfection, 293A cells were plated into 24-well plate at 1×10^5 cells per well. The cells should achieve 50 – 80% confluence in order to perform transfection. One microgram of *Pac*I-treated linearized adenovirus vectors were incubated with 8 µl Plus™ Reagent and serum-free DMEM at room temperature for 15 minutes. The mixture of 2 µl of Lipofectamine® in serum-free DMEM was added into the vector mixture and incubated at room temperature for another 15 minutes. The cell culture medium was removed and replaced with serum-free DMEM prior to transfection. The DNA-Lipofectamine mixture was gently dropped onto the cells. DMEM supplemented with 20% FBS was added to the cells after the 3-hour incubation at 37°C in 5% CO_2 atmosphere. The transfection efficiency will be observed *via* GFP expression. The comet formation exhibited the production of Ad5-CD4 and its ability to transduce the neighboring cells. The transduced cells were round and became less attached to the tissue culture plate, showing signs of cytopathic effects (CPE). Both cells and media were collected and followed by freezing/thawing process for four times. The adenovirus was kept at -70°C as viral stock. High titer viral stock were produced for the experiments.

2.8.4 Adenovirus amplification and concentration

To create a large viral stock for the experiments, 50 µl of frozen viral stock was transduced into 1×10^5 pre-plated 293A in 24-well plate to 1 ml total volume and incubated at 37°C with 5% CO_2. The transduced cells along with cultured media were harvested as they reached full CPE. The cells went through four cycle of freezing and thawing, creating the second viral stock. 293 A of 5×10^5 was transduced with 100 µl second viral stock in the total volume of 3 ml. After harvesting, freezing and thawing, this virus was called third viral stock. This stock was later transduced into 1×10^6 293A cells, creating fourth viral stock. The virus amplification moved on to 100 mm tissue culture dish with 80% confluence of 293A cells. The final stock of virus was amplified from three 150 mm tissue culture dish. The transduced cells were harvested and spinned down at 400 g for 10 minutes at 4°C to discard the culture supernatant. The pellet was resuspended in 1 ml of complete DMEM. Four freezing and thawing cycles were done in order to lyse the cells. The lysate went through centrifugation process at 400 g at 4°C for

10 minutes to remove the cellular debris. The supernatant was collected as the concentrated viral stock at -70°C for further experiments.

2.8.5 Adenovirus titration in HeLa cell line

To investigate the amount of adenovirus used in the experiments, HeLa cells were plated in 24-well tissue culture plate at 1×10^5 cells for 24 hours before transduction. Ad5-CD4 (WT), Ad5-CD4 (K35A) and Ad5-CD4 (K35AQ40A) were added to the cells at volume of 2.5, 5, 10, 15 and 20 µl. At 36 hours post transduction, the transduced cells were harvested and investigated the cells viability using 0.2% trypan blue. The expression of GFP were analyzed by flow cytometry.

2.8.6 Detection of CD4 (WT), CD4 (K35A) and CD4 (K35AQ40A) expression

To determine the expression of CD4 (WT), CD4 (K35A) and CD4 (K35AQ40A), the transduced HeLa cells were analyzed by flow cytometry. Twenty four hours prior transduction, HeLa cells were plated in 24-well tissue culture plate at 1×10^5 cells per well. At the cell confluence at 60 – 80%, the cells were transduced with the adenoviruses. The cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C for 36 h before being harvested. The transduced cells were washed twice before being blocked with 10% AB serum in 1% FBS-PBS-0.02% NaN_3 for 30 min on ice. The CD4 expressed on cell surface was probed with mouse anti-CD4 (MT4) (10 µg/ml). After 30-minute incubation on ice, the cells were washed once with 1% FBS-PBS-0.02% NaN_3. Rabbit anti-mouse immunoglobulins conjugated with RPE (5 µg/ml) was added to the cells then incubate on ice for 30 min. The cells were washed three times with 1% FBS-PBS-0.02% NaN_3. Cell pellets were resuspended in 1% paraformaldehyde in PBS before analyzing with flow cytometer.

2.9 Binding of CD4 (K35A) and CD4 (K35AQ40A) to CD4-specific DARPin

To compare the binding affinity of CD4-specific DARPin 23.2 to CD4 molecules, 1×10^5-plated HeLa cells were transduced with Ad5-CD4 (WT), Ad5-CD4 (K35A) or Ad5-CD4 (K35AQ40A), followed by 36 h incubation in a humidified 5% CO_2

atmosphere at 37˚C. The cells were harvested and washed twice with PBS. The transduced cells were block with 10% AB serum on ice for 30 min. Purified CD4-specific DARPin 23.2 (100 ng/ml) was added to the reaction. The cells were incubated on ice for 30 min. The cells were washed with 1% FBS-PBS-0.02% NaN_3. The CD4-specific DARPin was tracked by adding mouse anti-His tag (1 µg/ml). The reactions were incubated on ice for 30 min. After washing step, mouse anti-His tag were followed by RPE conjugated rabbit anti-mouse immunoglobulins. The transduced cell were washed three times before being resuspended with 1% paraformaldehyde in PBS. The cells were later analyzed by flow cytometry.