CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and equipments

Chemicals and equipments used in this study were shown in Appendix A and Appendix B.

2.2 *E. coli* strains and vectors

E. coli strains, TG-1 and HB2151 were kindly provided by Dr. A.D. Griffiths, MRC, Cambridge, UK. *E. coli* strains, Origami B and XL-1 Blue were purchased from Novagen (Madison, WI) and Stratagene (La Jolla, CA), respectively. The pComb3HSS, pComb3X, modified pAdTrack and pAdEasy vectors were generous gifts from Dr. Carlos F Barbas, The Scripps Research Institute, La Jolla, California, USA (Steinberger et al., 2000; Jendreyko et al., 2003). The pAK400cb vector was a kind gift from Dr. V. Santala (University of Turku, Finland). The modified pAdEasy-1/35 vector was generous gift from Prof. Xiaolong Fan (Nilsson et al., 2004), Department of Molecular Medicine and Gene Therapy, Lund University, Lund, Sweden.

2.3 Extraction and qualification of total RNA from M6-1B9 hybridoma cells

Hybridoma cells producing anti-CD147 mAb, M6-1B9 (isotype IgG3) was kindly provided from Prof. Dr. Watchara Kasinrerk, Division of Clinical

Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand (Kasinrerk et al., 1999). Cells were cultured in Iscove's Modified Dulbecco's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco), 40 μg/ml gentamicin and 2.5 μg/ml amphotericin B. Total RNA was extracted from 5×10^6 M6-1B9-producing hybridoma cells using TRIzol® (Invitrogen) according to the manufacturer's instructions. Initially, M6-1B9 hybridoma cells were washed and lysed by adding 1 ml TRIzol® reagent. Following homogenization, insoluble material was removed from the homogenate by centrifugation at 12,000 rpm for 10 min at 4 °C. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. The supernatant was transferred to a new microcentrifuge tube and incubated the homogenized samples for 5 min at room temperature (RT) to permit the complete dissociation of nucleoprotein complexes. Two-hundred microliter of chloroform per 1 ml of TRIzol® reagent was added and tubes were shaken vigorously by hand for 15 sec and incubated them at RT for 2-3 min. Then, the sample was centrifuged at 12,000 rpm for 15 min at 4 °C. The aqueous phase (clear) was collected to a fresh microcentrifuge tube and 500 μl of isopropanol was added to precipitate the total RNA from the aqueous phase. The solution was mixed by inversion and incubated for 10 min at RT. Centrifugation was performed at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded and the RNA pellet was washed once with 1 ml of 75% ethanol. Then the sample was mixed by vortexing and centrifuged at 7,500 rpm for 5 min at 4 °C. The RNA pellet was dried at RT for 5-10 min and finally dissolved in 20-30 μl of DEPC-treated water.

The amount of the total RNA was determined by UV spectrophotometer (1 OD at 260 nm is equal to 40 μg/ml).

2.4 RT-PCR amplification of Fd heavy chain and κ light chain cDNA fragments

First stranded cDNA was generated from 1 μg total RNA using random primers for Fab fragment construction. The set of oligonucleotides were synthesized according to the genes coding the κ light chain and the Fd region of the IgG3 heavy chain as shown in **Table 2.1** *Xho*I and *Sac*I restriction sites were added to the sense primers of the heavy and light chains, respectively. The antisense primers were introduced the *Spe*I site for heavy chains and the *Xba*I site for light chains.

The PCR amplification was performed in 50 μl reactions. Both sense and antisense primers were used at a concentration of 10 μM. Fourty cycles of the PCR were done as follows: denaturation at 94 °C for 15 sec, annealing at 52 °C for 50 sec, and extension at 68 °C for 1 min. An initial cDNA synthesis of 30 min at 55 °C and follow with denaturation step of 2 min at 94 °C and a final extension step of 5 min at 68 °C were also included. The PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV illumination.

Table 2.1 A list of primers use for constructing the Fab anti-CD147 (M6-1B9) cDNA.

Primers	Sequences
Heavy-chain Fd 3' primer	5'- GGG GGT act agt CTT GGG TAT TCT AGG CTC-3'
Heavy-chain variable 5' primer	
Hc1	5'- AGG TCC AGC TGc tcg agT CTG G-3'
Hc2	5'- AGG TCC AGC TGc tcg agT CAG G-3'
Hc3	5'- AGG TCC AGC TTc tcg agT CTG G-3'
Hc4	5'- AGG TCC AGC TTc tcg agT CAG G-3'
Hc5	5'- AGG TCC AAC TGc tcg agT CTG G-3'
Hc6	5'- AGG TCC AAC TGc tcg agT CAG G-3'
Hc7	5'- AGG TCC AAC TTc tcg agT CTG G-3'
Hc8	5'- AGG TCC AAC TTc tcg agT CAG G-3'
Hc9	5'- AGG TII AIC TIc tcg agT CAG G-3'
Hc10	5'- AGG TII AIC TIc tcg agT CTG G-3'
Murine κ light-chain 3' primers	5'- GCG CCG tct aga ATT AAC ACT CAT TCC TGT TGA A-3'
Murine κ light-chain 5' primers	
Lc1	5'- CCA GTT CCg agc tcG TTG TGA CTC AGG AAT CT-3'
Lc2	5'- CCA GTT CCg agc tcG TGT TGA CGC AGC CGC CC-3'
Lc3	5'- CCA GTT CCg agc tcG TGC TCA CCC AGT CTC CA-3'
Lc4	5'- CCA GTT CCg agc tcC AGA TGA CCC AGT CTC CA-3'
Lc5	5'- CCA GAT GTg agc tcG TGA TGA CCC AGA CTC CA-3'
Lc6	5'- CCA GAT GTg agc tcG TCA TGA CCC AGT CTC CA-3'
Lc7	5'- CCA GTT CCg agc tcG TGA TGA CAC AGT CTC CA-3'

Footnote: Restriction enzyme recognition sites are designated in small letters, with actagt for *Spe*I, ctcgag for *Xho*I, tctaga for *Xba*I and gagctc for *Sac*I. I in primer sequences is stand for inosine.

2.5 PCR product purification by QIAquick Extraction kit

The DNA fragment was excised from the agarose gel with a clean-sharp scalpel. The gel slice was later weighted in a colorless tube and calculated the volume by 100 mg estimated 100 µl. Three volume of the QG buffer to 1 volume of gel was added to dissolute the gel. The mixture was further incubated at 50 °C for 10 min or until the gel was completely dissolved. After dissolution, the mixture was transferred to QIAquick spin column, which was placed on a 2 ml collection tube. To bind the DNA, the spin column was subsequently centrifuged at 13,000 rpm for 1 min. The flow through was discarded. The QIAquick spin column was washed using 750 µl of PE buffer and centrifuged another 2 times at 13,000 rpm for 1 min in order to eliminate the flow through solution. Finally, the DNA binding column was eluted using 30 µl of distilled water and spun at 13,000 rpm for 1 min. The size of DNA was checked by 1% agarose gel electrophoresis.

2.6 Construction of phagemid expressing Fab-M6-1B9

2.6.1 Construction of pComb3H-SS possessing heavy chain gene

The Fd heavy chain DNA fragments of PCR products were digested with *Spe*I and *Xho*I (Fermentas), and 100 ng of digested PCR products was ligated with 210 ng of *Spe*I/*Xho*I sites of the linearized phagemid expression vector pComb3HSS in a total volume of 20 µl with 5 units of T4 DNA ligase enzyme (Fermentas). The reaction mixture was incubated at 4 °C for 16 h. The ligated product was transformed into the competent *E. coli* XL-1 Blue (Stratagene, La Jolla, CA). The

ligated DNA was co-incubated with 200 µl of cold-thawed $CaCl_2$ competent cells on ice for 1 h. The mixture was transferred into cooled screw cap tube and subsequently shocked at 42 °C for 1.5 min, then abruptly chilled on ice for 1 min. Three milliliter Luria-Bertani (LB) broth without antibiotic was added and bacteria was further cultured with shaking at 120 rpm, 37 °C for 3 h. The transformed bacteria were centrifuged at 2,500 rpm, RT for 10 min and plated on LB agar containing 100 µg/ml of ampicillin. The plates were incubated at 37 °C for 14-16 h.

2.6.2 Phagemid purification by using alkaline lysis method

An ampicillin resistant colony was picked and grown in 3 ml of LB broth containing 100 µg/ml of ampicillin with vigorous shaking (180 rpm) at 37 °C for 8 h. Half of culture volume was centrifuged at 10,000 rpm, 4 °C for 5 min. The supernatant was discarded and the bacterial cell pellet was lysed by adding 100 µl of 1× glucomix-lysozyme and vortexed vigorously. Two hundred microliters of freshly prepared NaOH/SDS was added and mixed by inverting. Then, 150 µl of potassium acetate was added and gently mixed by vortexing. The solution was centrifuged at 10,000 rpm, 4 °C for 5 min for collecting the clear supernatant. Nine hundred microliters of absolute ethanol was added and kept on ice for 2 min. The DNA was spun down at 10,000 rpm, 4 °C for 5 min and discarded the supernatant. The DNA pellet was reconstituted by 100 µl of steriled DW and followed by adding 50 µl of 7.5 M ammonium acetate and incubated at -70 °C for 10 min. The supernatant was collected by centrifugation at 10,000 rpm at 4 °C for 5 min. Three hundred microliters of absolute ethanol was added to the solution and incubated at -70 °C for 10 min. The solution was spun down to harvest the pellet. The pellet was cleaned up with 1 ml

70% ethanol by centrifugation at 10,000 rpm, 4 °C for 10 min. The DNA pellet was dried at 37 °C for 30 min, reconstituted with 30 µl of steriled DW and stored at -20 °C.

2.6.3 Characterization of recombinant clones

The purified phagemids were firstly checked by fractionating on 1% agarose gel electrophoresis. In order to verify the corrected *E. coli* clones, the purified phagemid from individual clone was characterized by digesting with *Spe*I and *Xho*I to identify the corrected band of insertion fragment. The PCR was used to confirm the corrected size of Fd heavy chain fragment. The newly constructed phagemid was named pComb3H-Fd.

2.6.4 Construction of pComb3H-Fd harboring light chain fragment

Phagemids containing the Fd heavy chain were prepared from overnight culture by QIAGEN Miniprep kit (Qiagen, Hilden, Germany) and digested with *Sac*I and *Xba*I restriction enzymes. The κ light chain fragments were also treated with the same restriction enzymes. After digestion, 1,200 bp light chain stuffer was removed by agarose gel extraction kit (Qiagen) and 4,000 bp linearized pComb3H-Fd was collected to further insert κ light chain. The ligation, transformation, phagemid purification of recombinant *E. coli* was performed as described above. Resriction fragment analysis of the purified plasmid was checked by *Sac*I and *Xba*I digestion. The PCR amplified product was checked for an insertion gene in the purified plasmid as described above. Finally, the DNA sequencing were prepared with specific reverse primers Hc1 (5'- AGG TCC AGC TGc tcg agT CTG G-3') and Heavy-chain Fd 3' primer (5'- GGG GGT act agt CTT GGG TAT TCT AGG CTC-3') for V_H and Lc7

primer (5'- CCA GTT CCg agc tcG TGA TGA CAC AGT CTC CA-3') and κ light-chain primer (5'- GCG CCG tct aga ATT AAC ACT CAT TCC TGT TGA A-3') for V_L genes, using the ABI automatic sequencer (Perkins Elmer), following manufacturers' instructions. The newly constructed phagemid harboring κ light chain fragment was named pCom3H-Fab-M6-1B9.

2.6.5 Plasmid Mini Prep (Qiagen)

An amplicillin resistant colony was picked and grown in 3 ml LB broth with 100 ug/ml of ampicillin with vigorous shaking (200 rpm) at 37 °C for 8 h. Cell suspension was spun at 10,000 rpm, 4 °C for 5 min. The supernatant was discarded and the pelleted bacterial cells were resuspended in 250 µl Buffer P1. Two hundred and fifty microliters of Buffer P2 were added and gently inverted the tube 4-6 times until the solution becomes viscous and slightly clear. Then, 350 µl Buffer N3 was added and mixed the solution gently but thoroughly, immediately for 4-6 times. The solution was centrifuged at 13,000 rpm for 10 min. The supernatant was collected, applied to the QIAprep spin column and centrifuged for 1 min at 13,000 rpm. QIAprep spin column was washed by adding 750 µl Buffer PE and centrifuging for 1 min. The flow-through was discarded, and centrifuged for an additional 1 min to remove residual wash buffer. Finally the QIAprep column was placed in a new microcentrifuge tube. DNA was eluted by adding 50 µl steriled DW to the center of each QIAprep spin column. The column was standed for 1 min and centrifuged at 13,000 rpm for 1 min. The DNA was stored at -20 °C.

2.7 Conversion of a M6-1B9 specific Fab into a single chain antibody fragment (scFv)

IgG-specific variable heavy (V_H) and light (V_L) chain gene fragments from purified pCom3H-Fab-M6-1B9 were amplified using PCR system (Eppendorf, England) for 30 cycles in first round (at 94 °C for 15 sec, at 56 °C for 30 sec, at 72 °C for 90 sec and 10 min at 72 °C for final extension), with each sense and anti-sense oligonucleotide primers set in **Table 2.2**. The fragments were isolated from a 1.5% agarose gel with the QIAGEN PCR purification kit (Qiagen). Then fragments were used as templates for the second round of PCR amplification to extend a linker, V_H fragments used MSCVH14 and MSCG3_B primers, V_L fragments used OmpSeq and MSCJK5-BL primers, respectively (Barbas, 2001). The amplified V_H-linker and V_L-linker PCR products were combined in a PCR reaction mixture. Twenty cycles (at 94 °C for 15 sec, at 56 °C for 30 sec, at 72 °C for 2 min and final extension for 10 min at the same temperature) were performed. These were gel-purified, digested with *Sfi*I, cloned into phagemid vector pComb3X and transformed into electro-competent *E. coli* TG1. The electroporated cells were then grown and plated onto LB agar with ampicillin. Colonies bearing the pComb3X-scFv-M6-1B9 construct were confirmed by *Sfi*I restriction enzyme digestion and PCR. Finally, the inserted gene fragment was sequenced using an ABI 3100 automatic sequencer.

Table 2.2 A list of primer used for constructing the scFv-M6-1B9.

Primers	**Sequences**
Heavy-chain variable (V_H) primer sets :	
Sense primer: MSCVH14	5’- GGT GGT TCC TCT AGA TCT TCC CTC GAG GTR AAG CTT CTC GAG TC -3’
Antisense primer: MSCG3_B	5’- CCT GGC CGG CCT GGC CAC TAG TGA CAG ATG GGG CTG TTG TTG T -3’
Light-chain variable (V_L) primer sets **:**	
Sense primer: OmpSeq	5’- AAG ACA GCT ATC GCG ATT GCA G -3’
Antisense primer: MSCJK5-BL	5’- GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA TTT CAG CTC CAG CTT GGT CCC -3’

2.8 Phage displaying scFv-M6-1B9 (or Fab-M6-1B9) *via* gpIII

2.8.1 Preparation of phage-displayed scFv-M6-1B9

A single colony of *E. coli* TG1 harboring pComb3X-scFv-M6-1B9 was chosen from an LB agar plate containing ampicillin for phage-displayed scFv-M6-1B9 preparation as previously described (Intasai et al., 2003). The transformed bacteria were grown in 10 ml of 2×TY broth containing ampicillin (100 μg/ml) at 37°C with shaking at 200 rpm. The precultured bacteria were subsequently transferred to the same medium containing 1% (w/v) glucose, 1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) and cultivated at 25 °C until the optical density at 600 nm (OD_{600}) reach 0.5. After induction, the bacterial culture was further infected with 10^{12} t.u./ml of VCSM13 helper phages and left at 37 °C for 30 min without shaking. Phage infected TG-1 was spun down at 3,000 rpm for 10 min at 4 °C. The pellet was resuspended in 30 ml 2×TY broth containing ampicillin (100 μg/ml) and kanamycin (70 μg/ml) and then transferred to 220 ml of the same broth and shaken at 180 rpm, 25 °C for 16 h.

2.8.2 Harvesting phage by polyethylene glycol (PEG) precipitation

Bacteriophage harboring scFv-M6-1B9 *via* gpIII was pelleted at 3,000 rpm, 4 °C for 30 min. The culture supernatant was further collected. The recombinant phages were harvested by 4% w/v of PEG 8,000 and 3% w/v of NaCl precipitation with shaking at 180 rpm, RT for 15 min or until PEG/NaCl completely dissolved. The supernatant was kept on ice for 30 min and centrifuged 10,800 rpm at 4 °C for 30 min. The pellet was air dried for 30 min and reconstituted with 2.5 ml of PBS pH 7.2.

The suspension was subsequently centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was preserved in 30% glycerol and stored at -70 °C.

2.8.3 Phage displaying scFv-M6-1B9 titration by *E. coli* infection

A single colony of *E. coli* TG1 was inoculated into 10 ml 2×TY broth and grown at 37 °C until the OD_{600} reach 0.6. One microliter of phage-displayed scFv-M6-1B9 was added into 999 ml of PBS pH 7.2 (dilution $1:10^3$) and then 1 µl of mixture was added into 1 ml of cultured bacteria (dilution $1:10^6$) and incubated for 15 min. The infected *E. coli* TG1 was further diluted into 10^8 and 10^{10} in PBS pH 7.2. Fifty microliters of each dilution was plated onto LB agar containing 100 µg/ml ampicillin and incubated at 37 °C overnight. The ampicillin resistant colonies were counted and calculated for the phage concentration using the formula at below.

$$A = B \times C \times (1000/V)$$

A = The original amount of phage obtained from preparation process (CFU/ml)

B = The number of ampicillin resistant colonies

C = Titer of viral infected bacteria

V = Volume (µl) of viral infected bacteria

In addition, *E. coli* TG1 harboring pCom3H-Fab-M6-1B9 which used for phage-displayed Fab-M6-1B9 *via* gpIII preparation was performed with the same method as described in section 2.8.1 - 2.8.3.

2.9 Immunoassay for detection of phage expressing recombinant M6-1B9

2.9.1 Determination the reactivity of phage expressing M6-1B9 by ELISA

Fifty microliters of 10 µg/ml avidin in carbonate/bicarbonate buffer pH 8.6 were coated in microtiter plates (NUNC, Roskilde, Denmark) at 4 °C overnight. The plate was then blocked with 200 µl of 2% skimmed milk in PBS pH 7.2 and incubated for 1 h at room temperature (RT). The wells were washed five times with 0.05% Tween-20 in PBS pH 7.2. After washing, 50 µl of 100 µg/ml BCCP fusion proteins, *i.e.* CD147-BCCP or SVV-BCCP (Tayapiwatana et al., 2006) in 2% skimmed milk, was added and the mixture was incubated for 1 h at RT. Unbound antigen was washed out and 50 µl of phage-displayed scFv-M6-1B9 (or phage-displayed Fab-M6-1B9 and VCSM13 phage) were added and incubated in a moist chamber for 1 h at RT. The plate was washed thoroughly with 0.05% Tween 20 in PBS pH 7.2 for 5 times, and peroxidase-conjugated anti-M13 phage mAb (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added to each well. Wells were then washed again prior to adding 100 µl 3, 3', 5, 5'-tetramethyl-benzidine (TMB) substrate. The OD at 450 nm was measured by an ELISA plate reader (TECAN, Austria) after adding 1 N HCl to stop the reaction. Monoclonal antibody M6-1B9 specific for CD147 was kindly provided by Prof. Dr. Watchara Kasinrerk, Division of Clinical Immunology, Department of Medical Technology, Facullty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand (Kasinrerk et al., 1999) and used as an antibody control in the ELISA system.

2.9.2 Determination of phage expressing M6-1B9 by SDS-PAGE and Western immunoblotting

10^{13} t.u/ml of recombinant phage-displayed scFv-M6-1B9 (or phage-displayed Fab-M6-1B9 and VCSM13 phage) were separated by SDS-PAGE under reducing conditions on a 12% polyacrylamide gel. The sample were boiled for 5 min and loaded onto the well of SDS-PAGE. Electrophoresis was carried out by applying constant voltage at 100 Volts using 0.025 M Tris/glycine, pH 8.5 containing 0.1% SDS as an electrophoretic buffer. After electrophoresis, the separated proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane. Blocking was performed for 2 h at room temperature with 5% skimmed milk in PBS pH 7.2 and further incubated with mouse anti-gpIII mAb (Exalpha Biologicals, Water town, MD) (dilution 1:2500) for 1 h. The membrane was washed five times with 0.05% Tween 20 in PBS pH 7.2 and then incubated with peroxidase-labeled goat anti-mouse immunoglobulins (KPL, Gaithersburg, MD) 1:8000 diluted in 5% skimmed milk in PBS pH 7.2 for 1 h. Unbound conjugate was washed out five times with 0.05% Tween 20 in PBS pH 7.2; the specific bands were visualized using an ECL chemiluminescent substrate detection system (Amersham Pharmacia) according to the manufacturer's protocol.

2.10 Preparation of soluble scFv-M6-1B9

E. coli TG1 harboring pComb3X-scFv-M6-1B9 was cultured in LB broth containing ampicillin and used for phagemid preparation by QIAGEN Miniprep Kit (Qiagen) (See in section 2.6.5). The phagemid, pComb3X-scFv-M6-1B9 was

transformed into the competent the non-suppressor *E. coli* strain HB2151 and incubated on ice for 1 h. The mixture was transferred into cooled screw cap tube and subsequently shocked at 42 °C for 1.5 min, then abruptly chilled on ice for 1 min. Three milliliters LB broth without antibiotic was added and bacteria was further cultured with shaking at 120 rpm, 37 °C for 3 h. the transformed bacteria was centrifuged at 2,500 rpm, RT for 10 min and plated on LB agar containing 100 µg/ml of ampicillin. The plates were incubated at 37 °C for 14-16 h. A single colony of transformed bacteria were grown in 10 ml of SB broth containing ampicillin (100 µg/ml) at 37 °C for 18 h. Ten microliters of precultured bacteria were subsequently transferred to 10 ml of the same medium containing 1% (w/v) glucose and ampicillin (100 µg/ml), then cultivated at 37 °C until the absorption at 600 nm reached 0.5. The precultured bacteria were then transferred to 90 ml of the same medium and cultivated at the same temperature until the OD_{600} reached 1.5. Then, IPTG was added to the culture at a final concentration of 1 mM. After induction, the bacteria were grown at 25 °C for 20 h. Cells were centrifuged at 15,000 g for 30 min at 4 °C to collect the supernatant (containing extracellular soluble scFv). Protein was precipitated with saturated $(NH_4)_2SO_4$ in an ice bath and concentrated with Amicon Ultra centrifugal filter units (Millipore, Cork, Ireland). Finally, the concentrated protein was reconstituted with 500 µl of 0.15 M PBS, pH 7.2.

2.11 Immunoassay for detection of the antigen- binding affinity of soluble scFv-M6-1B9

2.11.1 Binding assay of soluble scFv-M6-1B9 by ELISA

Fifty microliters of 10 µg/ml avidin in carbonate/bicarbonate buffer pH 8.6 were coated microtiter plates and incubated at 4°C overnight. The plates were then blocked with 200 µl of 2% bovine serum albumin (BSA) in PBS pH 7.2 for 1 h at RT. The wells were washed five times with 0.05% Tween-20 in PBS pH 7.2 and 50 µl of 100 µg/ml BCCP fusion proteins (Tayapiwatana et al., 2006) in 2% BSA were added and incubated for 1 h at RT. The unbound antigen was washed out. Fifty microliters of scFv-M6-1B9 at various dilutions were added and incubated in a moist chamber for 1 h at RT. The plates were washed thoroughly with 0.05% Tween 20 in PBS pH 7.2 for 5 times. Peroxidase-conjugated mAb anti-HA (Roche, Indianapolis, IN) was diluted to 1:100 and added to each well. The wells were then washed again prior to adding 100 µl TMB substrate and the OD at 450 nm measured after adding 1 N HCl to stop the reaction. mAb M6-1B9 was used as an antibody control in the ELISA system.

2.11.2 Competitive binding analysis of soluble scFv-M6-1B9 and mAb M6-1B9

Fifty microliters of 10 µg/ml avidin in carbonate/bicarbonate buffer pH 8.6 were coated microtiter plates and left overnight at 4 °C. The plate was then blocked with 200 µl of 2% BSA in PBS pH 7.2 for 1 h at RT. The wells were washed 5 times with 0.05% Tween-20 in PBS pH 7.2. Fifty microliters of 100 µg/ml BCCP fusion proteins in 2% BSA were added and incubated for 1 h at RT. The unbound

antigen was washed out. Fifty microliters of the mixture containing soluble scFv-M6-1B9 at dilution 1:250 and 20 µg/ml mAb M6-1B9 or mAb against survivin (MT-SVV3) at ratio 1:1 were added. After incubation in a moist chamber for 1 h at RT, the plate was washed thoroughly with 0.05% Tween 20 in PBS pH 7.2 for 5 times. Peroxidase-conjugated mAb anti-HA was diluted to 1:100 and added to each well. The wells were then washed again prior to adding 100 µl TMB substrate. The OD at 450 nm was measured after adding 1 N HCl to stop the reaction.

2.11.3 Culture of the human monocytic cell line (U937)

The human monocytic cell line (U937) was kindly provided from Prof. Dr. Watchara Kasinrerk, Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand. U937 cells were cultured in RPMI 1640 medium (Gibco), 10% FBS, penicillin (100 Units/ml), and streptomycin (100 µg/ml) and maintained in a humidified atmosphere of 5% CO_2 at 37°C.

2.11.4 Immunofluorescence analysis of the reactivity of soluble scFv-M6-1B9

U937 cells were adjusted to 1×10^7 cells/ml with 1% BSA-PBS-NaN_3 and blocked on ice with human AB serum at the ratio of 1:10 for 30 min. Fifty microliters of 1:10 dilution in 1% BSA-PBS-NaN_3 of soluble scFv-M6-1B9 were added to 50 µl of blocked cells and incubated on ice for 30 min. Cells were washed twice with 1% BSA-PBS-NaN_3. Subsequently, fifty microliters of 20 µg/ml mouse anti-HA-biotin (Sigma, St Louis, MO) were added and the cells were incubated on ice for 30 min. After washing, cells were resuspended with 20 µl 1% BSA-PBS-NaN_3.

FITC-conjugated sheep anti-mouse immunoglobulins antibody (Chemicon International, Melbourne, Australia) was then added. Cells were incubated on ice for another 30 min. Finally, cells were washed 3 times with 1% BSA-PBS-NaN_3 and fixed with 350 µl of 1% paraformaldehyde-PBS. Fluorescence reactivity of soluble scFv-M6-1B9 with CD147 on U937 cells was analyzed by flow cytometry.

2.11.5 Immunoblot analysis

To determine the binding activity of soluble scFv-M6-1B9, BCCP fusion proteins were separated in 12% polyacrylamide gel under reducing condition. The separated proteins were electroblotted onto polyvinylidene fluoride (PVDF) membrane for Western immunobloting. Blotted membrane was blocked at 4 °C overnight in 5% skimmed milk in PBS pH 7.2 and then incubated with the HRP-conjugated anti-HA antibody diluted in 5% skimmed milk in PBS pH 7.2 for 1 h at room temperature on a shaking platform. After washing step, the immunoreactive bands were then visualized by chemiluminescent substrate detection system.

2.11.6 Peripheral blood mononuclear cells isolation

For separation of peripheral blood mononuclear cells (PBMCs) from whole blood the density gradient centrifugation is performed. PBMCs were prepared by density centrifugation over IsoPrep solution (Robbins Scientific Corporation, Sunnyvale, CA). Twenty milliliters of heparinized blood were diluted with PBS pH 7.2 to 20 ml total volume. Two 50 ml tubes were filled with 10 ml IsoPrep each. Twenty milliliters of diluted cell suspension were over-layered on top of each 10 ml of IsoPrep. The tubes were spun at 1,500 rpm for 30 min without breaking. The white blood cell ring fractions from each tube were transferred to a new 50 ml tube using

steriled Pasteur's pipette. Cells were washed with 20 ml of PBS pH 7.2 for three times at 1,500 rpm for 10 min. The pellet was resuspended in 1 ml RPMI 1640 medium (Gibco), 10% FBS, penicillin (100 Units/ml), and streptomycin (100 μg/ml and counted the cell viability. The PBMCs were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

2.11.7 Cell proliferation assay

Cell proliferation was assessed using 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling. PBMCs were washed with PBS for 3 times and adjusted into 1×10^7 PBMCs/ml with PBS. CSFE (Molecular Probes, Eugene, OR) in the form of 5 mM stock solution in DMSO was added at final concentration of 0.5 μM for 10 min at 37 °C. To determine the effect of soluble scFv M6-1B9 on T-cell proliferation, triplicate aliquots of 1×10^5 PBMCs were cultured with immobilized CD3 mAb OKT3 (20 ng/ml) in the presence or absence of CD147 mAb clone M6-1B9 or soluble scFv M6-1B9. The culture was incubated for 5 days in a 5% CO_2 incubator at 37 °C. Cells from each treatment group were then washed twice with PBS, fixed with 1% formaldehyde in PBS and analyzed by a FACSCalibur flow cytometer (Becton Dickinson, Sunnyvale, CA).

2.12 Generation of recombinant adenoviruses expressing scFv-M61B9

2.12.1 Assembly of intrabody construct in pAdTrackCMV

The scFv coding regions were flanked by a human κ light chain leader sequence at the 5'-end, and a sequence encoding the HA tag (YPYDVPDYA) and the

ER retention signal (KDEL) at the 3'-end. The intrabody coding regions from pComb3X-scFv-M6-1B9 were then excised by digestion with *Sfi*I and cloned into modified pAdTrackCMV (Steinberger et al., 2000) and named pAdT-scFv-M6-1B9. This adapter fragment contains compatible *Sfi*I sites, which were used for cloning the scFv-M6-1B9 intrabody against CD147 into the adenovirus vector. The plasmid was transformed into the competent *E. coli* XL-1B and used for plasmid preparation by QIAGEN Miniprep Kit (Qiagen) as mentioned in section 2.6.5. Restriction enzyme analysis with *Sfi*I was verified the ligation.

2.12.2 ***Pme*I linearlization of the pAdT-scFv-M6-1B9** (He et al., 1998; Luo et al., 2007)

Four microgram of pAdT-scFv-M6-1B9 (a shuttle vector plasmid) was linearized with *Pme*I. The linearization was proceeded at 37 °C for 3 h. The linearized plasmid DNA was isolated and purified with QIAGEN Miniprep kit (See section 2.6.5).

2.12.3 Preparation of *E. coli* BJ5183 competent cells

The *E. coli* strain BJ5183 cells were prepared by using $CaCl_2$ method. An ampicillin resistant colony was selected from plate and further cultured at 37 °C for 8 h in 10 ml of LB broth containing 100 µg/ml ampicillin. The cultured bacteria were further pelleted at 2,500 rpm, 4 °C for 10 min and reconstituted by 10 ml of 0.1 M $CaCl_2$ at 4°C. Two rounds were repeatedly performed. Finally, the suspended bacteria were pelleted and further resuspended by 2 ml of 0.1 M $CaCl_2$. The BJ5183 competent cells were added by 0.4 ml of 85% glycerol and stored at -70°C.

2.12.4 Co-electroporation of pAdT-scFv-M6-1B9 and pAdEasy-1 vectors

The linearized and purified pAdT-scFv-M6-1B9 vector was co-electroporated with 100 ng of supercoiled pAdEasy-1 (Adenoviral backbone vector) in a total volume of 6.0 µl. Twenty microliters of electrocompetent *E. coli* BJ5183 cells were added, and electroporation was performed in 2.0 mm cuvettes at 2,500 V, 200 ohms, and 25 mF in a Bio-Rad Gene Pulser electroporator (**Figure 2.1**). The cells were immediately placed in 500 µl of SOC-medium and grown at 37 °C for 20 min with shaking at 200 rpm. One hundred twenty-five microliters of the cell suspension was plated on LB agar containing 50 µg/ml of kanamycin. After 16–20 h growth at 37 °C, 10-20 smallest colonies (which usually represented the recombinants) were picked and grown in 3 ml of LB broth containing 50 µg/ml of kanamycin. The plasmid DNA isolation and purification were performed with QIAGEN miniprep kit. Clones were first screened by analyzing their supercoiled sizes on agarose gels, comparing them to pAdEasy-1 control.

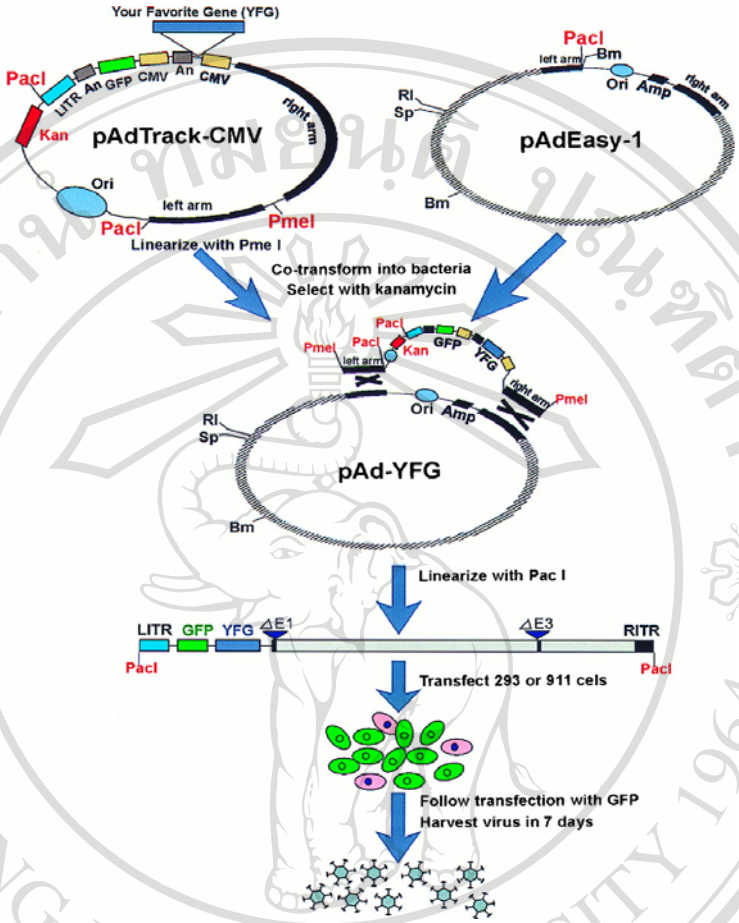

Figure 2.1 Schematic outline of the AdEasy system. The gene of interest is first cloned into a shuttle vector, *e.g.* pAdTrack-CMV. The resultant plasmid is linearized by digesting with restriction endonuclease *Pme*I, and subsequently cotransformed into *E. coli* BJ5183 cells with an adenoviral backbone plasmid, *e.g.* pAdEasy-1. Recombinants are selected for kanamycin resistance, and recombination confirmed by restriction endonuclease analyses. Finally, the linearized recombinant plasmid is transfected into adenovirus packaging cell lines, *e.g.* 293 cells. Recombinant adenoviruses are typically generated within 7 to 12 days. The left arm and right arm represent the regions mediating homologous recombination between the shuttle vector and the adenoviral backbone vector (He et al., 1998).

2.12.5 *Pac*I restriction analysis of pAdEasy-1 vector containing scFv-M61B9 gene

Three hundred nanograms of the purified vectors were digested with 5 U of *Pac*I at 37 °C for 3 h. The digested DNA fragments were checked by fractionating on 1% agarose gel electrophoresis to identify the band of corrected insert.

2.12.6 Electroporation of the recombinant pAdEasy-1 vector into *E. coli* DH10B

Those clones that had inserts were confirmed and electroporated into *E. coli* DH10B. One microliter of a 1:50 dilution of recombinant construct DNA was electroporated into 50 µl competent *E. coli* DH10B cells under conditions as described in section 2.12.4. One milliliter prewarmed SOC medium was added immediately and suspension was incubated at 37 °C for 1 h with shaking at 200 rpm. One hundred microliter cells were plated on LB agar containing 50 µg/ml of kanamycin and grown at 37 °C for overnight. At the following day two clones were picked, cultured in 10 ml SB medium containing 50 µg/ml of kanamycin and grown at 37 °C with shaking at 200 rpm for overnight. The plasmid DNA isolation and purification were performed with QIAGEN Midi prep kit. The DNA concentration was measured by spectrophotometry. The recombinant adenoviral vector DNA containing scFv-M61B9 gene was named pAdE-scFv-M6-1B9.

2.12.7 Purification of pAdE-scFv-M6-1B9 vector by using QIAGEN Plasmid Midi Kit

A single colony of transformant was picked and inoculated with a starter culture of 2-5 ml LB medium containing kanamycin (70 µg/ml). After incubation at 37 °C for 8 h with vigorous shaking, two hundred microliters of the starter culture was further grown in 100 ml of LB medium containing kanamycin (70 µg/ml) at 37 °C for 16 h with vigorous shaking. The bacterial cells were harvested by centrifugation at 6000 g at 4 °C for 15 min. The bacterial pellets were resuspended in 4 ml of P1 buffer. The bacteria were completely resuspended leaving no cell clump. The P2 buffer of 4 ml was added and the mixture was gently mixed but thoroughly by inverting for 4-6 times. The mixture was incubated at room temperature for 5 min. Four microliters of chilled P3 buffer were added to the lysate. The mixture was mixed immediately and gently by inverting for 4-6 times and poured into the barrel of the QIAfilter cartridge. The cartridge was incubated at RT for 10 min. A QIAGEN-tip 100 was equilibrated by applying 10 ml of QBT buffer. The column was allowed to empty by gravity flow. The cap from the QIAfilter nozzle was removed. The plunger was gently inserted into the QIAfilter. The cell lysate was filled into the previously equilibrated QIAGEN-tip until lysate passing through the QIAfilter cartridge. Approximately 10 ml of the lysate were generally recovered after filtration. The clear lysate was allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice with 10 ml of QC buffer. The QC buffer was allowed to move through the QIAGEN-tip by gravity flow. The DNA was eluted with 5 ml of QF buffer. The eluate was collected in a 10 ml tube. To precipitate DNA, 3.5 ml of isopropanol was

added to the eluted DNA at room temperature. After mixing and centrifuging immediately at 15,000 g for 10 min, the supernatant was carefully decanted without disturbing the pellet. The pellet was dried for 10 min and re-dissolved in suitable volume of DW.

2.12.8 Detection of pAdE-scFv-M6-1B9 by using polymerase chain reaction (PCR)

Twenty five nanograms of the pAdE-scFv-M6-1B9 vectors were amplified with 125 ng of each primer in a 20 µl of PCR mixture containing 2.5 U Taq polymerase (Eppendorf). The pAdE-scFv-M61B9 was proved with sense primer (ScFvM6cyt: 5'-GAG GAG GAG GTG TCG ACA TGG TGA TGA CCC AGA CTC C-3') and anti-sense primer (SVV3_Rcy: 5'-GAG GAG GAG CTG CGG CCG CTT AAG CGT AGT CCG GAA CGT C-3'). The amplification condition was initiated with jump start at 95 °C for 5 min followed by 35 rounds of 3 steps amplification: denaturation at 95 °C for 50 sec, annealing at 68 °C for 50 sec and extension at 72 °C for 1.5 min. Finally, the mixture was extended at 72 °C for 10 min. The amplified product was checked for the correct molecular size by 1% agarose gel electrophoresis (Pingmuang, 2008).

2.12.9 *Pac*I linearization of the pAdE-scFv-M6-1B9

One microgram of pAdE-scFv-M6-1B9 vector containing scFv-M61B9 gene was digested with 10 U of *Pac*I at 37 °C for 16 h. The *Pac*I digestion was heat inactivated at 70 °C for 10 min and stored at -20 °C.

2.12.10 Culture of an embryonic human kidney cell line (293A)

An embryonic human kidney transformed carrying sheared human adenovirus type 5 DNA cell line (293A)(Invitrogen, Carlsbad, CA) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10mM nonessential amino acids, 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cell line was maintained in a humidified atmosphere of 5% CO_2 at 37°C. Low passage 293 cells (less than passage 40) and high passage 293 cells were kindly obtained from Prof. Dr. Andre Lieber, University of Washington, Seattle, Washington, USA. Both cell lines cells were maintained in the same medium.

2.12.11 Transfection of 293A cells with *Pac*I linearized pAdE-scFv-M6-1B9

6×10^4 293A cells in 500 µl DMEM containing 10% FBS and antibiotics were plated on a polystyrene 24-well plate for 24 h before transfection, by which time they reached 60–70% confluence. Cells were washed once with 1 ml of DMEM (Gibco). Six micrograms of *Pac*I linearized pAdE-scFv-M6-1B9 were used for transfection. A transfection mixture was prepared by adding 1 µg of linearized pAdE-scFv-M6-1B9 DNA and 0.25 µl of Transfectin (Biorad) to 100 ul of DMEM (Gibco). After incubation at RT for 30 min, the transfection mixture was added to the cells. After 4–6 h in a humidified atmosphere of 5% CO_2 at 37 ^oC, the media containing the transfection mixture was removed and added 500 µl of prewarmed DMEM. The culture medium containing recombinant adenovirus was harvested after 7-10 days post transfection, when GFP plaques had appeared. After three cycles of

freezing in a methanol/dry ice bath (or liquid nitrogen) and rapid thawing at 37 °C, 1 ml of viral lysate was stored at -70 ^{o}C as virus stock.

2.12.12 Agar plaque purification of adenovirus

A method used for purification of virus stocks was the agar plaque purification method. Low passage 293 cells (less than passage 40) were plated in 10 cm diameter dish, by which the time they would achieve ~80 % confluence. One milliliter of virus serial dilution (in the range of 10^{-3} – 10^{-8}) was added and incubated at 37 ^{o}C. Twenty-four hours after infection, cells were carefully overlaid with 15 ml overlay media to each dish and allowed to solidify at room temperature before incubating at 37^{o}C. Overlay media for 293 cells is made by mixing 37 ^{o}C 2 × Minimum Essential Medium (supplemented with 25% fetal bovine serum, 4mM L-glutamine, 200 units/ml penicillin G, and 200μg/ml streptomycin sulfate) with an equal volume of 62 ^{o}C 1% UltraPURE agarose (GIBCO, Grand Island, NY). After 3 more days, 10 ml overlay media was added, and if necessary, 5 ml more were added 6 days later. Between 6 and 14 days after infection individual plaques were presented.

2.12.13 Plaque analysis

For each virus, 12 plaques were generally chosen to analyze. Plaques were picked as agarose plugs, mixed with 1 ml media, subjected to 4 freeze/thaw cycles in order to lyse cells and release virus, and added to 3×10^{4} low passage 293 cells seeded the day before in a 24 well plate. After the development of cytopathic effect (CPE) (usually 2-4 days), the virus was amplified by collecting the cells plus media, freezing and thawing four times, and adding them to 3×10^{5} low passage 293 cells along with 500 μl fresh media. After the development of CPE (usually 48 h),

1/3 of the cells and media were frozen at -70 ^{o}C for later amplification and 2/3 were used for DNA analysis. For DNA analysis, the cells were pelleted by centrifugation at 14,000 rpm for 2 min, and all but 200 µl of the supernatant was discarded. The pellet was resuspended by pipetting and slowly added to 200 µl pronase stock diluted 1:5 in pronase buffer (final concentration of 0.5 mg/ml pronase). The samples mixed by pipetting and vortexing and incubated at 37 ^{o}C for 2-6 h. Next, the sample was extracted with an equal amount of phenol/chloroform/isoamyl alcohol (400 µl), collecting the aqueous phase. The aqueous phase was extracted again with 400 µl chloroform. DNA was precipitated from the aqueous phase with 1/10 volume 3M Sodium acetate (pH 5.3) plus 3 volumes ethanol and incubation of at least one hour at –70 ^{o}C. The DNA was pelleted by centrifuging 12 min at 14,000 rpm and 4 ^{o}C, and the supernatant is discarded. The pellet was washed with 500 µl 70% ethanol and repelleted at 14,000 rpm for 5 min. The pellet was then air dried and dissolved in 50 µl TE. Twenty microliters of the sample was digested with *Hind*III and run on an agarose gel. The corrected restriction pattern was further amplified and purified.

2.12.14 Adenovirus amplification and purification

For a large scale preparation, the correct frozen 1/3 viral stock from above was subjected to 4 freeze/thaw cycles to release virus and added to 4×10^5 high passage 293 cells in one well of a 12 well plate with a final volume of 1.5 ml media. After the development of CPE, cells and media were collected, frozen/thawed four times, and this time added to 2×10^6 high passage 293 cells in a 6 cm dish with a final media volume of 5 ml. The next amplification step was performed in an 80%

confluent 10 cm dish of high passage 293 cells in 10 ml media. Then, a 95% confluent 15 cm dish was infected with 25 ml media. From one 15 cm dish, four 15 cm dishes were infected. The last amplification step was thirty 15 cm dishes. After the development of CPE this time, the cells were pelleted by centrifuging at 2,000 rpm for 10 min, and the supernatant was discarded. The cells were then resuspended in 1 ml Dulbecco's phosphate buffered saline (GIBCO, Grand Island, NY) and 10 mM $MgCl_2$ per 15cm dish. The cells were lysed by four freeze/thaw cycles. The lysates were centrifuged at 2,000 rpm for 10 min to remove cellular debris, and the supernatants were stored at -70 ^{o}C.

2.12.15 Cesium chloride gradient purification of adenovirus

The lysates were run on Cesium chloride (CsCl) step gradients for purification. CsCl was dissolved in 5mM Tris-HCl, 1mM EDTA, pH 7.8 at density of 1.25 g/cm^3 and 1.35 g/cm^3. The gradients were formed by layering 3.5 ml 1.25 g/cm^3 CsCl on top of 3.5 ml 1.35 g/cm^3 CsCl in a 12 ml Beckman ultra-clear tube and adding 5 ml lysate on top. The tube was then balanced, placed in a SW40Ti swing bucket rotor and centrifuged in a Beckman ultracentrifuge for 2 h at 35,000 rpm. After centrifugation two bands were observed in the gradient as shown in **Figure 2.2**. The lower band containing parental full-length virus was carefully collected from each tube, combined, and subjected to ultracentrifugation in an equilibrium gradient derived from 1.35 g/cm^3 CsCl for 18 h at 14 ^{o}C and 35,000 rpm. The full-length band was collected again and dialyzed twice against 1 liter dialysis buffer at 4 ^{o}C in the dark for 6-8 h each. Dialysis was carried out to remove CsCl from the virus preparation whilst keeping virus at a pH of 7.5 since lower pH storage buffers reduce adenoviral

titers significantly. Virus was then aliquoted into steriled microcentrifuge tubes and stored at –70 ^{o}C. The viral DNA was checked again by restriction analysis as described above using 50 µl virus diluted with 150 µl serum-free media.

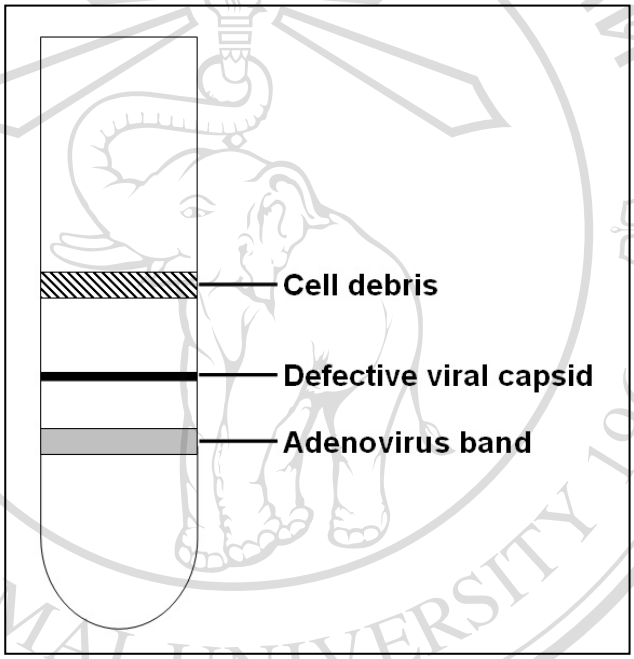

Figure 2.2 Cesium chloride separation of adenovirus from defective capsids and cell debris.

2.12.16 Calculation of Adenovirus titer

The titer can be determined by a spectrophotometer. Virus (dilution 1:20) was lysed by lysing solution (0.1% sodium dodecyl sulfate in TE) and measured their absorbance at 260 nm, where one optical density unit equals 10^{12} particles/ml.

2.12.17 Extraction of adenoviral DNA from CsCl purified virus

To an aliquot of CsCl purified adenovirus in a screw cap microcentrifuge, 200 µl of virion digestion buffer (solutions appendix) was added before incubating horizontally at 37 ^{o}C for 2 h. DNA was then extracted once with phenol:chloroform:isoamyl alcohol, ethanol precipitated (See section 2.12.13), washed with 70% ethanol, air dried and resuspended in 50 µl steriled DW.

2.12.18 Construction of recombinant pAdE/F35-scFv-M6-1B9

The modified pAdEasy-1/35 vector was generous gifts from Prof. Xiaolong Fan (Nilsson et al., 2004), Department of Molecular Medicine and Gene Therapy, Lund University, Lund, Sweden to Prof. Dr. Andre Lieber, Division of Medical Genetics, Department of Medicine, University of Washington, Seattle, Washington, USA. In the pAdEasy-1/F35, the Ad5 sequence from nt 30644 to 32781 (Genbank accession no. M73260/M29978) was replaced by the Ad35 fiber sequence from nt 129 to 972 (Genbank accession no. U10272) as shown in **Figure 2.3** (Shayakhmetov et al., 2000).

For generation of the pAdE/F35-scFv-M6-1B9 vector, the *Pme*I linearized and purified pAdT-scFv-M6-1B9 vector (See section 2.12.2) was co-electroporated with 100 ng of supercoiled pAdEasy-1/35. This shuttle plasmid was used to generate a recombinant adenoviral genome encoding Ad5F35-scFv-M6-1B9

by homologous recombination in *E. coli* BJ5183 with the fiber gene modified pAdEasy-1/F35 by using a standard method as described previously in section 2.12.4 (He et al., 1998). The genomic plasmid encoding Ad5F35-scFv-M6-1B9 was digested with *Pac*I (See section 2.12.9) and transfected into 293 cells by using the $CaPO_4$ precipitation method (He et al., 1998). The adenoviral vectors were expanded in 293 cells, purified, and titered in 293 cells by serial dilutions according to methods described previously in section 2.12.10-2.12.17.

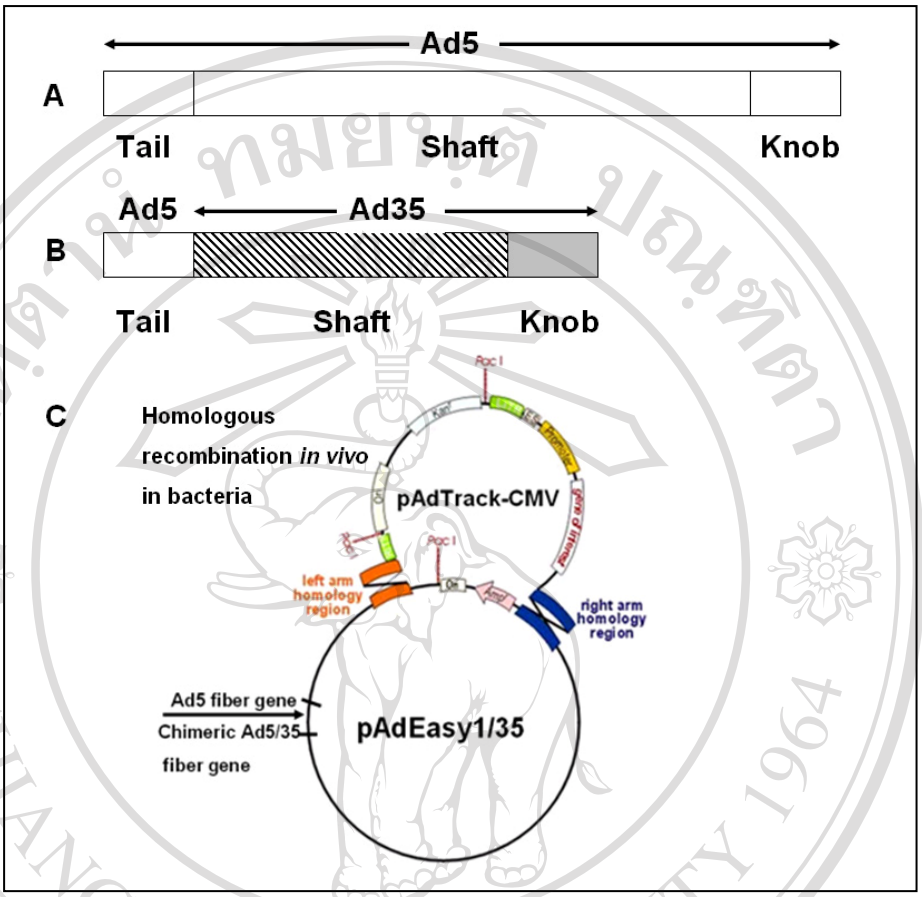

Figure 2.3 Construction of Ad5/F35 vectors. A) the Ad5 fiber gene. **B)** the chimeric fiber gene encoding the Ad5 fiber tail domain and Ad35 fiber shaft and knob domains. **C)** the fiber gene modified AdEasy system. The fiber gene in the pAdEasy-1 will be replaced with the indicated chimeric fiber gene. The fiber gene modified pAdEasy-1/F35 will be used to recombine with the shuttle plasmid encoding the *GFP* gene expression cassette in *E. coli* BJ5183 for generation of the recombinant adenoviral vector genome encoding the Ad5F35- scFv-M6-1B9-GFP vector (Nilsson et al., 2004).

2.12.19 Verification of Ad5-scFv-M6-1B9 and Ad5F35-scFv-M61B9 by using polymerase chain reaction (PCR)

One microliter of each purified Ad was amplified with 125 ng of each primer in a 50 µl of PCR mixture containing 2.5 U Taq polymerase (Fermentas). The specific primer sets for Ads knob amplification were used and listed in **Table 2.3**. The amplification condition was initiated with jump start at 95 °C for 5 min followed by 35 rounds of 3 steps amplification: denaturation at 95 °C for 50 sec, annealing at 57 °C for 50 sec for Ad5 knob (or 54.4°C for 50 sec for Ad35 knob) and extension at 72 °C for 1.5 min. Finally, the mixture was extended at 72 °C for 10 min. The amplified product was checked for the correct molecular size by 1% agarose gel electrophoresis.

Ad5-GFP and Ad35-GFP were used as control. These adenoviral vectors were kindly provided from Prof. Dr. Andre Lieber, University of Washington, Seattle, Washington, USA.

Table 2.3 A list of primer used for Ads knob and shaft amplification.

Primers	Sequences
Ad5 knob-For	5’-ACT GAA GGC ACA GCC TAT AC-3’
Ad5 knob-Rev	5’-AGT TGT GGC CAG ACC AGT CC-3’
Ad35 knob-For	5’-TCT TCT ACA GCG ACC AGT GA-3’
Ad35 knob-Rev	5’-ATG GCA TAG GCA ACA TTG GA-3’

2.12.20 Transduction of Ad5-scFv-M6-1B9 and Ad5F35-scFv-M61B9 in cell lines

Several cell lines (293A, HeLa, Jurkat, HepG2 and U937) were transduced with Ad5-scFv-M6-1B9 or Ad5F35-scFv-M6-1B9 at 100 and 1,000 MOI. After 48 h, the transduced cells were harvested, washed twice with 1% BSA-PBS-NaN_3 and resuspended with 350 µl 1% BSA-PBS-NaN_3. Percentage of GFP-positive cells was determined by flow cytometry.

2.12.21 CAR and αv-integrin staining

Cell lines (from section 2.12.19) were stained with mouse anti-CAR mAb (Clone RmcB) and mouse anti- αv-integrin mAb (Clone L230). These mAbs were generous gifts from Prof. Dr. Andre Lieber, University of Washington, Seattle, Washington, USA. PE-conjugated $F(ab')_2$ fragment of sheep anti-mouse immunoglobulins antibody were used as a secondary antibody. Finally, cells were washed 3 times with 1% BSA-PBS-NaN_3 and fixed with 1% paraformaldehyde-PBS. Fluorescence reactivity of the stained cells was investigated by flow cytometry (See section 2.11.4).

2.13 Immunological methods for functional analysis of intrabody against CD147 by adenoviral gene transfer

2.13.1 ScFv-M61B9 extraction by FractionPREP^{TM} Cell Fractionation System

293A cells were transduced at a multiplicity of infection (MOI) of 10 pfu/cell of Ad-scFv-M6-1B9 and harvested after 48 h. Transduced cells were fractionated using FractionPREP^{TM} Cell Fractionation System (BioVision, Mountain View, CA) according to the manufacturer's instructions. Briefly, the transduced cells were collected by centrifugation at 700 g for 5 min and washed with 5-10 ml of ice-cold PBS. After centrifugation at 700 g for 5 min, the pellet was resuspended in 1 ml of ice-cold PBS and transferred to a 1.5 ml microcentrifuge tube. The resuspended cells were spun for 5 min at 700 g and the supernatant was removed. The pellet was resuspended in 400 µl of Cytosol Extraction Buffer-Mix and incubated sample on ice for 20 min with gentle tapping 3-4 times every 5 min. After centrifugation at 700 g for 10 min, the supernatant (Cytosolic Fraction) was collected and kept on ice. The pellet was resuspended in 400 µl of ice-cold Membrane Extraction Buffer-A Mix and mixed well by vortex for 10-15 sec. Twenty two microliters of Membrane Extraction Buffer-B was added and then mixed by vertex for 5 sec. After incubation on ice for 1 min, the mixture was mixed by vertex for 5 sec again and centrifuged for 5 min at 1000 g. The supernatant (Membrane Fraction) was transferred immediately to a clean pre-chilled tube and kept on ice. The pellet was resuspended in 200 µl of ice-cold Nuclear Extraction Buffer Mix and mixed by vertex for 15 sec. The mixture was kept on ice

for 10 min with constant vortex for 15 sec every 10 min. After centrifugation at top speed in a microcentrifuge for 10 min, the sample was transferred to a clean pre-chilled tube (Nuclear Fraction). All Fractions were stored at -70 °C for future use.

2.13.2 Detection of scFv-M6-1B9 intrabody activity by Western blotting

The lysate fractions from section 2.13.1 were separated on a 12% SDS–PAGE gel under reducing conditions and then transferred onto a polyvinylidene-fluoride (PVDF) membrane. The membranes were blocked with 5% skimmed milk in PBS and traced by peroxidase-conjugated mAb anti-HA (Roche, Indianapolis, IN). The peroxidase reaction was visualized using an enhanced chemiluminescent substrate detection system.

To determine the binding activity of scFv-M6-1B9, biotin carboxyl carrier protein (BCCP) fusion proteins were separated on 12% SDS-PAGE, electroblotted onto PVDF membrane, probed with soluble lysate of 293A cells expressing scFv-M6-1B9 and traced by peroxidase-conjugated mAb anti-HA. The immunoreactive bands were visualized using an enhanced chemiluminescent substrate detection system.

2.13.3 Culture of the human cervical carcinoma cell line (HeLa)

The human cervical carcinoma cell line (HeLa) was kindly provided from Prof. Dr. Andre Lieber, University of Washington, Seattle, Washington, USA. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10mM nonessential amino acids, 10% fetal bovine serum, penicillin (100 U/ml) and

streptomycin (100 µg/ml) and maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

2.13.4 Flow cytometric analysis for CD147 surface expression

Five hundred microliters of 1.2×10^5 cells/ml 293A (or HeLa cells) were transduced with 10 MOI (~90% of the cells were infected) of adenovirus encoding scFv-M6-1B9 intrabody (Ad5-scFv-M6-1B9). After 36 h, 293A cells (or HeLa cells) were removed from 24-well tissue culture plates and washed 3 times with PBS. Cells were then blocked with human AB serum for 30 min on ice. Fifty microliters of 20 µg/ml purified mAb M6-1B9 in 1% BSA-PBS-NaN_3 were added to 50 µl of blocked cells and incubated on ice for 30 min. Cells were washed twice with 1% BSA-PBS-NaN_3 and resuspended with 20 µl 1% BSA-PBS-NaN_3. Subsequently, twenty-five microliters of PE-conjugated $F(ab')_2$ fragment of sheep anti-mouse immunoglobulins antibody were added and incubated on ice for 30 min. Finally, cells were washed 3 times with 1% BSA-PBS-NaN_3 and fixed with 1% paraformaldehyde-PBS. Fluorescence reactivity of the stained cells was investigated by flow cytometry. Adenovirus encoding scFv specific to survivin (scFv-SVV3) intrabody constructed by the same technique was used as transduction control.

2.13.5 Immunocytochemical analysis for CD147-intrabody colocalization

For analysis of CD147 and intrabodies on GFP-positive 293A, transfected cells were trypsinized and fixed for 10 min with 3.7% formaldehyde in PBS containing 50 mM $MgCl_2$. Fifty microliters of 1×10^6 fixed cells were placed on a silane-coated slide and air-dried. Following washing, cells were permeabilized with 0.2% Triton-X 100 for 12 min. Slides were then washed in PBS containing 50 mM

$MgCl_2$ and blocked with 1% BSA in SSC at RT for 5 min. Then, the fixed cells were incubated with a mixture of biotinylated anti-human extracellular matrix metalloproteinase inducer (EMMPRIN) mAb (0.1 μg/ml; R&D systems, Minneapolis, MN) and rabbit anti-HA mAb (Sigma) at 4 °C overnight. After washing, cells were blocked and then incubated with the mixture of Cy5-conjugated streptavidin (Amersham Life Sciences, Inc, Buckinghamshire, UK) and Cy3-conjugated anti-rabbit-IgG mAb (Sigma) at RT for 30 min. Nuclei were counterstained with DAPI. Imaging of stained cells was performed by using a Zeiss Apotome with an AxioCam HRM, AMCA, Cy3, Cy5 and FITC filters in combination with Planapo 63×/1.4 oil objective lens. Images were acquired by using AXIOVISION 4.4 (Carl Zeiss Canada Ltd., Toronto, ON, Canada) in multichanel mode.

12.13.6 Confocal analysis

The 1×10^4 HeLa cells were plated on eight-well Tissue-Tek chamber slides (Nalge Nunc International, Rochester, NY), and transduced with 10 MOI of Ad5-scFv-M6-1B9 for 48 h at 37 °C. Cells were washed, fixed in 4% formaldehyde in PBS containing 50 mM $MgCl_2$ and permeabilized with 0.1% Triton X-100. Slides were then washed in PBS containing 50 mM $MgCl_2$ and blocked with 2% skim milk in PBS containing 50 mM $MgCl_2$ at RT for 30 min. Then, the fixed cells were incubated with rabbit anti-HA antibody (Abcam, Cambridge, MA) at 37 °C for 1 h. After washing, cells were then incubated with Alexa Fluor 568-goat anti-rabbit IgG (Molecular Probes, Eugene, OR). The cellular nuclei were counterstained by DAPI. Images were acquired using

FLUOVIEW laser scanning confocal microscope (Olympus, FV1000; Olympus Optical, Japan).