CHAPTER III

RESULT

3.1 Immunogen preparation

In order to develop efficient methods for production of monoclonal antibody, three different approaches for immunogen preparations were adopted. In this study, CD4 protein antigen was used as a model. The immunogen preparation techniques applied were immunoprecipitate-based technique, CD4-BCCP beads technique and CD4-COS cell expression technique. Immunogen preparations by the three approaches were as follows.

3.1.1 Immunoprecipitated-base technique

To develop an immunoprecipitation technique for using as method for immunogen preparation, surface proteins of PBMCs were biotinylated with Sulfo-NHS-LC-biotin. Then, cell lysates were precipitated with various CD4 mAbs and control mAbs. In this experiment, CD4 mAbs clones L200 and VIT4 precipitated a band of biotinylated protein at the molecular weight of 55-60 kDa under non-reducing condition (Figure 3.1) which was corresponding to the CD4 protein. A CD4 mAb MT4, however, did not precipitate any protein. As control, the CD99 mAb, in contrast, precipitated a broad band at the molecular weight of 25-35 kDa corresponding to CD99 protein. CD8 mAb MT8, another isotype matched control mAb, did not precipitate any protein. The results indicated that the immunoprecipitation technique, using CD4 mAb L200 and VIT4 could be used to

prepare CD4 protien immunogen for immunization. mAb L200 was selected for further experiment in the preparation of CD4 immunogen.

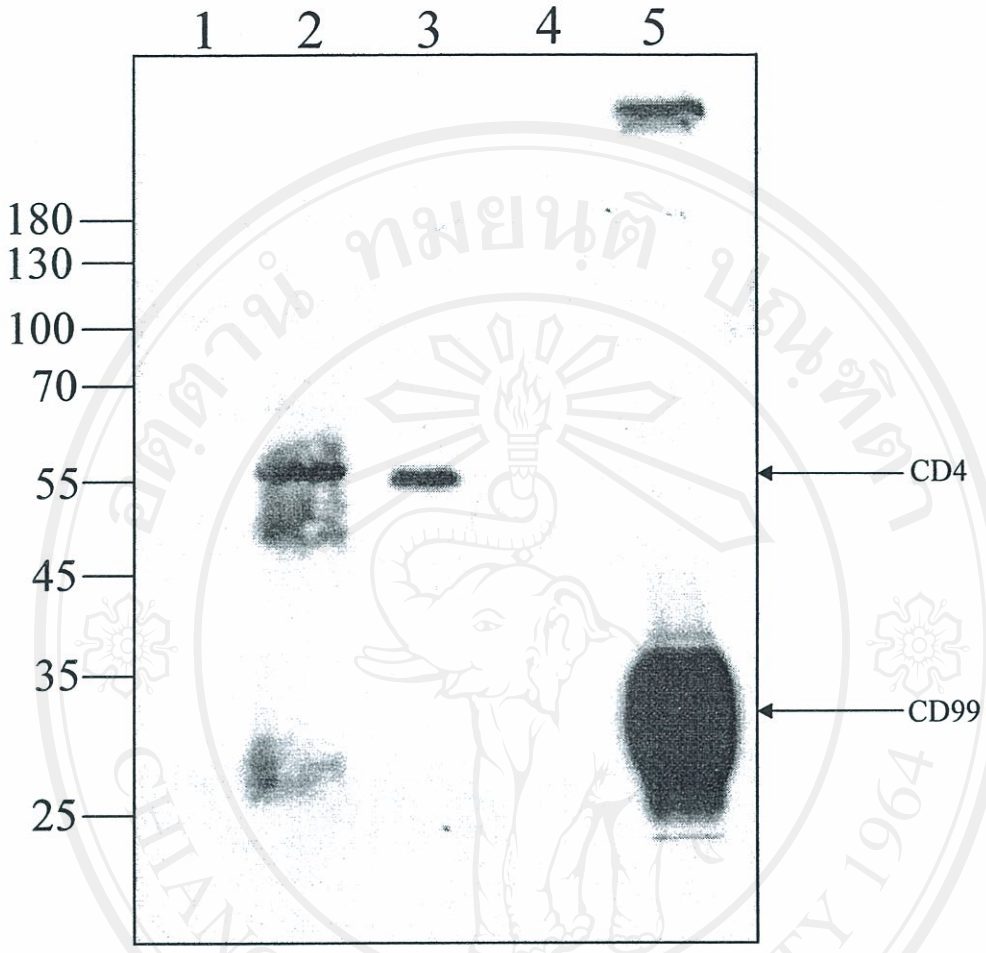

Figure 3.1 Immunoprecipitation for preparation of CD4 protein immunogen. Immunoprecipitation was performed using lysates of biotin labelled PBMCs and precipitated by CD4 mAb MT4 (lane 1), L200 (lane 2), VIT4 (lane 3), CD8 mAb MT8 (lane 4) and CD99 mAb MT99/3 (lane 5). Electrophoresis was performed under non-reducing condition. The positions of molecular mass markers are indicated on the left in kDa.

3.1.2 CD4-BCCP beads technique

3.1.2.1 Amplification of CD4 coding region

In order to produce recombinant CD4 protien, the plasmid harboring DNA encoding CD4 protein was first generated. To produce plasmid DNA encoding CD4-BCCP fusion protein, the extracellular domain of CD4 coding sequence was amplified from a plasmid vector πH3M-CD4 (Kasinrerk et al., 1996) using a set of primer that was designed to have the short restriction sequences for *Nde* I and *EcoR* I at the upstream and downstream of the CD4 gene, respectively. The amplified product of 1100 bp corresponding to cDNA encoding CD4 proteine was demonstrated by agarose gel electrophoresis (figure 3.2). The amplified CD4 cDNA fragment was purified and digested with *Nde* I and *EcoR* I, followed by another purification step (figure 3.3, lane 2). The digested PCR product was ligated into the appropriately cut pAK400CB plasmid vector (figure 3.3, lane 3). The resulting vector named pAk400CB-CD4 (Figure 3.4) was transformed into the *E. coli* XL-1 Blue and the chloramphenicol-resistant colonies were selected for purification of the plasmid vector. The correct of insertion of CD4 gene was verified again by restriction fragment analysis with restriction enzyme *Nde* I and *EcoR* I (Figure 3.5, lane 2), reamplification by PCR (figure 3.5, lane 3) and subjected for DNA sequencing. The nucleotide of the inserted sequence in pAk400CB-CD4 was completely identical to the nucleotide sequence of the known CD4 gene. Hence, the pAk400CB-CD4 could be use to produce biotinylated CD4. The produced CD4 recombinant protein was then used as immunogen in the further monoclonal antibody production experiments.

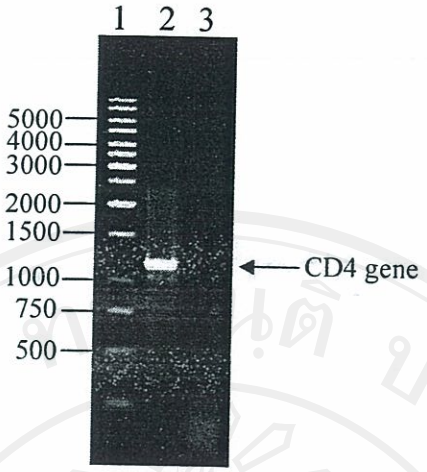

Figure 3.2 Agarose gel electrophoresis of amplified PCR product. CD4 gene was amplified from the πH3M-CD4 plasmid vector by PCR. DNA molecular weight marker (lane 1), amplified PCR products of CD4 gene at 1100 bp (lane 2) and product from non added template (lane 3). Sizes of standard DNA markers (bp) are indicated on the left.

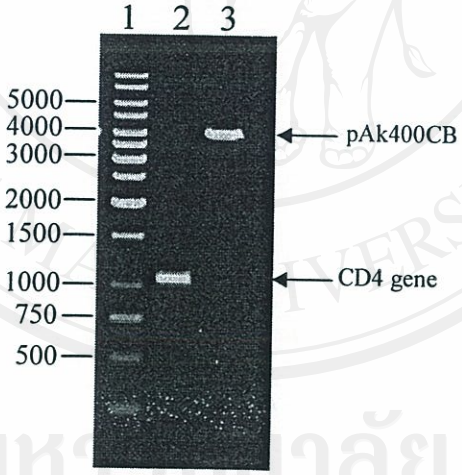

Figure 3.3 Agarose gel analysis of restriction of CD4 cDNA fragment and pAk400CB vector with *Nde* I and *EcoR* I. CD4 cDNA fragment (lane 2) and the pAk400CB vecter(lane 3) were digested with *Nde* I and *EcoR* I. DNA molecular weight marker was showed in lane 1. Sizes of standard DNA markers (bp) are indicated on the left.

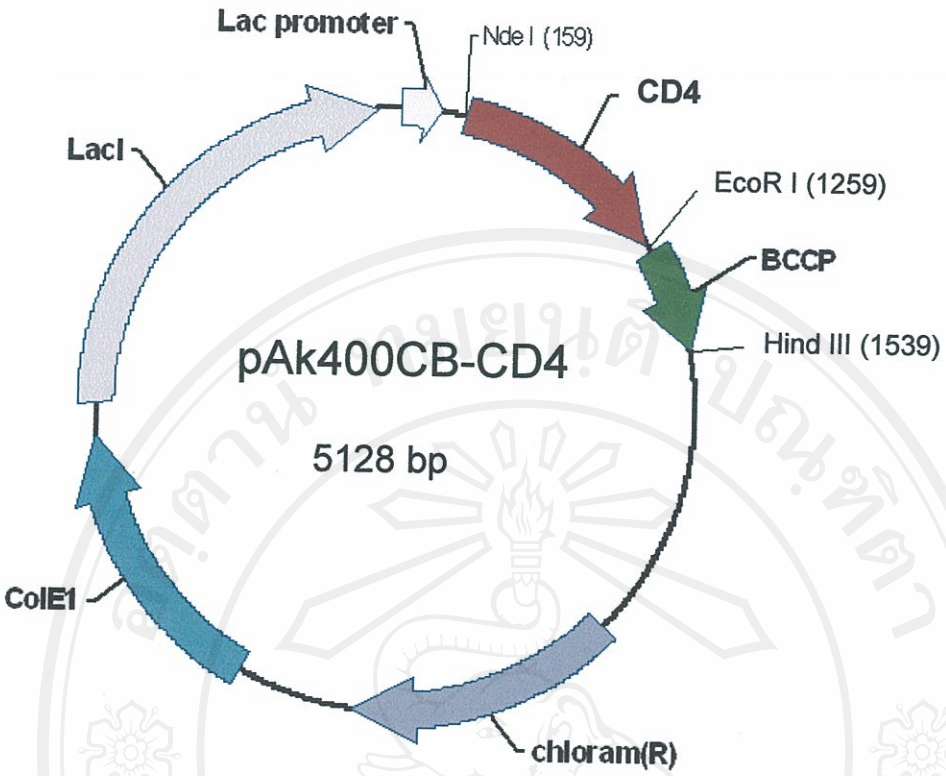

Figure 3.4 Schematic illustration represents the pAK400CB-CD4 vector. The CD4 was inserted between the *Nde* I and *EcoR* I restriction sites; the origin of replication (ColE1), lac promoter, lac repressor (lacI), chloramphenicol resistant gene (chloram(R)) and BCCP are shown.

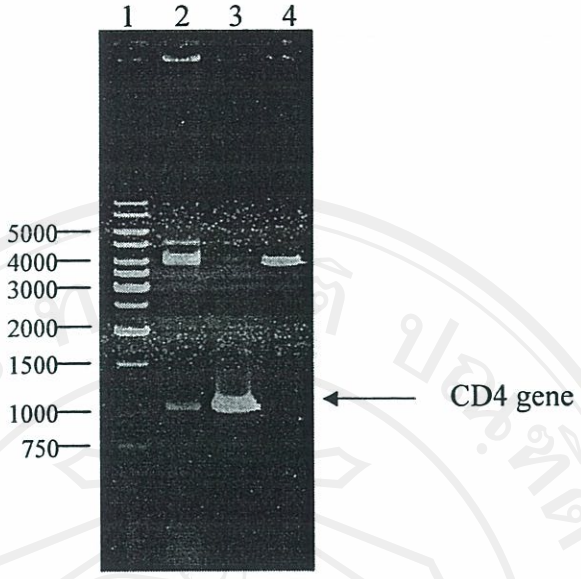

Figure 3.5 Verification of pAk400cb-CD4. The isolated pAk400CB-CD4 were digested with *Nde* I and *EcoR* I and reamplified by PCR followed by 1% agarose gel analysis. Standard DNA marker (lane 1), pAk400CB-CD4 plasmid vector digested with *Nde* I and *EcoR* I enzyme (lane 2), un-digested plasmid DNA (lane 4), amplified PCR product from pAk400CB-CD4 vector (lane 3). Sizes of standard DNA markers (bp) are indicated on the left.

3.1.2.2 Expression of biotinylated CD4-BCCP protein

The pAk400CB-CD4 plasmid was transformed into *E. coli* strain Origami B. To identify bacterial colonies that contain the pAk400CB-CD4 plasmid, the plasmid from individual clone were purified and characterized by reamplification by PCR and restriction fragment analysis with *Nde* I and *EcoR* I. The inserted fragment of CD4 at the molecular weight of 1100 bp was retrieved from re-amplification by PCR (Figure 3.6, lane 1) or the isolated plasmid after the restriction analysis with *Nde* I and *EcoR* I (Figure 3.6, lane 3). The results indicated pAk400CB-CD4 plasmid was transformed into *E. coli* strain Origami B and the selected clone of *E. coli* contains the pAk400CB-CD4 plasmid.

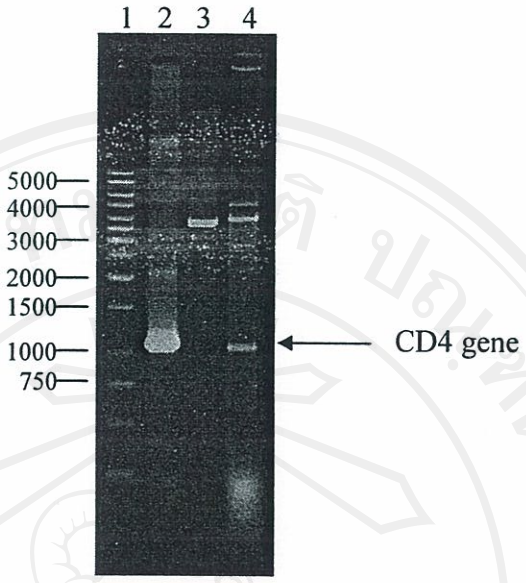

Figure 3.6 Characterization of pAk400CB-CD4. The isolated plasmid DNA were digested with both *Nde* I and *EcoR* I and reamplified by PCR followed by 1% agarose gel analysis. Standard DNA marker (lane 1), amplified PCR product from pAk400CB-CD4 vector (lane 2), un-digested plasmid DNA (lane 3), pAk400CB-CD4 plasmid vector digested with *Nde* I and *EcoR* I enzyme (lane 4). Sizes of standard DNA markers (bp) are indicated on the left.

3.1.2.3 Determination of biotinylated CD4-BCCP

E. coli Origami B containing the pAK400CB-CD4 plasmid were cultured in the shaking flask to allow protein expression. The bacterial cells were lysed in protein extraction reagent. To determine biotinylated CD4-BCCP products in the bacterial extract, indirect ELISA, Western blotting and immunofluoresce staining were performed.

3.1.2.3.1 Determination of biotinylated CD4-BCCP by indirect ELISA

The ELISA wells were coated with avidin for capturing the biotinylated CD4-BCCP, followed by addition of various CD4 mAbs and HRP-conjugated rabbit-anti-mouse immunoglobulins to detect the bound fusion proteins. As shown in Figure 3.7, CD4 mAbs (MT4 and leu3a) did not react to all concentrations of captured CD4-BCCP and CD147-BCCP. Polyclonal CD4 antibodies showed higher positive reactivity with CD4-BCCP than CD147-BCCP control. As expected, CD147-BCCP indicated positive reactivity with CD147 mAb M6-1B9. In addition, both of captured antigens strongly reacted with BCCP mAb BCCP-2. No signal was observed in conjugate control, which 0.05% PBS-Tween was used instead of antibody.

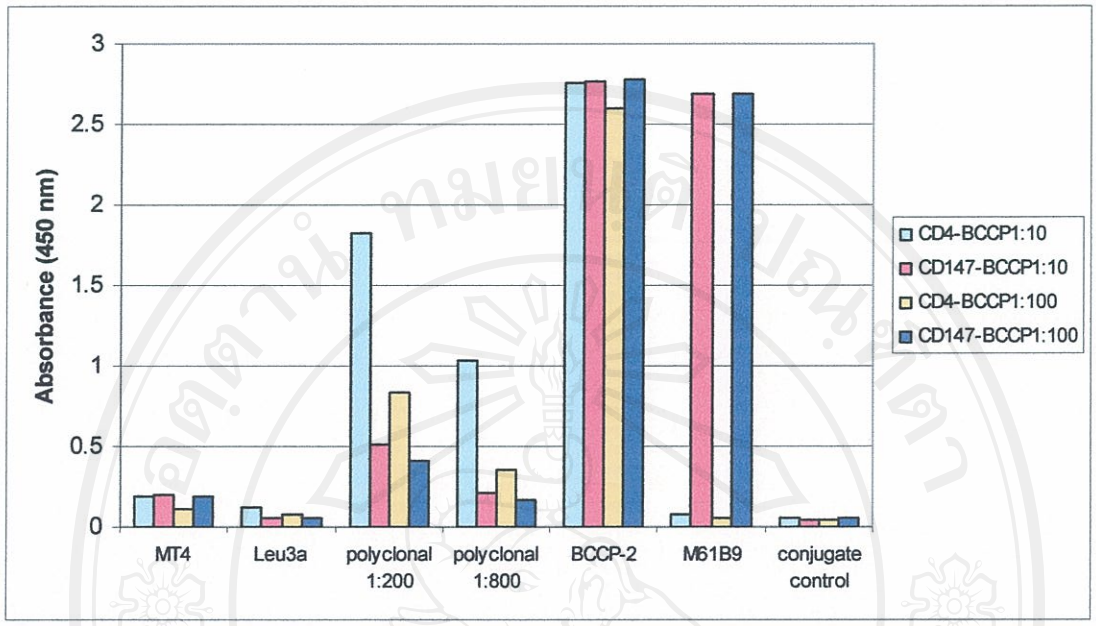

Figure 3.7 The absorbance of CD4-BCCP fusion protein or CD147-BCCP determined by capture ELISA using avidin coated plate and rabbit anti-mouse immunoglobulins-HRP as conjugate. Absorbance of conjugate control is the absorbance obtained from wells contained fusion proteins and conjugate.

3.1.2.3.2 Determination of biotinylated CD4-BCCP by Western immunoblotting

The protein components of bacterial extracts from Origami B transformed with pAK400CB-CD4, pAK400CB-CD147, and untransformed bacteria were separated in 10% polyacrylamide gel under non-reducing condition. After transfer to a nitrocellulose membrane, the blot was probed with CD4 antibodies. As shown in Figure 3.8, under non-reducing condition, all CD4 mAbs and polyclonal CD4 antibodies did not react with any protein band. Nevertheless, a band of protein with a molecular mass of 55 kDa, the size expected for CD4-BCCP protein was detected in Origami B-pAK400CB-CD4 extract when probed with BCCP mAb BCCP-2. In addition, the major band at 55 kDa was also obtained when probed with HRP-conjugated streptavidin (Figure 3.9). This band did not appear in the blot of pAK400CB-CD147 transformed Origami B and untransformed Origami B extracts. mAb BCCP-2 and HRP-conjugated streptavidin also recognized the biotinylated CD147-BCCP fusion protein at 30 kDa of the pAK400CB-CD147 transformed bacteria extract.

According to the results of ELISA and Western blotting, it turn out that the produced CD4-BCCP could not detected by CD4 mAbs. However, it was found that CD4-BCCP was detected by BCCP mAbs. Therefore, we speculated that the CD4-BCCP was produced and could be used as immunogen for further antibody production processes.

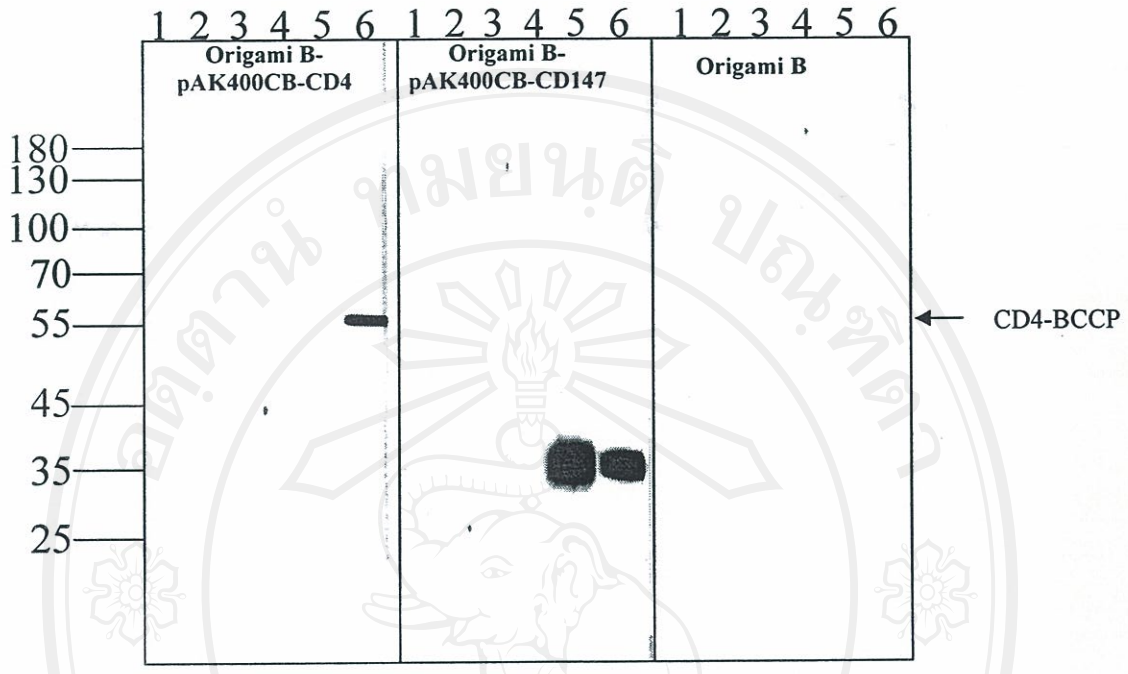

Figure 3.8 Western blot analysis of bacterial extracts obtained from various transformants using CD4 mAb MT4 (lane 2), polyclonal CD4 antibodies 1: 400 (lane3), polyclonal CD4 antibodies 1: 800 (lane 4), CD147 mAb M6-1B9 (lane 5) or BCCP mAb BCCP-2 (lane 6).

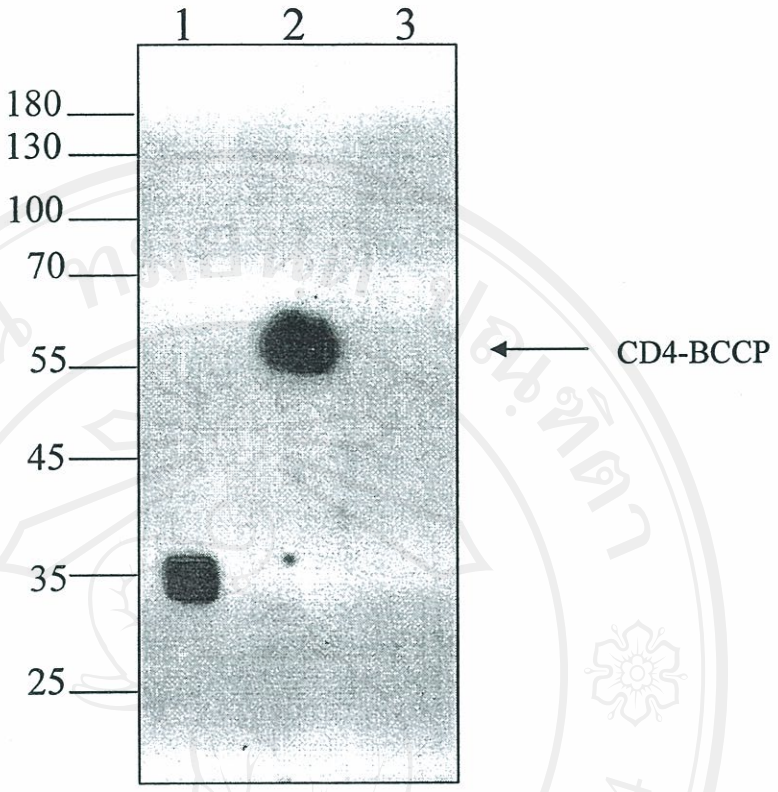

Figure 3.9 Detection of biotinylated fusion protein using HRP-conjugated streptavidin. The protein extracts from Origami B containing pAK400CB-CD147 (lane 1), pAK400CB-CD4 (lane 2), and untransformed Origami B (lane 3) were separated by non-reducing SDS-PAGE and subsequently transferred to nitrocellulose membrane. The membrane was reacted with HRP-conjugated streptavidin.

3.1.2.4 Preparation of CD4-BCCP beads

In an order to prepare CD4-BCCP coated on beads for immunization, the biotinylated CD4-BCCP fusion proteins were captured on the streptavidin-coated magnetic beads. The coated beads were separated from other bacterial components by magnetic concentrator. Indirect immunofluorescence staining was used to determine the presence of the fusion protein on the isolated beads. As shown in Figure 3.8, all CD4 mAbs (MT4, MT310 and leu-3a) did not react to the captured CD4-BCCP, CD147-BCCP or uncaptured streptavidin beads. The positive reactivity, however, observed in polyclonal CD4 antibodies was found higher with CD4-BCCP than CD147-BCCP. High positive reactivity was observed when stained CD147-BCCP beads with CD147 mAb. Moreover, both of captured antigens strongly reacted with BCCP mAb. No signal was observed in all of antibodies with uncaptured streptavidin beads. Hence, the CD4-BCCP beads were used for mouse immunization.

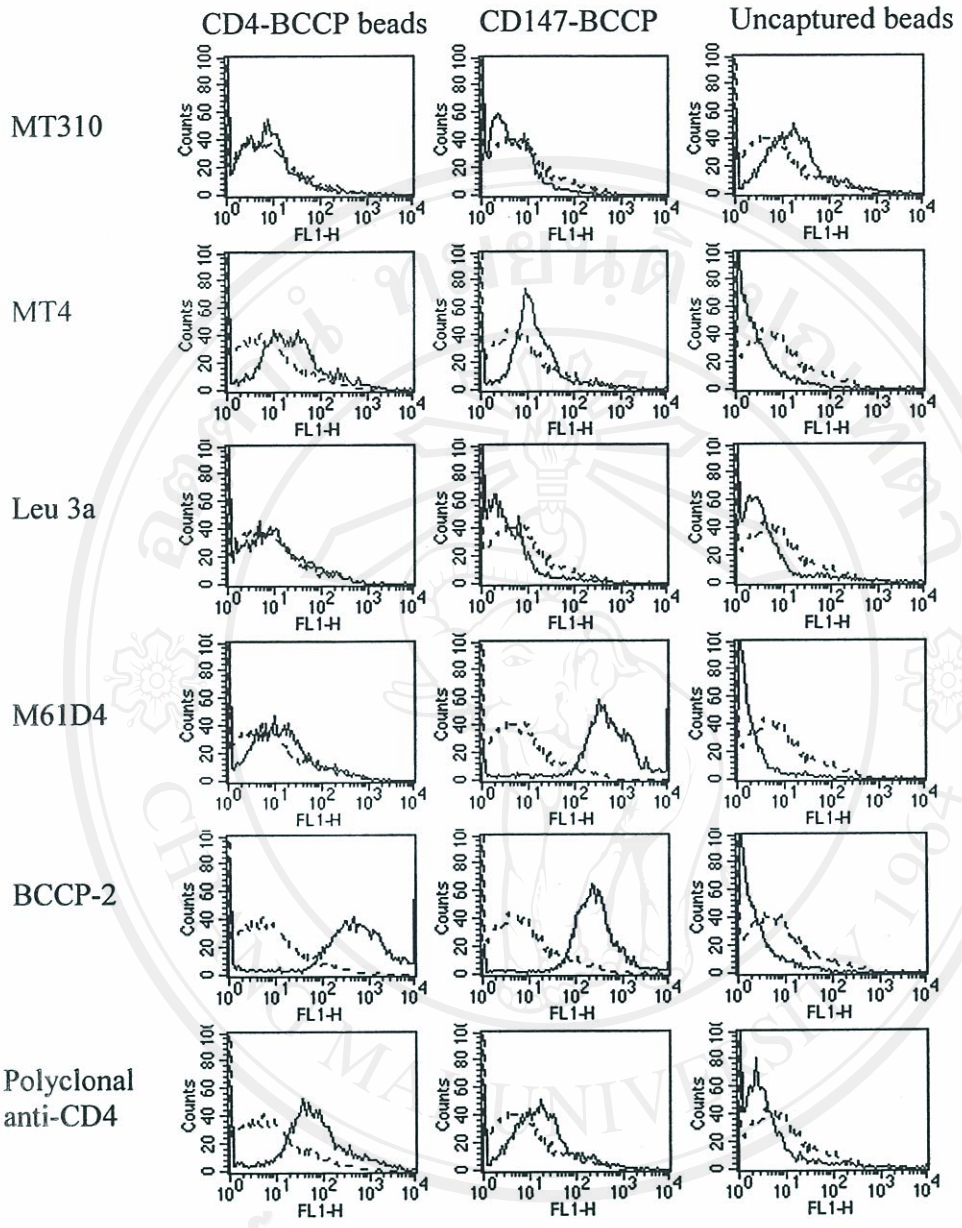

Figure 3.10 Flow cytometric analysis of captured or uncaptured streptavidin magnetic beads. CD4-BCCP, CD147-BCCP beads or uncaptured beads were stained with CD4 mAbs (MT310, MT4, Leu 3a), CD147 mAb (M6-1D4), BCCP mAb (BCCP-2) or CD4 polyclonal antibodies. Solid lines represent the immunofluorescence profiles of beads stained with indicated antibodies and dashed lines represent background fluorescence of conjugate control, which 1%BSA-PBS-NaN_3 was used instead of antibodies.

3.1.3 CD4-COS cell expression technique

3.1.3.1 Preparation of CD4 DNA

To prepare COS cell expressing CD4 protein for using as immunogen, the plasmid encoding CD4 protein, CD4-DNA, were prepared as described in materials and methods. The obtained CD4-DNA was proved for the expression of CD4 protein by using COS cells expression system and indirect immunofluorescent staining of the transfected COS cells. It was found that COS transfected with CD4-DNA were strongly positive with CD4 mAb MT4 (Figure 3.11). Untransfected COS cells were negative with the CD4 mAb. The CD4-DNA were, therefore, used to prepare CD4-COS cells in the further experiments.

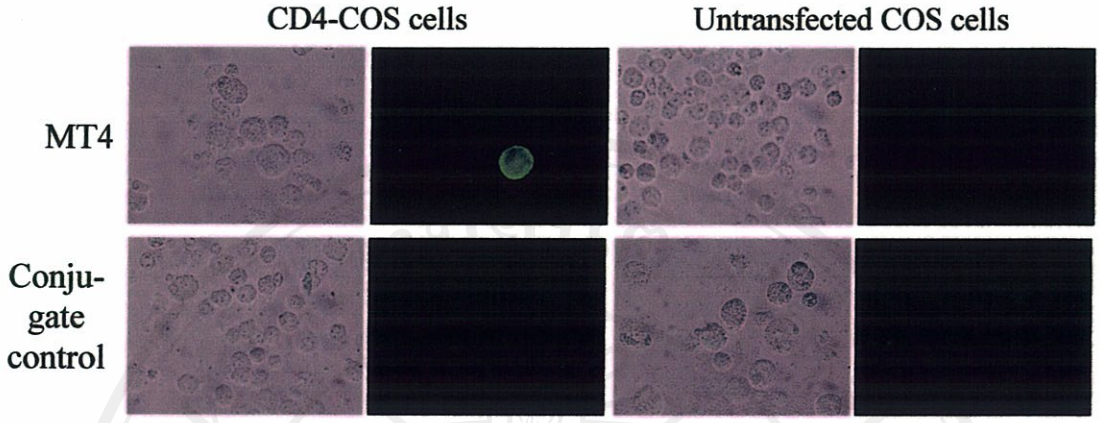

Figure 3.11 Immunofluorescence analysis of CD4-DNA transfeced COS cells. The CD4-DNA transfected COS cells and untransfected COS cells were stained with the CD4 mAbs MT4 and counterstained with FITC conjugated anti-mouse immunoglobulins. Stained cells were analyzed under a fluorescence microscope.

3.1.3.2 Preparation of COS cell expressing CD4 protein

To prepare COS cell expressing CD4 protein for using as immunogen, CD4-DNA were transfeced into COS cells by DEAE-dextran transfection method. The expression of CD4 protein on COS cells were confirmed by indirect immunofluorescence staining. The transfected COS cells were positive with CD4 mAb MT4 (Figure 3.12). Approximately 20 % of the transfected cells expressed CD4 protein in all preparations. After that the CD4 expressed COS cells were enriched by immunomagnetic beads sorting. After magnetic sorting, approximately 70% of CD4 positive cells were obtained. The obtained CD4 expressed COS cells were used to immunize mouse for generation of monoclonal antibody.

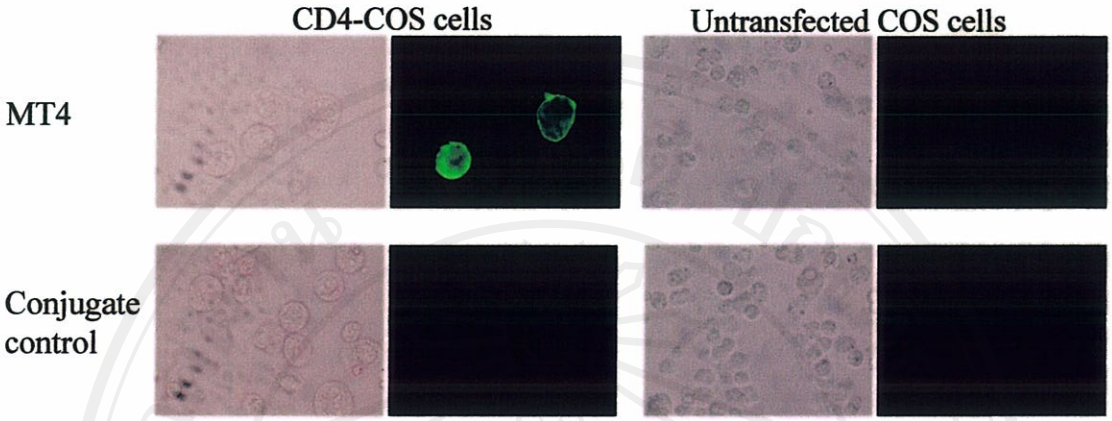

Figure 3.12 Immunofluorescence analysis showing expression of CD4 protein in transfected COS cells. The CD4-DNA transfected COS cells were stained with the CD4 mAbs MT4 and counterstained with FITC conjugated anti-mouse immunoglobulins. Stained cells were analyzed under a fluorescence microscope.

3.2 Mouse immunization

In an attempt to produce CD4 mAbs by using the prepared CD4 protein immunogens, the standard hybridoma technique was employed. Three groups of BALB/c mice (two mice per group) were immunized three times at one-week intervals with CD4 immunoprecipitated-beads (section 3.1.1), CD4-BCCP coated beads (section 3.1.2) or CD4 expressed COS cells (section 3.1.3). The first group, mice were immunized with CD4 immunoprecipitated-beads and named CD4-IP A and CD4-IP B. The second group, mice were immunized with CD4-BCCP beads and named CD4-BCCP A and CD4-BCCP B. The latest group, mice were immunized with CD4-COS cells and named CD4-COS A and CD4-COS B. Sera were collected form the immunized mice 7 days after each immunization. The presence of CD4 antibodies in serum was determined by indirect ELISA or indirect immunofluorescence technique.

For CD4-BCCP bead immunization, antibodies response was determined by indirect ELISA using CD4-BCCP as antigen. The CD4-BCCP was captured in avidin coated ELISA wells followed by addition of serial five-fold diluted mice sera, and the bound antibodies were detected by HRP-conjugated rabbit anti-mouse immunoglobulin antibodies. After the first immunization, CD4-BCCP antibodies were detected in the immunized mice sera, the antibody levels were increased after boosting (Figure 3.13). The antibody titer was up to 312,500 after the third immunization. The results demonstrated the success of induction of antibody production by immunization with CD4-BCCP beads.

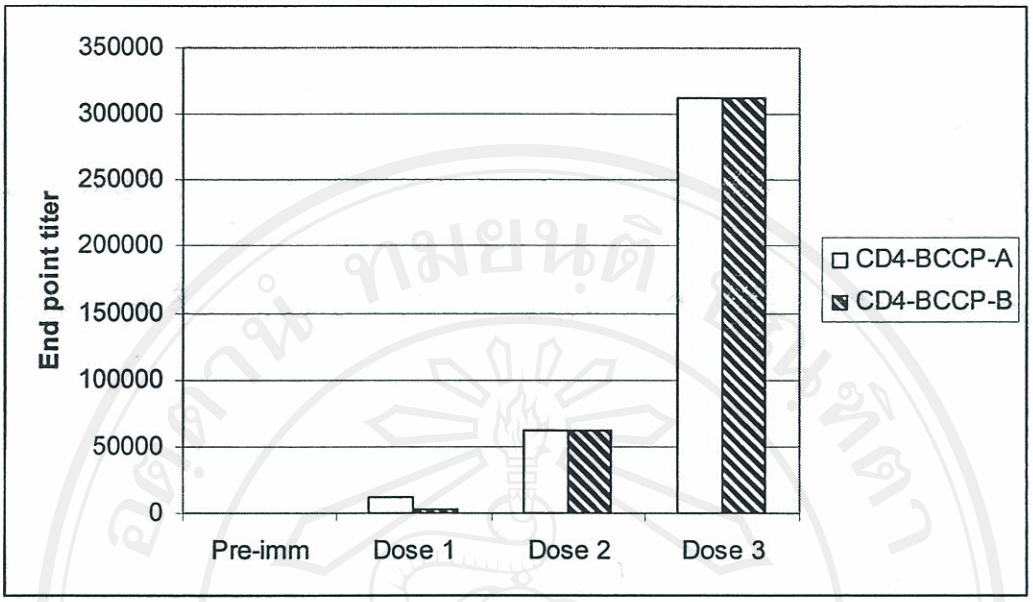

Figure 3.13 Analysis of antibody responses in mice sera immunized with CD4-BCCP beads. Sera were collected seven days after the indicated dose of immunization and determined for the presence of CD4-BCCP using indirect ELISA.

In addition to indirect ELISA, sera of all mice immunized with the three different immunogens were screened for the presence of CD4 antibodies by staining PBMCs surface protein by indirect immunofluorescence. As shown in Figure 3.14, all of pre-immunized sera did not react to PBMCs surface protein. In contrast, all of immunized mice sera showed positive reactivity. After the third immunization, the antibody titers were 1600 and 3200, 100 and 6400 for immunoprecipitated-beads, CD4-BCCP beads and CD4-COS cell immunization, respectively. The results demonstrated the success of induction of antibody production by immunization with three different immunogens.

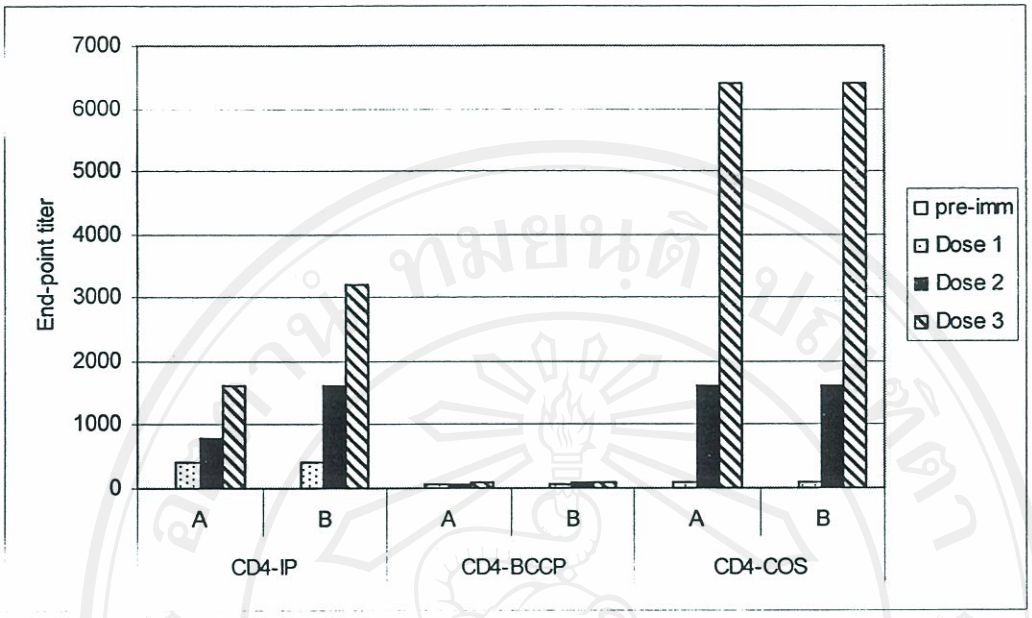

Fig 3.14 Determination of antibody responses in mice sera after immunization with CD4-immunoprecipitated beads, CD4-BCCP beads or CD4-COS cells. PBMCs were stained with various dilutions of pre-immunized sera or sera collected 7 day after indicated dose of immunization, using indirect immunofluorescene technique.

To confirm whether the detected antibodies in mice sera were specifically reacted to CD4 protein, the COS cell expression system was applied. CD4-DNA were transfected into COS cells. Then, the CD4 expressing COS cells and untransfected COS cells were stained with mice sera. Positive reactivities were detected after immunization with all immunogen used (Figure 3.15-3.17). As experimental controls, transfected COS cells were stained with standard CD4 mAb MT4. Strongly positive reactivity was observed (Figure 3.18), no signal was observed in conjugate control, which 1%BSA-PBS Azide was used instead of mAb MT4 (Figure 3.18). After the third immunization, the CD4 antibody titers in all immunized sera were determined by staining of CD4 expressing COS cells. The results were summarized in Figure 3.19. These results confirmed that the antibodies generated by immunization with all immunogen preparations are specifically reacted to CD4 protein.

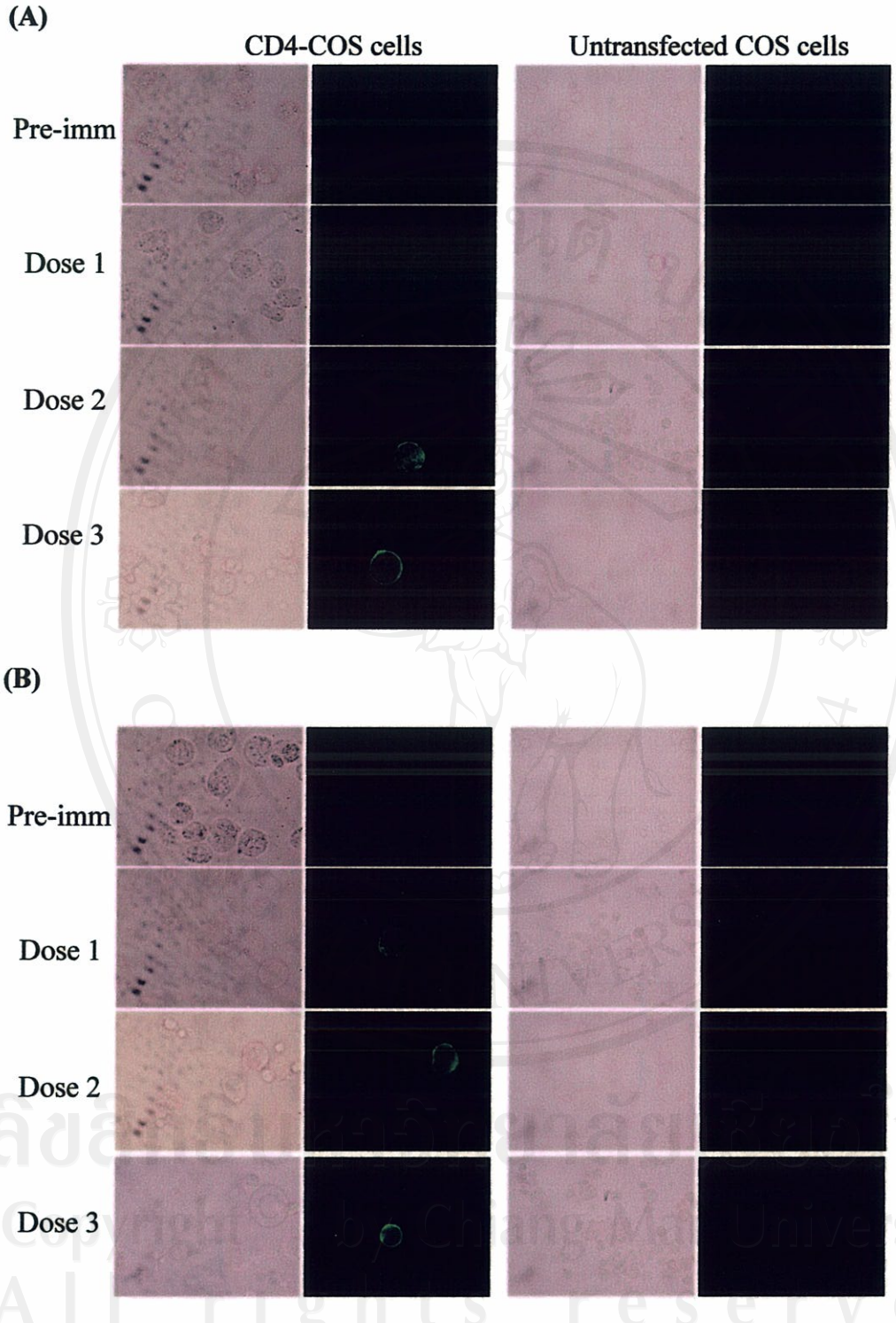

Figure 3.15 Immunofluoescence analysis of CD4 antibodies in sera of CD4-IP A (A) and CD4-IP B (B). CD4-DNA transfected COS cells (CD4-COS cells) or untransfected COS cells were stained with 1:50 diluted pre-immunized serum or serum collected 7 days after the indicated dose of immunization.

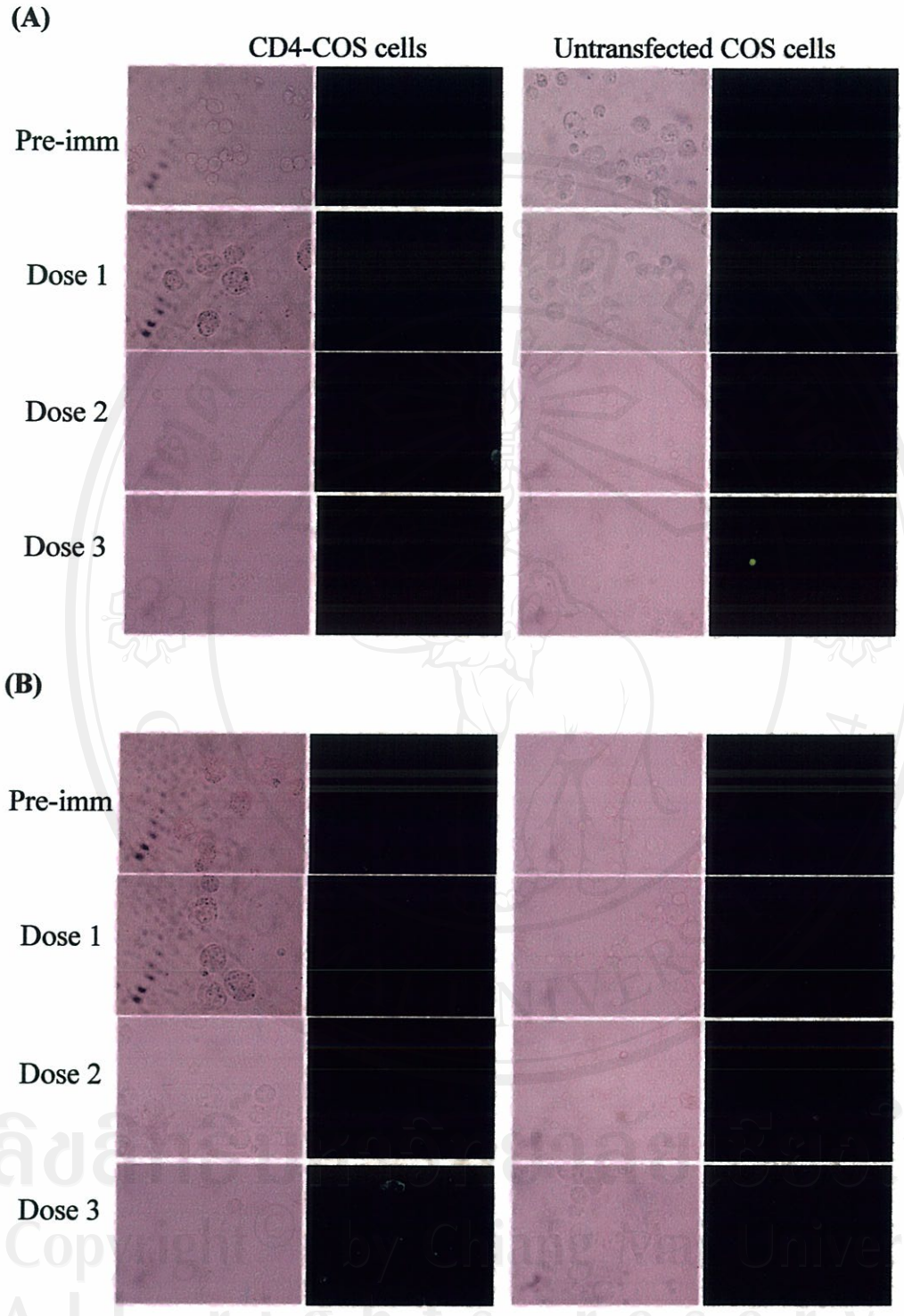

Figure 3.16 Immunofluoescence analysis of CD4 antibodies in sera of CD4-BCCP A (A) and CD4-BCCP B (B). CD4-DNA transfected COS cells (CD4-COS cells) or untransfected COS cells were stained with 1:50 diluted pre-immunized serum or serum collected 7 days after the indicated dose of CD4-BCCP beads immunization.

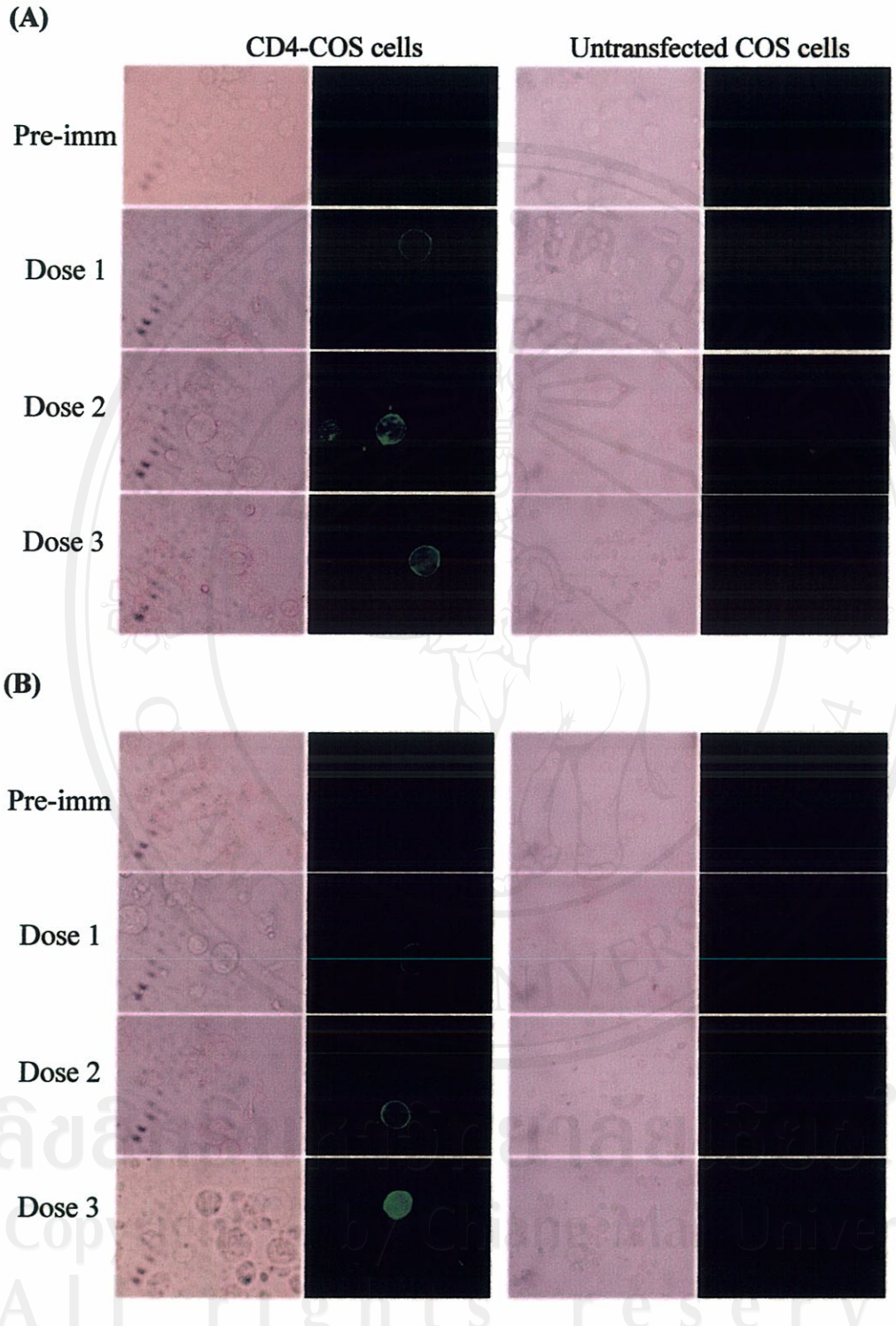

Figure 3.17 Immunofluorescence analysis of CD4 antibodies in sera of CD4-COS A (A) and CD4-COS B (B). CD4-DNA transfected COS cells (CD4-COS cells) or untransfected COS cells were stained with 1:50 diluted pre-immunized serum or serum collected 7 days after the indicated dose of CD4-COS cells immunization.

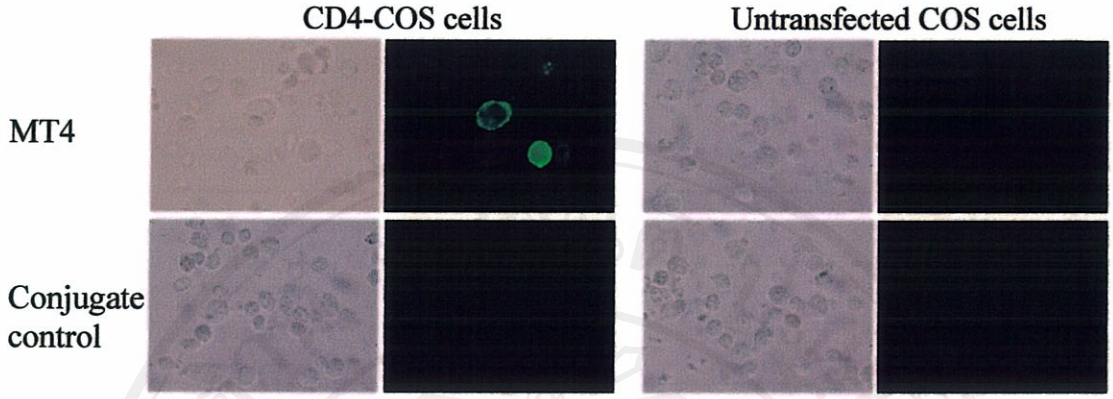

Figure 3.18 Immunofluorescence of CD4 transfected COS cells with CD4 mAb MT4. CD4-DNA transfected or untransfected COS cell were stained with CD4 mAb MT4, using indirect immunofluorescence technique. Stained cells were analyzed under a fluorescence microscope.

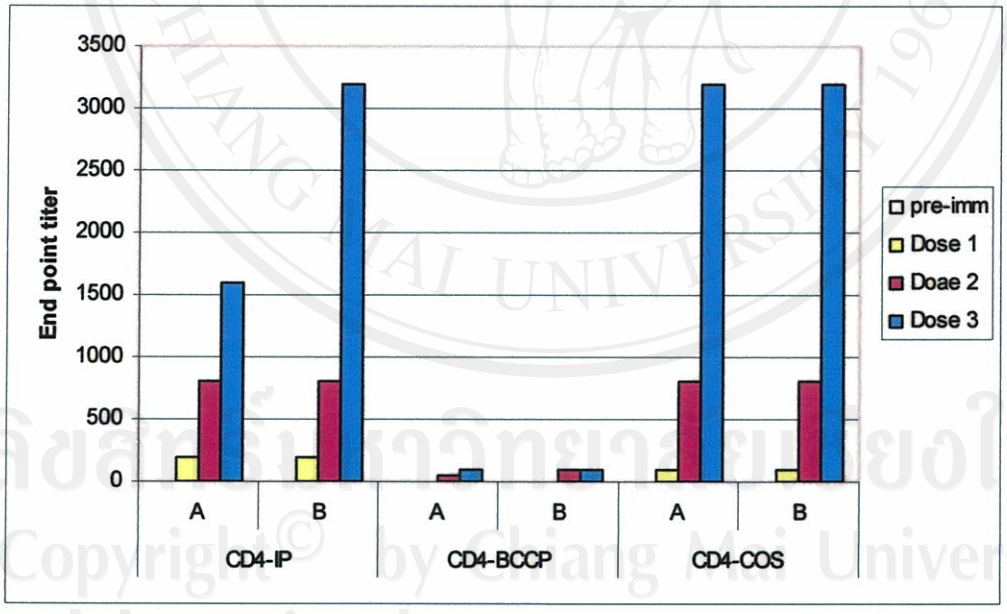

Figure 3.19 Determination of CD4 antibody titer in sera of the immunized mice. CD4-DNA transfected or untransfected COS cell were stained with various dilution of pre-immunized serum or serum collected 7 days after the indicated dose of immunization.

3.3 Hybridoma productions

To generate CD4 hybridomas, an spleen cells from immunized mouse selected from each group were fused with myeloma cells using standard hybridoma techniques.

By immunoprecipitated-beads immunization, 60% of the seed wells developed single or multiple hybridomas. The cultures supernatants from 400 hybridoma containing wells were screened for antibodies by staining of PBMC surface protein using indirect immunofluorescent technique. By this technique, supernatant from 1 hybridoma containing wells showed positive with all population of PBMCs, supernatant from 1 hybridoma containing well showed positive only with monocytes and 3 supernatant showed positive with a lymphocyte subpopulation but negative or weakly positive with monocytes. To obtain CD4 antibody-secreting hybridomas, the culture supernatants which showed positive reactivity with a lymphocyte subpopulation, were further screened by staining of CD4-DNA transfected COS cells. By this way, well containg CD4 antibody will positive with CD4 expressing COS but negative with untransfected COS cells. It was found that only one hybridoma containing well showed positive reactivity to CD4-DNA transfected COS cells with no reactivity to COS cells. The hybridomas in this positive well were then re-cloned by limiting dilution. The healthy single clone were propagated and named MT4/4.

For hybridoma production by CD4-BCCP beads immunization, 90% of the seeded wells developed single or multiple colonies. The culture supernatant from 700 hybridoma containing wells were screened firstly by indirect ELISA using CD4-BCCP fusion protein as antigen. To eliminate any hybridomas specific to BCCP part, the culture supernatant were counter screened with CD147-BCCP. By this screening,

200 hybridomas that indicated the difference of absorbance greater than 0.3 when tested with CD4-BCCP and CD147-BCCP were further screened by indirect immunofluorescence technique using PBMCs as antigen. It was found that, supernatant from only 1 hybridoma containing wells showed positive with a lymphocyte subpopulation but negative or weakly positive with monocytes. The hybridomas in these positive wells were then re-cloned by limiting dilution. Unfortunately, this clone lost their activity during cultivation. Additionally, two hybridomas which were strongly positive with CD4-BCCP without cross reacted to CD147-BCCP were selected for single cell cloning by limiting dilution. Of two clones, one turned negative in the re-screening. The positive hybridoma clone which gave positive reactivity only with CD4-BCCP were propagated and named CD4-BCCP-1 (Figure 3.20).

For hybridoma production using CD4-COS cells immunization, 60% of the seeded wells developed single or multiple colonies. The cultures supernatant from 580 hybridoma containing wells were screened for antibodies by staining of PBMCs using indirect immunofluoresceence staining. It was found that supernatant from 18 hybridoma containing wells showed positive with all population of PBMCs, supernatant from 35 containing well showed positive only with monocytes and 71 supernatant showed positive with a lymphocyte subpopulation but negative or weakly positive with monocytes. Out of 71 hybridomas, two clones showed strong immunofluorescence staining only with CD4-DNA transfected COS cells. The positive hybridomas were re-cloned by limiting dilution. The healthy single clones which gave positive reactivity with CD4 expressing cells were propagated and named MT4/2, MT4/3, respectively.

According to three hybridoma productions, the summary of hybridoma production was shown in Table 3.1. To this end, one CD4 hybridoma producing cell, MT4/4, derived from immunoprecipitated-beads immunization and two CD4 hybridoma producing cells, MT4/2 and MT4/3, derived from CD4-COS cells immunization were obtained. Furthermore, one CD4-BCCP hybridoma producing cells named CD4-BCCP-1, which recognized CD4-BCCP fusion protein but negative to CD4 expressing cells, derived from CD4-BCCP beads immunization was obtained.

Table 3.1 Summary of CD4 monoclonal antibody productions using three different immunogens

	Immunogen		
	CD4 immunoprecipitated-beads	CD4-BCCP beads	CD4-COS cells
Percentage of hybridoma [a] containing wells	60	90	60
Total number of screened [b] hybridoma	400	700	580
Total number of hybridomas reacted to PBMCs	1	0	18
Total number of hybridomas reacted to monocytes	1	0	35
Total number of hybridomas reacted to lymphocyte subpopulation	3	1	71
Total number of hybridomas reacted to CD4 protein expressed on COS cells	1	0	1
Total number of hybridomas specifically reacted to CD4-BCCP		1	

[a]Number of wells containing hybridoma divided by number of wells seeded. Plating density of the fused cells was comparable for all hybridoma production (3×10^4 cells/well)

[b]Total number of hybridomas which were screened for CD4 mAb producing

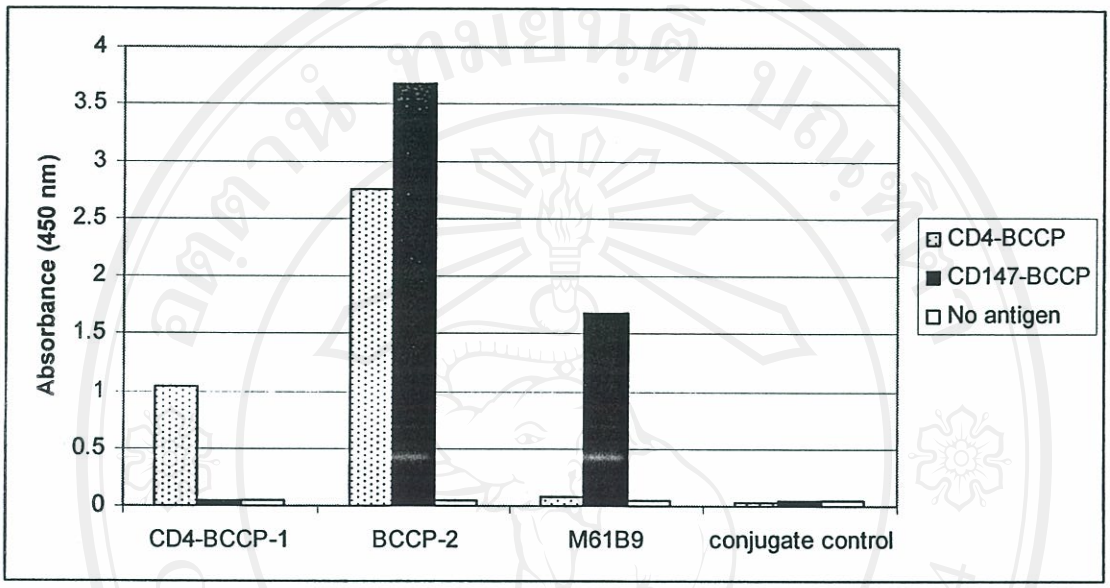

Figure 3.20 ELISA analysis of the reactivity of mAb CD4-BCCP-1 with CD4-BCCP and CD147-BCCP. Absorbance of conjugate control is the absorbance obtained from wells containing fusion proteins and conjugate.

3.4 Characterization of the generated mAbs

The reactivity of generated CD4 mAbs was examined by indirect immunofluorescence, Western immunoblotting and immunoprecipitation technique.

3.4.1 Specificity of generated mAbs characterization

The specificity of generated monoclonal antibodies was confirmed by indirect immunofluorescence technique using CD4-DNA transfected COS cell as positive target cell and CD8-DNA transfected COS cells as negative control cells. As shown in Figure 3.21 and Table 3.2, MT4/2, MT4/3, MT4/4 and CD4 mAb MT4 strongly reacted to CD4-DNA transfected COS cells but did not reacted to CD8-DNA transfected COS cells. In contrast, CD8 mAb MT8 strongly reacted only to CD8-DNA transfected COS cells. No signal was observed in mAb CD4-BCCP-1 and conjugate control. The result indicated that the generated CD4 mAbs, MT4/2, MT4/3 and MT4/4, specifically reacted to recombinant CD4 protein expressed on CD4-DNA transfected COS cells.

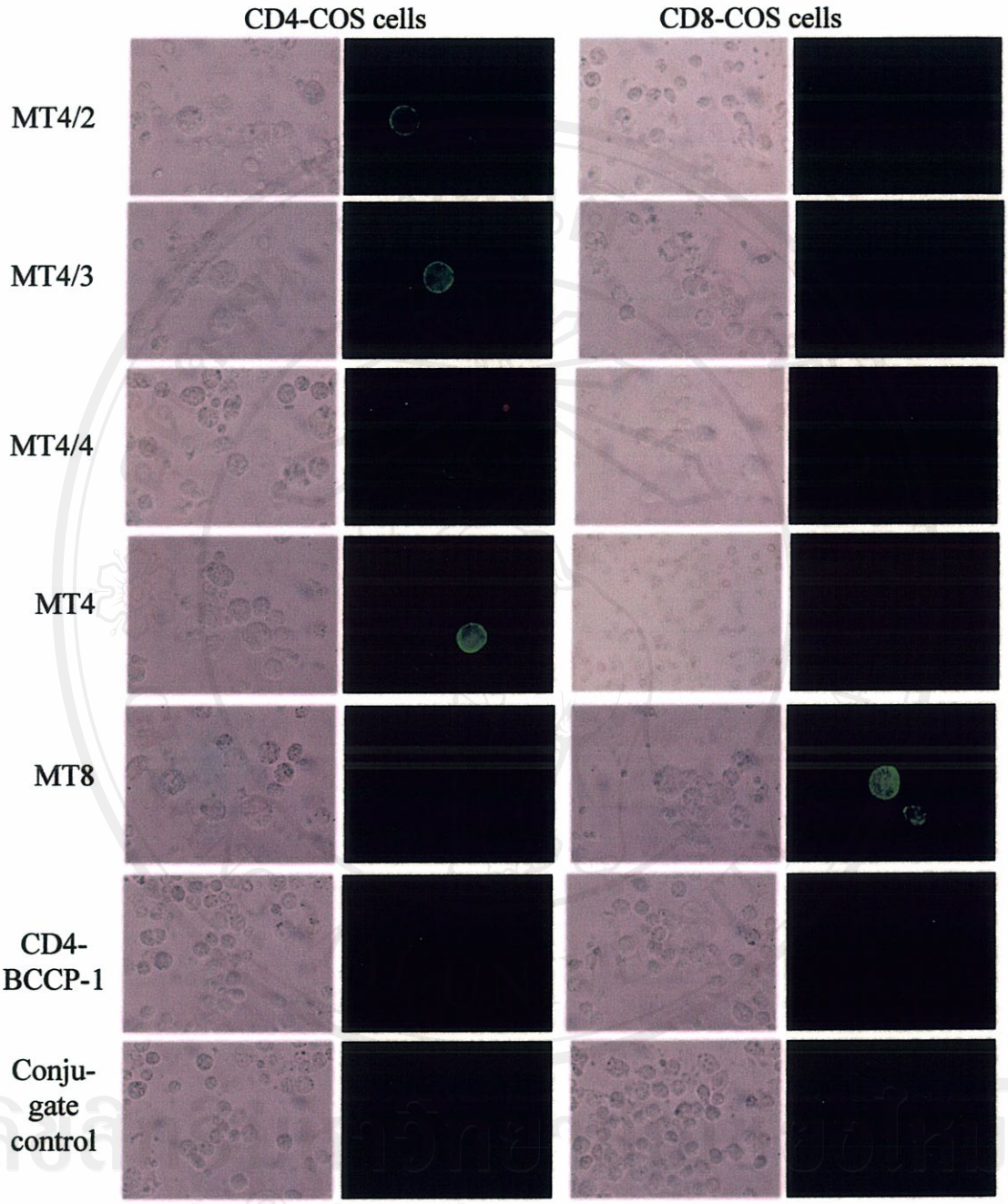

Figure 3.21 Immuofluorescence analysis of specificity of generated monoclonal antibodies. CD4-DNA transfected COS cells (CD4-COS cells) or CD8 transfected COS cells (CD8-COS cells) were stained indicated mAbs and counterstained with FITC conjugated sheep anti-mouse immunoglobulins. The stained cells were analyzed under a fluorescence microscope.

Table 3.2 The reactivity of generated mAbs with CD4 transfected COS cells and CD8 transfected COS cells

mAb	Immunofluorescence reactivity	
	CD4 transfected COS cells	CD8 transfected COS cells
MT4/2	+*	-**
MT4/3	+	-
MT4/4	+	-
MT4***	+	-
MT8***	-	+
CD4-BCCP-1	-	-
Conjugate control	-	-

* Positive reactivity

** Negative reactivity

*** mAb MT4 and MT8 are CD4 and CD8 mAbs previously generated in our department.

3.4.2 Characterization of the generated CD4 mAbs by Western immunoblotting and immunoprecipitation

3.4.2.1 Western immunoblotting

The PBMCs lysates was separated by SDS-PAGE under non-reducing and reducing conditions. After blotting on nitrocellulose membrane, the membrane was probed with the generated CD4 mAbs and isotype-matched control. All CD4 mAbs including standard CD4 mAb leu3a did not react to any protein under both reducing and non-reducing condition (data not shown). These results may indicated that the generated CD4 mAbs are the mAbs that react to the conformational epitops on the CD4 protein.

3.4.2.2 Immunoprecipitation

We, then, performed immunoprecipitation. The generated CD4 mAbs were used to precipitate CD4 protein from PBMCs lysates. In these experiments, standard CD4 mAb L200 and generated CD4 mAbs, MT4/2, MT4/3 and MT4/4 precipitated a band of biotinylated protein at the molecular weight approximately of 55 kDa corresponding to the CD4 protein (Figure 3.22). In contrast, mAb MT4 isotype matched control mAb did not precipitate any biotinylated protein. In addition, positive control CD99 mAb MT99/3 precipitated CD99 protein of the molecular weight approximately of 34.5 kDa. The results indicated that the generated CD4 mAbs, MT4/2, MT4/3 and MT4/4, reacted to native CD4 protein presented on PBMCs.

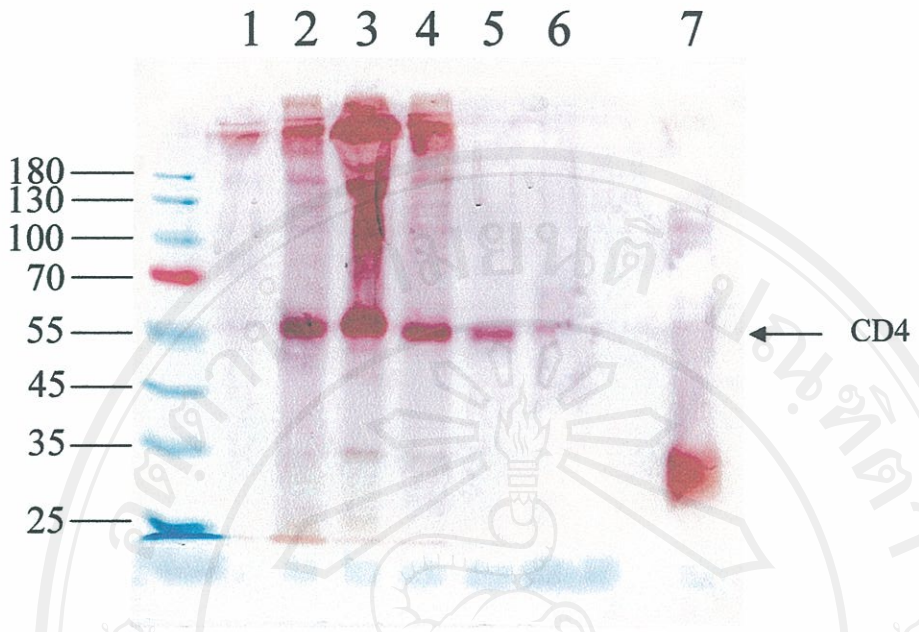

Figure 3.22 Immunoprecipitation of CD4 protein from PBMCs using the generated CD4 mAbs. MT4 mAb (lane 1), MT4/2 mAb (lane 2), MT4/3 (lane 3), MT4/4(lane 4), L200 mAb (lane 5), VIT4 mAb mAb (lane 6), and MT99/3 (lane 7) mAb coated beads were shown. Electrophoresis was performed under non-reducing condition. The positions of molecular mass markers are indicated on the left in kDa.

3.4.3 Determination of isotype of the generated monoclonal antibodies

The isotype of monoclonal antibodies produced by MT4/2, MT4/3, MT4/4 and CD4-BCCP-1 clones were determined by capture ELISA. As show in figure 3.23, the isotype of MT4/2, MT4/3, MT4/4 and CD4-BCCP-1 were IgM, IgG2a, IgM and IgG2b, respectively.

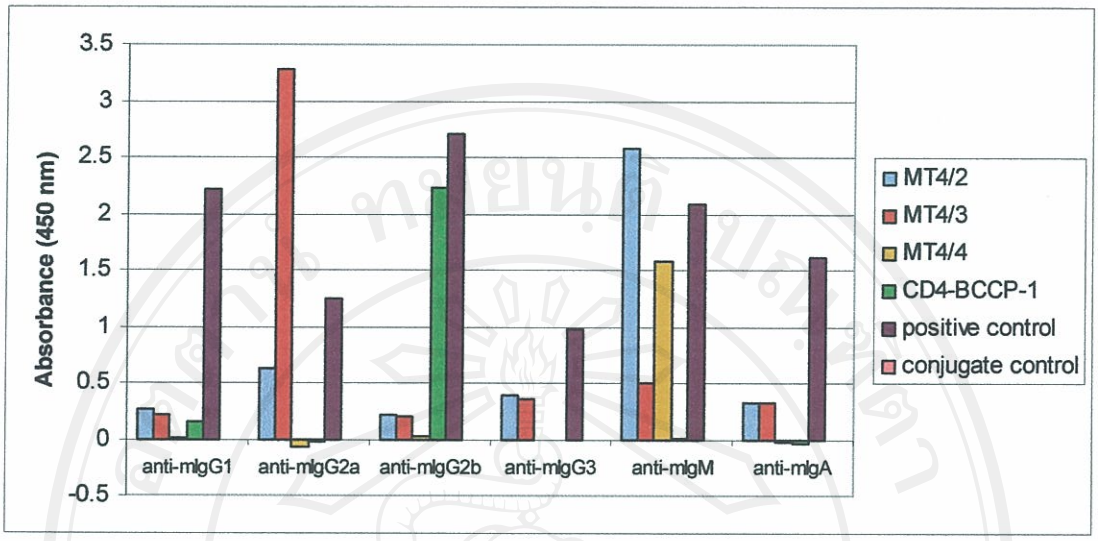

Figure 3.23 Determination of the isotype of the generated monoclonal antibodies by capture ELISA. Tissue culture supernatants were added into each well of mcrotiter plate coated with isotype specific antibodies (goate anti mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA), and antigen-antibody reaction was visualized by using peroxidase labeled rabbit anti-mouse immunoglobulins and TMB substrate. The results were read absorbance at 450 nm.

3.4.4 Enumeration of the percentage of $CD4^+$ cells in peripheral blood lymphocytes by using the generated CD4 mAbs

The generated CD4 mAbs were used for the enumeration of $CD4^+CD3^+$ cells in peripheral blood lymphocytes of 3 healthy subjects by lysed whole blood immunofluorescence staining. The percentage of $CD4^+CD3^+$ cells in lymphocyte population was analyzed by flow cytometry and compared to those using CD3-FITC/CD4-PE standard reagent purchased from Becton Dickinson. As shown in Table 3.3, the percentage of CD4 positive cells obtained by using the generated mAbs was very similar to those obtained using the standard CD4 mAb. The results suggested that the generated CD4 mAbs can be used for enumeration of $CD4^+$ cells in peripheral blood lymphocytes.

Table 3.3 Determination of the percentage of CD4+ lymphocytes using the generated CD4 mAbs and standard reagent

Subject No.	Percentage of CD4/CD3 positive cells in lymphocytes			
	MT4/2 mAb	MT4/3 mAb	MT4/4 mAb	Standard reagent
1	23.15	29.12	33.09	27.27
2	27.13	28.33	22.88	23.41
3	35.72	32.36	36.86	32.68