CHAPTER III

RESULTS

3.1 Production and purification of mAbs to CD147 molecule

To produce anti-CD147 mAbs, M6-2F9, M6-2B1, M6-1F3, M6-1D4, M6-1B9 and M6-1E9 hybridroma clones were injected into the intraperitoneal cavity of Balb/c mice. Ascitic fluids were collected 2-3 weeks after injection. The mAbs M6-2F9, M6-2B1, M6-1F3, M6-1D4, M6-1B9 and M6-1E9 present in the ascitic fluids were purified by affinity chromatography. In this study, M6-2F9, M6-2B1, M6-1F3 and M6-1D4 which are of IgM isotype were purified by anti-mouse IgM coated Sepharose column and mAb M6-1B9 and M6-1E9 which are of IgG isotype were purified by Protein A coated Sepharose column. In this study, from 400 µl of starting ascitic fluids, the yields of purified mAbs M6-2F9, M6-1F3, M6-2B1, M6-1D4, M6-1B9 and M6-1E9 were 1.21, 1.00, 0.62, 1.43, 1.67 and 1.77 mg, respectively.

3.2 Determination of activity and specificity of the purified anti-CD147 mAbs

To determine the activity of the purified anti-CD147 mAbs, U937 cell line was stained with every purified mAbs by indirect immunofluorescence. As shown in Figure 3.1, mAbs M6-2F9, M6-2B1, M6-1F3, M6-1D4, M6-1B9 and M6-1E9 showed positive reactivity with U937 cells. These results indicated that all mAbs still have binding activities after purification process.

To confirm that the purified mAbs were specifically reacted to CD147 molecule, the COS cell expression system was used. COS cells were transfected with M6 (CD147)-DNA or CD8-DNA. Then, the CD147 and CD8 expressing COS cells were stained with purified mAbs M6-2F9, M6-2B1, M6-1F3, M6-1D4, M6-1B9, M6-1E9 and MT8 (CD8). All of the purified anti-CD147 mAbs showed positive reactivity to CD147 expressing COS cells (Figure 3.2), with no reactivity to CD8 expressing COS cells. In contrast, MT8 mAb showed positive reactivity only with CD8 expressing COS cells. The results indicated that all purified anti-CD147 mAbs are specifically reacted to CD147 molecule.

3.3 Determination of epitopes recognized by anti-CD147 mAbs

3.3.1 Cross-blocking analysis using U937 cells

To study the epitope mapping, FITC conjugated anti-CD147 mAbs were necessary. Therefore, the first step of epitope mapping study was the to label anti-CD147 mAbs with FITC. FITC to protein (F/P) ratios were determined by OD at 495 nm and at 280 nm ratios. As shown in Table 3.1, in this study, the F/P ratios of FITC-conjugated anti-CD147 mAbs ranged from 0.9-1.375.

After FITC conjugation, the FITC-conjugated anti-CD147 mAbs were titrated to obtain the optimal concentration for using in epitope mapping assay. As shown in Figure 3.3, all FITC-labeled mAbs reached the saturated concentration at 40 μg/ml which no significantly change of fluorescence intensity when reduced the concentration to 20 μg/ml. This concentration was therefore selected for cross-blocking analysis.

To determine the epitopes of CD147 molecule that were reacted to different anti-CD147 mAbs, U937 cells were first stained with un-labeled anti-CD147 mAbs and subsequently with FITC-conjugated anti-CD147 mAbs. The cross-blocking analysis was calculated from the mean fluorescence intensity between cells stained with and without un-labeled anti-CD147 mAb. The level of inhibition of more than 50% would suggest that the first un-labeled mAb (blocking antibody) can inhibit the binding of the second labeled mAb (FITC labeled mAb). The percent inhibitions between each pair of anti-CD147 mAbs were shown in Table 3.2. MAb M6-2F9 inhibited the binding of FITC-conjugated M6-2F9, M6-2B1 and M6-1F3 but did not inhibit the binding of mAbs M6-1D4, M6-1B9 and M6-1E9. MAb M6-1D4 inhibited the binding of FITC-conjugated M6-1D4, M6-2F9, M6-2B1 and M6-1F3 but enhanced the binding of the mAbs M6-1B9 and M6-1E9. Whereas mAbs M6-2B1 and M6-1F3 did not inhibit the binding of any anti-CD147 mAbs. The mAbs M6-1E9 and M6-1B9 inhibited binding of all anti-CD147 mAbs. Surprisingly, in the experiments, mAbs M6-2B1, M6-1F3 and M6-1B9 did not demonstrate self-inhibition. According to their binding capabilities, the studied anti-CD147 mAbs could be clustered into 4 epitopes: 1) M6-2F9, 2) M6-2B1 and M6-1F3, 3) M6-1D4 and 4) M6-1B9 and M6-1E9.

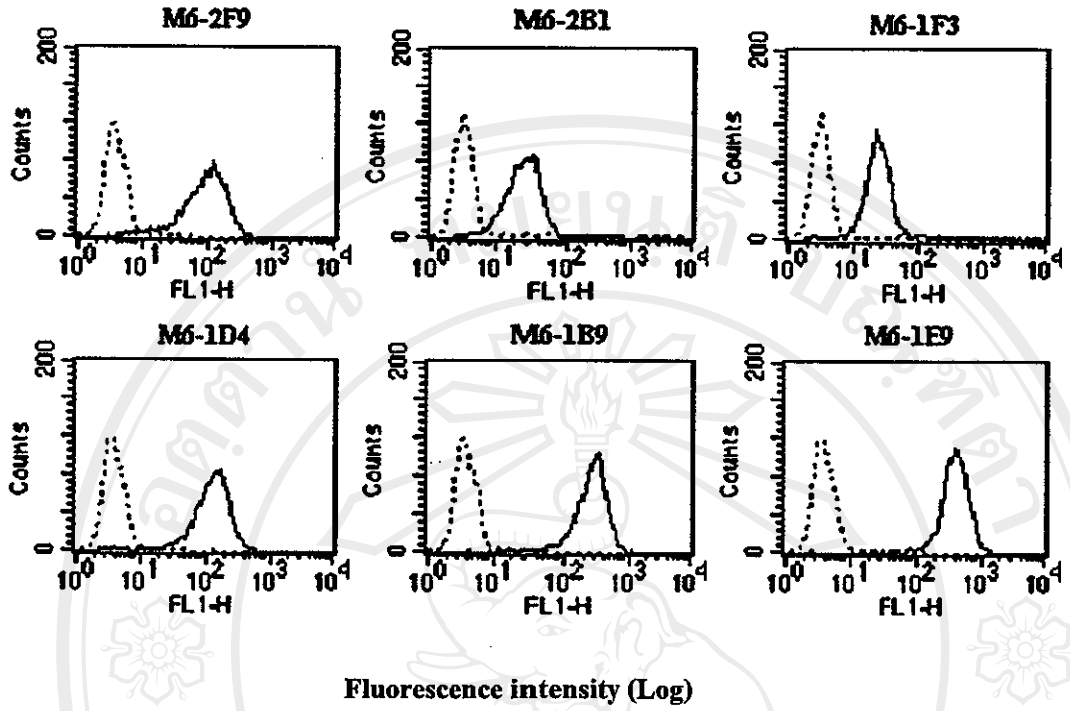

Figure 3.1 The immunofluorescence profiles of activity of purified anti-CD147 mAbs. U937 cells were stained with purified mAbs M6-2F9, M6-2B1, M6-1F3, M6-1D4, M6-1B9 and M6-1E9 by indirect immunofluorescent method and analyzed by flow cytometry. Solid lines represent the immunofluorescent profiles of cells stained with the indicated mAbs. Dot lines represent the immunofluorescent profiles of cells stained with the conjugate control.

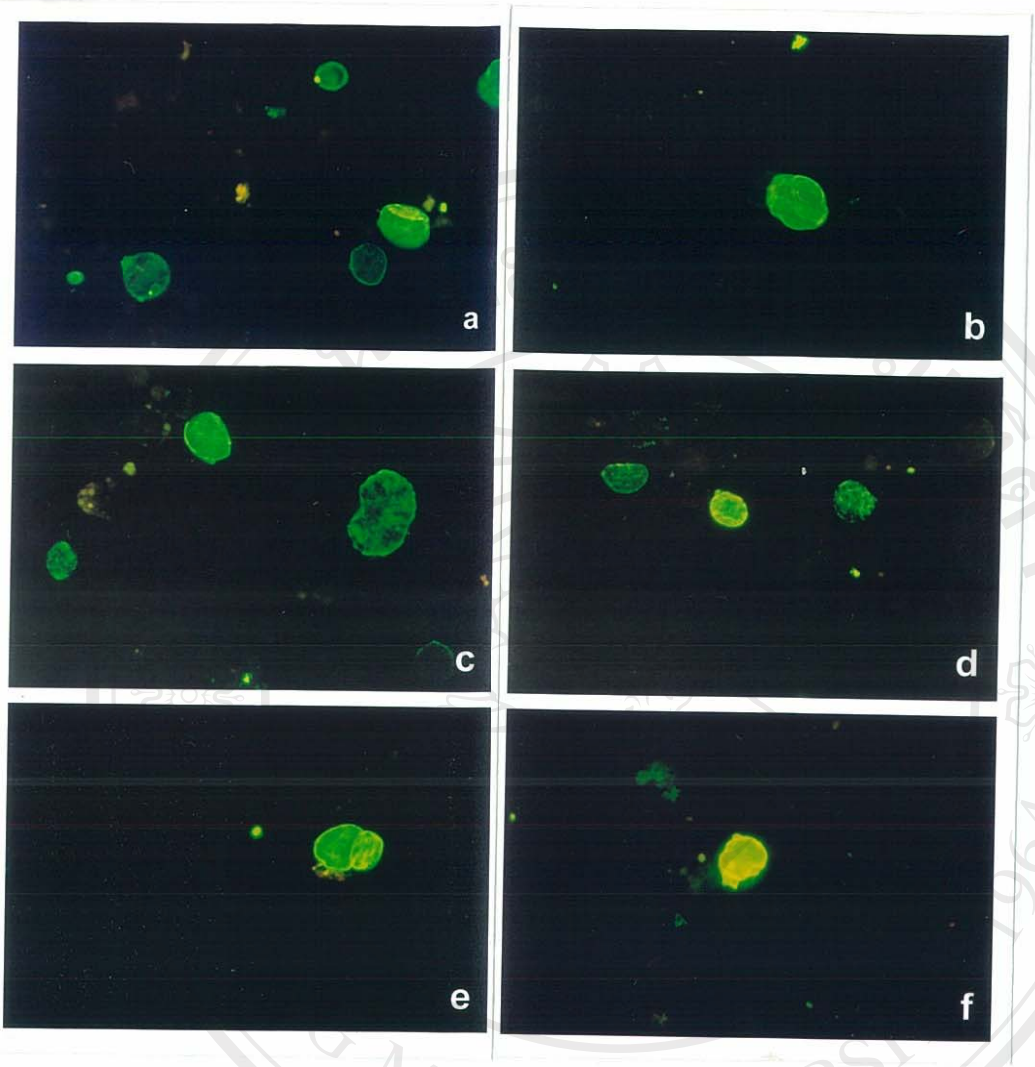

Figure 3.2 Reactivity of purified anti-CD147 mAbs with CD147 expressing COS cells. M6-DNA transfected COS cells were stained with mAbs M6-2F9 (a), M6-2B1 (b), M6-1F3 (c), M6-1D4 (d), M6-1B9 (e) and M6-1E9 (f).

Table 3.1 F/P ratio of FITC-conjugated anti-CD147 mAbs

Antibody	F/P ratio
M6-2F9	1.195
M6-2B1	0.91
M6-1F3	0.9
M6-1D4	1.09
M6-1B9	1.375
M6-1E9	1.112

F/P ratio: fluorescent to protein ratio

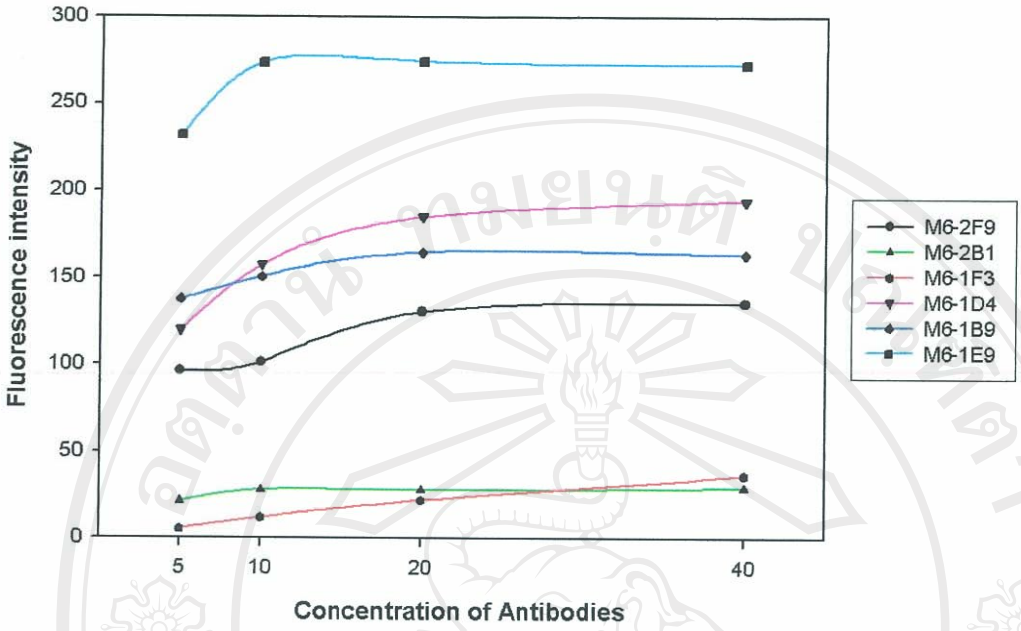

Figure 3.3 Titration for optimal concentration of FITC-conjugated anti-CD147 mAbs. The FITC conjugated anti-CD147 mAbs was used to stain U937 cells at the final concentrations of 5, 10, 20 and 40 μg/ml by direct immunofluorescent assay. The fluorescence intensity was analyzed by flow cytometer.

Table 3.2 Cross-blocking analysis of anti-CD147 mAbs

		% Inhibition					
		FITC- conjugated anti-CD147 mAb					
		M6-2F9 FITC	**M6-2B1 FITC**	**M6-1F3 FITC**	**M6-1D4 FITC**	**M6-1B9 FITC**	**M6-1E9 FITC**
Un-labeled anti-CD147 mAbs (Blocking antibody)	**M6-2F9**	80.11	98.59	87.06	6.11	-9.08	9.95
	M6-2B1	2.55	36.50	39.92	2.66	3.70	4.06
	M6-1F3	19.91	38.94	10.95	8.15	3.05	5.44
	M6-1D4	99.95	99.89	92.18	91.23	-62.49	-27.23
	M6-1B9	90.40	99.71	96.59	56.48	6.24	78.65
	M6-1E9	98.91	100	95.63	53.95	83.50	80.44

Three independent experiments were performed in each cross-blocking assay.

The % inhibition was calculated from the mean fluorescence intensity between cells stained with and without un-labeled anti-CD147 mAb.

3.3.2 Localization of epitope recognized by anti-CD147 mAbs

3.3.2.1 Production of plasmid DNA encoding domain 1 or domain 2 of CD147 molecule

To produce plasmid DNA encoding domain 1 or domain 2 of CD147 molecule, plasmid DNAs were transformed into *E. coli* and grown in LB agar containing antibiotics. Then, 2 colonies from each transformed *E. coli* were selected. The plasmid DNA was isolated and cut with *Xba* I enzyme. As shown in Figure 3.4, the isolated plasmid DNA showed the same pattern. Two DNA bands of 1.75 and 3.0 kb were observed after enzyme digestion, suggesting that the plasmid DNA contain cDNA insert. The sizes of inserted DNA were corresponding to those reported for cDNA encoding domain 1 or domain 2 of CD147 molecule (Stockinger, un-published observations). Then, the selected colonies encoding domain 1 and 2 were grown in 500 ml of LB broth and the plasmid DNA were isolated by QIAGEN plasmid midi kit. After isolation, the concentrations of plasmid DNA encoding domain 1 was 1.832 mg/ml and for domain 2 was 444 μg/ml. The OD 260/280 ratios of plasmid DNA encoding domain 1 or 2 were 1.832 and 1.85, respectively, suggesting that the purity of plasmid DNA were high. To confirm that the isolated plasmid DNA contained cDNA encoding the corresponding proteins, the plasmid DNA were digested with restriction enzyme. After agarose gel electrophoresis, 2 bands of 1.75 and 3.0 kb corresponding to the vector and inserted DNA, respectively, were obtained from each plasmid DNA (figure 3.5) indicating that the isolated plasmid DNA could be used in further experiment.

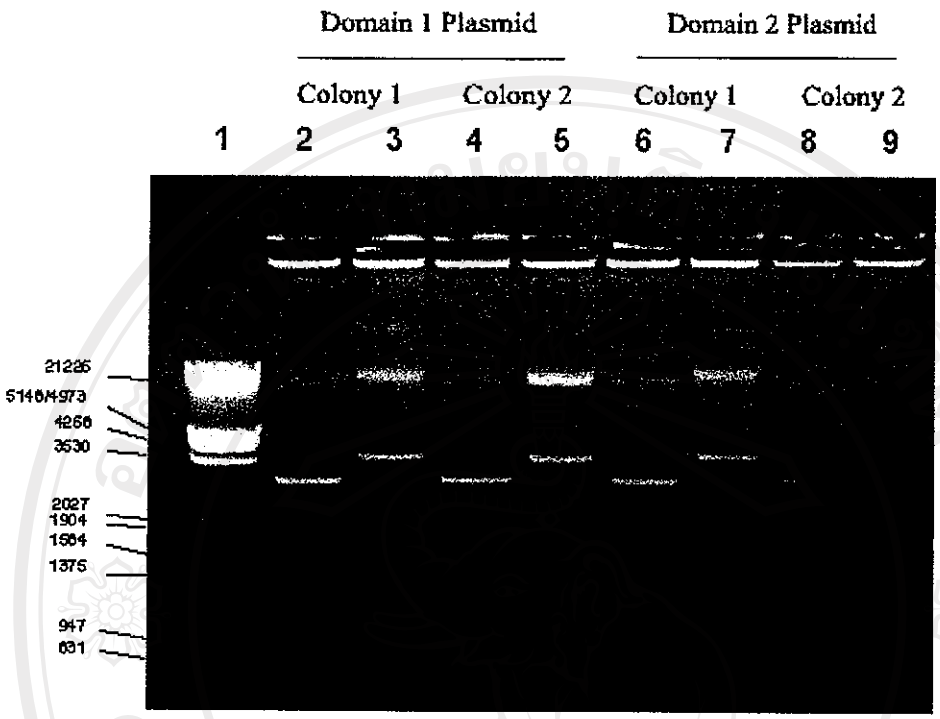

Figure 3.4 Characterization of the plasmid DNA encoding domain 1 or domain 2 of CD147 molecule isolated from various bacterial colonies. The isolated plasmid DNA were digested with *Xba* I and analyzed by 1% agarose gel electrophoresis. DNA obtained from colony 1 and 2 of domain 1 of CD147 molecule were shown in lanes 2-5 whereas DNA from colony 1 and 2 of domain 2 of CD147 molecule were shown in lanes 6-9. Lane 1 is standard DNA marker, lanes 2, 4, 6, and 8 are plasmid DNA digested *with Xba* I and lanes 3, 5, 7 and 9 are un-digested plasmid DNA. The positions of the standard DNA size in kilobases are indicated on the left.

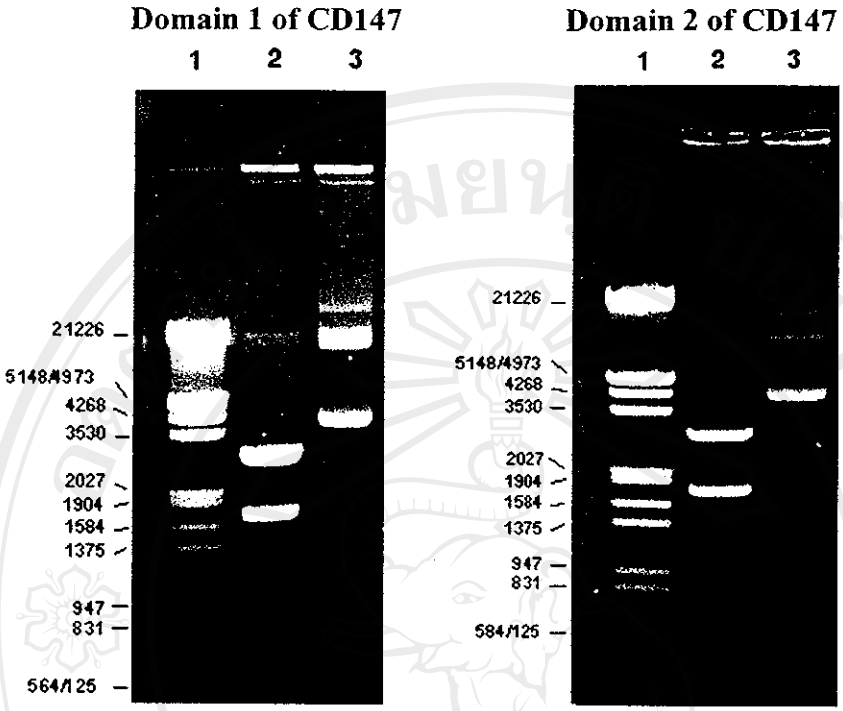

Figure 3.5 Characterization of the plasmid DNA encoding domain 1 or domain 2 of CD147 molecule isolated from the selected bacterial colonies. The isolated plasmid DNA were digested with *Xba* I and analyzed by 1% agarose gel electrophoresis. Lane 1 is a standard DNA marker, Lane 2 represents plasmid DNA digested *with Xba* I enzyme and Lane 3 is un-digested plasmid DNA. The positions of the standard DNA sizes in kilobases are indicated on the left.

3.3.2.2 Localization of epitope recognized by anti-CD147 mAbs

To determine the epitopes which reacted with anti-CD147 mAbs locate whether on domain 1 or domain 2 of the CD147 molecule, the CD147 domain 1 or domain 2 expressing COS cells were used in this study. COS cells were transfected with cDNA encoding whole CD147 molecule, domain 1 or domain 2 of CD147, and CD8 molecule. The transfected COS cells were stained with purified CD147 or CD8 mAbs by intracellular staining. As shown in Figure 3.6-3.8, mAb M6-2F9, M6-2B1, M6-1D4, M6-1B9 and M6-1E9 reacted to intact CD147 expressing COS cells and domain 1 of CD147 expressing COS cells but did not react to domain 2 or CD8 expressing COS cells. The mAb M6-1F3 reacted only to intact CD147 expressing COS cells but did not react to domain 1, domain 2 of CD147 or CD8 expressing COS cells. As control, mAb MEM-M6/6, a anti-CD147 mAb which reacted to domain 2 of CD147 (Koch *et al.*, 1999), reacted to intact CD147 and domain 2 expressing COS cells but did not react to domain 1 and CD8 expressing COS cells. MT8 (CD8) mAb was also used as a control, it reacted only to CD8 expressing COS cells. These results indicated that mAb M6-2F9, M6-2B1, M6-1D4, M6-1B9 and M6-1E9 reacted to the epitope located on the domain 1 of CD147 molecule. MAb M6-1F3 reacted to epitope neither on domain 1 nor domain 2 of CD147 molecule. It may react to the epitope which is located on the position out side the domains.

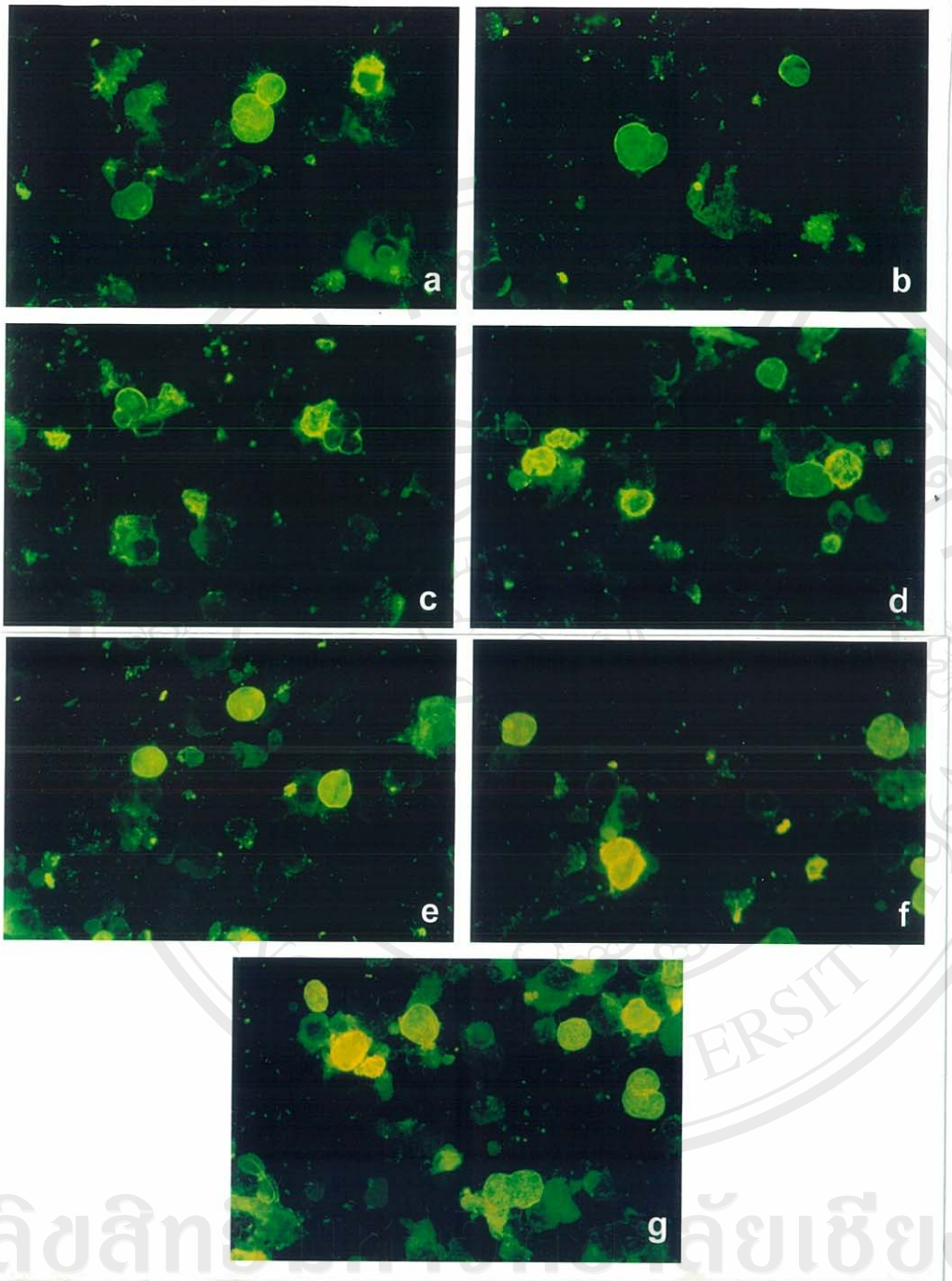

Figure 3.6 Reactivity of mAbs M6-2F9 (a), M6-2B1 (b), M6-1F3 (c), M6-1D4 (d), M6-1B9 (e), M6-1E9(f), and MEM-M6/6 (g) with intact CD147 expressing COS cells.

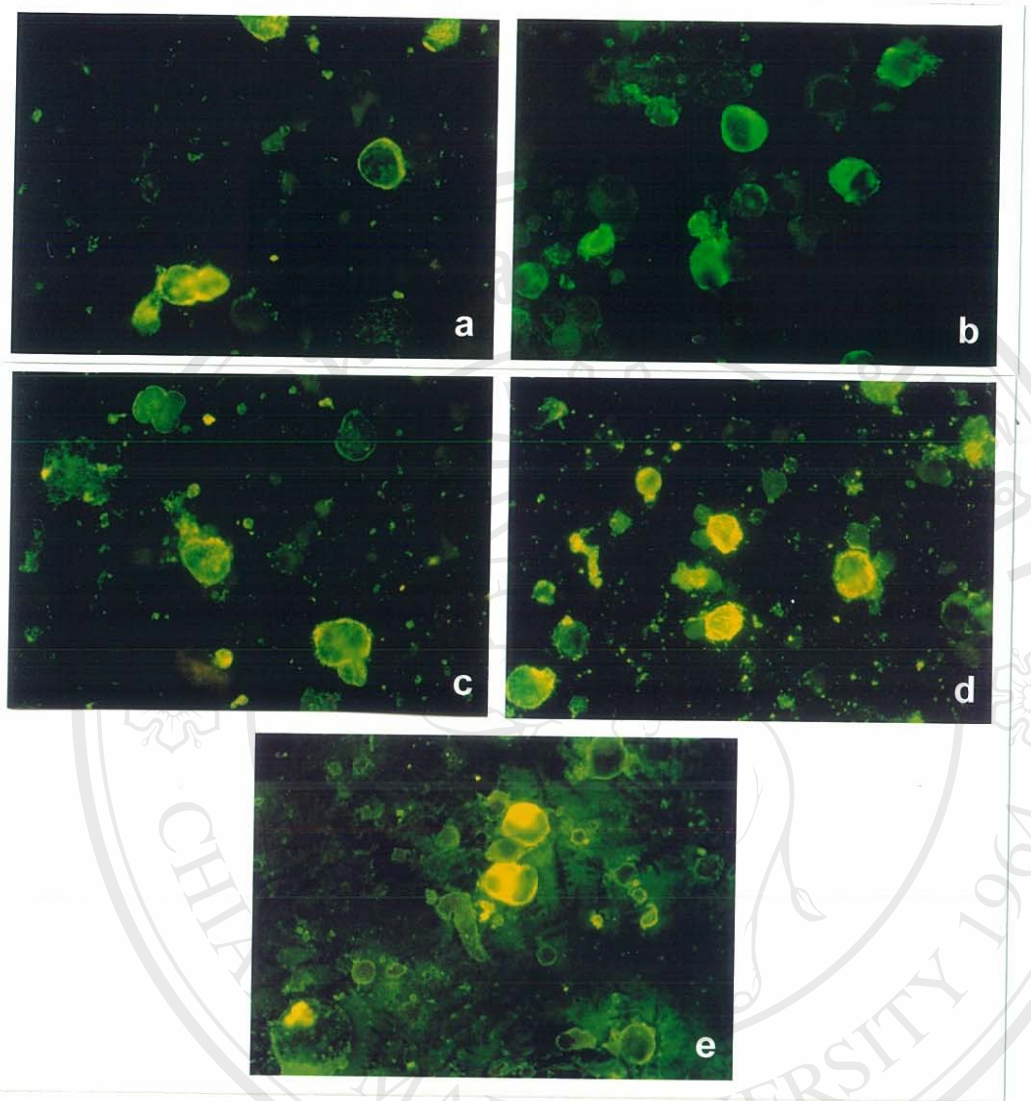

Figure 3.7 Reactivity of mAbs M6-2F9 (a), M6-2B1 (b), M6-1D4 (c), M6-1B9 (d) and M6-1E9 (e) with CD147 domain 1 expressing COS cells.

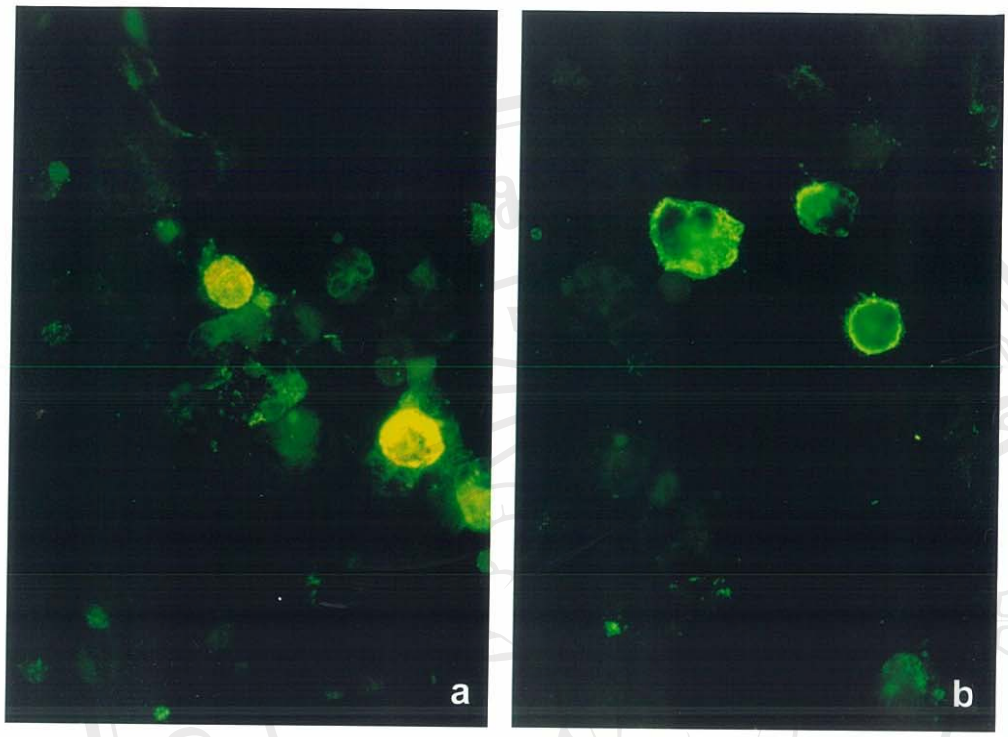

Figure 3.8 Reactivity of mAb MEM-M6/6 with CD147 domain 2 expressing COS cells (a) and mAb MT8 with CD8 expressing COS cells (b).

3.4 Characterization of anti-CD147 mAb by Western blotting and immunoprecipitation

3.4.1 Western blotting

The U937 cell lysate was separated by SDS-PAGE under non-reducing and reducing conditions. After blotting on nitrocellulose membrane, the membrane was probed with anti-CD147 mAbs and isotype-matched control. As shown in Figure 3.9, under non-reducing condition, mAbs M6-2F9, M6-1D4, M6-1B9 and M6-1E9 reacted to a protein band at the molecular weight of approximately 50 kDa. M6-1F3 did not react with any protein band. Whereas, M6-2B1 showed several non-specific bands. Under reducing condition, M6-1D4, M6-1B9 and M6-1E9, but not M6-2F9, M6-2B1 and M6-1F3, reacted to a protein band at the molecular weight approximately of 53.5 kDa (Figure 3.10). With isotype matched control antibodies, under non-reducing conditions, mAb MT54 (CD54) and MT99/3 (CD99) reacted to protein bands at the molecular weight of 82 and 35 kDa, respectively (Figure 3.9). Under reducing condition, MT99/3 mAb reacted to protein band at the molecular weight approximately of 34.5 kDa (Figure 3.10). In contrast, purified MT8 (CD8) and BF1-4G2 did not react to any proteins under non-reducing (Figure 3.9) and reducing conditions (Figure 3.10). The results indicated that mAbs M6-2F9, M6-1D4, M6-1B9 and M6-1E9 reacted to linear epitope of the CD147 molecule. M6-2F9 reacted to linear epitope that was altered by treatment with 2ME. In contrast, mAbs M6-2B1 and M6-1F3 may react to conformational epitope on CD147 molecule.

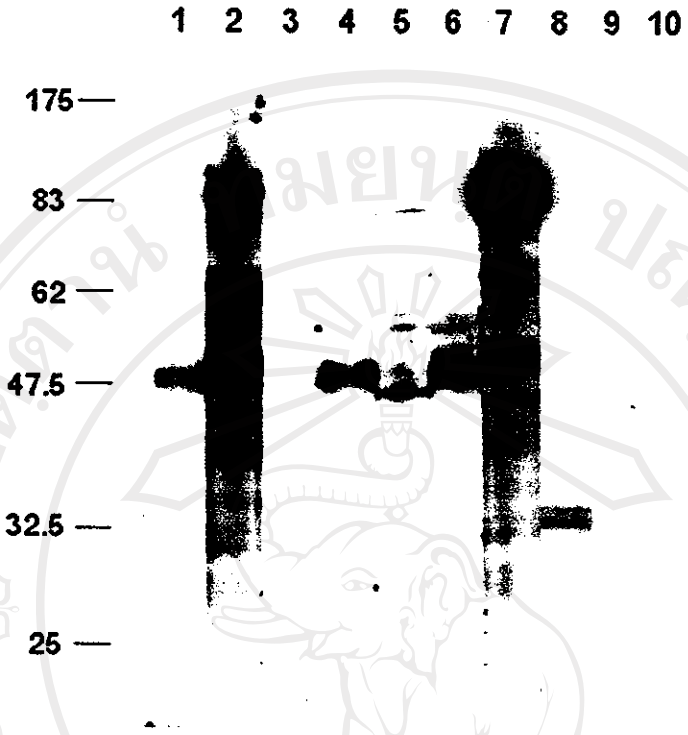

Figure 3.9 Analysis of anti-CD147 mAbs by Western blotting under non-reducing condition. Lysate of U937 cells were separated by SDS-PAGE under non-reducing condition and transferred to a nitrocellulose membrane. The membrane was incubated with mAbs M6-2F9 (lane 1), M6-2B1 (lane 2), M6-1F3 (lane 3), M6-1D4 (lane 4), M6-1B9 (lane 5), M6-1E9 (lane 6), MT54 (lane 7), MT99/3 (lane 8), MT8 (lane 9) and BF1-4G2 (lane 10). Standard molecular weight markers (kDa) are indicated on the left.

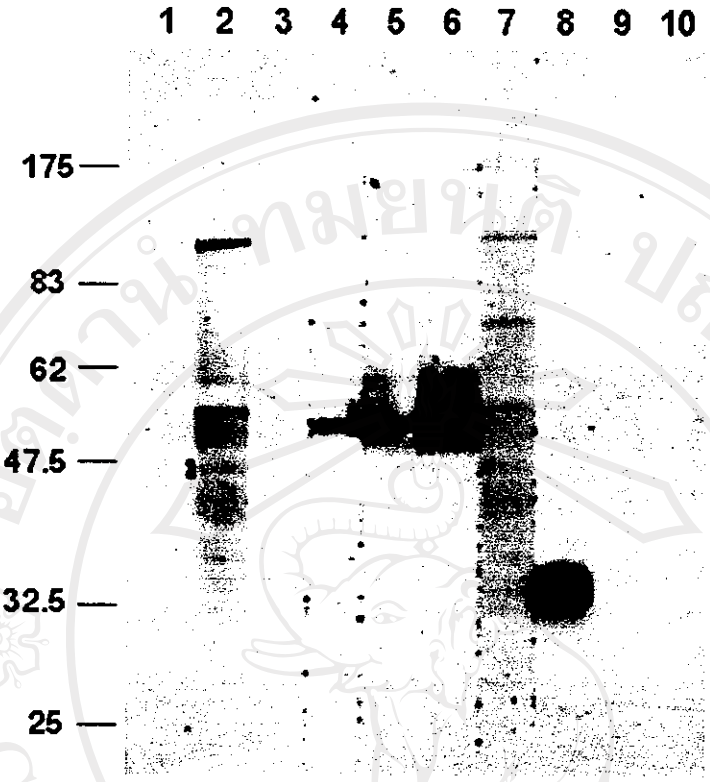

Figure 3.10 Analysis of anti-CD147 mAbs by Western blotting under reducing condition. Lysate of U937 cells were separated by SDS-PAGE under reducing condition and transferred to a nitrocellulose membrane. The membrane was incubated with mAbs M6-2F9 (lane 1), M6-2B1 (lane 2), M6-1F3 (lane 3), M6-1D4 (lane 4), M6-1B9 (lane 5), M6-1E9 (lane 6), MT54 (lane 7), MT99/3 (lane 8), MT8 (lane 9) and BF1-4G2 (lane 10). Standard molecular weight markers (kDa) are indicated on the left.

3.4.2 Immunoprecipitation

As hydrophobic stretch of the transmembrane region of CD147 molecule was interrupted by a charge residue, glutamic acid, as well as containing a leucine-zipper motif (Kasinrerk *et al.*, 1992). It was suggested that this molecule might interact with other proteins within the plasma membrane. To characterize the molecule reacted with anti-CD147 mAb, immunoprecipitation technique was performed. The cell surface proteins of U937 cells were biotinylated with Sulfo-NHS-LC-biotin. Then, cell lysate was precipitated with anti-CD147 mAbs and isotype-matched control mAbs. In these experiments, mAbs M6-1D4, M6-1B9 and M6-1E9 precipitated a broad band of biotinylated protein at the molecular weight of 50-66 kDa under reducing condition (Figure 3.11) and 45.5-61.7 kDa under non-reducing condition (Figure 3.12). MAbs M6-2F9, M6-2B1 and M6-1F3 did not precipitate any biotinylated protein under either non-reducing or reducing conditions. The results indicated that, in our immunoprecipitation conditions, no co-precipitation of protein was observed.

According to the immunoprecipitation, Western blotting and epitope mapping studies, the epitopes on CD147 molecules that were recognized by anti-CD147 mAbs were summarized in Table 3.3.

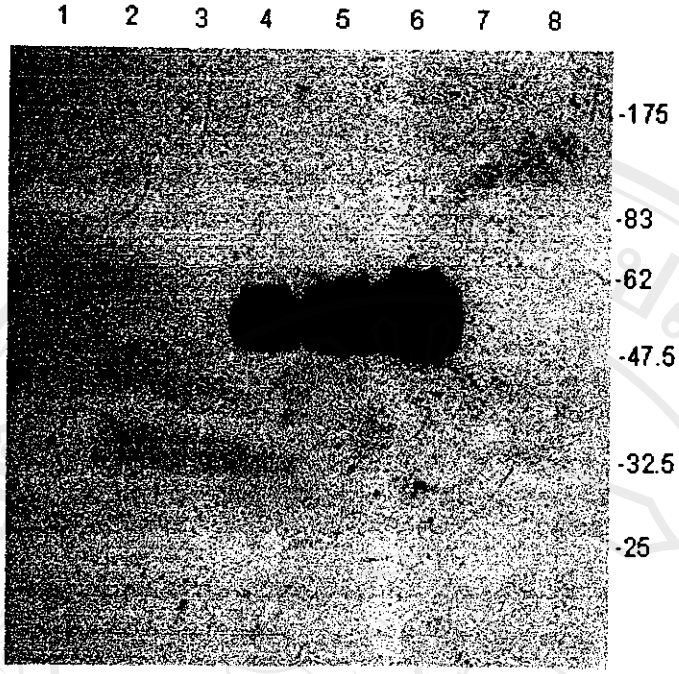

Figure 3.11 Immunoprecipitation of cell surface molecule recognized by anti-CD147 mAbs under reducing condition. SDS-PAGE analysis of immunoprecipitates obtained from biotin-surface-labeled U937 cells using mAbs M6-2F9 (lane 1), M6-2B1 (lane 2), M6-1F3 (lane 3), M6-1D4 (lane 4), M6-1B9 (lane 5), M6-1E9 (lane 6), MT8 (lane 7) and BF1-4G4 (lane 8). Standards molecular weight markers (kDa) are indicated on the right.

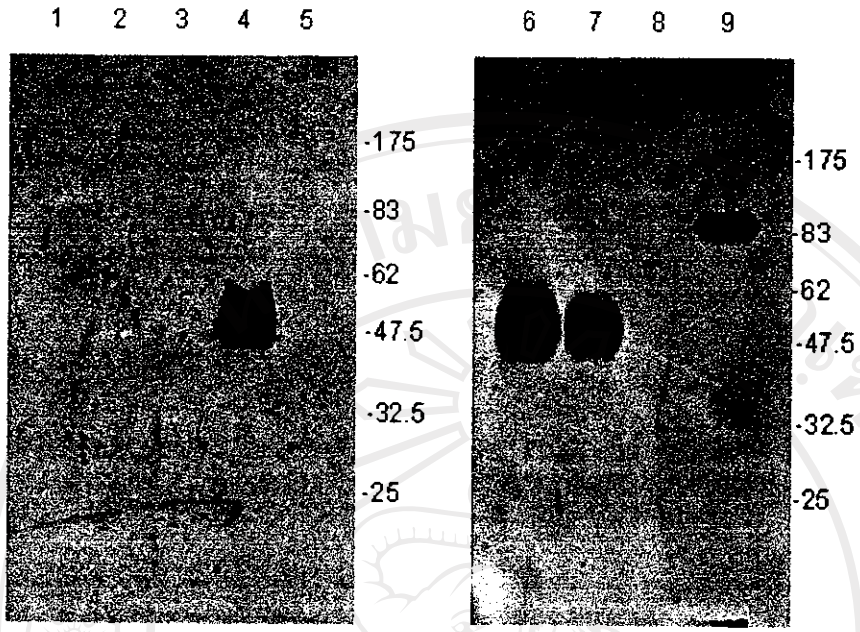

Figure 3.12 Immunoprecipitation of cell surface molecule recognized by anti-CD147 mAbs under non-reducing condition. SDS-PAGE analysis of immunoprecipitates obtained from biotin-surface-labeled U937 cells using mAbs M6-2F9 (lane 1), M6-2B1 (lane 2), M6-1F3 (lane 3), M6-1D4 (lane 4), M6-1B9 (lane 6) and M6-1E9 (lane 7) and mAb MT8 (lane 5) and BF1-4G4 (lane 8), an isotype matched control and mAb MT54 (lane 9), a positive control. Standards molecular weight markers (kDa) are indicated on the right.

Table 3.3 Summary of epitopes recognized by anti-CD147 mAbs

Anti-CD147 mAb	Epitope recognition
M6-2F9	The mAb reacts to a linear epitope located on domain 1 of the CD147 molecule, which altered after treatment with 2ME.
M6-2B1	The mAb reacts to conformational epitope located on domain 1 of the CD147 molecule.
M6-1F3	The mAb reacts to conformational epitope. The location of the recognized epitpoe is still un-clear.
M6-1D4	The mAb reacts to linear epitope located on domain 1 of the CD147 molecule.
M6-1E9 and M6-1B9	Both mAbs recognize the same or overlapping epitopes. This epitope(s) is linear epitope and located on domain 1 of the CD147 molecule.

3.5 Functional study of CD147 molecule involving the induction of apoptosis

To study the function of CD147 molecule that involves in the induction of apoptosis, U937, KG1a and Sup-T1 cell lines were activated with mAbs against different epitope of CD147 molecule. The apoptotic cells were detected by Annexin V-FITC Apoptosis detection kit. As shown in Figure 3.13-3.15, all anti-CD147 mAbs did not induce apoptosis of all cell line tested. MAb CA-1H4 was used as positive control as this mAb induced apoptosis in Sup-T1 cell line (Figure 3.14). The results indicated that CD147 molecule may not involved in the induction of apoptosis.

3.6 Functional study of CD147 molecule that involves anti-CD3 mAb induced cell proliferation

3.6.1 Titration of anti-CD3 mAb for induction of T cell proliferation

The experiment was started with the determination of the sub-optimal concentration of immobilized anti-CD3 mAb for induction of T cell proliferation. Heparinized whole blood was collected from 3 healthy donors and the PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. Then, the PBMCs were activated with immobilized mAb OKT3 (anti-CD3 mAb) at concentrations of 3.9 ng/ml to 4 μg/ml. Cell proliferation was determined by measuring of ^{3}H-thymidine incorporation. As shown in Figure 3.16, the immobilized OKT3 mAb induced T cells proliferation in a dose-dependent fashion. The concentration at 20 ng/ml was shown to be the sub-optimal dose and was selected in the further experiments.

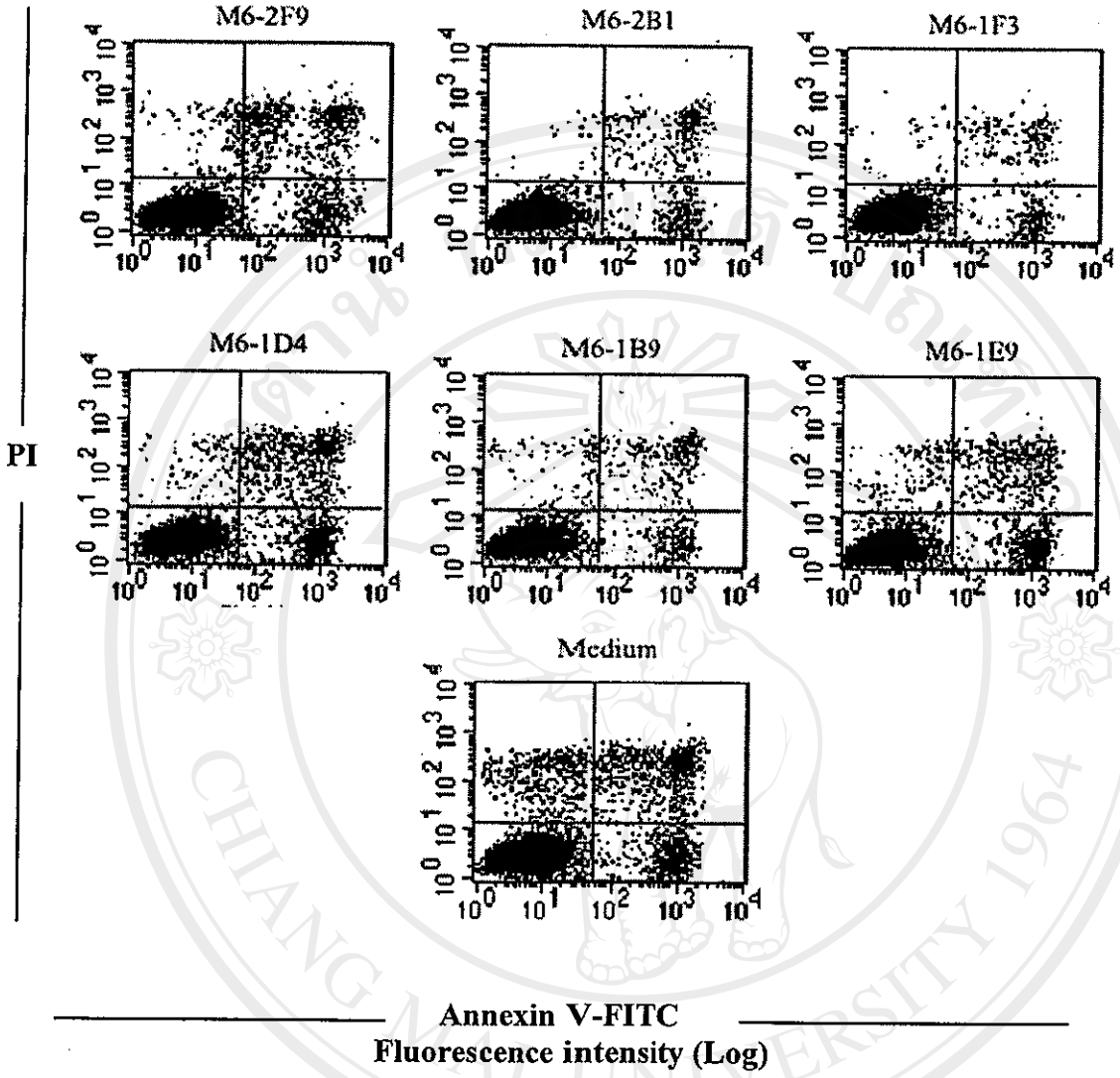

Figure 3.13 The immunofluorescence profiles of anti-CD147 mAbs for apoptotic induction of U937 cell line. The U937 cells were activated with 20 μg/ml of the indicated mAbs or medium alone for 4 hours. The apoptotic cells were detected by Annexin V-FITC apoptosis detection kit.

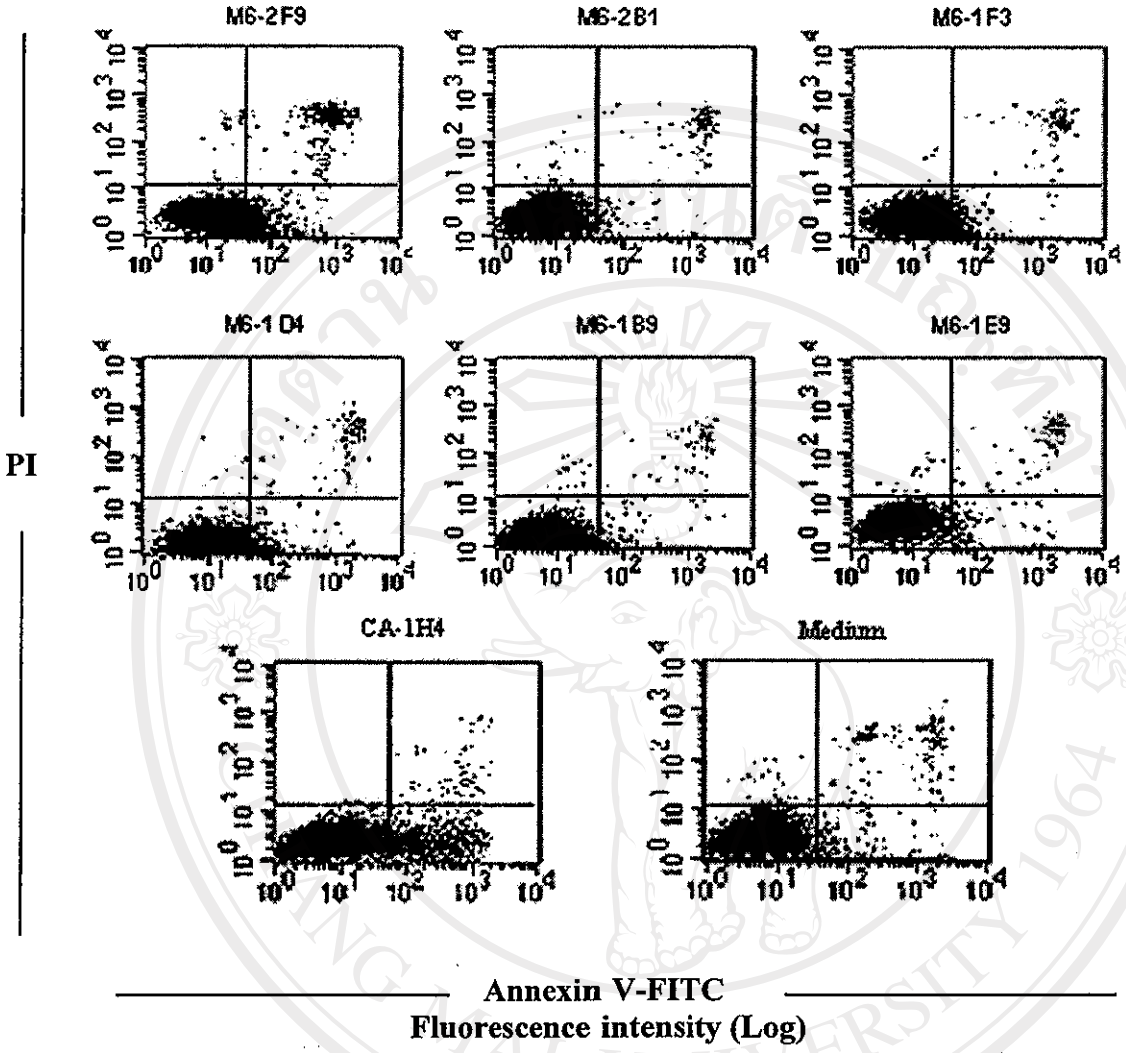

Figure 3.14 The immunofluorescence profiles of anti-CD147 mAbs for apoptotic induction of Sup-T1 cell line. The Sup-T1 cells were activated with 20 μg/ml of indicated mAb or medium alone for 4 hours and the apoptotic cells were detected by Annexin V-FITC apoptosis detection kit.

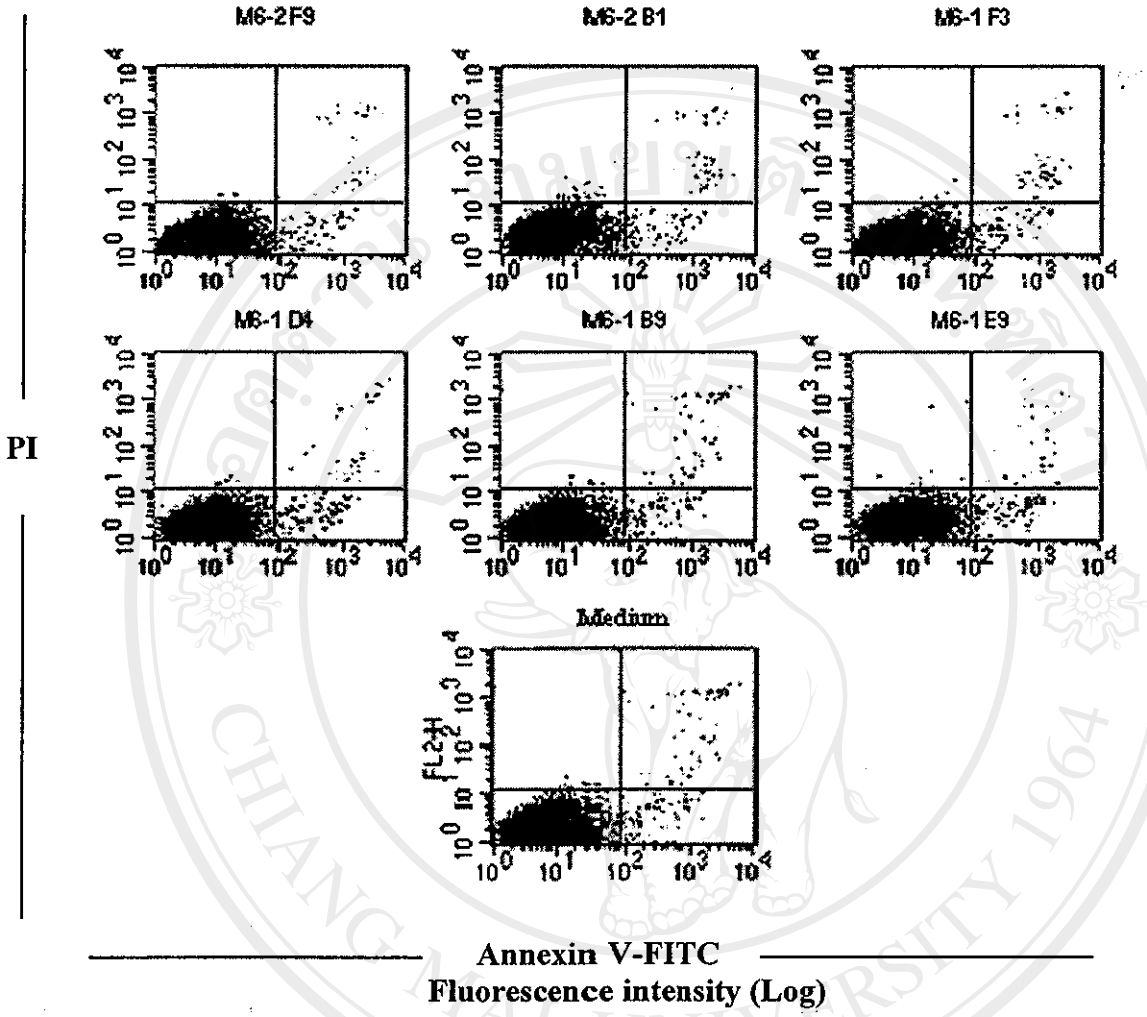

Figure 3.15 The immunofluorescence profiles of anti-CD147 mAbs for apoptotic induction of KG1a cell line. The KG1a cells were activated with 20 μg/ml of indicated mAbs or medium alone for 4 hours and the apoptotic cells were detected by Annexin V-FITC apoptosis detection kit.

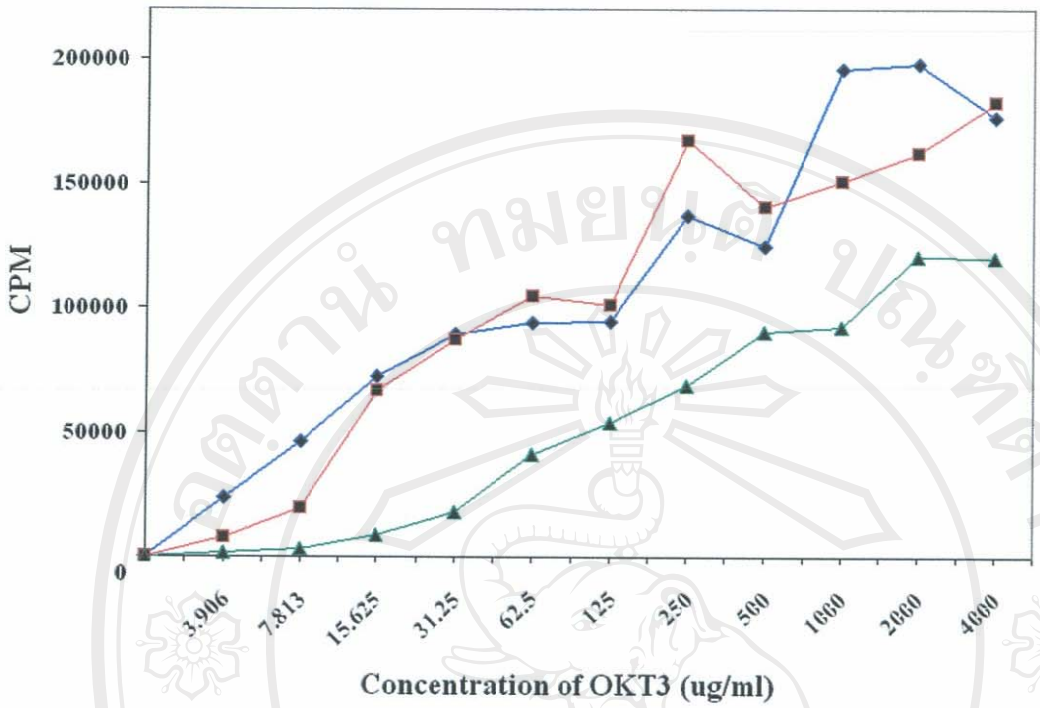

Figure 3.16 Titration of immobilized mAb OKT3 concentration for T cells proliferation assay. PBMCs were stimulated with 3.9 ng/ml to 4 µg/ml of immobilized mAb OKT3. T cells proliferation was assessed at 18 hours after activation by measurement of ^{3}H-thymidine incorporation. The data were obtained from 3 separate experiments.

3.6.2 Functional study of CD147 molecule involving the regulation of anti-CD3 mAb induced cell proliferation

PBMCs isolated from 3 healthy donors were activated with 20 ng/ml immobilized OKT3 mAb and cultured in the presence or absence of various concentrations (0.05, 0.1 and 1 μg/ml) of anti-CD147 mAbs. Cell proliferation was determined by ^{3}H thymidine incorporation. As shown in Figure 3.17, the mAbs M6-1B9 and M6-1E9, which recognizing the same epitope, inhibited OKT3-induced T cell proliferation. The percentages of inhibition were 88%, 77% and 53% for M6-1B9 and 92%, 93% and 90% for M6-1E9 at 1, 0.1 and 0.05 μg/ml, respectively. In contrast, mAbs M6-2F9, M6-2B1, M6-1F3 and M6-1D4 and isotype-matched control mAbs (FE-1H10 and UPC-10) did not deliver any inhibitory effected on OKT3-mediated T cell proliferation. The results indicated that CD147 molecule involved in the regulation of CD3-mediated T cell activation and certain epitope on the molecule is responsibility for the regulation.

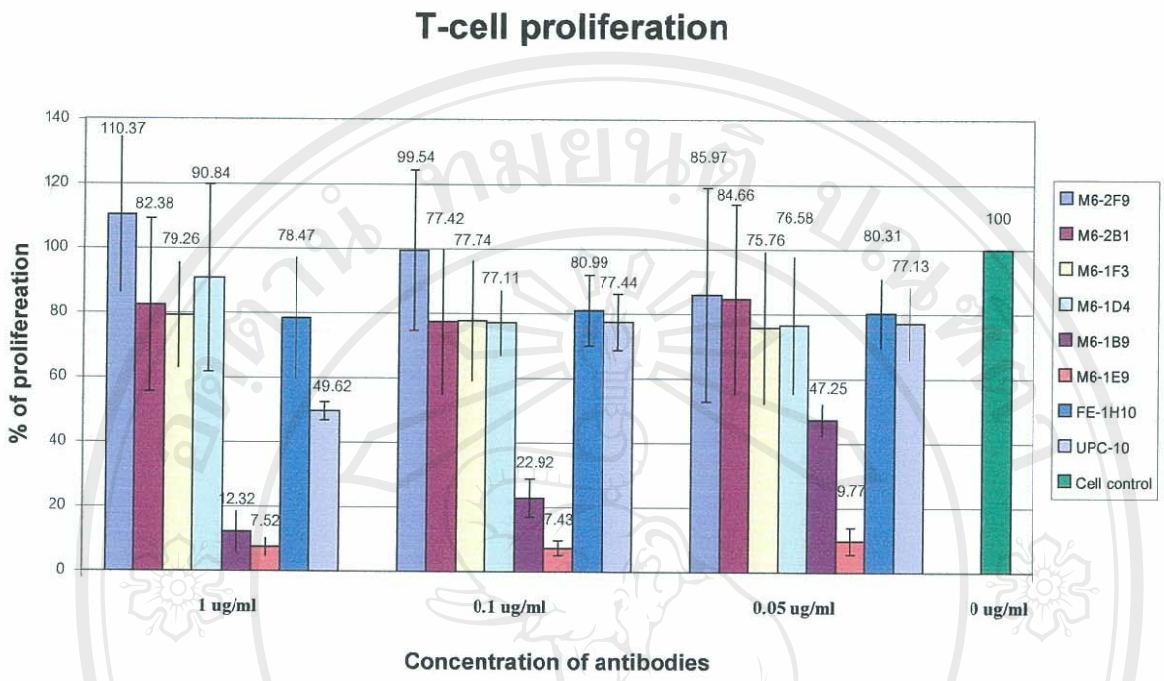

Figure 3.17 Involvement of CD147 molecule in the regulation of CD3-mediated T cells proliferation. PBMCs were stimulated with 20 ng/ml of immobilized mAb OKT3 and cultured with 0, 0.05, 0.1 and 1 μg/ml of various anti-CD147 mAbs or isotype matched control mAbs. T cells proliferation was assessed at 18 hours of culture by measurement of ^{3}H-thymidine incorporation. Three consecutive experiments were performed. Results are shown in mean ± SD of % proliferation.

3.7 Functional study of CD147 molecule involving PHA-induced cell proliferation

Sub-optimal concentration of PHA for the induction of T cell activation was determined. PBMCs were isolated from 3 healthy donors and activated with PHA at final concentrations of 0, 0.625, 1.25, 2.5 and 5 μg/ml. Cell proliferation was determined by measuring of ^{3}H-thymidine incorporation. As shown in Figure 3.18, the degree of T cell proliferation was in a dose-dependent fashion. The concentration of PHA at 0.625 μg/ml was shown to be the sub-optimal concentration and was selected for further studies.

To verify whether CD147 molecule involves in the regulation of PHA-induced cell proliferation. PBMCs isolated from 3 donors were cultured with sub-optimal dose of PHA in the presence of anti-CD147 mAbs against different epitopes. As shown in Figure 3.19, all anti-CD147 mAbs tested did not show any inhibitory effect on PHA-induced cells proliferation. In the presence of anti-CD147 mAbs, the degree of cell proliferation was at the similar level as was observed when using isotype-matched control mAbs (FE-1H10 and UPC-10). The results indicated that CD147 molecule had no relevant role in the regulation of PHA-mediated cell activation.

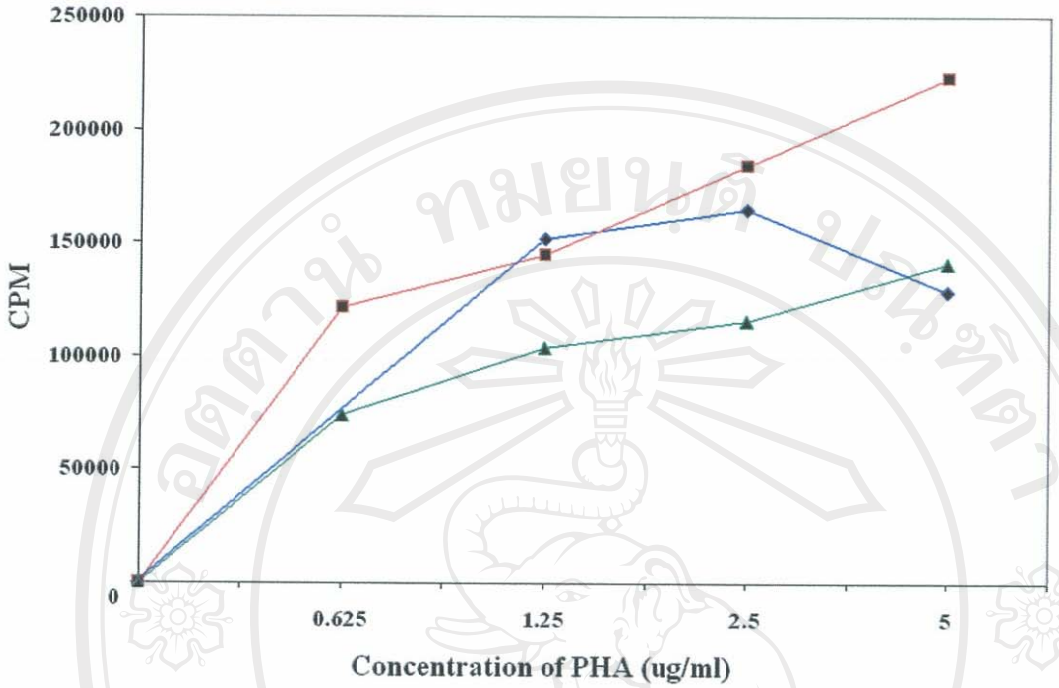

Figure 3.18 Titration of PHA for T cells proliferation assay. PBMCs were stimulated with PHA at 0.625 to 5 μg/ml. Cell proliferation was assessed at 18 hours of culture by measurement of ^{3}H-thymidine incorporation. The data were obtained from 3 separated experiments.

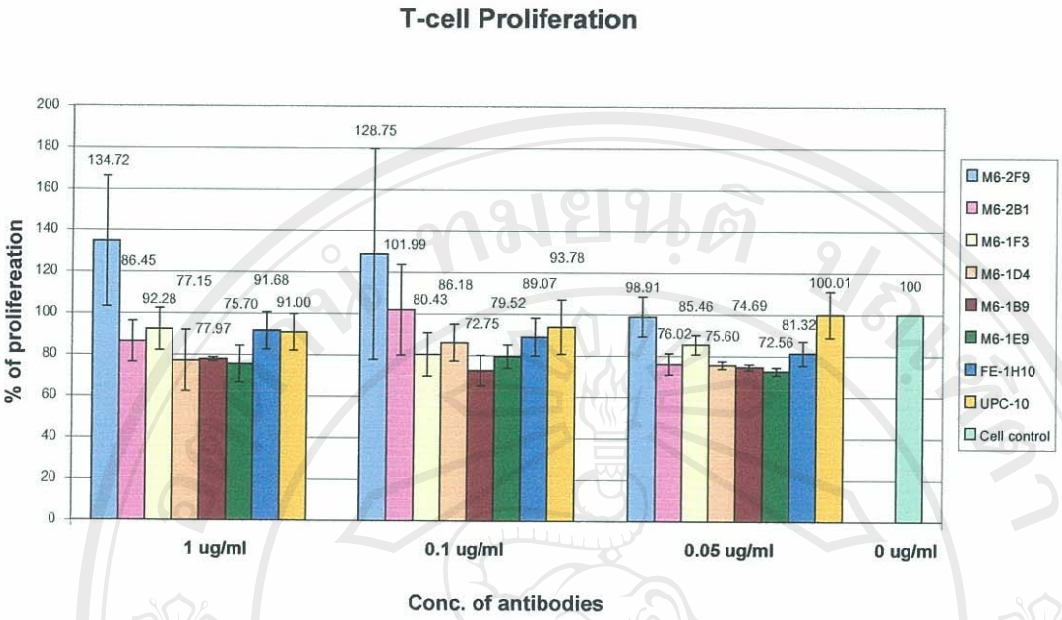

Figure 3.19 Involvement of CD147 molecule in the regulation of PHA-mediated cells proliferation. PBMCs were stimulated with 0.625 μg/ml of PHA and cultured with 0, 0.05, 0.1 and 1 μg/ml of various anti-CD147 mAbs or isotype matched control mAb. Cells proliferation was assessed at 18 hours of culture by measurement of ^{3}H-thymidine incorporation. Three consecutive experiments were performed. Results are shown in mean ± SD of % proliferation.