CHAPTER III

RESULTS

3.1 Specificity of anti-CD4 monoclonal antibodies used in this study

Four clones of anti-CD4 mAbs, namely MT4, MT4/2, MT4/3, and MT4/4, were recently produced in our laboratory (Pata *et al*., 2009). In order to employ the produced anti-CD4 mAbs for functional characterization of CD4 molecule, hybridomas producing the four clones of mAbs were propagated to produce mAbs in the form of culture supernatants. The obtained culture supernatants were then confirmed for their specificity. In this study, cDNA encoding CD4 and CD8 molecules (Kasinrerk *et al*., 1999) were transfected into COS cells. The transfected COS cells were then used for proving of the specificity of the mAbs. CD4 and CD8 transfected COS cells were stained with the produced culture supernatants by indirect immunofluorescent technique and analyzed by a fluorescence microscope. Three of tested clones, including MT4, MT4/2 and MT4/3, reacted to the CD4 transfected COS cells but did not react to the CD8 transfected COS cells (Figure 3.1). While anti-CD4 mAb clone MT4/4 neither reacted to CD4 nor CD8 transfected COS cells (Figure 3.1). This is not surprise since the mAb MT4/4 was obtained by using recombinant CD4 protein produced from *E. coli* as immunogen and was reported not react to the native CD4 molecule (Pata *et al*., 2009). In this experiment, an anti-CD8 mAb clone MT8 was used as a control for the CD8 expression on CD8 transfected COS cells. The mAb MT8 reacted to the CD8 transfected COS cells, but not CD4 transfected COS cells, indicating the correct expression of CD8 on CD8 transfected COS cells.

Taken together, our results indicated that anti-CD4 mAb clones MT4, MT4/2 and MT4/3 specifically reacted to CD4 molecule and could be used in further experiments.

3.2 Large scale production and purification of anti-CD4 mAbs

For functional study of CD4 molecule, large amount of purified anti-CD4 mAbs was required. To produce large amount of anti-CD4 mAbs, hybridomas (clones MT4, MT4/2, and MT4/3) were inoculated into peritoneal cavity of BALB/C mice to generate ascitic fluids. Ascitic fluids containing anti-CD4 mAbs were collected and the antibodies were purified by affinity chromatography. The obtained purified mAbs were then checked for their purity and reactivity.

For purity verification, the purified mAbs were subjected for SDS-PAGE under reducing and non-reducing conditions. Under reducing condition of SDS-PAGE, anti-CD4 mAb clones MT4, MT4/2 (IgM isotype), and MT4/3 (IgG isotype) were separated into two protein bands corresponding to immunoglobulin heavy chain and light chain and no other protein bands were observed (Figure 3.2 lane 1-3). Under non-reducing condition, only one major band corresponding to immunoglobulin intact molecule was observed in the gel (Figure 3.2, lane 4-6). These results indicated that the obtained purified anti-CD4 mAbs were pure and could be used in further experiments.

The reactivity of the purified anti-CD4 mAbs was then determined. By using the COS cell transfection technique as mentioned above, the purified anti-CD4 mAbs MT4, MT4/2, and MT4/3 specifically reacted to the CD4 transfected COS cells, but did not react to the CD8 transfected COS cells (Figure 3.3). These results indicated

that all purified anti-CD4 mAbs (MT4, MT4/2, and MT4/3) specifically react to CD4 molecule.

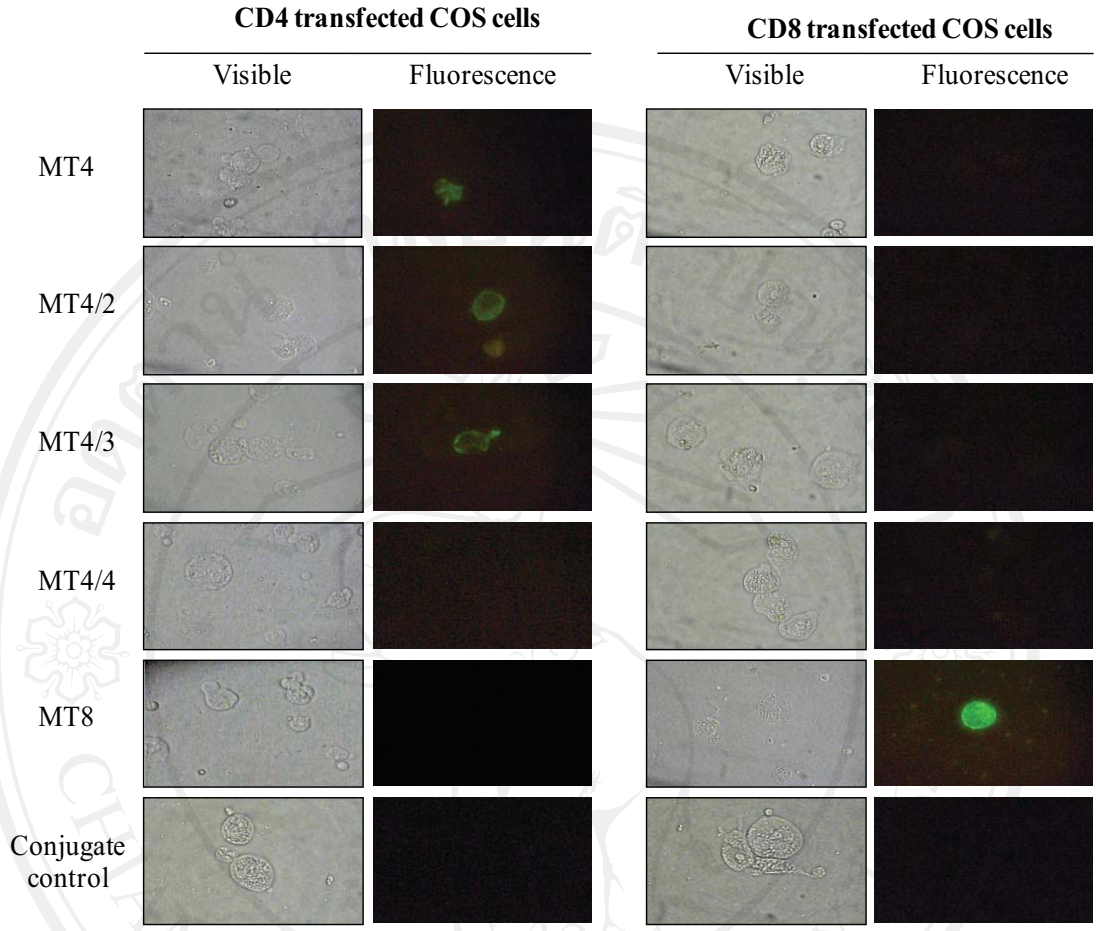

Figure 3.1 Immunofluorescence analysis of specific reactivity of anti-CD4 mAbs. COS cells were transfected with plasmid DNA encoding CD4 or CD8 protein. CD4 and CD8 transfected COS cells were stained with culture supernatants obtained from four hybridoma clones (MT4, MT4/2, MT4/3, and MT4/4) by indirect immunofluorescent staining. The stained cells were analyzed by a fluorescence microscope under visible light and fluorescent light. MT8 is the mAb generated in our laboratory which is specific to CD8 molecule.

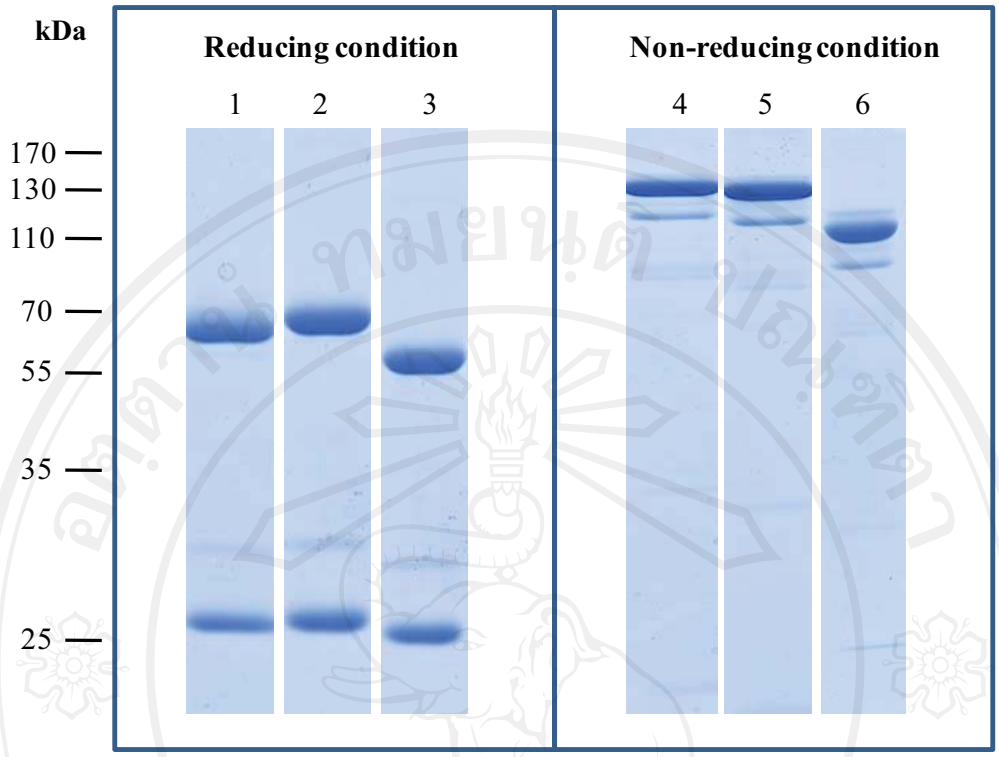

Figure 3.2 SDS-PAGE analysis of the purified anti-CD4 mAbs. The purified anti-CD4 mAb clones MT4 (lane 1, 4), MT4/2 (lane 2, 5), and MT4/3 (lane 3, 6), after separation through affinity chromatography, were analysed using a 10% SDS-PAGE under reducing and non-reducing conditions. The gel was stained with Coomassie Brilliant Blue after electrophoresis. The molecular weight markers are indicated on the left.

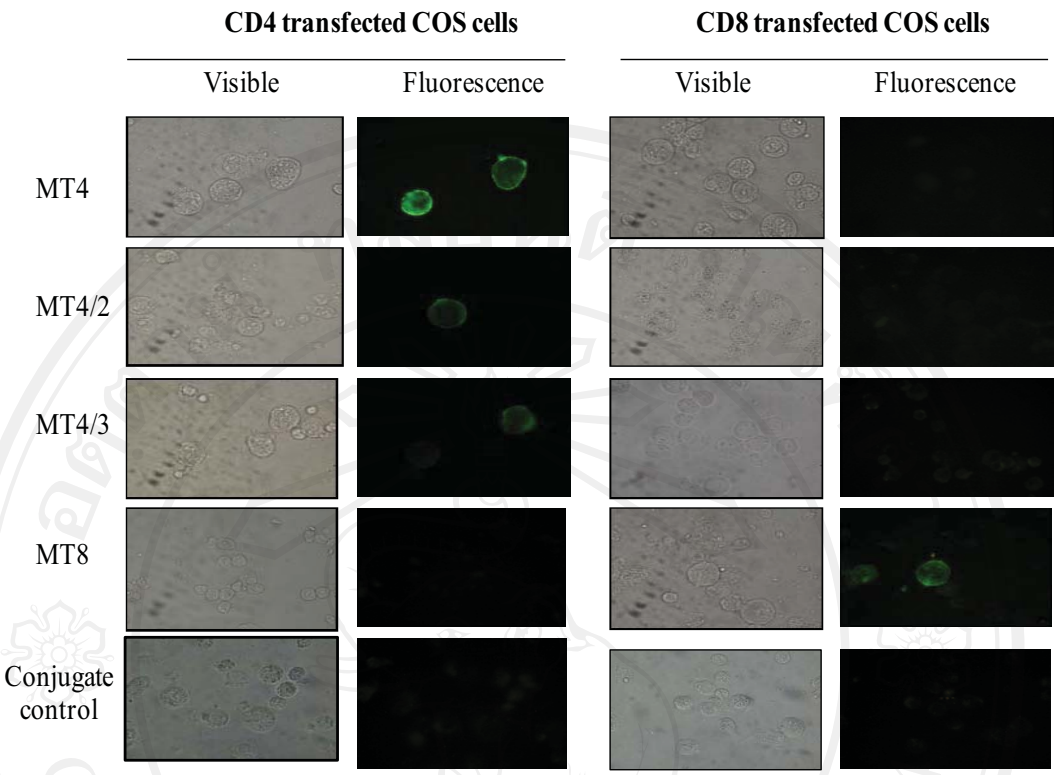

Figure 3.3 Immunofluorescence analysis of specific reactivity of purified anti-CD4 mAbs. COS cells were transfected with plasmid DNA encoding CD4 or CD8 protein. CD4 transfected and CD8 transfected COS cells were stained with purified anti-CD4 mAb clones MT4, MT4/2, and MT4/3 by indirect immunofluorescent staining. The stained cells were analyzed by a fluorescence microscope under visible light and fluorescent light. MT8 is the mAb generated in our laboratory which is specific to CD8 molecule.

3.3 Immunoprecipitation of CD4 molecule using purified anti-CD4 mAbs

To determine specific reactivity of purified anti-CD4 mAb clones MT4, MT4/2, and MT4/3, immunoprecipitation was employed. The surface proteins of a CD4 positive cell line, Sup T1, were biotinylated with Sulfo-NHS-LC-biotin. The biotinylated cell lysates were immunoprecipitated using purified anti-CD4 mAbs. A protein of the molecular weight of 55 kDa corresponding to CD4 protein was precipitated by the purified anti-CD4 clones MT4, MT4/2, and MT4/3 (Figure 3.4, lane 4-6). In contrast, IgM and IgG isotype matched control mAbs did not precipitate any protein (lane 2-3). In addition, positive control anti-CD99 mAb clone MT99/3 precipitated CD99 protein of the molecular weight of 25-30 kDa (lane 1).

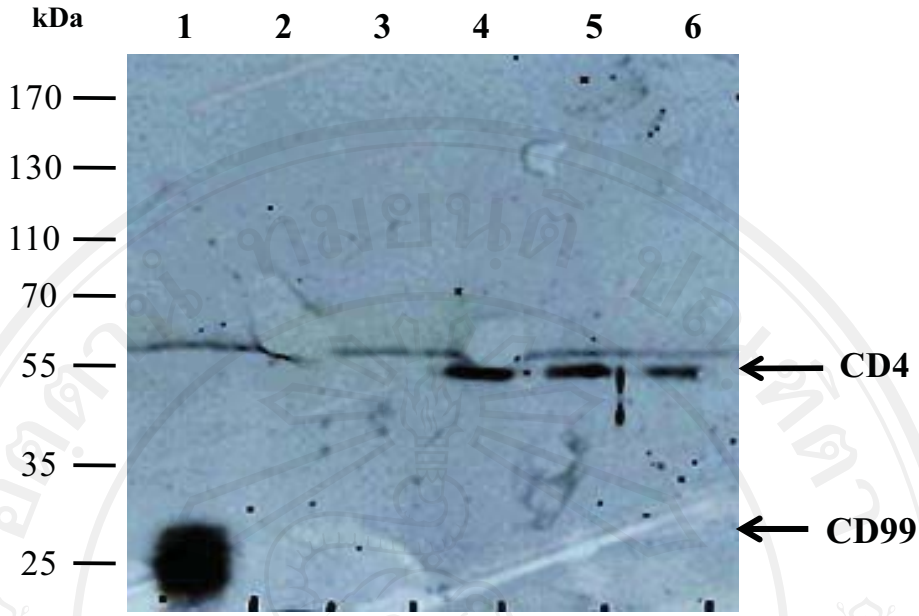

Figure 3.4 Immunoprecipitation of CD4 molecule by purified anti-CD4 mAbs. The surface proteins of Sup T1 cell line were biotinylated using Sulfo-NHS-LC-biotin. Biotinylated cell lysates were precipitated with purified MT4 mAb (lane 4), MT4/2 mAb (lane 5), MT4/3 mAb (lane 6), anti-CD99, MT99/3 (lane 1), IgM isotype match control mAb, Hb1a (lane 2), and IgG isotype match control mAb, Thal N/B (lane 3). The precipitated proteins were electrophoresed under reducing condition. The protein bands were visualized with peroxidase conjugated streptavidin and chemiluminescence detection system. The standard molecular markers are indicated on the left.

3.4 Epitope recognition by anti-CD4 mAbs

To analyze the epitope recognized by anti-CD4 mAb clones MT4, MT4/2, and MT4/3, cross blocking analysis was performed. PBMCs of four individuals were firstly stained with purified anti-CD4 mAbs (MT4, MT4/2, and MT4/3) and then counterstained with FITC-conjugated anti-CD4 mAbs (MT4, MT4/2, and MT4/3). The stained cells were then analyzed by a flow cytometer. Mean fluorescence intensity of each FITC-conjugated anti-CD4 mAb was measured and the percent inhibition was calculated. The percent inhibition between each anti-CD4 mAbs pair were shown in Table 3.1. According to the cross blocking pattern, anti-CD4 mAb clones MT4 and MT4/2 inhibited each other and showed similar inhibitory patterns. While anti-CD4 mAb clone MT4/3 did not inhibit other clones. The results indicated that anti-CD4 mAb clones MT4 and MT4/2 react to the same or adjacent epitope on CD4 molecule. Whereas, MT4/3 mAb reacts to the un-related epitope.

Table 3.1 Cross blocking analysis of anti-CD4 mAbs.

	% Inhibition		
Unlabeled monoclonal antibody	**FITC-conjugated monoclonal antibody**		
	MT4	MT4/2	MT4/3
MT4	92.71 %	79.60 %	8.35 %
MT4/2	61.38 %	80.38 %	-4.27 %
MT4/3	11.24 %	-4.02 %	83.96 %

The % inhibition was calculated from the mean fluorescence intensity between cells incubated with and without unlabeled anti-CD4 mAbs. The data was representative of four independent experiments.

3.5 Reactivity of anti-CD4 mAbs with CD4 molecules expressed on lymphocytes and monocytes

3.5.1 The saturated concentration of anti-CD4 mAbs for immunefluorescence staining

To determine the saturated concentration of anti-CD4 mAb clones MT4, MT4/2, and MT4/3 for measurement of CD4 expression on lymphocytes and monocytes, PBMCs were stained with 400, 200, 100, and 50 μg/ml of purified anti-CD4 mAbs by indirect immunofluorescent staining. The stained cells, both lymphocytes and monocytes, were then analyzed by flow cytometry. The FACS profiles obtained are shown in Figure 3.5. Mean fluorescence intensities of the lymphocyte and monocyte positive cells obtained from each mAb concentration were compared. The results showed that the saturated concentration of all three anti-CD4 mAbs tested was 200 μg/ml (Figure 3.6). This concentration was used in the further experiments for determining the reactivity of anti-CD4 mAbs to CD4 molecules expressed on the surface of lymphocytes and monocytes.

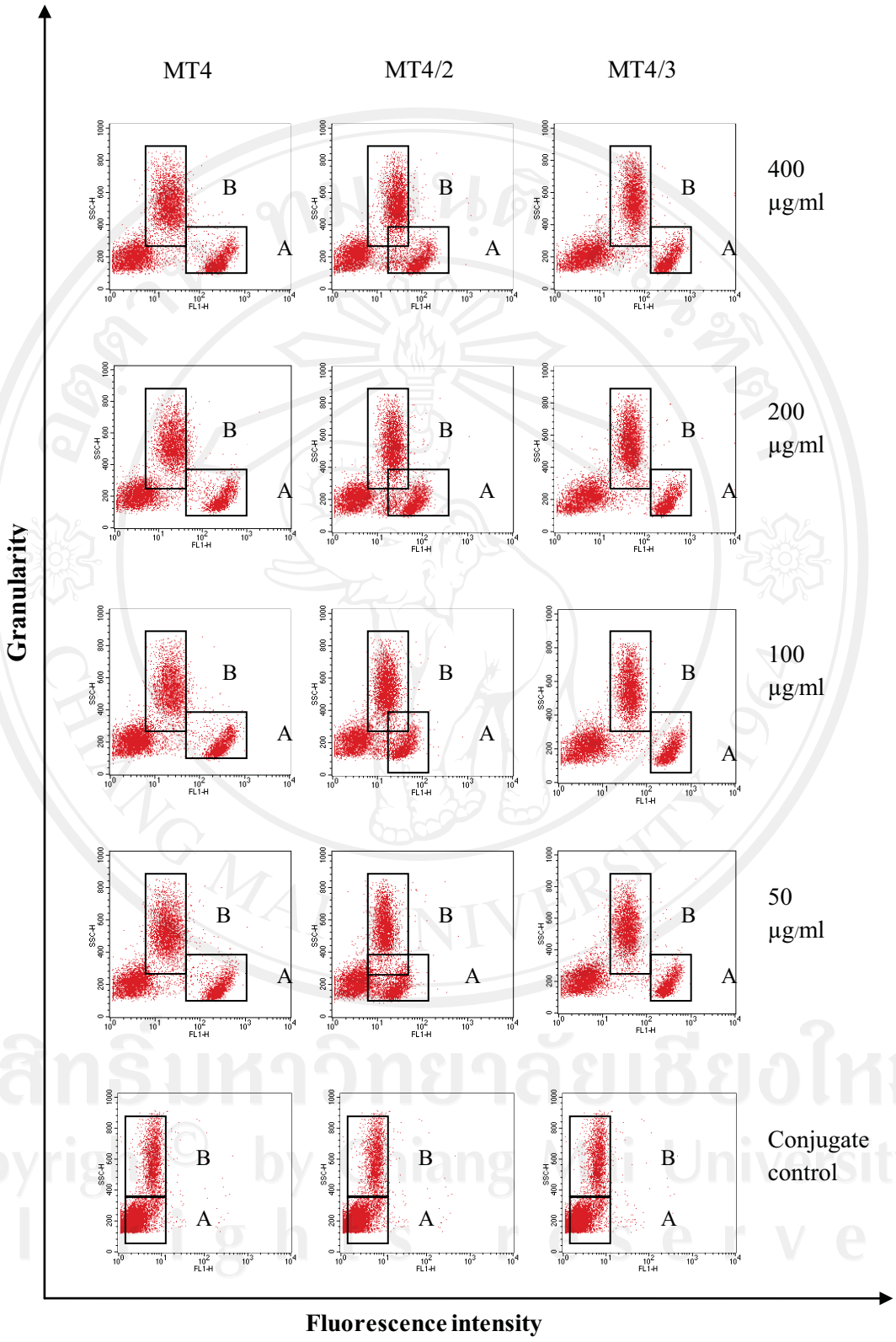

Figure 3.5 Flow cytometric analysis of lymphocytes and monocytes stained with various concentrations of anti-CD4 mAbs. PBMCs were stained with 400, 200, 100, and 50 μg/ml of purified anti-CD4 mAbs clones MT4, MT4/2, and MT4/3 by indirect immunofluorescent staining. The stained cells were analyzed by flow cytometry. Granularity (SSC) and FITC fluorescence (FL-1) were plotted to show the binding of each concentration of mAbs to lymphocyte (A) and monocyte (B) populations. The results were representative of four independent experiments.

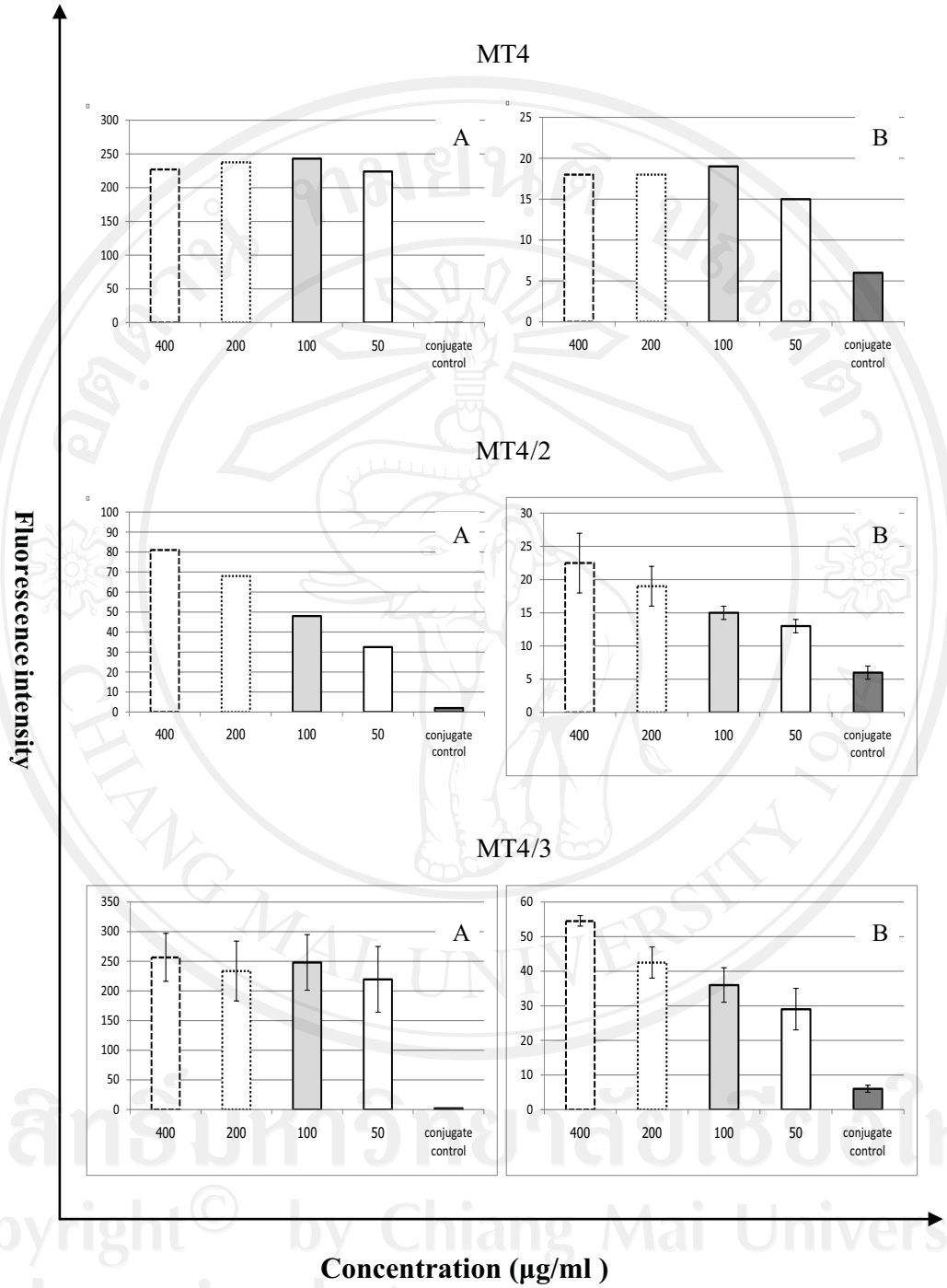

Figure 3.6 The saturated concentration of anti-CD4 mAbs for determination of CD4 molecules expressed on lymphocytes and monocytes. PBMCs were stained with purified anti-CD4 mAb clones MT4, MT4/2, and MT4/3 at different concentration (50-200 µg/ml) by indirect immunofluorescent staining. Each value represents the mean ± SD (n=4) of mean fluorescence intensity from lymphocyte positive cells (A) and monocyte positive cells (B).

3.5.2 Reactivity of anti-CD4 mAbs to CD4 molecules expressed on lymphocyte and monocyte surfaces

CD4 protein was reported to differently express on surface of lymphocytes and monocytes (Lynch *et al*., 1996; 1999). We were engrossed whether our generated anti-CD4 mAbs can recognized the CD4 expressing between lymphocytes and monocytes. PBMCs obtained from 20 healthy donors were stained with 200 µg/ml (saturated concentration) of purified anti-CD4 mAb clones MT4, MT4/2, and MT4/3 and counterstained with FITC-conjugates. The stained cells were then analyzed by flow cytometry. The results showed that MT4 mAb strongly reacted to CD4 molecule on lymphocyte cell surfaces, however, did not react or very weakly reacted to CD4 molecule on monocytes (Figure 3.7). MT4/2 mAb weakly reacted to CD4 molecule on lymphocytes and monocytes (Figure 3.7). In contrast, MT4/3 mAb strongly reacted to CD4 molecules on both lymphocytes and monocytes (Figure 3.7). The mean fluorescence intensities of the lymphocyte and monocyte positive cells of all tested donors were illustrated in Figure 3.8. From the results, MT4 mAb reacted to CD4 molecule expressed on lymphocytes but did not react to those expressed on monocytes indicating CD4 molecule expressed on lymphocytes is different from those expressed on monocytes. This difference can be identified by the MT4 mAb.

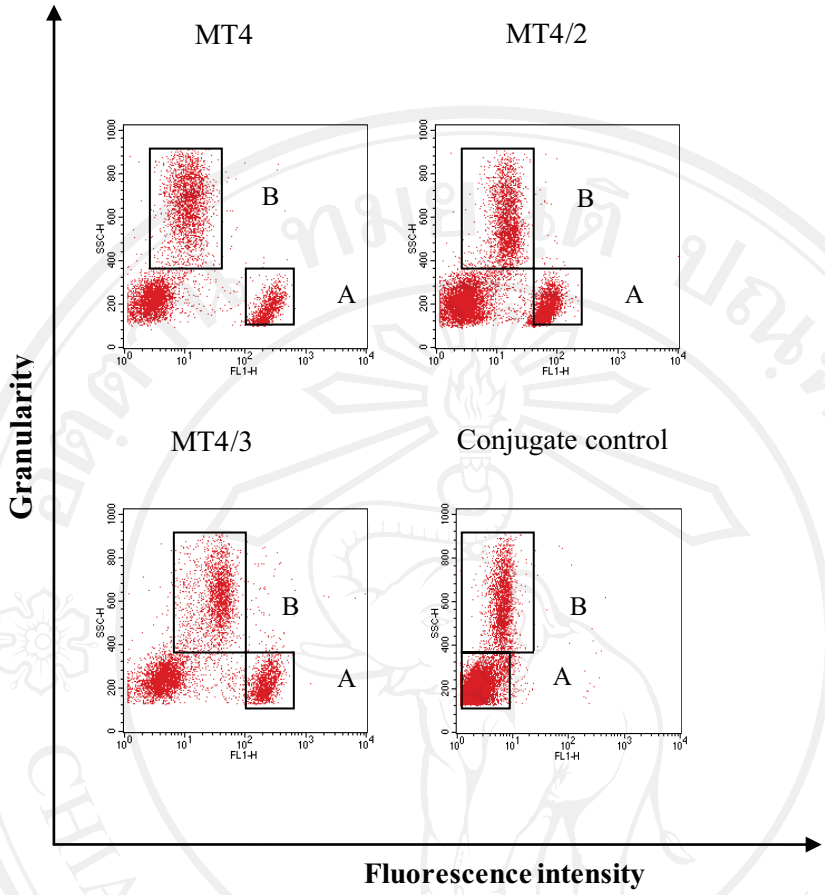

Figure 3.7 Flow cytometric analysis of lymphocytes and monocytes by stained with anti-CD4 mAbs MT4, MT4/2, and MT4/3. PBMCs were stained with 200 µg/ml of purified anti-CD4 mAbs MT4, MT4/2, and MT4/3 by indirect immunofluorescent staining. The stained cells were analyzed by flow cytometry. Granularity (SSC) and FITC fluorescence (FL-1) were plotted to show the binding of each mAb to lymphocyte (A) and monocyte (B) populations. The results were representative of twenty independent donors.

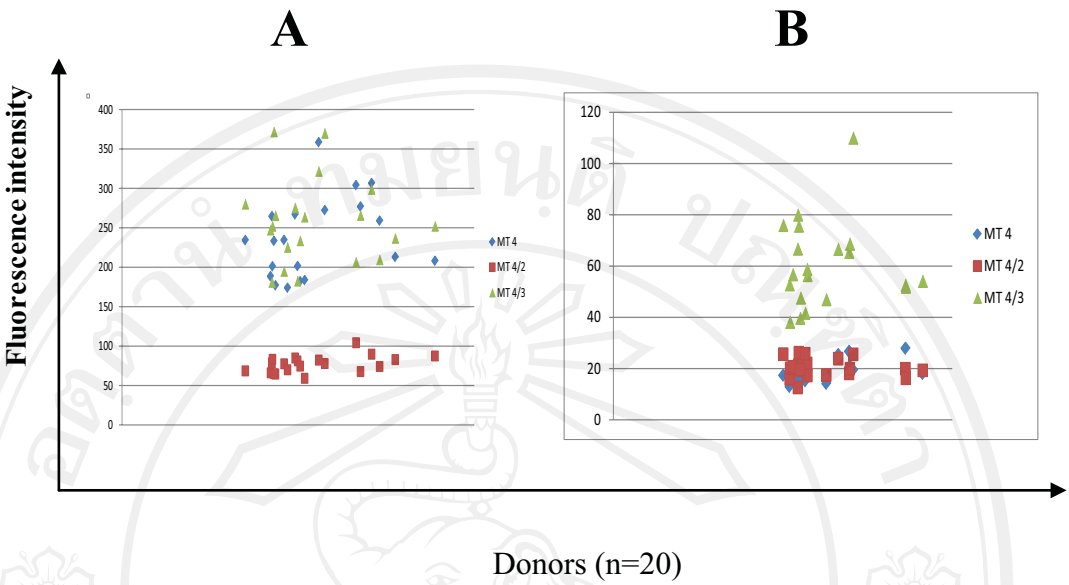

Figure 3.8 The reactivity of anti-CD4 mAbs MT4, MT4/2 and MT4/3 on lymphocytes (A) and monocytes (B). PBMCs were stained with purified anti-CD4 mAbs (MT4, MT4/2, and MT4/3) by indirect immunofluorescent staining. The stained cells were analyzed by flow cytometry. Mean fluorescence intensities (Y axis) from twenty healthy donors (X axis) of the positive cells were compared.

3.6 Functional study of CD4 molecule

3.6.1 Effect of the generated anti-CD4 mAbs on anti-CD3 induced PBMC proliferation

Although the functions of CD4 molecule on lymphocytes are well characterized, the function of CD4 on monocytes is still ambiguous. Since we have generated several anti-CD4 mAbs which have different reactivity property, these mAbs were employed for functional analysis of CD4 molecule. In this study, the effect of anti-CD4 mAbs on anti-CD3 induced lymphocyte proliferation was determined. PBMCs were activated with immobilized anti-CD3 mAb OKT3 and cultured in the presence or absence of anti-CD4 mAbs MT4, MT4/2, and MT4/3. As shown in Figure 3.9, in all cultivation times, anti-CD4 mAb clones MT4 and MT4/2 have no effect on anti-CD3 induced lymphocyte proliferation. While, MT4/3 mAb showed inhibitory effect on anti-CD3 induced lymphocyte proliferation (Figure 3.9). As predicted, IgM isotype matched control mAb (FE-1H10) and IgG isotype matched control mAb (13M-1F) have no effect on anti-CD3 induced lymphocyte proliferation (Figure 3.9). In this study, an inhibitory mAb (anti-CD147 mAb, M6-1E9) was included in the experiments as positive control and showed the inhibitory effect on anti-CD3 induced lymphocyte proliferation (Figure 3.9). These results indicate that anti-CD4 mAb clone MT4/3, which strongly reacted to CD4 molecules on both lymphocytes and monocytes, could inhibit anti-CD3 induced lymphocyte proliferation.

In addition, the effect of mAbs MT4, MT4/2, and MT4/3 on lymphocyte activation was investigated. All tested mAbs themselves did not induced cell proliferation (Figure 3.10)

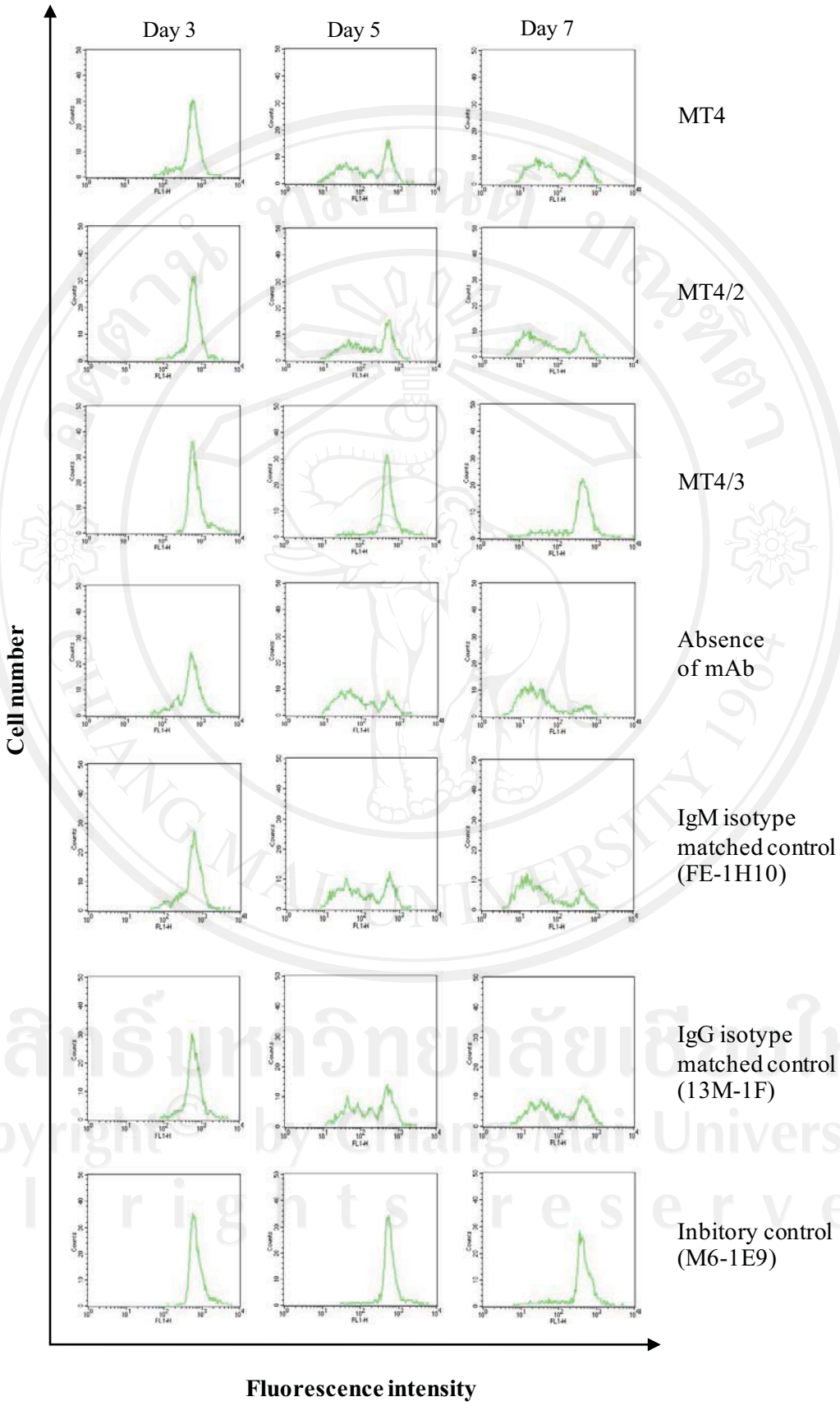

Figure 3.9 Effect of anti-CD4 mAbs on anti-CD3 induced lymphocyte proliferation. PBMCs were labeled with CFSE. CFSE labeled PBMCs were induced for cell proliferation by immobilized anti-CD3 mAb (OKT3) in the presence of anti-CD4 mAbs MT4, MT4/2 and MT4/3, IgM isotype matched control (FE-1H10), IgG isotype matched control (13M-1F), and inhibitory control anti-CD147 mAb (M6-1E9), or in the absence of mAb. After day 3, 5 and 7 of cultivation, cells were harvested and determined for the reduction of fluorescence intensity by flow cytometry. The results were representative of three independent experiments.

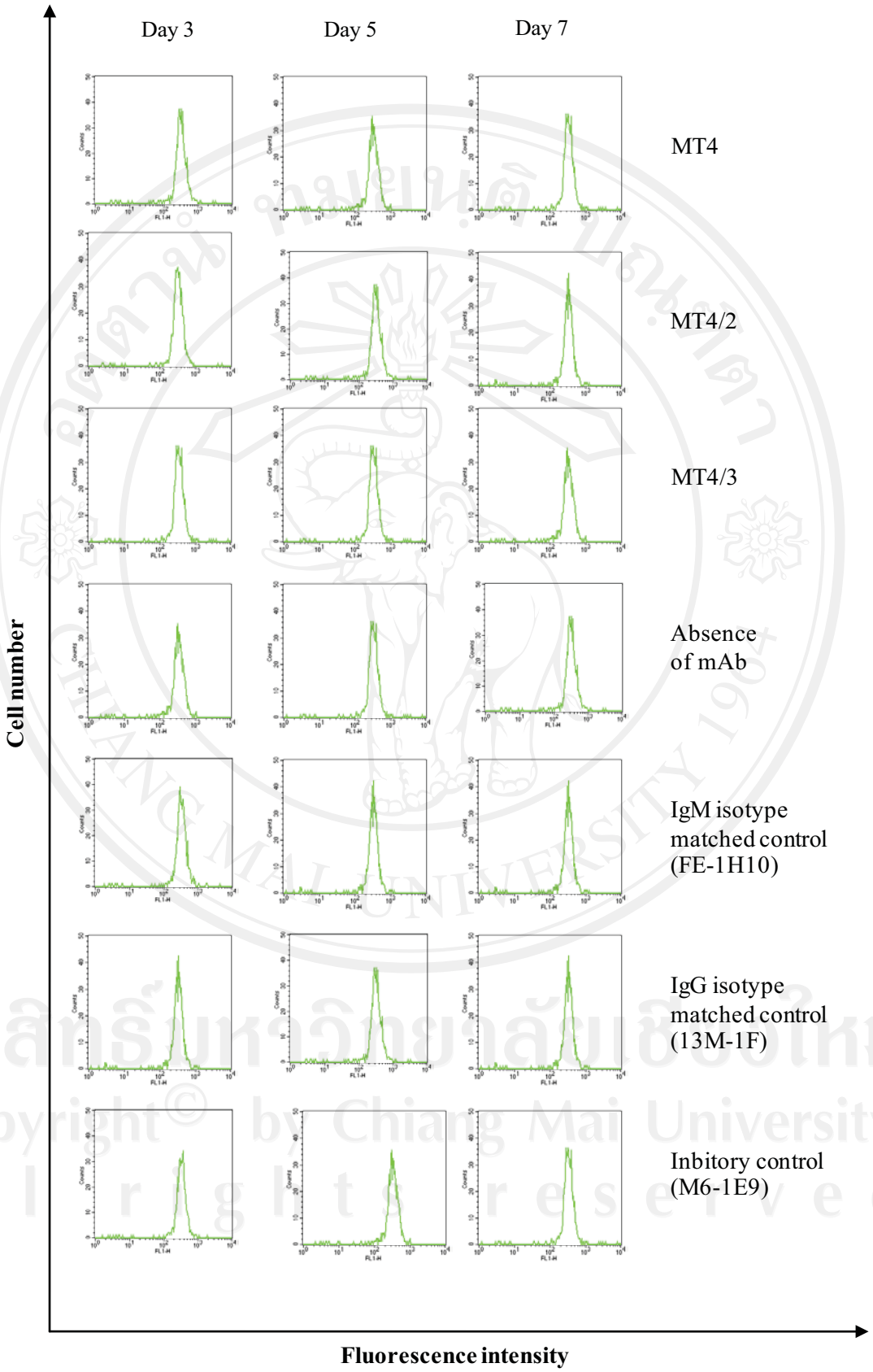

Figure 3.10 Effect of anti-CD4 mAbs on lymphocyte proliferation. PBMCs were labeled with CFSE. CFSE labeled PBMCs were cultured in the presence of anti-CD4 mAbs MT4, MT4/2, and MT4/3, IgM isotype matched control (FE-1H10), IgG isotype matched control (13M-1F), and inhibitory control anti-CD147 mAb (M6-1E9), or in the absence of mAb. After day 3, 5 and 7 of cultivation, cells were harvested and determined for the reduction of fluorescence intensity by flow cytometry. The results were representative of three independent experiments.

3.6.2 Effect of the generated anti-CD4 mAbs on anti-CD3/CD28 induced monocyte-depleted lymphocyte proliferation

In the above study, anti-CD4 mAb MT4/3 that react to CD4 molecules on both lymphocytes and monocytes suppressed anti-CD3 induced PBMC proliferation. However, anti-CD4 mAb MT4 that react to only CD4 on lymphocytes did not alter anti-CD3 induced PBMC proliferation. We, therefore, hypothesized that CD4 molecule expressed on monocytes may responsible for the regulation of T cell proliferation. To address this hypothesis, the effect of mAbs MT4, MT4/2, and MT4/3 on monocyte-depleted lymphocyte activation was determined. In this study, monocytes were depleted from PBMCs by cell adhesion. The obtained monocyte-depleted lymphocytes were confirmed for their purity by staining with PE-conjugated anti-CD14 mAb. It was found that less than 3% of monocytes were remained in the monocyte-depleted lymphocyte population. Monocyte-depleted lymphocytes were stimulated with anti-CD3 plus anti-CD28 mAb in the presence or absence of anti-CD4 mAb clones MT4, MT4/2, and MT4/3 and cell proliferation were determined by CFSE staining. As shown in Figure 3.11, at all cultivation times, all anti-CD4 mAbs including MT4, MT4/2, and MT4/3 have no effect on anti-CD3/CD28 induced monocyte-depleted lymphocyte proliferation. Since anti-CD4 mAb MT4/3 suppressed PBMC (containing lymphocytes and monocytes) proliferation but did not has inhibitory effect on monocyte-depleted lymphocyte (containing only lymphocytes) proliferation. The results point out that CD4 expressed on monocytes play a role in the suppression of lymphocyte activation.

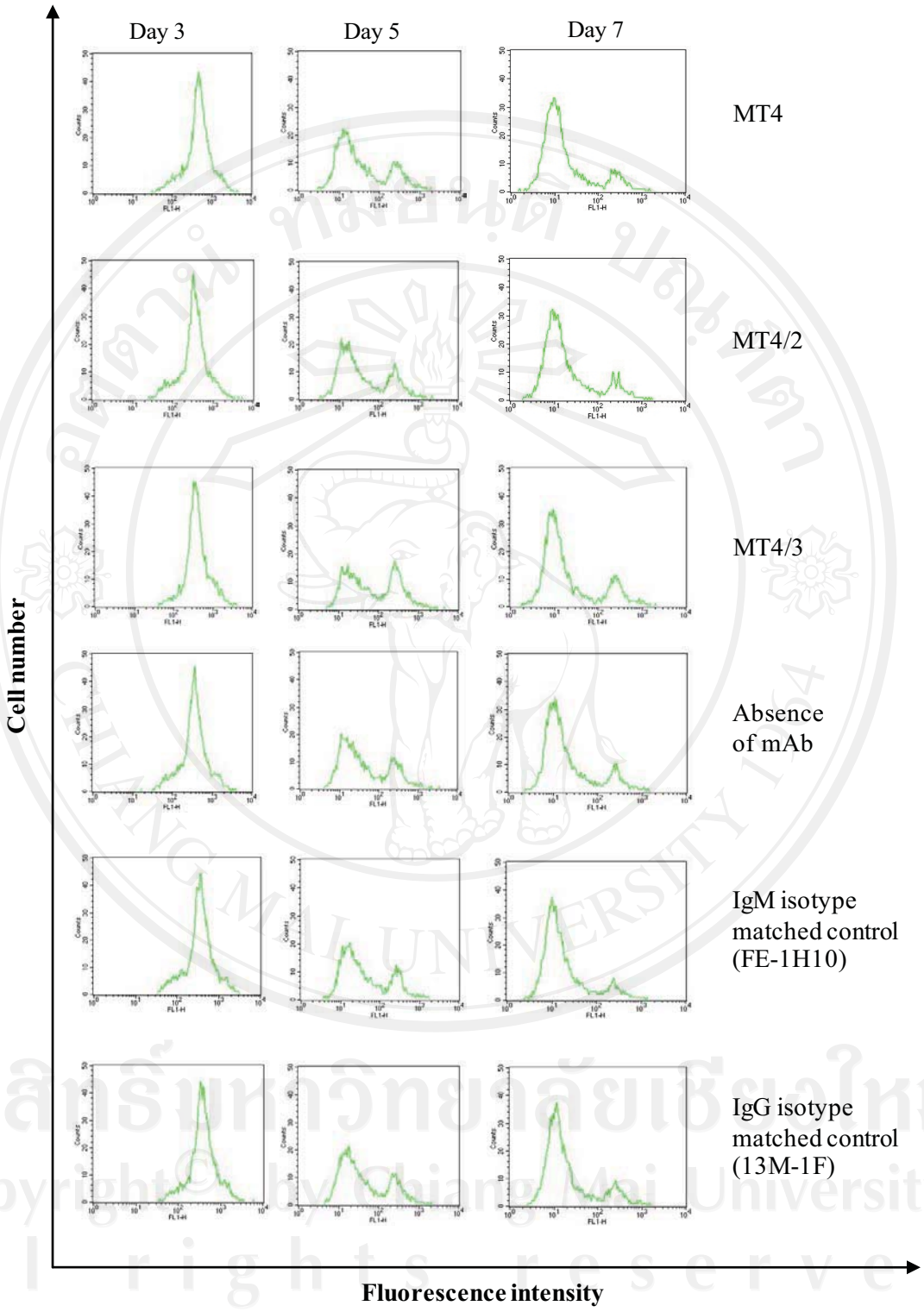

Figure 3.11 Effect of anti-CD4 mAbs on anti-CD3/CD28 induced monocyte-depleted lymphocyte proliferation. CFSE labeled monocyte-depleted lymphocytes were activated by immobilized anti-CD3 mAb plus anti-CD28 mAb in the presence or absence of anti-CD4 mAbs MT4, MT4/2, and MT4/3, IgM isotype matched control (FE-1H10), and IgG isotype matched control (13M-1F). After day 3, 5 and 7 of cultivation, cells were harvested and determined for cell proliferation by determination of the reduction of fluorescence intensity by flow cytometry. The results were representative of three independent experiments.

3.6.3 Effect of the generated anti-CD4 mAbs on oxidative burst induction

As CD4 expressed on monocytes was demonstrated to be involved in regulation of lymphocyte proliferation, we further investigated the function of CD4 on other monocyte function. In this study, the effect of anti-CD4 mAbs on monocyte oxidative burst induction was determined. Whole blood was incubated with mAbs MT4, MT4/2, and MT4/3, then monocyte oxidative burst was measured using DHE staining. Mean fluorescence intensities of oxidized DHE in the presence or absence of mAbs MT4, MT4/2, and MT4/3 were compared. In this study, PMA, an oxidative burst induction agent was used as control. As shown in Figure 3.13**,** all tested anti-CD4 mAbs MT4, MT4/2 and MT4/3 did not induce monocyte oxidative burst. While, PMA control showed strongly induce monocyte oxidative burst (Figure 3.12).

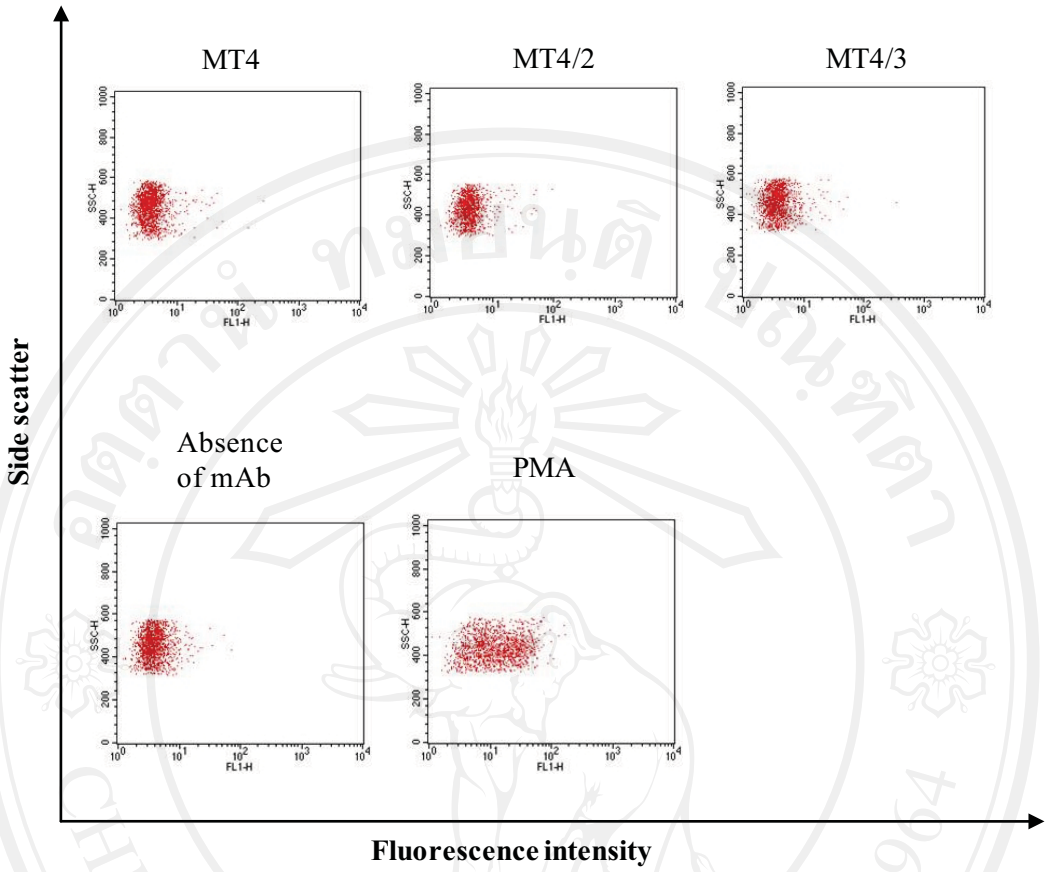

Figure 3.12 Effect of anti-CD4 mAbs MT4, MT4/2, and MT4/3 in the induction of monocyte oxidative burst. Oxidative burst were determined using DHE-lysed whole blood staining method. Mean fluorescence intensities of oxidized DHE in the presence of anti-CD4 mAb clones MT4, MT4/2 and MT4/3 or absence of mAb were measured by flow cytometry. PMA was used as positive control. The results were representative of three independent experiments.