CHAPTER III

RESULTS

3.1 Large scale production and purification of mAbs MT3 and COSA2A

Mabs MT3 and COSA2A were recently produced in our laboratory. These two mAbs were screened and found to be reacted with a population of lymphocytes (un-published observation). To further study the cellular expression, biochemisty properties and function of the molecules recognized by mAbs MT3 and COSA2A, purified mAbs were firstly prepared.

Ascities containing mAbs MT3 and COSA2A were induced by inoculation of the hybridomas into mouse peritoneal cavity and ascitic fluids were collected. Then, the mAbs were isolated from the obtained ascitic fluids by affinity chromatography. The obtained mAbs were checked for its activity by indirect immunofluorescence staining and for its purity by SDS-PAGE.

For mAb MT3, the results indicated that purified mAb MT3 still has good reactivity after purification process (Figure 3.1A). This mAbs reacted to a population of lymphocytes as was previously observed (un-published observation). For purity examination, under reducing condition of SDS-PAGE, mAb MT3 was separated into two protein bands corresponding to heavy chain and light chain and no other protein band was observed (Figure 3.2 lane 1). Under non-reducing condition, no protein band was observed in the gel (Figure 3.2, lane 1). This is because of that the intact mAb MT3 has a very large molecular weight, it could not migrate into the separating

gel (Figure 3.2, lane 1). These results indicated that the obtained purified mAb MT3 was pure and could be used for further experiments.

For mAb COSA2A, the purified mAb COSA2A has also good reactivity after purification, as was shown Figure 3.1B. This mAbs reacted to a population of lymphocytes as was previously observed (un-published observation). Surprisingly, in the examination of the purity of mAb COSA2A by SDS-PAGE, only one major protein band corresponding to immunoglobulin light chain, was observed (Figure 3.2 lane 2). As the purified mAb COSA2A still has antigen binding property, this purified mAb was then used in further experiment.

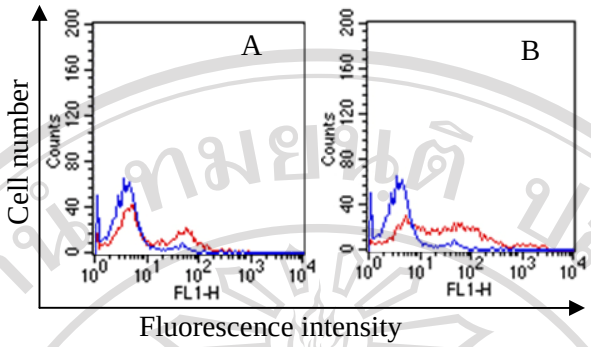

Figure 3.1 The reactivity of purified monoclonal antibodies, MT3 and COSA2A. Peripheral blood mononuclear cells (PBMCs) were stained with purified mAbs MT3 (A) and COSA2A (B) by indirect immunofluorescence and analyzed by flow cytometry. Lymphocyte population was gated according to their size and granularlity, and the fluorescence of the stained cells was determined. Blue lines represent the conjugate control and red lines represent the reactivity of purified mAb MT3 (A) and COSA2A (B).

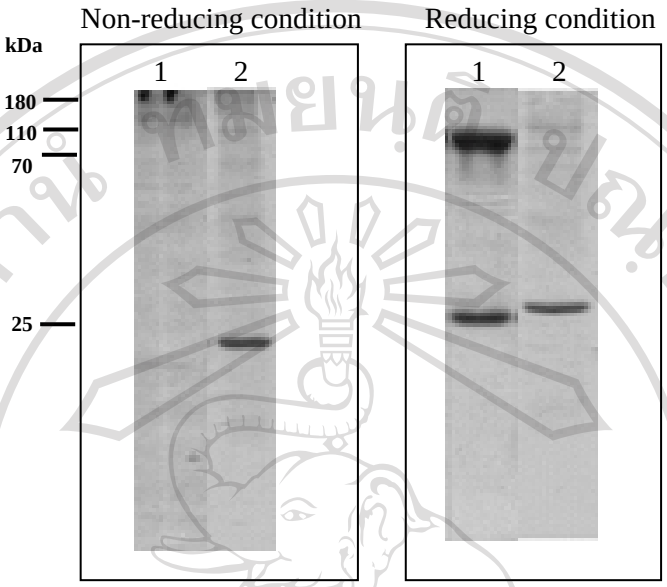

Figure 3.2 SDS-PAGE analysis of the purified mAbs MT3 and COSA2A. The purified mAbs MT3 (lane 1) and COSA2A (lane 2) were run on a 12.5% SDS-PAGE under non-reducing and reducing conditions. The gels were stained with PAGE blue after electrophoresis. The molecular weight markers are indicated on the left in kDa.

3.2 Cellular distribution of the molecule recognized by mAbs MT3 and COSA2A.

To determine the expression of molecule recognized by both mAbs, various cell types were stained with mAbs MT3 and COSA2A by indirect immunofluorescence staining and analyzed by flow cytometry.

3.2.1 The expression of MT3 molecules and COSA2A molecules on peripheral blood cells.

To examine the expression of MT3 and COSA2A molecules on peripheral blood cells all leukocyte populations and red blood cells were isolated and stained with mAbs MT3 or COSA2A.

Within leukocyte populations, mAb MT3 reacted with a population of lymphocytes, but did not react with monocytes and granulocytes (Figure 3.3). With RBC, this mAb also showed positive reactivity. Surprisingly, 3 of 10 RBC donors showed weakly positive staining pattern indicating the different expression level of the MT3 molecules on RBC (Figure 3.3).

With mAb COSA2A, a population of lymphocytes showed positive reactivity. Granulocytes, monocytes and RBC were all negative (Figure 3.4).

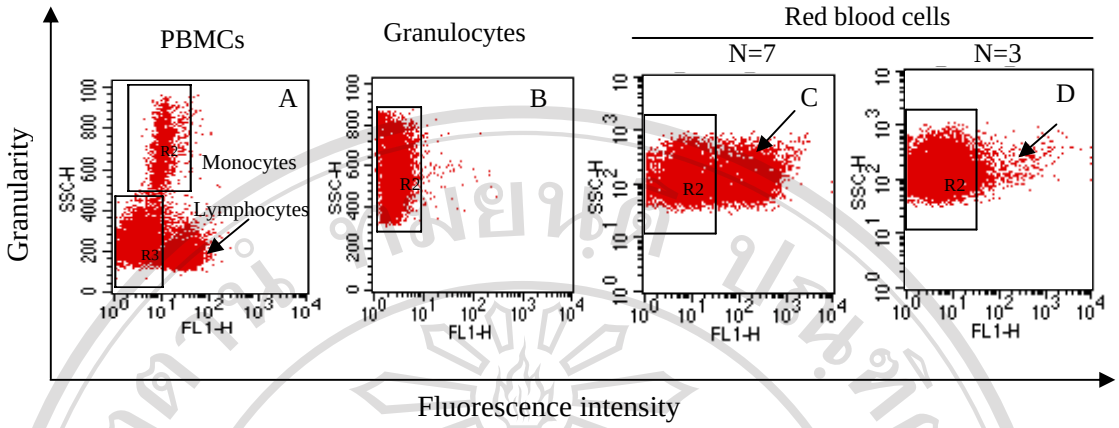

Figure 3.3 Expression of MT3 molecules on peripheral blood cells. PBMCs including lymphocytes and monocytes, granulocytes and red blood cells were stained with mAb MT3 by indirect immunofluorescence. mAb MT3 reacted to a sub-population of lymphocytes (A, arrow) and red blood cells (C, arrow), but not with monocytes (A) and granulocytes (B). Red blood cells of some donors show weakly positive with mAb MT3 (D, arrow). For leukocytes, data are representative of 3 independent experiments. For red blood cells, data are representative of 10 independent experiments.

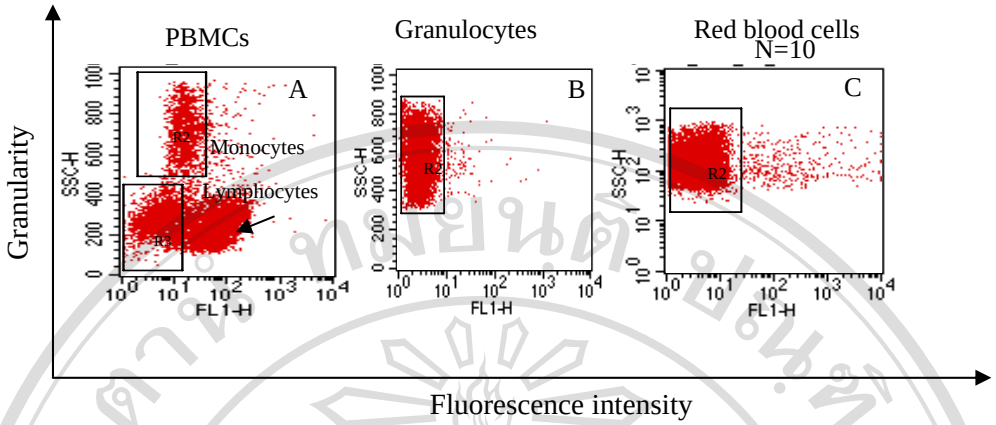

Figure 3.4 Expression of COSA2A molecules on peripheral blood cells. Peripheral mononuclear cells (PBMCs) including lymphocytes and monocytes, granulocytes and red blood cells were stained with mAb COSA2A by indirect immunofluorescence. mAb COSA2A was reacted to a sub-population of lymphocytes (A, arrow). mAb COSA2A did not react to monocytes (A), granulocytes and red blood cells (B and C). Data are representative of 3 independent experiments for leukocytes and representative of 10 independent experiments for red blood cells.

3.2.2 The expression of MT3 and COSA2A molecules on hematopoietic cell lines.

Four types of hematopoietic cell lines were determined for the expression of MT3 and COSA2A molecules. The erythro-myelocytic cell line (K562 cell), human leukemic monocyte lymphoma cell line (U937 cell), human Burkitt's lymphoma cell line (Daudi cell) and human acute lymphoblastic leukemia cell line (Molt4 cell and SupT1 cell) were assessed by indirect immunofluorescence staining.

The results showed that mAb MT3 reacted with only T lymphocyte lines, Molt4 and SupT1, while K562, U937 and Daudi cells show negative reactivity (Figure 3.5). In contrast, mAb COSA2A did not react to any tested cell lines (Figure 3.6).

3.3 The expression of MT3 and COSA2A molecules on lymphocyte sub-populations.

As mAbs MT3 and COSA2A reacted to a population of lymphocytes (figure 3.3 and 3.4), the reacted lymphocyte sub-populations were further identified. To distinct lymphocytes sub-populations, CD molecules were used as specific markers. In this study, CD19 and CD56 were used as markers for B lymphocytes and NK cells, respectively. CD3 molecule was used as a marker for T lymphocytes. CD4 and CD8 molecules were used as the markers for T lymphocyte subsets.

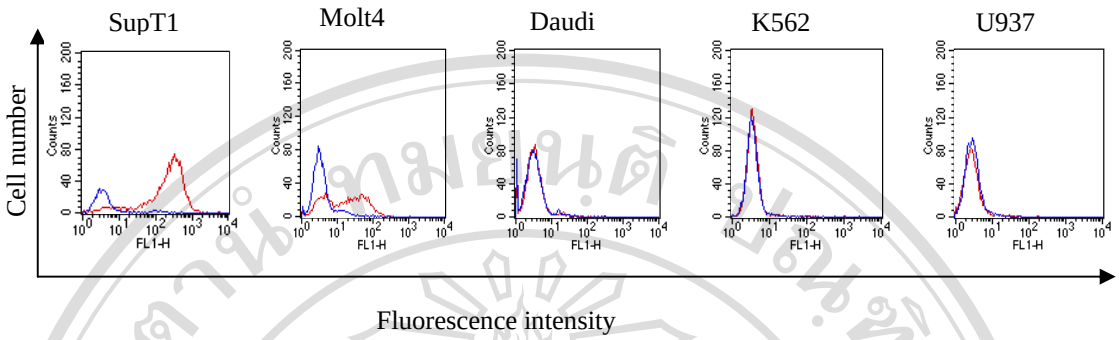

Figure 3.5 Expression of MT3 molecules on various hematopoietic cell lines. Hematopoietic cell lines including SupT1 (T cell line), Molt4 (T cell line), Daudi (B cell line), K562 (erythro-myelocytic cell line) and U937 (monocytic cell line) were stained with mAb MT3 (red line) and conjugated control (blue line) by indirect immunofluorescence and analyzed by flow cytometry.

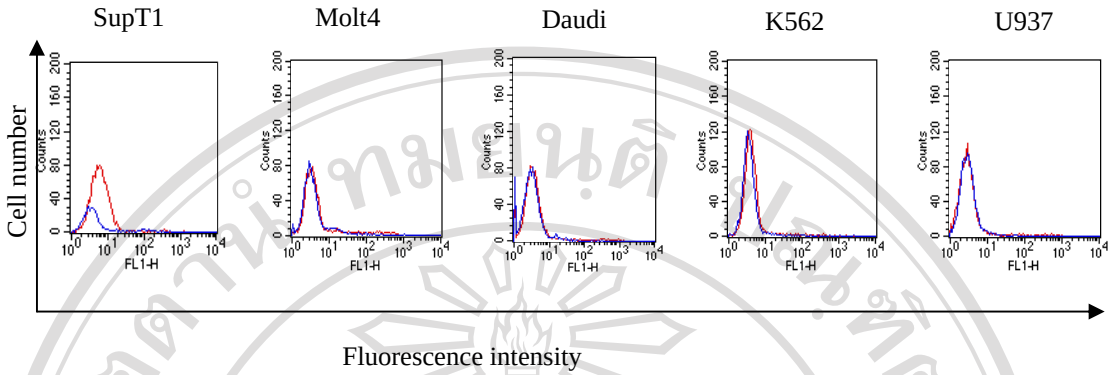

Figure 3.6 Expression of COSA2A molecules on various hematopoietic cell lines. Hematopoietic cell lines including SupT1 (T cell line), Molt4 (T cell line), Daudi (B cell line), K562 (erythro-myelocytic cell line) and U937 (monocytic cell line) were stained with mAb COSA2A (red line) and conjugated control (blue line) by indirect immunofluorescence and analyzed by flow cytometry.

3.3.1 Study on B lymphocytes and NK cells

To determine the expression of MT3 and COSA2A molecules on B lymphocytes and NK cells, PBMCs were stained with anti-CD19-PE or anti-CD56-PE and FITC indirect labeling to mAbs MT3 or COSA2A.

It was found that mAbs MT3 did not react to either $CD19^+$ and $CD56^+$ cells indicating MT3 molecules neither express on B lymphocytes nor NK cells (Figure 3.7). With mAb COSA2A, a small population of $CD19^+$ and $CD56^+$ cells showed positive reactivity. The majority of B lymphocytes and NK cells, however, were negative with mAb COSA2A (Figure 3.7).

3.3.2 Study on T lymphocytes and T lymphocyte subsets

To determine the expression of MT3 and COSA2A molecules on T lymphocytes ($CD3^+$ cells) and T lymphocyte subsets ($CD4^+$ and $CD8^+$ cells). PBMCs were stained with mAb MT3 (IgM isotype) or COSA2A (IgM isotype) and anti-CD4 mAb (IgG isotype) or anti-CD8 mAb (IgG isotype). Then, cells were re-stained with anti-mouse IgM-PE and anti-mouse IgG-FITC.

The results showed that mAb MT3 reacted with a population of $CD3^+$ T lymphocytes as shown in Figure 3.8. Moreover, mAb MT3 also reacted with a population of $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes (Figure 3.8). Percentage of $CD3^+MT3^+$ cells, $CD4^+MT3^+$ cells and $CD8^+MT3^+$ cells are shown in table 3.1.

The COSA2A molecules were expressed on almost $CD3^+$ T lymphocytes (Figure 3.9). All sub-population of $CD4^+$ T lymphocytes and most of $CD8^+$ T lymphocytes were found to express COSA2A molecules (Figure 3.9). Percentage of $CD3^+COSA2A^+$ cells, $CD4^+COSA2A^+$ cells and $CD8^+COSA2A^+$ cells are shown in table 3.2.

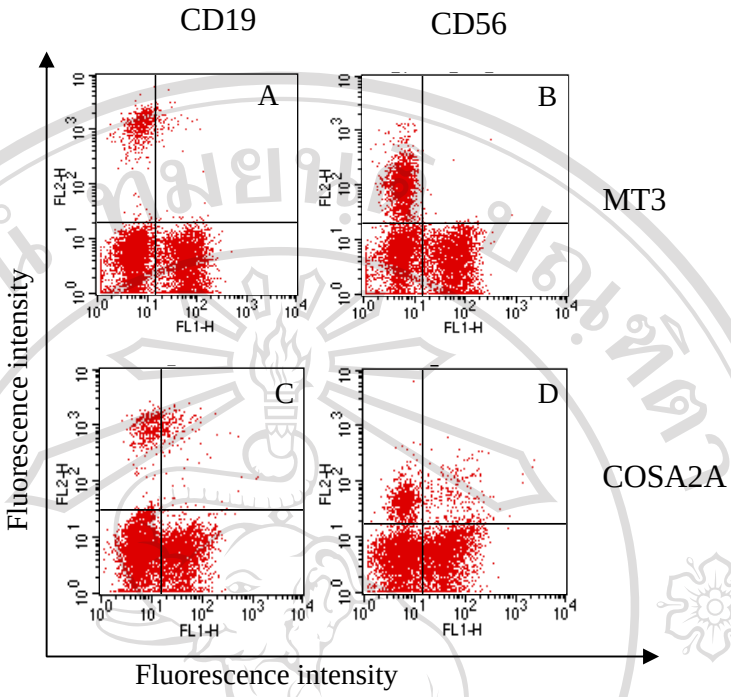

Figure 3.7 Expression of MT3 and COSA2A molecules on B lymphocytes and NK cells. PBMCs were stained with mAbs MT3 (upper panel; A and B) or COSA2A (lower panel; C and D) and anti-mouse Igs-FITC followed with anti-CD19-PE (A and C) or anti-CD56-PE (B and D). Data are representative of 3 independent experiments.

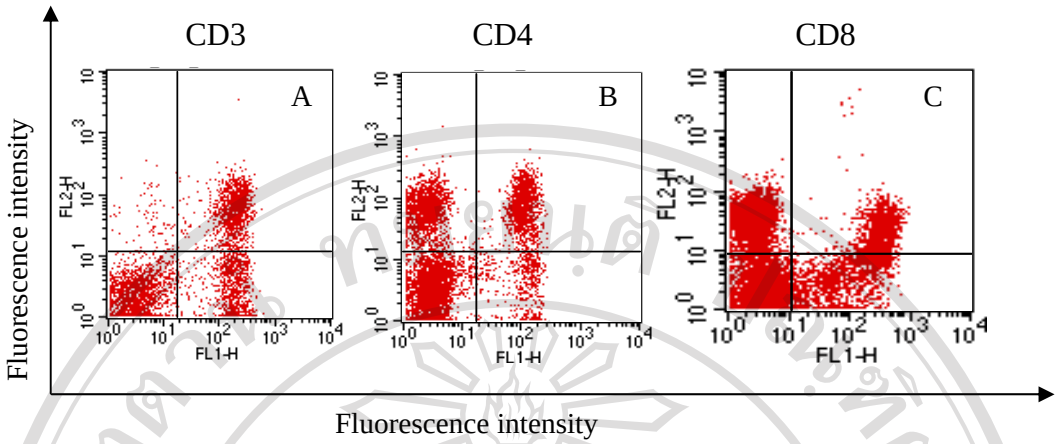

Figure 3.8 Expression of MT3 molecules on T lymphocytes and sub-population of T lymphocytes by two-color immunofluorescence analysis. PBMCs were stained with mAb MT3 and anti-mouse IgM-PE and followed with the second color, anti-CD3-FITC (A) or anti-CD4 mAb clone MT4/3 (isotype IgG) and anti-mouse IgG-FITC (B) or anti-CD8 mAb clone MT8 (isotype IgG) and anti-mouse IgG-FITC (C). Data are representative of 3 independent experiments.

Table 3.1 Percentage of $MT3^+$ cells in lymphocytes and sub-population of lymphocytes.*

Sample numbers	Within lymphocytes								
	$CD3^+MT3^-$	$CD3^+MT3^+$	$CD3^-MT3^+$	$CD4^+MT3^-$	$CD4^+MT3^+$	$CD4^-MT3^+$	$CD8^+MT3^-$	$CD8^+MT3^+$	$CD8^-MT3^+$
N1	20.28	52.92	0.56	7.21	19.52	25.43	17.79	12.45	28.08
N2	29.42	39.16	1.60	9.94	25.72	10.30	12.89	19.51	30.54
N3	24.91	31.27	2.24	7.02	26.38	14.99	17.54	21.29	26.06
Mean±SD	24.87±4.57	41.12±10.95	1.47±0.84	8.06±1.63	23.87±3.78	16.91±7.74	16.07±2.75	17.75±4.67	28.23±2.24

*Lymphocytes were gated and positive cells were calculated.

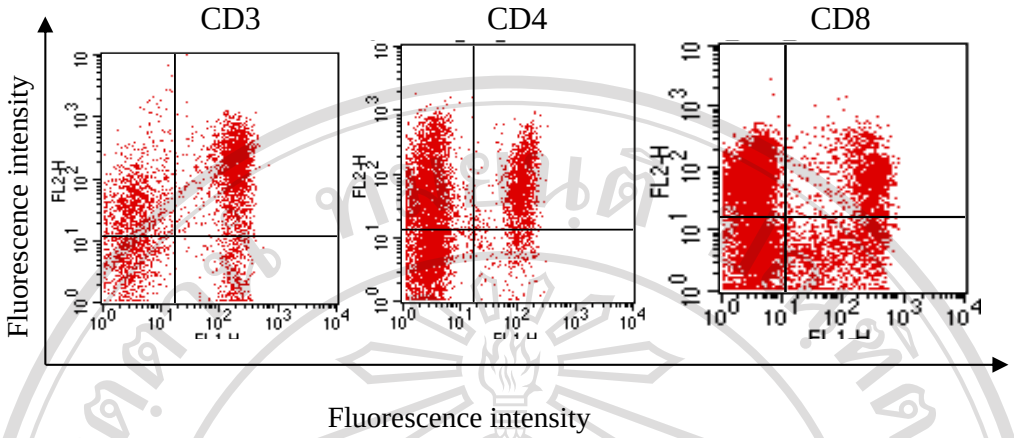

Figure 3.9 Expression of COSA2A molecules on T lymphocytes and sub-population of T lymphocytes by two-color immunofluorescence analysis. PBMCs were stained with mAb COSA2A and anti-mouse IgM-PE and followed with the second color, anti-CD3-FITC (A) or anti-CD4 mAb clone MT4/3 (isotype IgG) and anti-mouse IgG-FITC (B) or anti-CD8 mAb clone MT8 (isotype IgG) and anti-mouse IgG-FITC (C). Data are representative of 3 independent experiments.

Table 3.2 Percentage of COSA2A molecules on lymphocytes and sub-population of lymphocytes.*

Sample numbers	Within lymphocytes								
	$CD3^+$ $COSA2A^-$	$CD3^+$ $COSA2A^+$	$CD3^-$ $COSA2A^+$	$CD4^+$ $COSA2A^-$	$CD4^+$ $COSA2A^+$	$CD4^-$ $COSA2A^+$	$CD8^+$ $COSA2A^-$	$CD8^+$ $COSA2A^+$	$CD8^-$ $COSA2A^+$
N1	8.33	63.40	7.34	9.04	23.08	28.12	13.14	20.40	36.05
N2	4.05	63.82	18.90	4.33	22.30	42.51	14.80	21.29	32.58
N3	9.52	49.69	24.27	4.55	29.12	26.91	15.55	15.90	43.23
Mean±SD	7.30±2.88	58.97±8.04	16.84±8.65	5.97±2.66	24.83±3.73	32.51±8.68	14.50±1.23	19.20±2.89	37.29±5.43

*Lymphocytes were gated and positive cells were calculated.

3.4 The expression of MT3 and COSA2A molecules on CD45RO$^+$, RA$^+$ and RB$^+$ cells.

To determine the expression of MT3 and COSA2A molecules on naïve T lymphocytes (CD45RA$^+$ and CD45RB$^+$ cells) and memory T lymphocytes (CD45RO$^+$ cells). PBMCs were stained with mAb MT3 mAb (IgM isotype) or COSA2A mAb (IgM isotype) and anti-CD45RO clone UCHL-1, anti-CD45RA clone MEM93 and anti-CD45RB clone MEM55 conjugated with FITC, after that the stained cells were re-stained with anti-mouse IgM-PE.

As shown in Figure 3.10, mAb MT3 reacted to a subset of CD45RA$^+$, CD45RB$^+$, but not to CD45RO$^+$ cells. The mAb COSA2A showed the same staining pattern as was observed with mAb MT3 (Figure 3.10).

Taken together, from the cellular distribution studies, it can be concluded that MT3 molecule is expressed on a sub-population of T lymphocyte. This molecule is expressed on both of T lymphocyte subsets, CD4$^+$ and CD8$^+$ T lymphocyte.

COSA2A molecules express on a sub-population of B lymphocytes, NK cells and T lymphocytes. This molecules show broadly expression on both subsets of T lymphocytes, CD4$^+$ and CD8$^+$ T lymphocyte.

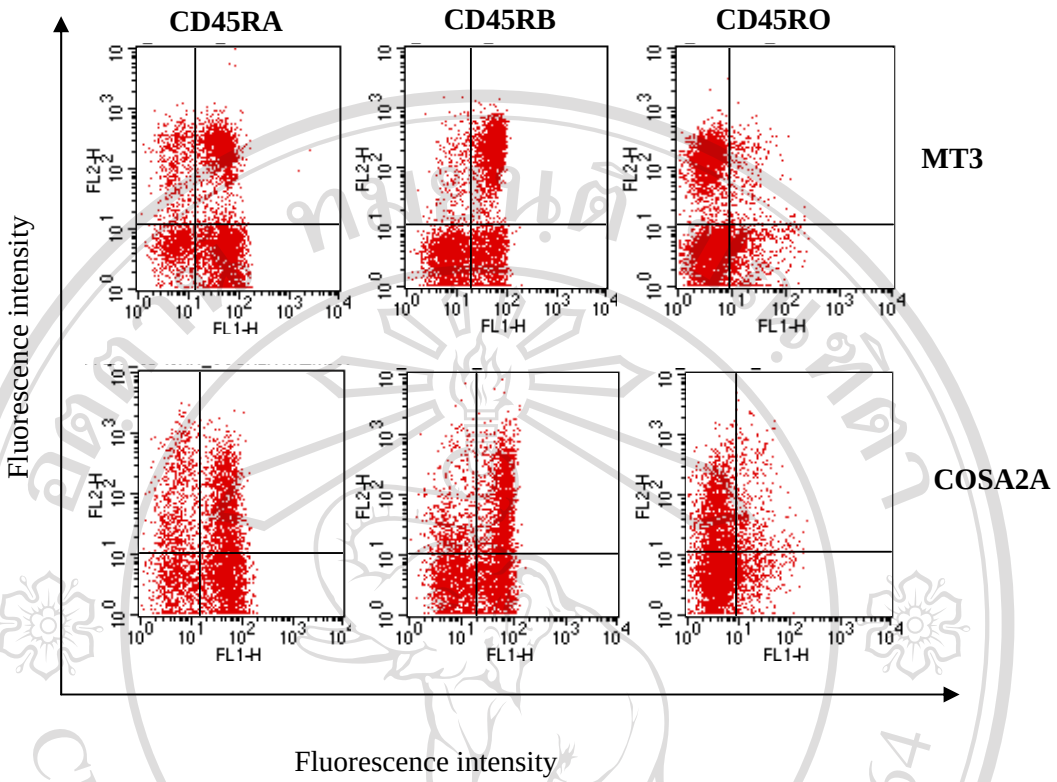

Figure 3.10 Expression of MT3 and COSA2A molecules on CD45RA$^+$, CD45RB$^+$ and CD45RO$^+$ cells. PBMCs were stained with FITC-anti-CD45RA mAb clone MEM93, FITC-anti-CD45RB mAb clone MEM55 and FITC-anti-CD45RO mAb clone UCHL1. Then the cells were stained mAb MT3 (upper panel) and mAb COSA2A (lower panel), subsequently stained with anti-mouse IgM-PE. Data are representative of 3 independent experiments.

3.5 Biochemical study

3.5.1 Determination of the molecular weight of MT3 and COSA2A molecules by Western immunoblotting

To determine the molecular weight of the molecules recognized by mAb MT3 and COSA2A. PBMCs were lysed by using 1% NP-40 lysis buffer. The cell lysates were separated in 10% SDS-PAGE under non-reducing and reducing conditions. Then, the proteins were transferred to a nitrocellulose membrane. The membrane was then probed with mAbs MT3 and COSA2A. As shown in Figure 3.11A and B, mAb MT3 did not react with any protein band under non-reducing and reducing conditions. The mAb COSA2A reacted with two major protein bands of the molecular weight of 55 kDa and 36 kDa under non-reducing condition. However, under reducing condition, no protein band was observed (Figure 3.11B). As control, a CD99 mAb, MT99/3, reacted to a broad protein band at the molecular weight of 25-35 kDa corresponding to CD99 protein

3.5.2 Determination of the molecular weight of MT3 molecules by Immunoprecipitation

To further determine the molecular weight of MT3 molecules, immunoprecipitation was employed. The surface proteins of PBMCs were biotinylated with Sulfo-NHS-LC-biotin and cell lysates were prepared. The biotinylated lysates were immunoprecipitated by mAb MT3. By using mAb MT3, a very large protein (M.W > 180 kDa) were precipitated in both reducing and non-reducing conditions (Figure 3.12). As control, the CD4 mAb clone MT4/2 precipitated a broad protein band at the molecular weight of 55 kDa corresponding to

CD4 protein. As the second control, CD4 mAb clone MT4 did not precipitate any protein. Moreover, under reducing condition, many non-specific precipitated proteins were found in all lanes. The results indicated that molecule recognized by mAb MT3 has molecular weight larger than 180 kDa.

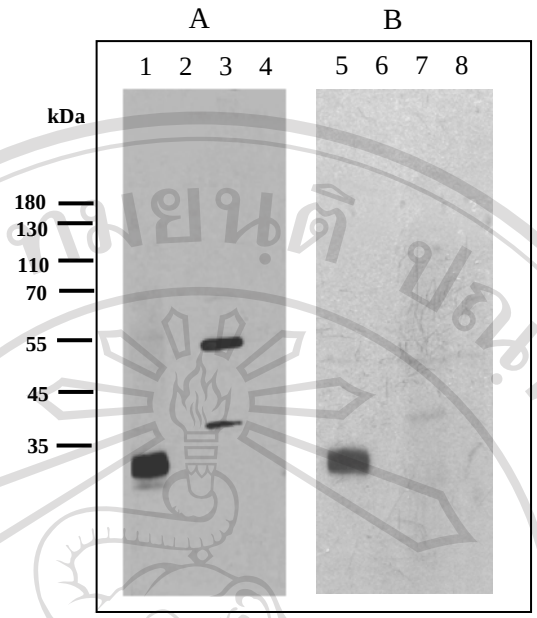

Figure 3.11 Western immunobloting analysis of cell surface molecules recognized by mAbs MT3 and COSA2A. PBMCs lysate were separated under non-reducing condition (A) and reducing condition (B) by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were reacted to CD99 mAb MT99/3 as positive control (lane 1 and lane 5), conjugated control (lane 2 and lane 6), mAb COSA2A (lane 3 and lane 7) and mAb MT3 (lane 4 and lane 8). The molecular mass markers are indicated on the left in kDa.

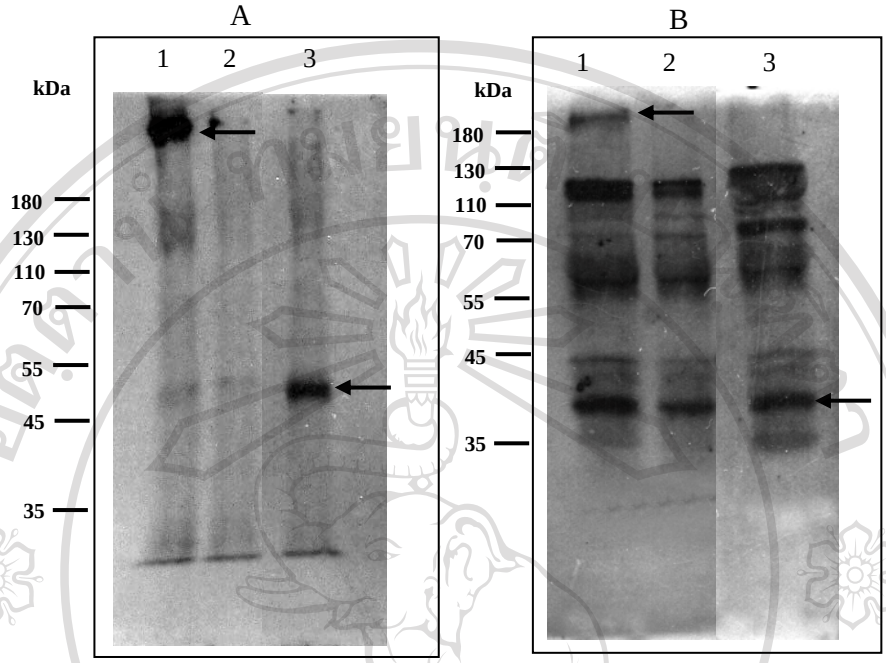

Figure 3.12 Immunoprecipitation for characterization of cell surface molecules recognized by mAb MT3. Immunoprecipitation was performed using biotinylated PBMCs lysates and precipitated by mAb MT3 (lane 1, arrow), CD4 mAb MT4 as isotype control (lane 2) and CD4 mAb MT4/2 as positive control (lane 3, arrow). Electrophoresis was performed under non-reducing (A) and reducing conditions (B). The molecular mass markers are indicated on the left in kDa.

3.6 Surface expression of MT3 and COSA2A molecules on PHA-activated PBMCs

To determine whether the molecule recognized by mAbs MT3 and COSA2A were up-regulated or down-regulated upon lymphocyte activation. PBMCs were activated with a mitogen, PHA, for 1 and 3 days. The activated PBMCs were then stained with mAbs MT3 and COSA2A by indirect immunofluorescence. As shown in Figure 3.13, the expression of MT3 molecules on PHA activated cells was reduced compare to the unstimulated cells. Similarly, after PHA activation, the expression of COSA2A molecule was reduced when compare with unstimulated staining cells (Figure 3.14). These results indicate that MT3 and COSA2A molecules are an activation associated molecules.

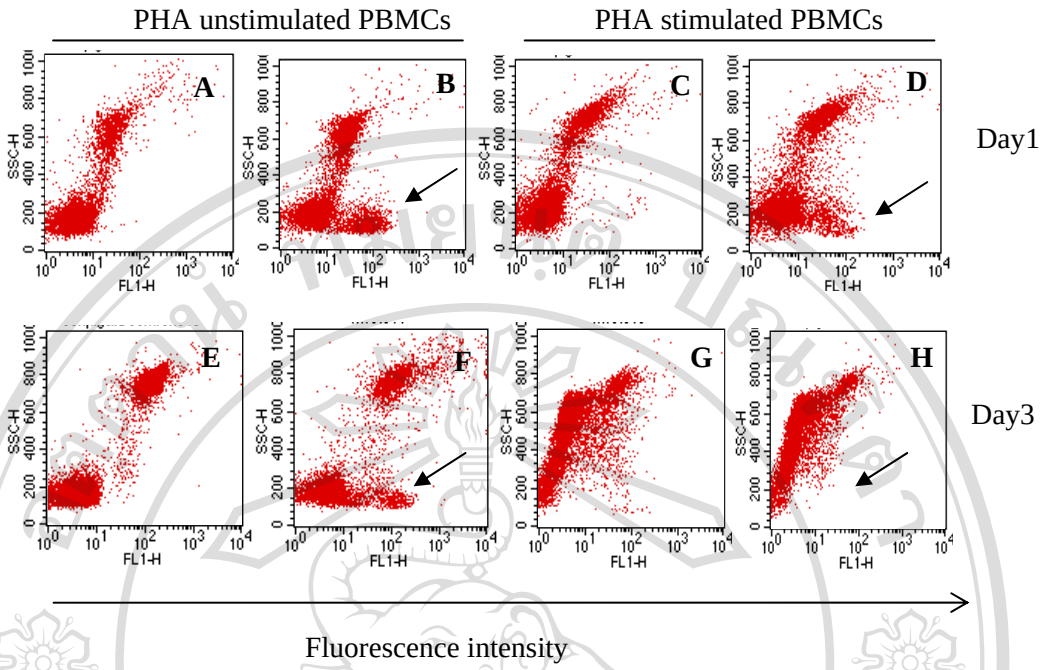

Figure 3.13 Expression of MT3 molecules on PHA-activated PBMCs. PBMCs were stimulated with 2.5 ug/ml of PHA. The expression of MT3 molecules on unstimulated PBMCs (B and F) and PHA activated PBMCs after 1day (D) and 3 days (H) of cultivation was examined by staining with mAb MT3 using indirect immunofluorescence and analyzed by flow cytometry. A, C, E and G were a conjugated control. The mAb MT3 positve cells were indicated by arrows. Data are representative of 3 independent experiments.

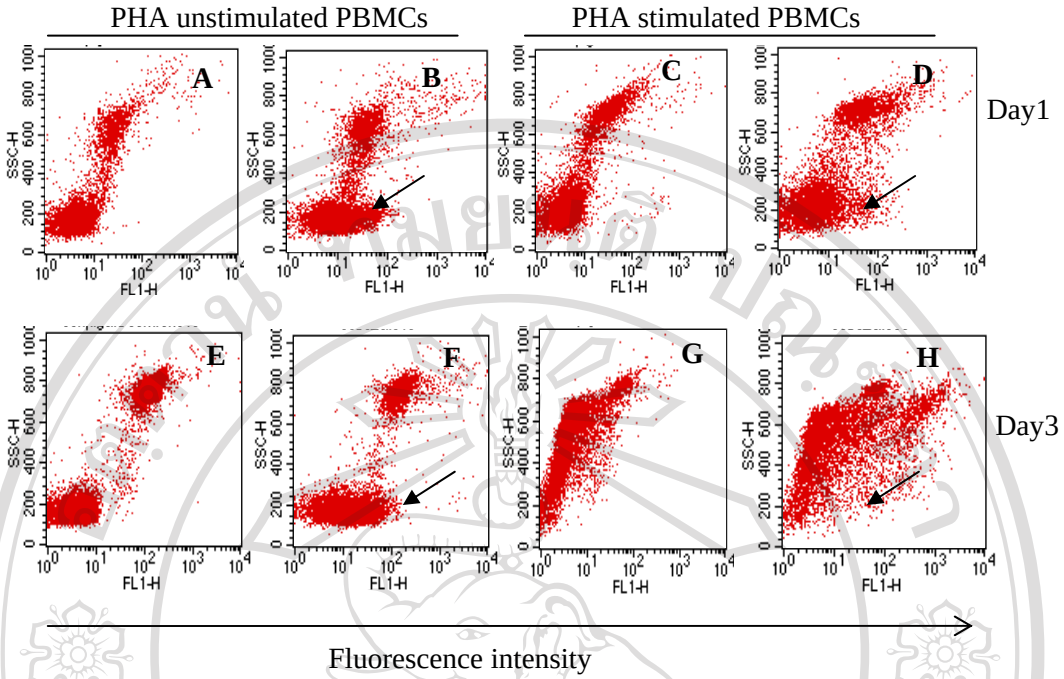

Figure 3.14 Expression of COSA2A molecules on PHA-activated PBMCs. PBMCs were stimulated with 2.5 ug/ml of PHA. The expression of COSA2A molecules on unstimulated PBMCs (B and F) and PHA activated PBMCs after 1 day (D) and 3 days (H) of cultivation was examined by staining with mAb MT3 using indirect immunofluorescence and analyzed by flow cytometry. A, C, E and G were a conjugated control. The mAb COSA2A positve cells were indicated by arrows. Data are representative of 3 independent experiments.

3.7 Regulation of anti-CD3 induced lymphocyte proliferation by mAbs MT3 and COSA2A

To determine the involvement of mAbs MT3 and COSA2A on the regulation of lymphocyte activation, anti-CD3 induced T lymphocyte proliferation in the presence or absence of the soluble mAbs MT3 or COSA2A were determined. As shown in Figure 3.15, 3.16 and 3.17, 3.18 in all studied cultivation times, mAb MT3 and COSA2A have no effect on anti-CD3 induced T lymphocyte proliferation. As predicted, isotype matched control mAb (FE-1H10) has also no effect on OKT3 induced T lymphocyte proliferation. In contrast, an inhibitory control mAb, M6-1B9, showed the inhibitory effect on anti-CD3 induced T lymphocytes proliferation.

PBMCs were also incubated with mAb MT3 and COSA2A without adding OKT3 mAb. As was shown in Figure 3.16 and Figure 3.18, mAb MT3 and COSA2A did not induce cell proliferation.

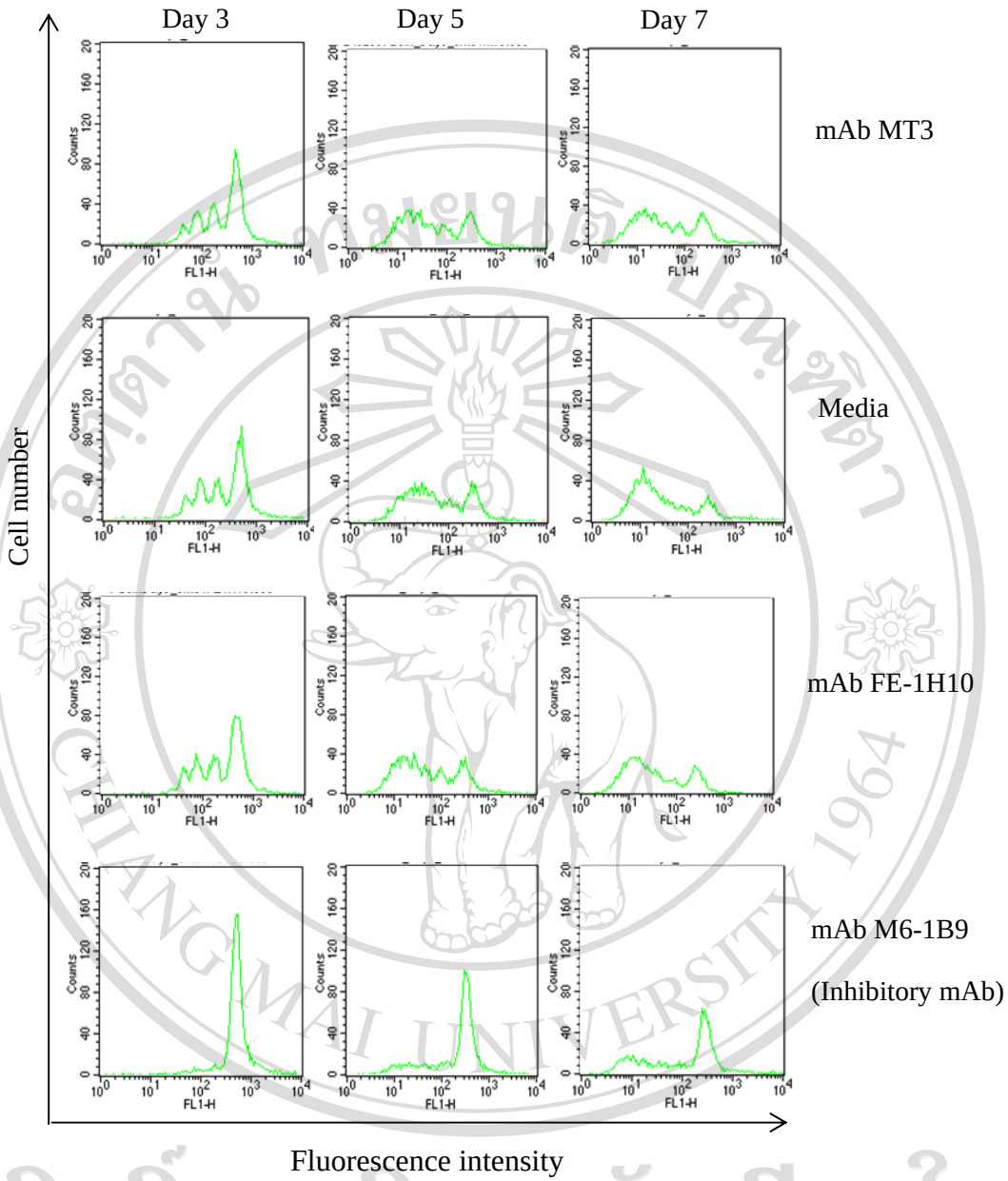

Figure 3.15 Effect of mAb MT3 on OKT3-induced T lymphocyte proliferation. CFSE labeled PBMCs were induced for cell proliferation by immobilized anti-CD3 mAb, OKT3 in the presence of mAbs including MT3, FE-1H10 (isotype matched control) and M6-1B9 (Inhibitory control) or absence of mAb. After day 3, 5 and 7 of cultivation, cells were harvested and determined for the reduction of fluorescence intensity by flow cytometry. The results were representative of 3 independent experiments.

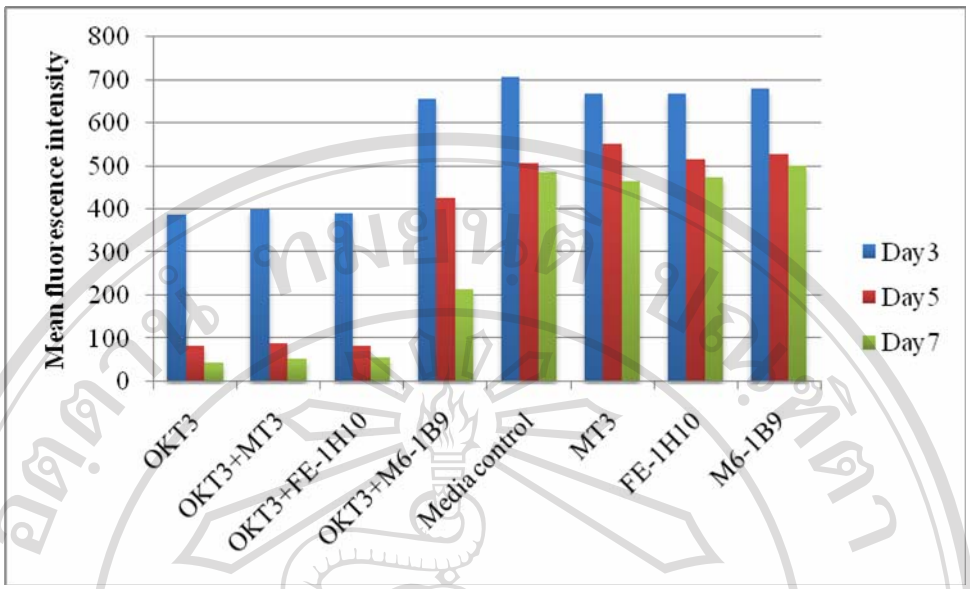

Figure 3.16 MT3 has no effect in T lymphocyte proliferation. PBMCs were activated with the immobilized mAb OKT3 (anti-CD3) at 60 ng/ml in the presence of 20 µg/ml of mAb MT3 or isotype matched control mAb (FE-1H10) or inhibitory control mAb (M6-1B9) or medium. The results also show the effect of mAb MT3 or isotype matched control mAb (FE-1H10) or inhibitory control mAb (M6-1B9) without OKT3 (anti-CD3) by incubating PBMCs with those mAb at 20 µg/ml into each. The results represent in mean fluorescence intensity of CFSE labeled T lymphocytes. The bars represent mean of 3 healthy donors.

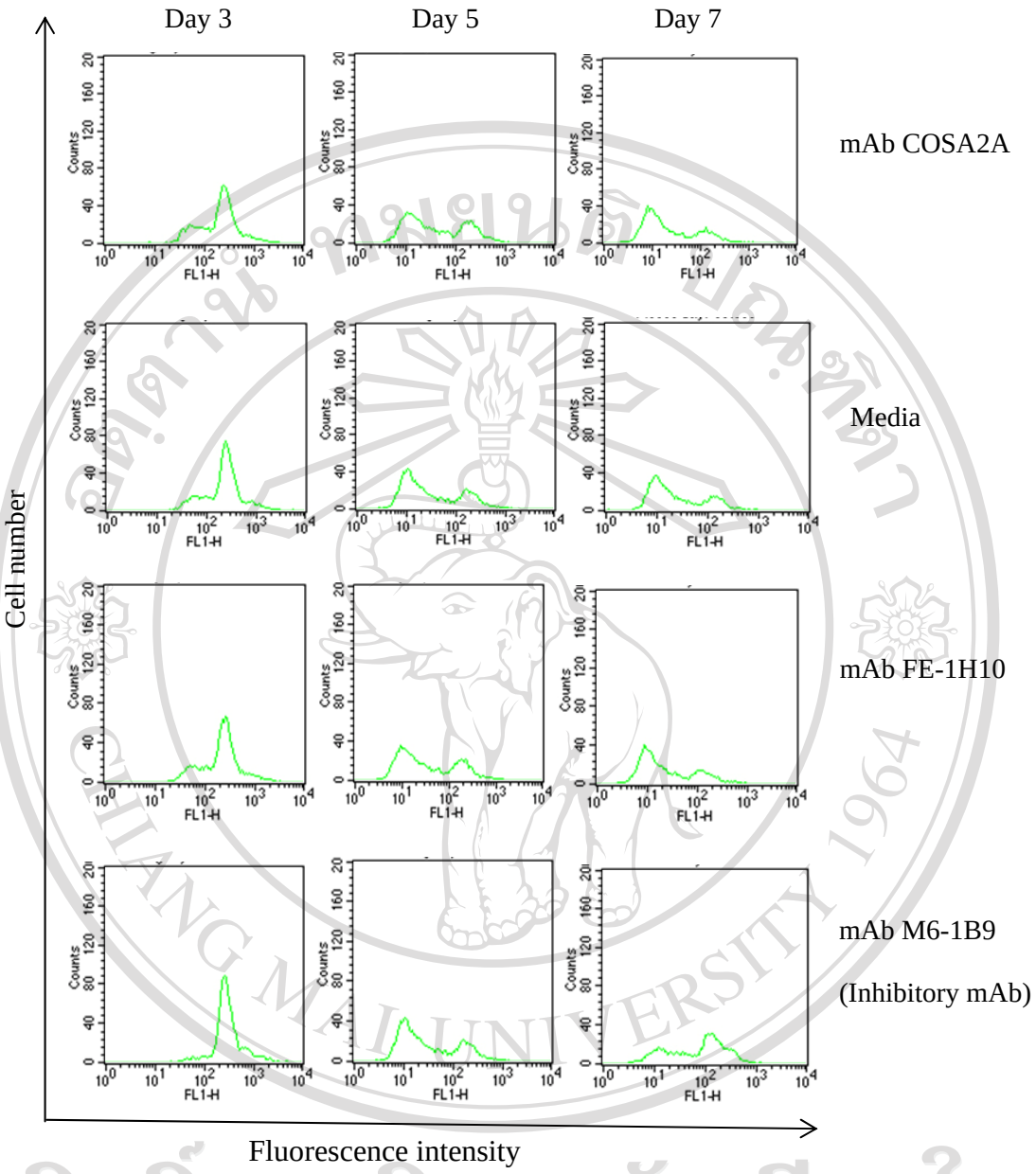

Figure 3.17 Effect of mAb COSA2A on OKT3-induced T lymphocyte proliferation. CFSE labeled PBMCs were induced for cell proliferation by immobilized anti-CD3 mAb, OKT3 in the presence of mAbs including COSA2A, FE-1H10 (isotype matched control) and M6-1B9 (Inhibitory control) or absence of mAb. After day 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 3 independent experiments.

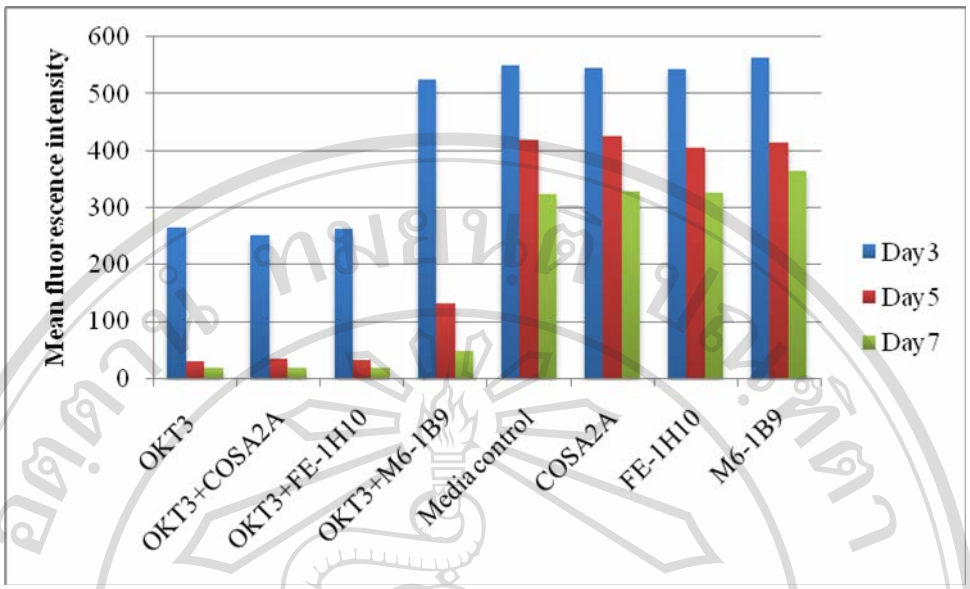

Figure 3.18 COSA2A has no effect in T lymphocyte proliferation. PBMCs were activated with the immobilized mAb OKT3 (anti-CD3) at 60 ng/ml in the presence of 20 µg/ml of mAb COSA2A or isotype matched control mAb (FE-1H10) or inhibitory control mAb (M6-1B9) or medium. The results also show the effect of mAb COSA2A or isotype matched control mAb (FE-1H10) or inhibitory control mAb (M6-1B9) without OKT3 (anti-CD3) by treating PBMCs with those mAb at 20 µg/ml into each. The results represent in mean fluorescence intensity of CFSE labeled T lymphocytes. The bars represent mean of 3 healthy donors.