CHAPTER IV

DISCUSSIONS AND CONCLUSIONS

In an attempt to identify new leukocyte surface molecules that involve in the function of T lymphocytes, several monoclonal antibodies to leukocyte surface molecules were produced in Prof. Dr. Watchara Kasinrerk's laboratory at the department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. By immunoprecipitation based immunization, CD3 monoclonal antibodies were coated on Separose beads and used to precipitate CD3 complex from T lymphocytes. The immunoprecipited proteins were immunized and monoclonal antibodies were produced. By this strategy, a monoclonal antibody termed MT3 was produced. Preliminary studies indicated that the mAb MT3 reacted to a population of lymphocytes. In addition to the mAb MT3, by immunization of monkey kidney COS7 cells, a mAb named COSA2A was generated. This mAb was also reacted to a population of lymphocytes. Thus, both mAbs, MT3 and COSA2A, were interested and selected for further investigations. Perhaps, the molecule recognized by each mAb can be a novel undefined lymphocyte surface molecule.

In this study, hybridoma clones MT3 and COSA2A were propagated and used to induce ascitic fluids in Balb/c mice for further large-scale production of the monoclonal antibodies. The ascitic fluid containing mAbs MT3 and COSA2A were confirmed to have the mAb activity by indirect immunofluorescence staining. Then the produced ascites were purified by affinity chromatography. The SDS-PAGE analysis was performed for checking the purity of both mAbs. The results indicated

that the purified mAb MT3 was separated into two bands of heavy chain and light chain under reducing conditions. While only one band of intact immunoglobulin was observed under non-reducing conditions. Surprisingly, only one protein band that expected to be light chain was found in the purified mAb COSA2A. The activities of both purified mAbs were re-checked by indirect immunofluorescence staining. The purified mAb MT3 and COSA2A showed the same reactive patterns as was previously observed (unpublished observation). Although, SDS-PAGE results indicated that the purified mAb COSA2A contains only light chain, it still remained a good antibody binding activity. As antigen binding sites of antibodies are formed by the variable regions of light and heavy chains, of antigen interactions with the individual light and heavy chain of antibodies was demonstrated (Hopper and Papagiannes, 1986, Stevens *et al*., 1991). Light chains purified from polyclonal antibodies could display binding activity. In addition, dimers of light chain secreted by myeloma cells are known to bind haptens. Mei and Lan demonstratedthe binding properties of a light chain purified from a monoclonal antibody to VIP (vasoactive intestinal polypeptide) (Sun *et al*., 1994). They observed that this light chain displays sequence-specific and high affinity binding of VIP (Sun *et al*., 1994). These reports indicated that only light chain still remained binding activity to specific antigen. As the purified of both mAbs, MT3 and COSA2A, have antigen binding activity, the obtained purified mAbs were then used in further experiments for characterization of its recognized molecules.

The expression of the molecules recognized by mAbs MT3 and COSA2A on various cell types were determined by indirect immunofluorescence staining and flow cytometry.

In the study of mAbs MT3, this mAb specifically reacted to human T cell lines, SupT1 and Molt4, but not with other cell lines, K562, U937 and Daudi cells. Moreover, this mAb reacted with a sub-population of lymphocytes, but not with other leukocytes. Then, the expression of MT3 molecule on lymphocyte sub-populations was determined. In this study, CD19, CD56 and CD3 molecules were used as markers for B lymphocytes, NK cells and T lymphocytes, respectively. The MT3 molecule was demonstrated to express on a population of T lymphocytes ($CD3^+$ cells) but not on B and NK cells. Exclusive expression of molecule MT3 on T lymphocytes is in concordance with its expression on only T cell lines. From the obtained results, we concluded that mAb MT3 recognizes a molecule express on a population of T lymphocytes. As it recognized a population of $CD3^+$ cells, we would emphasize here that the MT3 molecule is not CD3 molecule.

As T lymphocytes can be divided into 2 sub-populations, $CD4^+$ and $CD8^+$ lymphocytes, depend on the expression of CD4 and CD8 molecules. We therefore raised the question whether MT3 molecule is CD4 and CD8 molecules. To address this question, expression of MT3 molecules on $CD4^+$ and $CD8^+$ lymphocytes were elucidated. The results indicated that the molecule recognized by mAb MT3 expressed on a population of either $CD4^+$ T lymphocyte and $CD8^+$ T lymphocyte. These data suggested that mAb MT3 recognized neither CD4 nor CD8 molecules, but

may be a novel surface molecule. By using mAb MT3, a new sub-population of T lymphocytes can be identified.

Surprisingly, MT3 molecule also express on RBCs membrane. As CD3, CD4 and CD8 molecules are restrictively expressed on T lymphocytes, the MT3 molecule therefore is not CD3, CD4 or CD8 proteins. On red blood cells, however, quantitative polymorphic expression of the MT3 molecules was observed. Majority of tested RBCs express MT3 molecules, but few showed very weakly expression. The expression of MT3 on RBCs may linked to the expression of other RBC antigens as was observed with CD99 molecule (Kasinrerk *et al*., 2000; Khunkaewla *et al*., 2007).

In the study of mAbs COSA2A, we found that mAb COSA2A reacted with a sub-population of lymphocytes, but showed negative reactivity with all other tested leukocytes and RBCs and also negative with all tested hematopoietic cell lines. In addition of T lymphocytes, the mAb COSA2A was reacted with a small population of B lymphocytes and NK cells. Both subsets of T lymphocyte include $CD4^+$ T lymphocyte and $CD8^+$ T lymphocyte, expressed molecule recognized mAb COSA2A. These data indicated that mAb MT3 and COSA2A recognized different molecules.

CD45 was first reviewed by Thomas in 1989 and by Trowbridge and Thomas in 1994 (Thomas, 1989; Trowbridge and Thomas, 1994; McNeill *et al*., 2004). CD45 was the prototypic receptor-like protein tyrosine phosphatase (PTP) and an essential regulator of signal transduction pathways in immune cells. CD45 plays an important function role in immune cells by regulation of phosphatase activity. CD45 is one of the most abundant cell surface glycoproteins, comprising up to 10% of the cell surface

area. On cell surface, the CD45 extracellular domain is expressed as multiple isoforms. The alternatively spliced isoforms of CD45 differentially homodimerize in primary T lymphocytes (Trowbridge and Thomas, 1994; Hermiston *et al*., 2003). Five isoforms have been reported as proteins expressed at significant levels in human or murine lymphocytes: CD45RABC, CD45RAB, CD45RBC, CD45RB and CD45RO (McNeill *et al*., 2004). The smallest isoform RO dimerizes are expressing on memory T lymphocytes. CD45RA and RB are expressed on naïve T lymphocytes (Hermiston *et al*., 2003). In this study, we determined whether MT3 and COSA2A molecules were expressed on naïve or memory T lymphocytes. Two color staining and flow cytometry was performed to identify mAbs MT3 and COSA2A recognized molecules on memory or naïve T lymphocytes. The results indicated that MT3 and COSA2A molecules were expressed on naïve T lymphocytes.

To understand more detail on MT3 and COSA2A molecules, its biochemical characteristics were verified. The molecular weight of MT3 and COSA2A molecule were studies by Western immunoblotting analysis. The PBMC lysates were separated by SDS-PAGE under reducing and non-reducing conditions and Western immunbloting was used to determine molecular weight. In reducing condition, 2-mercaptoethanol (2-ME) was added to the protein lysate. 2-ME is a reducing agent that destroys covalent bonds such as disulfide bond. Therefore, under this condition proteins were straightened by reducing its disulfide bond and migrate slower than in the non-reducing condition. In this study, mAb COSA2A reacted with two major bands of proteins with the molecular weight at 55 kDa and 36 kDa under non-reducing condition. The mAb COSA2A, however, did not react to any protein band

under reducing condition. These data indicated that mAb COSA2A recognized an epitope which disappear after disruption of disulfide bond. In another hand, it may indicate that the COSA2A molecule consist of intramolecule disulfide bond(s). Two reactive protein bands observed on Western immunoblotting indicated that the COSA2A molecule, in its native form, may form homodimerization. This speculation is needed to be further attested. In contrast to COSA2A, mAb MT3 did not react to any protein band on both non-reducing and reducing condition indicating the mAb MT3 recognize a conformational epitope.

To further determine the molecular weight of MT3 molecule, immunoprecipitation was employed. The results showed that mAb MT3 precipitated a protein band with a molecular weight that larger than 180 kDa under both reducing and non-reducing conditions. However, one can observe that the precipitated protein is larger under non-reducing condition compare to reducing condition. The results suggested that MT3 molecule may associated with other molecule. As the mAb MT3 was produced by immunization of mouse with the precipitated proteins obtained from CD3 mAb precipitation, it can be that the MT3 molecule is a novel protein in the TCR-CD3 complex. This speculation is really needed to be further confirmed.

When T lymphocyte activation, several events are occurred. These include the changes of some surface molecule expression. Some leukocyte surface molecules were up regulated upon T lymphocyte activation, such as CD25 (IL-2 receptor) (Kasinrerk *et al*., 1999), CD147 (Kasinrerk *et al*., 1999) and CD54 (Alegre *et al*., 2001; Rossmann *et al*., 2003). By the way some molecules may be down-regulated expression level including chemokine receptors such as CXCR4 (Colantonio et al.,

2002) and CCR6 (CD186) (Colantonio *et al*., 2002; Koprak *et al*., 2003). The expression of MT3 and COSA2A molecule on mitogen-stimulated PBMC were therefore investigated. In this study, PHA was used to activate lymphocytes. PHA is a mitogen that cross-links human T lymphocyte surface molecules including TCR, therefore, it induces polyclonal activation of T lymphocytes. We found that that expression of MT3 and COSA2A molecules on T lymphocytes were reduced after PHA activation. This data suggested that the MT3 and COSA2A molecules are activation associated molecules of T lymphocytes. The down regulation of these molecules on activated lymphocytes may involve in the regulation of lymphocyte functions, either in enhancing and reducing cell function.

After antigen activation, T lymphocytes will be activated via CD3-TCR complexes. Activation of CD3-TCR complex cause cell activation which are often associated with the expression newly surface proteins, cytokines production and proliferation (MacDonald and Nabholz, 1986; Straus and Weiss, 1993). On the other hand, instead of antigen activation, CD3 mAb can be used as polyclonal activators of T lymphocytes. To study the involvement of MT3 and COSA2A molecules on the regulation of T lymphocyte activation, in this study, the effect of mAbs MT3 and COSA2A on anti-CD3 induced T lymphocyte proliferation was carried out. CD3 mAb clone OKT3 was used to activate T lymphocyte proliferation in the presence and absence of mAbs MT3 or COSA2A. The results indicated that neither mAbs MT3 nor COSA2A have effect on OKT3 induced T lymphocyte proliferation. From these results, it can be roughly concluded that engagement of MT3 and COSA2A molecules with mAbs MT3 and COSA2A did not effect on anti-CD3 induce T lymphocytes

proliferation. However, the effect of these mAbs on cytokines production still does not investigate. Involvement of MT3 and COSA2A on regulation of T lymphocyte activation are also one point that have to be done in the further studies.

In conclusion, in this study, various monoclonal antibodies against leukocyte surface molecules were produced. Among the produced mAbs, two mAbs named MT3 and COSA2A were found to be of interest as it reacted with a sub-population of lymphocyte. In this study, mAbs MT3 and COSA2A and its recognized molecules were characterized. Monoclonal antibody MT3 reacts with a sub-population of T lymphocytes. This lymphocyte sub-population seems to be a new T lymphocyte sub-population. Whereas, mAb COSA2A was able to react with a population of T lymphocytes, B lymphocytes and NK cells. Interestingly, the expression of MT3 and COSA2A molecules on T lymphocytes were reduced after PHA activation. Functional studies indicated that both mAbs MT3 and COSA2A did not effect on anti-CD3 induce T lymphocyte proliferation. For more characterization details, including amino acid sequencing, of these molecules are under examined.