CHAPTER IV

DISSCUSSION & CONCLUSION

CD4 molecule is a leukocyte surface glycoprotein expressed on various cells types, including T lymphocytes and monocytes (Beare *et al*., 2008; Littman, 1996). On T lymphocytes, the best known for CD4 function is to interact and stabilize TCR interactions with peptide–MHC II complexes on APC during the induction of adaptive immunity, and mediation of intracellular T cell signaling (Beare *et al*., 2008; Littman, 1996). Upon T lymphocyte activation, large multiprotein clusters of CD4, TCR and CD3 are localized to lipid rafts and provide a focal point for intracellular signaling, through TyK, Lck interactions with the cytoplasmic tail of CD4, CD3 phosphorylation, recruitment of ZAP-70, Lck autophosphorylation and PKC feedback serine phosphorylation of Lck and CD4. However, little is known about the function of CD4 on monocytes. Several forms of CD4 molecules are reported to be expressed on cell surfaces (Beare *et al*., 2008; Moldovan *et al*., 2006; Lynch *et al*., 1999). Lynch *et al*. (2006) recently reported that the CD4 proteins of lymphocytic and monocytic cells are significantly different. CD4 expressed on monocytes is predominantly as 110 kDa covalent homodimers, but in lymphocytes mainly as 55 kDa monomers (Lynch *et al*., 1996; 1999). Oligomers of CD4 molecule expressed on cell surface have also been described, which involve noncovalent dimers of D1 CDR-3 loops (Briant *et al*., 1997; Langedijk *et al*., 1994), covalent disulfide dimers of D2 (Matthias *et al*., 2002), and noncovalent (Wu *et al*., 1997) or disulfide-mediated covalent 110 kDa

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homodimers of D4 (Lynch *et al*., 1996; 1999; 2003). According to its different structures expressed on lymphocytes and monocytes, we and other speculate that CD4 on monocytes may play an important role that differ from its function on lymphocytes. In order to determine the function of CD4 expressed on monocytes, a set of anti-CD4 mAbs are required. We, therefore, decided to generate anti-CD4 mAbs. In our studies, three different CD4 immunogens, including native CD4 proteins, recombinant CD4 proteins expressed by mammalian COS cells and *E. coli* were produced. The three CD4 protein types were used as immunogens for immunization of mice for production of anti-CD4 mAbs. In our experiments, four clones of anti-CD4 mAbs, namely MT4, MT4/2, MT4/3, and MT4/4, were generated (Pata *et al*., 2009). The produced anti-CD4 mAbs were then employed for the functional analysis of the CD4 molecule.

In this report, the generated hybridomas producing anti-CD4 mAbs were propagated and tested for specificity using culture supernatants obtained from the propagated hybridomas reacting with COS transfected with cDNA encoding CD4 and CD8 molecules. The results indicated that anti-CD4 mAb clones MT4, MT4/2, and MT4/3 specifically reacted to CD4 expressing COS cells, but not to CD8 expressing COS cells. While anti-CD4 mAb clone MT4/4 did not react to both CD4 and CD8 expressing COS cells, these results are corresponding with the results of Pata *et al*. (2009). Since anti-CD4 mAb clone MT4/4 was prepared from the immunization of BALB/c mice with recombinant CD4 protein produced from *E. coli*, by which bacterial expression systems do not have the ability to carry out normal post-translational modifications such as intra- and inter-chain disulfide bond formation, signal peptide cleavage, and addition of O- and N-linked carbohydrates (Seed, 1995).

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The recombinant CD4 produced from *E. coli* may have a structure that is different from the native CD4 expressed on mammalian cells. By immunization with the *E. coli* recombinant protein, the produced anti-CD4 mAbs may react to the epitope that does not exist or hidden in the native CD4 molecule. As anti-CD4 mAb clone MT4/4 did not react to the native CD4 molecule, this mAb was discarded from our study. Hence, anti-CD4 mAb clones MT4, MT4/, and MT4/3 were selected in further studies.

For employing mAbs for functional study of leukocyte surface molecules, large amount and purified mAbs are required. In this study, in order to produce large amount of anti-CD4 mAbs, hybridoma clones MT4, MT4/2, and MT4/3 were propagated and used to induce ascitic fluids in BALB/c mice (Harlow and Lane, 1988). Then, the produced ascitic fluids were subjected for mAb purification by affinity chromatography. The SDS-PAGE analysis was performed for checking the purity of obtained purified mAbs. The results showed that the purity of all purified anti-CD4 mAbs were satisfactory. The specific reactivity of purified anti-CD4 mAbs were then checked by reacting to CD4 and CD8 transfected COS cells (Kasinrerk and Tokrasinwit, 1999). All purified anti-CD4 mAbs showed the specific reactivity to CD4 expressing COS cells. Afterward, the purified mAbs were further determined for their reactivity by immunoprecipitation. In the study, the purified anti-CD4 mAb clones MT4, MT4/2, and MT4/3 precipitated a protein of 55 kDa corresponding to CD4 protein under reducing condition (Zola *et al*., 2007). From our several experiments, it was firmly indicated that the obtained purified anti-CD4 mAb clones MT4, MT4/2, and MT4/3 are pure and specifically react to CD4 molecule. They, therefore, could be used in the further experiments.

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The epitope recognition of anti-CD4 mAb clones MT4, MT4/2, and MT4/3 were determined by cross blocking analysis (Chiampanichayakul *et al*., 2006). In this study, PBMCs were pre-stained with unlabeled anti-CD4 mAbs (MT4, MT4/2, and MT4/3) and counterstained with all FITC-conjugated anti-CD4 mAbs. The stained cells were then analyzed by a flow cytometer. Inhibition of FITC conjugates by the un-labeled mAbs was calculated. The results showed that 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 suggest that anti-CD4 mAb clones MT4 and MT4/2 may react to the same or adjacent epitope of CD4 molecule. Whereas, anti-CD4 mAb clone MT4/3 reacts to the un-related epitope. In previous study, the cross blocking experiments have been sucessfully employed for the determining epitope recognized by mAbs of interest (Chiampanichayakul *et al*., 2006). Anti-CD4 mAbs that recognized different epitopes on the CD4 molecule have been reported (Lynched *et al*., 2006). Engagements of these anti-CD4 mAbs may induce different CD4+ lymphocyte function.

The purified mAbs MT4, MT4/2, and MT4/3 were then employed for the determination of CD4 expressed on lymphocytes and monocytes. We wondered if our generated mAbs react to the CD4 epitope that differently expressed between lymphocytes and monocytes. To do this, we first determined the saturated concentration of anti-CD4 mAb clones MT4, MT4/2, and MT4/3 in reacting to CD4 molecule. PBMCs were stained with various concentrations of purified mAbs MT4, MT4/2, and MT4/3 by indirect immunofluorescent staining and analyzed by flow cytometry. The results showed that the saturated concentration of purified anti-CD4 mAbs clones MT4 and MT4/3 was 200 μg/ml. For the MT4/2 mAb the saturated

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concentration was seem to be over 400 μg/ml. In this study, however, 200 μg/ml of all mAbs was used for determining the reactivity of anti-CD4 mAbs on lymphocyte and monocyte surfaces. The saturated concentration (200 μg/ml) of mAbs is concerned because we want to make sure that there have been the excess mAbs during cell staining. This is therefore ensured that during CD4 surface staining, all CD4 molecule expressed will be occupied by the stained mAbs.

Purified MT4, MT4/2, and MT4/3, at the saturated concentration, were used to stained PBMCs obtained from 20 healthy donors by indirect immunofluorescent method. The results showed that three clones of anti-CD4 mAbs reacted to CD4 molecules on lymphocyte and monocyte surfaces in different manners. Anti-CD4 mAb MT4 reacted to CD4 molecule on lymphocyte surfaces strongly, but very weakly react to CD4 molecule on monocytes. While, anti-CD4 mAb MT4/3 strongly reacted to CD4 molecules on both lymphocytes and monocytes. The reactivity of mAb MT4/3 is the same of other anti-CD4 mAbs reported anywhere. For anti-CD4 mAb clone MT4/2, it reacted to CD4 molecules on lymphocytes and monocytes with very weakly reaction. This mAb may has low affinity. It also can be that the epitope recognized by this mAb is hidden in the CD4 molecule. Concerning to the reactivity of mAb MT4 which strongly react to CD4 on lymphocytes, but weakly or not at all to CD4 expressed on monocytes, indicated that MT4 is a unique anti-CD4 mAb which never been repoted. We, so far, have no experimental results that bear on the question of why mAb MT4 fails to bind to monocytes. It is possible to speculate that mAb MT4 may be binding to a particular epitope of CD4 molecule which is exposed on lymphocytes but is sterically or conformationally obstructed on monocytes. The epitope variable, perhaps due to conformation or to protein association differences

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between CD4 on lymphocytes and monocytes was suggested (Lynch *et al*., 2006). The different binding patterns between mAbs MT4 and MT4/3 to CD4 expressed on monocytes may not be due to of its different affinity. The reactivity of mAb MT4 was observed even higher than those of MT4/3 when staining with CD4 molecule expressed on lymphocytes. This therefore can not be implied that mAb MT4 has low affinity, so it fails to identify the low CD4 expression on monocytes. From the obtained results, anti-CD4 mAb clone MT4 reacted to CD4 molecule expressed on lymphocytes but did not react to CD4 molecule expressed on monocytes, it is concluded that CD4 molecule expressed on lymphocyte surfaces is different from those expressed on monocyte surfaces. This difference can be identifying by the MT4 mAb. When the % CD4+ lymphocytes were determined using the mAb MT4, MT4/2, and MT4/3. In all 20 tested donors, the percentage of CD4+ cells obtained by all mAbs is not different. These results indicated that all anti-CD4 mAbs can be to enumeration of CD4+ lymphocytes. However, as mention above, they react to CD4 molelcules on lymphocytes and monocytes with different pattern.

After antigen activation, T lymphocytes will be activated via CD3-TCR complexes. Activation of CD3-TCR complex causes cell activation which is often associated with the expression of new surface proteins, cytokine production and cell proliferation (MacDonald and Nabholz, 1986; Straus and Weiss, 1993). On the other hand, instead of using antigen for activation, anti-CD3 mAb can be used as a polyclonal activator of T lymphocytes. Although the function of CD4 molecule on lymphocytes has been well characterized, the function of CD4 molecule 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

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of CD4 molecule. In this study, the effect of anti-CD4 mAb clones MT4, MT4/2, and MT4/3 on anti-CD3 induced PBMC proliferation was carried out. PBMCs were activated with anti-CD3 mAb in the presence or absence of mAbs MT4, MT4/2, and MT4/3. It was found that anti-CD4 mAbs clones MT4 and MT4/2 have no effect on anti-CD3 induced lymphocyte proliferation. While MT4/3 has inhibitory effect on anti-CD3 induced lymphocyte proliferation. As anti-CD4 mAb clone MT4/3 reacted to CD4 molecules on both lymphocyte and monocyte cell surfaces, we therefore raised the question whether CD4 molecule expressed on monocytes may responsible for the observed inhibitory effect. To address this question, monocytes were depleted from PBMCs by cell adhesion. By this procedure, as was monitor by CD14+ cell staining, the monocytes remained less than 3%. Then, the effect of anti-CD4 mAbs MT4, MT4/2, and MT4/3 on anti-CD3/CD28 induced monocyte-depleted lymphocyte proliferation was investigated. The results showed that all tested anti-CD4 mAbs did not have any effect on anti-CD3/CD28 induced monocyte-depleted lymphocyte proliferation. Since anti-CD4 mAb MT4/3 could suppressed PBMC proliferation, which contain lymphocytes and monocytes, but did not has inhibitory effect on monocyte-depleted lymphocyte (contain only lymphocytes) proliferation. From these results, it can be concluded that CD4 molecule expressed on monocytes play a role in the suppression of lymphocyte activation. Different function of CD4 molecules on T lymphocytes and monocytes is speculated. Tyrosine kinase, Lck is associated to the CD4 molecule expressed on T lymphocytes. However, this kinase is lacking in CD4 of non-lymphocytic cells (Rudd *et al*., 1988; Foti *et al*., 1997; Pelchen-Matthews *et al*., 1998; Lynch *et al*., 2006). CD4 signaling may occur in monocytic cells, but perhaps through the interaction of other tyrosine kinase. A tyrosine kinase, Hck, was

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demonstrated to be associated with CD4 in monocytic cells, but absent in lymphocytic cells (Lynch *et al*., 2005). Hck may responsible for the signal transduction via CD4 molecule on monocytes.

As CD4 expressed on monocytes was demonstrated to be involved in regulation of lymphocyte proliferation, we further investigated the role of CD4 on other monocyte function. Monocytes are one of the professional phagocytic cells in our body and oxidative burst is important function of monocytes in order to kill the phagocytosed pathogens. In this study, the effect of anti-CD4 mAbs on the induction of oxidative burst in monocytes was determined. It was found that none of the tested anti-CD4 mAbs induced monocyte oxidative burst. These results indicated that although CD4 on moncytes involve in the regulation of lymphocyte activation, it does not involve in other function, such as oxidative burst.

In conclusion, in this studies, we confirmed that the expression of CD4 molecule on lymphocyte and monocyte surfaces are different. Since anti-CD4 mAb clone MT4 recognizes CD4 molecule expressed on lymphocytes but does not react to CD4 molecule on monocytes, this mAb is unique and can be used for differentiate the difference of CD4 molecule. By using the generated anti-CD4 mAbs, we demonstrated the first time that CD4 molecule expressed on monocytes play a role in the suppression of lymphocyte activation. The engagement of CD4 on monocytes with a certain mAb induce an intracellalur signaling that activated the production of certain lymphocyte regulatory monokines, such as IL-10. The details mechanism of CD4 molecule expressed on monocytes in the suppression of lymphocyte activation is of interest for further investigation.

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