CHAPTER IV

DISCUSSION AND CONCLUSION

CD147 molecule is a leukocyte surface molecule, which was clustered as CD147 molecule at the 6th International Workshop on Human Leukocyte Differentiation Antigen (HLDA workshop) (Stockinger *et al.*, 1997). This molecule also known as M6 Ag (Kasinrerk *et al,* 1992), extracellular matrix metalloproteinase inducer (Biswas *et al,*. 1995) or basigin (Miyauchi *et al.*, 1991), is a 50-60 kDa type I transmembrane glycoprotein belonging to the immunoglobulin superfamily. CD147 is widely expressed on hemopoietic and non-hemopoietic cells. It is strongly up-regulated on T cells upon activation, indicating a function in T cell biology (Kasinrerk *et al.*, 1992, Stockinger *et al.*, 1997). Function of CD147 molecule, however, is so far un-clear. Concerning in the regulation of T cell responses, Kirsch *et al.* (1997) showed that mAbs specific to CD147 molecule had no effect on T cells proliferation. However, Koch *et al* (1999) demonstrated that a anti-CD147 mAb, MEM-M6/6, has capability to inhibit human T cell proliferation in allogeneic mixed lymphocyte responses as well as in T cell receptor (TCR)/CD3 plus CD28 driven highly purified T cell cultures. The regulation of T cell proliferation via CD147 molecule is therefore controversial. At the Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University, Dr. Watchara Kasinrerk had generated 6 mAbs specific to CD147 molecules (Kasinrerk *et al.*, 1999; un-published observations). As several anti-CD147 mAbs were available, in the present study, the function of CD147 molecule in the regulation of T cell responses was investigated.

In this study, purified anti-CD147 mAbs were first prepared for further used in the functional analysis. To produce the anti-CD147 mAbs, the ascitic fluid containing anti-CD147 mAbs were induced in Balb/c mice by inoculation of six of hybridoma clones including M6-2F9, M6-2B1, M6-1F3, M6-1D4, M6-1B9 and M6-1E9. Anti-CD147 mAbs presented in ascitic fluids were purified by affinity chromatography. MAbs M6-1B9 and M6-1E9, which are IgG isotype, were purified by Protein A coated Sepharose column (Harlow and Lane, 1988). Protein A is an approximately 42 kDa polypeptide derived from cell wall of certain strains of β-hemolytic

Streptococci aureus (Tebbutt *et al.*, 1976; Harlow and Lane, 1988). Protein A can bind Fc region of IgG isotype, but not IgM, of difference species including human, mouse, rat, rabbit and horse (Nilsson *et al.*, 1976; Harlow and Lane, 1988). This protein has four potential binding sites for antibodies; however, only two of them can be used at one time (Harlow and Lane, 1988). Binding of Protein A to Ig can be separated with lowering the pH of the buffer (Harlow and Lane, 1988). As mAbs M6-2F9, M6-2B1, M6-1F3 and M6-1D4, which are IgM isotype, they can not bind Protien A sepharose column. Sepharose coated with rat monoclonal antibody specific to mouse IgM were therefore used in the purification process.

Before using the purified anti-CD147 mAbs, the activity and specificity of the obtained purified mAbs was confirmed by immunofluorescent staining assay. All purified anti-CD147 mAbs reacted to U937 cells which expressed CD147 molecules on their surface (Kasinrerk *et al.*, 1999) indicating all purified anti-CD147 mAbs still have its binding activity. MAbs M6-2B1 and M6-1F3 showed lower positive reactivity compare to others, indicating the low binding affinity. During immunofluorescent staining process, the low affinity antibodies are removed during the washing steps and, therefore, showed weak or negative staining results (Koch *et al.*, 1999). The purified anti-CD147 mAbs were further proved for their specificity by staining with the CD147 expressing COS cells. As expected, all purified anti-CD147 mAbs reacted to CD147 expressing COS cells but not to mock transfectants. These results indicated that the purified anti-CD147 mAbs were antibodies directed against CD147 molecule.

Epitope mapping analysis could be explained whether antibodies react to the same or different epitopes on the antigen molecule. Several methods can be for this purpose, such as real-time biospecific interaction analysis (Koch *et al.*, 1999), saturation transfer difference NMR spectroscopy (Murata *et al.*, 2003), binding to synthetic peptides (Kovacs-Nolan *et al.*, 2003) and cross-blocking analysis (Earl *et al.*, 1997; Tuckwell *et al.*, 2000; Hou *et al.*, 2001). In the present study, the cross-blocking analysis was carried out. The purified anti-CD147 mAbs were first conjugated with a fluorochrome, FITC. FITC, an fluorescent compound, has maximal excitation wavelength at 492 nm and emission light at 516-525 nm (Diamadis and Christopoulos, 1996). Several methods have been described for labeling FITC to protein of interest (Feteanu, 1978; Harlow and Lane, 1988; Diamadis and Christopoulos, 1996; Hemmila, 1991). In this study, the isothiocyanate labeling method, which coupled with FITC through S-C-N group, was used

(Feteanu, 1978). By this method, the isothiocyanate compounds bind to protein at pH 9.0-9.5 in aqueous solution with minimum loss of antibody activity. After labeling, the FITC-conjugated anti-CD147 mAbs were then titrated for optimal concentration for further cross-blocking experiment. U937 cells were stained with various concentrations of FITC-conjugated anti-CD147 mAbs and analyzed by flow cytometry. The results indicated that the concentration of 40 μg/ml of each FITC-conjugated anti-CD147 mAb was shown to be the saturated concentration. This concentration was therefore selected for cross-blocking analysis in epitope mapping experiments. As predicted, mAbs M6-2B1 and M6-1F3, which have low binding affinity, showed low fluorescent positive intensity at concentration of 40 μg/ml. The low fluorescent positive intensity of FITC-conjugated antibody can create erroneous cross blocking results.

In our cross blocking experiment. U937 cells were incubated with un-labeled anti-CD147 mAbs and subsequently with FITC-conjugated anti-CD147 mAbs. The cross-inhibition of one mAb with other mAbs suggested a sterical/spatial relationship of the epitopes reacted with the studied mAbs (Harlow and Lane, 1999; Koch *et al,* 1999). In this studies, the epitopes recognized by anti-CD147 mAbs could be clustered into 4 types. Among those, mAbs M6-1B9 and M6-1E9 were demonstrated to react to the same or very close epitope. In the cross blocking experiment, mAbs M6-2B1, M6-1F3 and M6-1B9 did not show self-inhibition. As mAbs M6-2B1 and M6-1F3 have low binding affinity, they did not stable bind to its specific epitope on the CD147 molecule and therefore can not block the binding of FITC labeled antibody. Moreover, because FITC-conjugated M6-1B1 and M6-1F3 showed very low positive fluorescence intensity. It is possible that they had self-inhibition but the limitation of fluorescence intensity was too low to show the clearly inhibitory results when compared the intensity of non-inhibited control and background. In the case of mAb M6-1B9 which is a high affinity antibody, showing of no self-inhibition may due to the defect of FITC conjugate activity.

CD147 molecule consists of 2 extracellular domains (Kasinrerk *et al.*, 1992). The first domain is homologous to domain 3 of IL-1 receptor and the second domain was found to be significantly related to domain 5 of a chain of CD22 (Kasinrerk *et al.*, 1992). In this study, the domain of CD147 molecule which anti-CD147 mAbs bound was investigated. The COS cell expression system with cDNA encoding domain 1 or 2 of CD147 molecule and indirect immunofluorescent staining method were used in this study. COS cells were transfected with

plasmid carrying cDNA encoding domain 1 or 2 of CD147 molecule. As the expressed domain 1 or 2 were not expressed on cell surface of the transfectants, the intracellular staining method was employed. In this study, we found that mAbs M6-2F9, M6-2B1, M6-1D4, M6-1B9 and M6-1E9 reacted to the epitope located on the domain 1 of CD147 molecule, whereas, mAb M6-1F3 did not react either domain 1 or 2 expressed on transfectants. It is likely that mAb M6-1F3 reacted to epitope neither located on domain 1 nor domain 2 of CD147 molecule or the folding of domains expressed on transfected COS cells is different from the native molecule.

The biochemical characterization of CD147 molecule and epitopes that anti-CD147 mAbs bound was further studied using Western blotting and immunoprecipitation technique. Both techniques can inform about structural molecule, epitopes that were reacted with antibodies and the associated molecules. By Western blotting, proteins of U937 cells were separated with SDS-PAGE under reducing and non-reducing conditions. In reducing condition, 2-ME in reducing buffer will act as a reducing agent, which annihilate covalent bond such as disulfide bond. In this study, the results showed that anti-CD147 reacted to a protein band with molecular weight 50 kDa under non-reducing condition and 53.5 kDa under reducing condition. The obtained molecular weight of CD147 molecule was in same range as described in previous reports (Kasinrerk *et al.*, 1992; Staffler and Stockinger, 2000; Koch *et al.*, 1999). Moreover, these results suggested that mAbs M6-2F9, M6-1D4, M6-1B9 and M6-1E9 reacted to linear epitope whereas mAbs M6-2B1 and M6-1F3 may reacted to conformational epitope on CD147 molecule. In addition, the mAb M6-2F9 reacted to epitope that was altered by treatment with 2ME. According to the epitope mapping and Western blotting experiments, the studied anti-CD147 mAbs reacted to 5 different epitopes on CD147 molecule. MAbs M6-2F9, M6-2B1, M6-1F3 and M6-1D4 react to the different epitopes while mAbs M6-1B9 and M6-1E9 seem to recognize to the same epitope or overlapping epitopes.

Since Kasinrerk *et al.* (1992) found that the hydrophobic stretch of the transmembrane region of CD147 molecule was interrupted by a charge residues, glutamic acid, and contained a leucine-zipper motif. It was suggested that CD147 molecule might interact with other proteins on cell surface. To identify the molecule that associate with CD147 molecule, immunoprecipitation technique was carried out. The biotinylated cell surface proteins from U937 cell lysate were precipitated with anti-CD147 mAbs. Mabs M6-1D4, M6-1B9 and M6-1E9 precipitated a protein

band with molecular weight of 50-66 kDa under reducing condition and these bands was shifted to 45.5-61.7 kDa under non-reducing condition. MAbs M6-2F9, M6-2B1 and M6-1F3 did not precipitate any proteins. These might be because of low affinity of the antibodies. FITC-conjugated mAbs M6-2F9, M6-2B1 and M6-1F3 showed low fluorescence intensity comparing to FITC-conjugated mAbs M6-1D4, M6-1B9 and M6-1E9 were observed in the previous experiment. From these experiments, with our immunoprecipitation conditions, the association of other protein(s) with CD147 molecule could not be demonstrated.

In 1997, Kirsch *et al.* studied the involvement of CD147 molecule in apoptosis induction. They described that the binding of mAb 8D6, which is a CD147 mAb, did not induce apoptosis. As only one CD147 mAb were used in that studied, we therefore confirm their result by using the generated anti-CD147 mAbs. For detection of apoptosis, Fadok *et al.* established a apoptosis detection method by detecting phosphatidylserine (PS) on the plasma membrane of apoptotic cells. By this technique, Annexin V was used as a detecting agent (Fadok *et al.*, 1992; Waander *et al.*, 2000). Annexin V was originally isolated from the human umbilical cord artery by virtue of its anticoagulant activity (Reutelingsperger *et al.*, 1985). It can specifically bind PS, after binding to the phospholipid surface, Annexin V forms two-dimensional lattices, which are stabilized by protein–protein interactions (Andree *et al.*, 1992; Mosser *et al.*, 1991). In the present study, U937, KG1a and Sup-T1 cell lines were activated with anti-CD147 mAbs and apoptotic cells were detected with Annexin V-FITC Apoptosis detection kit. The results showed that all of anti-CD147 mAbs did not involve in the induction of apoptosis. These are in agreement as was described by Kirsch *et al.* (1997) and indicated that CD147 molecule did not regulate cell apoptosis.

Since the first identification of CD147 or M6 characteristics (Kasinrerk *et al.*, 1992; Miyauchi *e t al*, 1991), several studies were carried out for its immunoregulation functions. Recently, Kasinrerk *et al.* (1999) reported the effects of anti-CD147 mAbs in inducing homotypic cell aggregation of U937. Interestingly, not all of the mAbs tested showed that effect. This subsequently discovered that the mechanism was depended on LFA-1/ICAM-1 pathway (Kasinrerk *et al.*, 1999) and the signaling was through protein kinases (Khunkeawla *et al.*, 2001). On T cell activation, Kirsch *et al.* (1997) reported that a anti-CD147 mAb, 8D6, had no effect on T cells proliferation. In contrast, Koch *et al.* (1999) reported that anti-D147 mAb, MEM-M6/6,

inhibited human CD3-mediated T cell proliferation. The discrepancy results may come from that the mAbs used in their studies reacted to different epitopes on the CD147 molecule.

Since several anti-CD147 mAbs, which reacted to different epitopes, were available in our laboratory, clarification of the function of CD147 on T cell activation was therefore could be done. In this study, PBMC were activated with sub-optimal dose of OKT3 mAb or PHA in the presence of various anti-CD147 mAbs. OKT3 is a mAb specific to the CD3ε portion of the TCR complex and can activate T cells through TCR complex by cross-linked CD3 molecules (Smith and Bluestone, 1997). In the present study, mAbs M6-1B9 and M6-1E9 which react to the same epitope, but not other anti-CD147 mAbs, inhibited OKT3-induced T cell proliferation. This results indicated that certain epitopes on CD147 molecule is involved in T cell activation.

Phytohaemagglutinin (PHA) is a lectin isolated from red kidney bean, *Phaseolus vulgaris*. PHA can activate T cells through interacts with the α/β or γ chains of the TCR, but not with CD3 (Licastro *et al.*, 1993). Previous study reported that the inhibition of the T cell receptor-mediated signal transduction by microinjection of anti-Lck mAb into T-cells did not suppress phytohaemagglutinin-induced calcium increase (Nakamura *et al.*, 1994). This result suggests that PHA, which induces T cell activation, did not involve the activation of T cells at the proximal signaling events of TCR-mediated T cell activation. In our study, all anti-CD147 mAbs did not inhibit PHA-induced cells proliferation. Taken together with the results obtained from OKT3-induce T cell proliferation, the CD147 molecule is involved in T cell activation at early signal transduction of TCR-mediated T cell activation.

From our results, we propose that CD147 molecule may contain some bioactive domains. Activation of the bioactive domain, by specific mAbs or its natural ligand, induce signal transduction and cellular responses will be occurred. In this study, stimulation of the certain epitope on CD147 molecule by specific mAb down-regulate T cell activation. The effect of anti-CD147 mAbs on CD147 molecule that inhibited T cell activation may come from two hypotheses. First, anti-CD147 mAb reacted to specific epitope, which is a signaling receptor for induction of negative signal to inhibit T cells proliferation. Second, anti-CD147 mAb may react to specific epitope of CD147 molecule which is the receptor for positive signal. Blocking of this positive receptor therefore inhibit T cell response. Further investigation on the mechanisms of CD147 molecule in inhibition of lymphocyte activation may lead to a better understanding of

immune regulation, which may provide new avenues for clinical intervention. It will be also invaluable in the disease diagnosis and treatment and may be also let to development of new drug for regulation of immune responses.

In conclusion, in this study the function of CD147 was investigated. It was found that CD147 molecule is involved in the regulation of T cell activation, but it does not play role in the induction of apoptosis. The function of this molecule is control via specific bioactive domains on the molecule. We demonstrated here that CD147 molecule is a molecule that involves in the regulation of immune system.