CHAPTER I

INTRODUCTION

1.1 Statement of problems

DNA immunization refers to the induction of an immune response to a protein expressed *in vivo*, subsequent to the introduction of its encoding DNA. In contrast to classical protein immunization, which antigens are administered, DNA immunization involves the administering of genetic material encoding the antigen. The antigen is, therefore, produced within the cells of the immunized individual and induces the immune responses (Donnelly *et al*., 1997). Several investigators have demonstrated the feasibility of using direct injection of plasmid DNA for the induction of protective immunity against various pathogens (Lewis and Babiuk, 1999) and the production of specific antibodies (Kasinrerk *et al.*,1999; Moonsom *et al.,* 2001; Kasinrerk *et al.,* 2002; Puttikhunt *et al.,* 2003). Immunization with DNA-based plasmid has been successfully attempted in several tissues by various routes of administering. However, the intramuscular injection has been demonstrated as the most efficient route to transfer an aqueous solution of plasmid DNA (Davis and Whalen, 1995). The DNA immunization technique has several advantages over classical protein immunization. Large scale production of plasmid DNA is easier and less expensive to be produced and maintained its quality control than protein antigens. DNA, in addition, is highly stable material. DNA based immunization, therefore, promises to

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

be an attractive alternative to the classical vaccines and an immunizing agent for the preparation of induction of immune response and production of hyperimmune serum.

More recently, phage display technology was developed and has proven to be a very powerful technique for the production of interested proteins or peptides. The produced proteins or peptides of interest are expressed as a fusion product to a range of phage structural proteins (Perham *et al.*, 1995; Yip *et al.*, 2001; Kiel *et al.*, 2000). Recently, it was demonstrated the possible to deliver fusion protein for vaccine immunization by phage display carrier system. The phage display carrier systems have substantial eliciting strong immune responses, able to mediate prevention in animal models (D'Mello *et al.*, 1997; Wan *et al.*, 2001). The use of phage display could increase the half-life of the peptide in the circulation and providing T-cell help for the induction of a strong antibody response, even without added adjuvant (Yip *et al.*, 2001). The phage expressing protein of interest can be produced inexpensively in large quantities in correctly folded conformation (Yip *et al.*, 2001; Forrer *et al.*, 1999). This phage display technique is, therefore, able to use as immunizing agent for production of specific antibody.

The immune system is an important system of the body for maintenancing health. It functions as a protective system, which plays roles to defend and eliminate pathogens. The basis of immunity is the immune system's ability to recognize foreign antigen and react to them, while at the same time tolerant to the body's own tissues (Male *et al.*, 1987). The immune system composes of many types of cells that work together. However, leukocytes are known to be the group of cells which play a major role in the immune system. Leukocytes consist of lymphocytes, monocytes, granulocytes and natural killer cells (NK cells). They have to cooperate with cells of

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

the other systems. At the present, it has become clear that cell cooperation is mediated by molecules on their surface (Barclay *et al.*, 1997).

Leukocytes express a large number of different molecules on their surfaces called leukocyte surface antigen. Several of them function as ligand receptors which are of critical importance in the development of the immune response. The recognition of foreign antigens by antigen-specific receptors on lymphocytes is the basis for initiation of the immune response. Antigen-specific receptors of B-lymphocytes are surface immunoglobulins, which are mostly of the IgM and IgD isotypes. Specific interaction of these surface immunoglobulins with foreign antigens results in signal transduction and finally induction of antibody production (Cambier and Ransom 1987). Antigen-specific receptors of T-lymphocytes are expressed exclusively on the surface of these cells (Clevers *et al.*, 1988). T-lymphocyte recognition of antigens through T cell receptors is the basis for a range of immunological phenomena including T cell helper and suppressor activities, cytotoxicity and lymphokine production. These interactions cause the communication between these cells. Both cell-cell and ligand-receptor interactions lead to the signal transduction into cells, which are finally activated to make up the immune system.

Human CD147 molecule was also known as basigin (Miyauchi *et at.*, 1991), M6 antigen (Kasinrerk *et al.*, 1992) and extracellular matrix metalloproteinase inducer (EMMPRIN) (Biswas *et al.*, 1995). The CD147 molecule is broadly expressed on both hemopoietic and non-hemopoietic cells, endothelial cells and human peripheral blood cells (Stockinger *et al.*, 1997; Kasinrerk *et al.*, 1999). It is more strongly expressed on thymocytes than on mature peripheral blood T-cells (Kirsch *et al.*, 1997). In peripheral blood, CD147 expression is up regulated T cell activation

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

(Kasinrerk *et al.,* 1999). CD147 is, therefore, speculated to be an essential molecule in the immune system. By using COS cell expression system, Kasinrerk *et al.*, (1992) cloned a cDNA encoding M6 protein. Interestingly, the hydrophobic stretch of the transmembrane region of M6 is interrupted by a charged residue, a glutamic acid and contains a leucine-zipper that are potential protein-protein interaction motifs. The strong conservation of the molecule suggest an important functional role for this region perhaps in interactions with other proteins within the plasma membrane or signal transduction.

In this thesis, both DNA based and phage display immunization will be studied and compared for the production of polyclonal antibody against CD147 molecule. Plasmid DNA encoding CD147 and phage-displayed CD147 molecule will be prepared and used as immunizing agent for BALB/c mice. The kinetics of anti-CD147 antibody responses will be studied and compared. These findings will be very useful for knowing the possibility of utilizing new technique for production of hyperimmune serum and will be applied in monoclonal antibody production in future. The generated antibodies can also be used as a tool for functional study of CD147 molecule.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

1.2 Literature reviews

1.2.1 Immune system

The immune system is a complex network of cells and organs that recognize foreign substances in the body and destroy them. It protects the body from pathogens or infectious agents such as bacteria, viruses, fungi and parasites. In addition, this

system also recognizes and destroys pathogens. The immune system is divided into two parts, which are innate immunity and acquired or adaptive immunity.

1.2.1.1 Innate immunity

Innate immunity is the primitive system of host defense against the pathogens. It presents at birth and persists throughout life. It consists of a number of physical, cellular and chemical barriers. The physical and chemical barriers are the first defense mechanisms against infection. Skin and mucous membranes physically impede invasion. Chemical barriers include gastric pH and enzymes such as lysozyme in tear and saliva and other biologically active molecules such as interferones and blood protein complements (Kaiser, 2002). Cells such as natural killer (NK) cells and polymorphonuclear leukocytes (PMNs) are integral components of this aspect of protection (Aderem and Uderhill, 1999; Abraham and Arock, 1998).

1.2.1.2 Adaptive immunity or Acquired immunity

Acquired immunity is the next line of host defense for antigen or immunogen (Sela, 1969). Antigens are substances in microorganisms or tissues that the immune system considers foreign. It refers to an antigen-specific defense mechanisms in which the process takes several days to cause microbial protection and elimination of a specific antigen. Immune responses are normally direct against the antigen that provoked them. The characteristic of acquired immunity is the use of antigen-specific receptors on T or B cells to drive targeted effector responses in two stages. First, the antigen is presented to and recognized by the antigen specific T or B cells leading to cell priming, activation and differentiation, which usually occurs within the

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

specialized environment of lymphoid tissue (Brodsky and Guagliardi, 1991). Second, the effector response take place, either due to the activated T cells leaving the lymphoid tissue and homing to the disease site or due to the release of antibody from plasma cells (activated B cells) into blood and tissue fluids and then to the infective focus. This is the immunity that one develops throughout life.

Innate immunity differs from acquired immunity in each of these characteristics as are shown in Table 1.1. (Lydyard *et al.*, 2000).

Table 1.1 Comparison between innate and acquired immunity (Lydyard *et al.*, 2000)

Character	Cells	Molecules
1. Innate immunity		
-Responds rapidly	-Phogocytes	-Cytokines
-Has some specificity	-Natural Killer cells	-Complement
-No memory		-Acute phase protein
2. Acquired immunity		
-Slow to start	-T and B cell	-Antibodies
-Highly specific		-Cytokines
-Memory		

The acquired immunity is the capacity for memory, which enables the host to mount a more rapid immune response on re-exposure to the same antigen. The basis for immunologic memory and central principle of acquired immunity is the antigen-

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

driven clonal expansion of T and B cells. The acquired immunity can be divided into two major branches. There are cell-mediated immunity which is the province of T cells and humoral immunity mediated by antibodies.

1.2.1.2.1 Humoral mediated immunity (HMI)

Humoral mediated immunity is the line of host defense for extracellular microorganisms and prevents the spread of intracellular infections. Antibodies are specific glycoproteins produced by B cell-differentiated plasma cell (Podack, 1995). Antibodies, however, perform their effector functions distantly from their production sites. The first exposure to an antigen, such as virus, bacteria and microbial toxins, leads to the activation of naïve B-lymphocytes, as a consequence, B-lymphocytes different into either antibody-producing cells or memory cells. The antibodies may be derived from long-lived antibody-producing cells or memory B cells that migrate to the bone marrow and persist there leading to secondary immune response. These memory B cells circulate throughout the body waiting to encounter with the same antigen once it enters the body. Memory B-lymphocytes are capable for inducing anamnestic response, a heightened secondary response to the antigen (Parslow *et al.*, 2001; Kaiser, 2002). The effector function of antibodies is neutralization and elimination of infected microbes and microbial toxins (Parslow *et al.*, 2001). In viral neutralization, antibodies are made against viral capsid or envelope to prevent virus from adsorbing to host cells as well as preventing viral re-infection. For bacteria, antibodies are made against pili, capsules and adhesions to prevent bacteria from adhering to and colonizing host cells. Additionally, antibodies of IgG isotype which bind to a surface antigen of the microbes will promote phagocytosis through the

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

binding to Fc receptor on phagocytes. Alternatively, antibodies of IgG and IgM isotype can activate the classical complement pathway resulting in the generation of C3b or C4b that can also and promote phagocytosis. Furthermore, IgG b inds to a surface antigen of microbes, it promotes microbial cell lysis by NK cells through antibody-dependent cellular cytotoxicity or ADCC (Abbas *et al.*, 2000; Kaiser, 2002).

1.2.1.2.2 Cell-mediated immunity (CMI)

The cell-mediated immunity is the results of effector function of T lymphocytes. In an acquired immune response, the naive T cells must be induced to proliferate and differentiate into cells capable of contributing to the removal of pathogens. Naive T cells need to be activated in order to carry out their function. At the molecular level naïve T cells receive massage from outside the cell via interaction with a cell surface receptor (Hogg and Landis, 1993). These signals are then passed through the cytoplasm to the nucleus to induce the gene transcription required for cell proliferation, synthesis and release of effector molecules such as cytokines (Jain *et al.*, 1995; Minami *et al.*, 1993). Thus, binding of the T lymphocyte to an antigen presented by antigen presenting cells (APCs), such as macrophage, dendritic cell, B-cell or cells which can express MHC class I or/and class II molecule (Abbas *et al.*, 2000; Kaiser, 2002), via its antigen receptor, T c ell receptor (TCR) and to CD4 or CD8 coreceptor (the first signal) is necessary for initiation of the cell activation (Guerder *et al.*, 1994; Fields *et al.*, 1996; Parslow *et al.*, 2001; Kaiser, 2002). Additionally, the second signal, the binding of accessory cell surface molecules including LFA-1, CD2 and CD28 on T cells to their counter receptors on APCs, is essential for the interactions increase the avidity of cell-cell interaction and required

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

in order to prevent T cell angry. Interaction of co-stimulatory molecules, such as B7 molecules, on the APC and CD28 on CD8^{+} T cells is essential for the activation of naïve CD4^{+} T cell to $T_{H}O$ (Medzhitov and Janeway, 1997; Roitt, 1997; Rudd, 1996).

Regulatory CD4^{+} T cells can be divided into 2 different types based on the cytokines they produce. The $T_{H}1$ cells recognize antigens presented by macrophages and have primarily function to activate and promote CMI by producing cytokines such as IL-2, IFN-γ and TNF-β. Conversely, $T_{H}2$-lymphocytes recognize antigens presented by macrophage and B-lymphocytes. They produce cytokines such as IL-4, IL-5, IL-9, IL-10 and IL-13, which promote antibody production. Additionally, the same cytokines may function as growth factor to expand the respective subsets (Abbas *et al.,* 2000, Kaiser, 2002).

In contrast, CD8^{+} T cells are involved in antiviral and antitumor activity (Henkart, 1994; Squier and Cohen, 1994). After activation CD8^{+} T cell inserts perforins into the cell membrane after binding to virus infected cells or tumor cells. Cytoplasmic granules containing granzymes pass through the pores from the cell into the target cytoplasm and induce target cell death. Additionally, CD8^{+}-suppressor T cells help to turn off both HMI and CMI by destroying activated B-lymphocytes and T-lymphocytes through the FasL/Fas interaction in apoptosis with a progammed cell suicide (Abbas *et al.,* 2000; Kaiser, 2002).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

1.2.1.3 Cell involved in immune system

The haematopoietic stem cells, from which all leukocytes, erythrocytes and platelets are ultimately derived, initially develop in the yolk sac, the in fetal liver and spleen, and later on in the bone marrow. All leukocytes in the blood are critical to

antibody defense. They consist of neutrophils, lymphocytes, basophils, eosinophils and monocytes. In additional, macrophages, dendritic cells and mast cells are the defense cells in tissue.

1.2.1.3.1 Phagocytes

The phagocytes compose of neutrophils, basophils, eosinophils, monocytes, dendritic cells and macrophages. Neutrophils, monocytes, dendritic cells and macrophages are very important effectors for phagocytic destruction of antigens which is targeted by antibody and complement and predominant in acute inflammatory response, especially against extracellular bacteria (Tomlinson, 1993). Furthermore, dendritic cells and macrophages can help adaptive immune responses by processing antigens to T-lymphocytes (Palucka and Banchereau, 2002) and produce a variety of cytokines that plays numerous roles in body defense. Basophils and mast cells can release histamine, leukotrienes and prostaglandins (Kaiser, 2002) that promote inflammation by causing vasodilatation, increasing capillary permeability and increasing mucous production (Hart, 2001). Eosinophils are specialized for destruction of parasitic worms, protozoa and participate in late-phase allergic inflammation (Capron and Dessaint, 1992).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่

1.2.1.3.2 Lymphocytes

Copyright© by Chiang Mai University
All rights reserved

Lymphocytes consist of T cells, B cells and NK cells (David and Huston, 1997; Parkin and Cohen, 2001). T cells are generally quiescent and requite antigen stimulation to progress from naïve cells. In order to activate T cells, the signaling from the T cell antigen receptor (TCR) complex through TCR and co-stimulatory

receptors are mainly pathway in requirement of T cell activation (Weiss and Littman, 1994). For B cells which are phenotypically defined by their cell surface expression of transmembrane immunoglobulins (David and Huston, 1997). Natural killer cells (NK cells) are lymphocytes that lack B-cells receptors and T-cell receptor. They kill cells bound by antibody-dependent cell cytotoxicity or cells that lack MHC-I molecules on their surface (Parslow *et al.*, 2001).

1.2.2 Cell co-operation in immune system

The immune system have evolved in most responses require the participation of more than one cell type. Although many of cells in the immune system are separated from each other, they maintain communication through cell contact and molecules secreted by them (Noelle and Snow, 1991; Paul and Seder, 1994). The various cells of immune system works together through direct contact with other by surface molecules interaction or interactions between chemical mediators, the chemokines and cytokines, with their specific receptors trigger the function of the cell.

Leukocytes like mononuclear phagocytes or macrophage, in which integrated microorganisms persist, contribute to the adaptive immune response by acting as professional APC. The professional APC must be to present peptide fragments of the both classes of MHC molecule, and to deliver a co-stimulatory signal through various surface proteins. Because macrophages have a variety of surface receptors for microbial constituents, including mannose receptor and the scavenger receptor (Linehan *et al.*, 2000), once bound, microorganisms are engulfed and degraded in the cells, generating peptides that can be presented by MHC class II molecule on the cell

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

surface. At the same time, MHC class II and other accessory molecules are induced on the surface of the macrophage. Thereafter, cell-cell interaction between macrophage and T cells provided by cell surface proteins on both cells leading to stimulate T cell proliferation and differentiation (Razi-Wolf *et al.,* 1992). Then immune response is initiated.

In the case of T cell activation, it requires an interaction between the TCR of the T cell and MHC-peptide complex on the surface of other cell. Furthermore, interaction between B cell-T cell which is essential for antigen recognition by the immune system and its response to the antigenic stimulus by production of antibody (Noelle and Snow, 1991). The principal interaction between B and T cells is through cell surface proteins, the B cell surface class II-antigenic peptide complex and the helper T cell surface receptor for antigen. This interaction will trigger T cells to secrete various cytokines that induce B cell to proliferate, differentiate and secrete antigen-specific antibodies (Parker, 1993).

Moreover, the migration of naïve T cells through the lymph nodes also involves cell co-operation (Picker and Butcher, 1993; Hogg and Landis, 1993). This migration is initiated at the interactions between T cells and antigen presenting cells (APC) involves antigen non-specific binding to other cells. Similar interactions eventually guide the effector T cells into the peripheral tissues and play an important part in interactions with their target cells. Binding of T cells to other cells is controlled by an array of adhesion molecules on the surface of the T cells (Hogg and Landis, 1993). These cell surface proteins recognize a complementary array of adhesion molecules on the surface of the cells with which the T cell interacts.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

1.2.3 CD147 molecule

The CD147 molecule is a leukocyte surface protein, which found on the surface of various cells. It was designated as cluster of differentiation (CD) system in 1997 at the Sixth International Workshop and Conference of Human Leukocyte Differentiation Antigens (HLDA workshop) (Stockinger *et al.*, 1997). The aliases of CD147 molecule are basigin (Miyauchi *et at.*, 1991), M6 antigen (Kasinrerk *et al.*, 1992) and extracellular matrix metalloproteinase inducer (EMMPRIN) (Biswas *et al.*, 1995). It is a glycoprotein of type 1 transmembrane protein of the immunoglobulin superfamily composing of 269 amino acids with a molecular mass of 50-60 kDa (Stockinger *et al.*, 1997). Amino acid sequences comparison showed that human CD147 is the species homologue of rat OX-47 antigen (Fossum *et al.*, 1991) or CE9 (Nehme *et al.*, 1995) antigen and a chicken antigen HT7 (Seulberger *et al.*, 1990), 5A11 antigen (Fadool *et al.*, 1993), a mouse protein termed gp42 (Altruda *et al.*, 1989) frequency Oka blood group antigen (Spring *et al.*, 1997). The CD147 gene has been mapped to band p13.3 of chromosome 19 (Kaname *et al.*, 1993) and the mouse gene has been found to consist of seven exons (Cheng *et al.*, 1994). The CD147 has typical features of type I integral membrane protein. The putative extracellular domain composes of two Ig-like domains most probably of the domain V and C2 types as determined by comparison with other Ig domains. Domain 1 of the molecule is homologous to domain 3 of IL-1 receptor and domain 2 was found to be significantly related to domain 5 of a chain of CD22 (Kasinrerk *et al.*, 1992). Endoglycosidase F treatment of immunoprecipitates resulted in a mobility shift from 54 kDa to 28 kDa showed that the majority of basigin (Miyauchi *et al.*, 1990) or neurothelin (Schlosshauer *et al.*, 1990). In addition, the CD147 bears high-the

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

oligosaccharide chains are N-linked (Kasinrerk *et al.*, 1992). The 21 amino acid of the putative transmembrane region are absolutely identical in the human, rat and chicken homologues and, with the exception of one amino acid, also in the mouse and rabbit form. Interestingly, the hydrophobic stretch of the transmembrane region of CD147 is interrupted by a charged residue, a glutamic acid, and contains a leucine-zipper that are potential protein-protein interaction motifs. This and the strong conservation of the molecule point to an important functional role for this region perhaps in interactions with other protein within the signal transduction or plasma membrane. From all characterizations of the lines, it is the lymphocyte activation molecule and structure of the molecule that suggest the important function of this protein.

In the Sixth International Workshop and Conference of Human Leukocyte Differentiation Antigens, several mAbs were submitted and clustered. The submitted mAbs were recognized several distinct epitopes of the human CD147, such as AAA6 (Felzmann *et al.*, 1991), UM-8D6, HI197, HIM6 (Pharmingen) and H84. All of these mAbs recognize determinants in the N-terminal Ig domain (D1) of the CD147 molecule (Stockinger *et al.*, 1997). Additionally, The CD147 monoclonal antibodies (mAbs) AAA6 and UM-8D6 inhibited homotypic aggregation of the estrogen-dependent breast cancer cell line MCF-7, as well as MCF-7 cell adhesion to type IV collagen, fibronectin and laminin (Staffler and Stockinger, 2000; Schiavone *et al.*, 1997). Furthermore, CD147 mAbs could activate the homotypic cell aggregation of U937 cell line by LFA-1/ICAM-1 dependent pathway (Kasinrerk *et al.*, 1999) through the activation of protein kinases and reorganization of the cytoskeleton (Khunkeawla *et al.*, 2001).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

The CD147 appears to involve also in regulation of T cell functions. The first indication for its T cell regulatory role was given by the finding that it is strongly up-regulated on T cells upon activation (Kasinrerk *et al.,* 1992; Koch *et al.,* 1999). Furthermore, one CD147 mAb, MEM-M6/6, was found to prevent 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 (Koch *et al.,* 1999).

1.2.4 DNA immunization

DNA based immunization has provided a promising new technology that was developed during the last decade. The delivery of DNA vaccine may affect the nature of the immune response induced against antigens encoded by plasmid DNA. The plasmid DNA introduced into muscle of the host leads to expression of antigen in the host cells when transcription is driven by eukaryotic control elements (Donnelly *et al.,* 1997).

1.2.4.1 Processing and presentation of genetically introduced antigens

Immunization with plasmid DNA has been shown to activate both humoral and cellular immune responses, including the generation of antigen-specific $CD8^+$ cytotoxic T cells as well as $CD4^+$ T helper cells (Donnelly *et al.,* 1997). $CD8^+$ cytolytic T lymphocytes recognize a non-covalent trimeric complex consisting of a MHC class I heavy chain allele, β_2-microglobulin, and an 8-12 amino acid long peptide antigen. $CD4^+$ T helper lymphocytes, in contrast, recognize 12-25 amino acid long peptide antigens presented in the context of dimeric MHC class II molecules (Lowrie and Whalen, 2000). Most of the peptides presented by MHC class I

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

molecules are derived from endogenously synthesized protein antigens processed in the cytosol by multicatalytic proteasomes. The short peptides are delivered into the endoplasmic reticulum by the transporter associated with antigen presentation (TAP). In the endoplasmic reticulum, compatible peptides interact with nascent class I heavy chains and β_2-microglobulin-generating transport-competent trimeric complexes that are subsequently delivered to the cell surface where they become accessible to $CD8^+$ T cell scrutiny. In contract, exogenous proteins taken up into endocytic compartments from the extracellular milieu are the major source of antigens presented in the context of MHC class II molecules. Internalized antigen is denatured by low pH and degraded by endosomal and lysosomal proteases, exposing peptide segments that bind to MHC class II molecules. The resulting class II-peptide complexes are transported to the membrane for detection by $CD4^+$ T cells. One result of this general segregation of processing pathway is that protein antigens introduced into the extracellular fluids typically do not gain access to the cytosol and are therefore generally excluded from the MHC class I processing pathway. This represents a theoretical hurdle for protein-based vaccine design and may explain why the elicitation of strong cytotoxic T lymphocyte responses with non-living protein antigens has generally been problematic. Several studies, however, have found that cell-associated antigens can elicit $CD8^+$ T cell responses *in vivo*, and accumulating evidence suggests that some phagocytic cell types, such as macrophages or immature dendritic cells, can take up exogenous protein for class I-restricted presentation (Watts, 1997).

Once a protein antigen is denatured and/or digested, the ability of a given peptide to bind to, and be presented by, a given MHC class I or class II allele is determined by structural motifs within the peptide sequence. These motifs allow for

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

sufficient compatibility between peptide-binding groove of the MHC molecule (Rammensee, 1995). The degree of intermolecular compatibility determines the affinity of peptide for an individual MHC molecule, the corresponding half-life of peptide-MHC complexes and, to a large degree, the likelihood that the peptide-MHC complex is immunogenic.

In addition to the recognition of peptide-MHC ligand by the T-cell receptor, T-cell priming requires the presentation of antigen by professional antigen presenting cells. The presentation of antigens to T cells in the absence of appropriate costimulatory signals can result in activation induced cell death by apoptosis (Boise *et al.,* 1995). Dendritic cells are the most potent APC identified to date (Steinman, 1991; Cella *et al.,* 1997). They are capable of capturing antigens in the periphery, and then migrating to T-cell-rich areas of secondary lymphoid organs, where they can efficiently stimulate naïve or quiescent T cells. Upon encounter with antigen, dendritic cells undergo a maturational process, which is associated with their capacity to activate T cells. This includes the upregulation of MHC, costimulatory, and adhesion molecule expression on their cell surface (Lowrie and Whalen, 2000).

1.2.4.2 A critical role for professional antigen presenting cells

The genetic immunization can elicit both humoral and cellular immune responses, the DNA-encoded antigen presumably gains access to both the MHC class I and class II antigen processing pathways, and is also capable of interacting with immunoglobulin molecules on the surface of B-cells. Initially, the efficient induction of $CD8^+$ cytotoxic T cells was considered to be result of endogenous antigen synthesis *in vivo*, which presumably permitted antigen to access the class I restricted antigen

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

processing pathway of transfected cells. However, the predominant transfected cell type following intramuscular DNA injection is the myocyte (Lowrie and Whalen, 2000).

DNA vaccines can either be coated onto gold particle and introduced into the dermis via a gene gun (Tang *et al.,* 1992) or injected as an aqueous solution by an intramuscular route (Ulmer *et al.,* 1993). Bone marrow derived antigen presenting cells (APC), especially dendritic cells, seem to be essential for the immune responses observed after vaccination by gene gun inoculation as well as intramuscular injection (Dupuis *et al.,* 2000). Interestingly, intramuscular or intradermal injection of DNA in saline has been found to preferentially induce a T_H1 bias of the immune response in mice, with the expansion of IFN-γ-producing $CD4^+$ T cells and the production of antibodies of the IgG2a isotype (Pertmer *et al.,* 1995; Raz *et al.,* 1996; Pertmer *et al.,* 1996). Surprisingly, plasmid DNA immunization using the gene gun targeting skin or muscle (Pertmer *et al.,* 1995; Pertmer *et al.,* 1996) induces immune response with a T_H2 bias. There also appear to be difference in the dependency of the immune response on gene expression in the target tissue for intramuscular needle vs. epidermal gene gun DNA delivery (Torres *et al.,* 1997), however, both routes of application induce efficient humoral and cellular immune responses against a variety of pathogens, tumor antigens, allergens (Dietrich *et al.,* 1999) and therapy of infectious diseases or mediate prevention in small animal models (Jing *et al.,* 2002; Darji *et al.,* 1997; Cui and Mumper, 2001). An increasing number of studies using experimental animal models have demonstrated that plasmid DNA immunization can promote effective immune responses against numerous viruses, including influenza, rabies, HIV, HBV and HCV, and the several bacteria, *Mycobacterium tuberculosis*,

Copyright© by Chiang Mai University
All rights reserved

Mycoplasma pulmonis and *Borrelia burgdorferi* as well as parasites, such as malaria and leishmania (Donnelly *et al.,* 1997). Importantly, antigen-specific antibodies and cytotoxic T lymphocyte reactivity could be detected in rodents longer than 1 year after immunization (Pardoll and Beckerleg, 1995).

1.2.4.3 Genetic engineering of the immune response

There are several examples of attempts to modulate the immune response elicited by plasmid DNA immunization through the co-delivery and expression of antigen with other immunologically relevant molecules. For examples, simultaneous delivery of plasmid DNA encoding GM-CSF enhances immune responses resulting from plasmid DNA immunization (Xiang and Ertl, 1995; Conry *et al.,* 1996). This is consistent with the involvement of dendritic cells in the activation of the immune system since GM-CSF is important for the growth, differentiation, and maturation of dendritic cells, at least *in vitro*. In addition, several groups have recently shown the adjuvant effects of co-delivery of plasmids encoding immunostimulatory cytokines including IL-2, IL-7 or IL-12 (Kim *et al.,* 1997; Tsuji *et al.,* 1997; Chow *et al.,* 1997; Iwasaki *et al.,* 1997). Adjuvant administration of plasmids encoding T-cell growth factors such as IL-2 and IL-7, and T_H1 biasing cytokines such as IFN-α and IL-12, may be expected to selectively enhance the induction of cell-mediated immunity.

Another potential strategy for the enhancement of T-cell responses is the adjuvant administration of plasmids encoding costimulatory molecules such as B7.1 or B7.2, presumably to provide the second signal required for optimal T-cell activation (Lenschow *et al.,* 1996; Chanbers *et al.,* 1997). Co-delivery of costimulatory molecule genes for B7.1, and particularly for B7.2, has been shown to

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

enhance the induction of cell- mediated immunity following intramuscular injection of plasmid-encoded antigen (Conry *et al.,* 1996; Iwasaki *et al.,* 1997; Kim *et al.,* 1997). In addition, B7 family members also appear to induce cytokine production, particularly IFN-γ, by NK cells leading to enhanced immune response. Furthermore, the immunostimulatory DNA sequences (ISS) containing unmethylated CpG dinucleotide motifs in a particular base context can contribute to effective induction of an immune response is particularly relevant for DNA vaccine. For example, the bacterial DNA containing ISS is capable of activating NK cells and macrophages *in vitro* leading to the production of IFN-α/β, IFN-γ, TNF-α, IL-1, IL-6, IL-12 and IL-18 (Sato *et al.,* 1996; Klinman *et al.,* 1996; Ballas *et al.,* 1996; Stacey *et al.,* 1996; Pisetzki, 1996; Klinman *et al.,* 1997), and direct inducting B cell proliferation and immunoglobulin secretion. Thus, injection of high dose of ISS-containing plasmid DNA activates innate immunity, and leads to the production of T_H1 biasing cytokines within the skin or muscle, promoting cell mediated immunity. Interestingly, the potent immunostimulatory effects of Freund's adjuvant may depend on the ISS-enriched mycobacterial DNA. Indeed, sequence motifs, CpG immunostimulatory motifs, in some plasmids are adjuvants and immunostimulatory that could eventually be added to DNA vaccines (Klinman *et al.,* 1997; Krieg, 1999; Stratford *et al.,* 2001). There are two different approaches that could be used to do this, either administration of CpG-containing synthetic oligonucleotides (ODN) with the DNA vaccine or direct cloning of the CpG sequences into the DNA vaccine vector. It has been reported that the immunostimulatory effects include direct induction of B cell proliferation and immunoglobulin secretion (Krieg *et al.,* 1995), as well as activation of monocytes, macrophages and dendritic cells to upregulate their expression of costimulatory

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

molecules, which drive immune responses, and secreting a variety of cytokines, including high levels of IL-12 (Klinman *et al.,* 1996; Halpern *et al.,* 1996). Thus, the plasmid DNA immunization by biolistic cutaneous delivery induces immune responses with a T_H2 bias (Pertmer *et al.,* 1996; Feltquate *et al.,* 1997).

In additional, vaccines consisting of naked plasmid have several potential advantages over alternative immunization approaches relying on the delivery of purified or recombinant proteins, or live attenuated or recombinant viruses. They offer the promise of a readily deliverable, molecularly defined reagent that results in antigen synthesis *in situ,* but that is neither infectious nor capable of replication. Importantly, both humoral and cell-mediated immune responses may be elicited against multiple defined antigens simultaneously. Furthermore, it may become possible to manipulate the nature of the resulting immune response through the co-delivery of gene encoding immunomodulating cytokines or costimulatory molecules. Genetic constructs can be modified, allowing for the removal or insertion of transmembrane domains, signal sequences, or other residues that affect the intracellular trafficking and subsequent processing of antigen. The sequence may also be modified by site directed mutagenesis, permitting single amino acid exchanges designed to enhance the antigenic potency of individual epitopes or to abolish unwanted physiologic effects of the wild type protein. Importantly, plasmid DNA encoding suitable antigens or immune modulators, can readily and economically be constructed and produced in large quantities with a high degree of purity and stability. However, disadvantages of naked DNA vaccination include the uptake of DNA by only a minor fraction of muscle cells, exposure of DNA to deoxyribonuclease in the interstitial fluid thus necessitating the use of relatively large quantities of DNA, and,

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

in some cases, injection into regenerating muscle in order to enhance immunity (Davis *et al.,* 1993; Manickan *et al.,* 1997; Gregoriadis, 1998). Therefore, several groups have recently shown the DNA immunization via liposomes could circumvent the need of muscle involvement and instead facilitate (Gregoriadis, 1995) uptake of DNA by APC infiltrating the site of injection or in the lymphatics, at the same time protecting DNA from nuclease attack (Gregoriadis *et al.,* 1996). Moreover, transfection of APC with liposomal DNA could be promoted by the co-entrapment, together with DNA, of plasmids expressing appropriate cytokines, such as IL-2, or immunostimulatory sequences. When immunization of BALB/c mice by a variety of routes with cationic liposomal DNA leads to much greater humoral and cell mediated immune responses than those obtained with naked DNA or DNA complexed to similar pre-formed liposomes (Gregoriadis *et al.,* 1997; Gregoriadis, 1998). The cationic lipid is essential for the promotion of strong responses and that phosphatidylethanolamine (PE) and dioleoyl phosphatidylcholine (DOPE) are equally effective in inducing immune responses in genetic vaccination (Gregoriadis *et al.,* 1997; Perrie and Gregoriadis, 1998; Gregoriadis, 1998). Furthermore, the DNA-containing liposomal suspensions can be freeze-dried in the presence of a cryoprotectant without significant loss of material from within the vesicles on reconstitution with 0.9% NaCl (Gregoriadis *et al.,* 1987; Gregoriadis, 1993). In addition, DNA vaccination is a simple methodology and a broader spectrum of immune responses for achieving greater efficacy similar to natural infection, live attenuated or recombinant viral vaccines without the safety hazards of inoculation of live virus (Shi *et al.,* 1999). They are relatively inexpensive and easy to produce.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

1.2.5 Phage display system

More recently, phage display has proven to be a very powerful technique for obtaining libraries containing of protein. It is a very effective way for producing large numbers of diverse protein, peptides and molecule that perform specific functions (Griffith, 1993; Barbas, 1993; Winter *et al.,* 1994; Burton, 1995; Ladner, 1995; Neri *et al.,* 1995; Hoogenboom, 1998; Ladner, 2000; Seigel, 2001). This technique can also be used to study receptor and antibody binding site (Griffith, 1993; Winter *et al.,* 1994), protein-ligand interactions (Cesareni, 1992), and to improve or modify the affinity of proteins for their binding partners (Neri *et al.,* 1995; Ladner, 1995; Burton, 1995). The fusion proteins have also been made between peptides and a range of phage structural proteins at positions which preserve the biological properties of fusion partner protein (Perham *et al.,* 1995), glutathione S-fusion protein (Yip *et al.,* 2001), and filamentous hemaglutinin fusion protein (Kiel *et al.,* 2000). In the phagemid system, it is based on a vector containing only genes for gpIII or gpVIII and bacterial and viral origins of replication. DNA sequences of inserted into a location in the genome of filamentous bacteriophage, such as Ff filamentous phage particles (strain M13, fl and fd), that the encoded protein is expressed on the surface of filamentous phage as a fusion product to one of the phage coat proteins. Therefore, instead of having to genetically engineer peptides or proteins variant one-by-one and then express, purify and analyze each variant, phage display containing several billion variants can be constructed simultaneously. These libraries can be easily used to select and purify a specific phage particle bearing a sequence with desired binding specificity from the non-binding variants.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

The development of antibody phage display technology was essential two major keys. First, the demonstration that foreign DNA inserted into filamentous phage gene III (gpIII) or filamentous phage gene VIII (gpVIII) is expressed as a fusion protein and displayed on the surface of the phage (Smith, 1985). Second, the successful expression of functional antibody fragment in the periplasmic space of *E. coli* (Skerra and Plückthun, 1988; Better *et al.*, 1998).

The phage coat protein gpIII and gpVIII are involved in the cloning and detection of recombinant phage antibodies and peptides. The folded proteins and recombinant antibodies are typically expressed as gpIII fusion protein and displayed at the tip of filamentous phage. For example, the correct size of CD147-truncated gpIII fusion protein is 38 kDa and both the *E. coli* strain and growth condition were important for obtaining the correct conformation of CD147 displayed on phage particle (Tayapiwatana *et al.*, 2003). When these antibodies bind to the antigen bound phage is detected with a HRP-labeled antibody that recognizes the gpVIII coat protein. Since several thousand copies of gpVIII exist on the phage surface, it effectively amplified the detection signal. In contrast, peptide may be displayed as fusions to either gpIII or gpVIII. If peptides were fused to gpVIII, bound phage can be detected by rat monoclonal antibodies that recognize an epitope localized in the N-terminal portion of gpIII (Dente *et al.*, 1994). In additional, it was possible to deliver fusion protein for vaccine immunization with phage display carrier system. The phage display carrier systems have substantiated to elicit strong immune responses, able to medicate prevention in animal models (D'Mello *et al.*, 1997; Wan *et al.*, 2001). The use of phage display has the additional advantage of increasing the half-life of the peptide in the circulation and providing T-cell help for the induction of a

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

strong antibody response, even without added adjuvant (Yip *et al.*, 2001). Finally, the phage display carrier system can be produced inexpensively in large quantities and increased correctly folded peptides (Yip *et al.*, 2001; Forrer *et al.*, 1999).

1.3 Objectives

1. To prepare plasmid DNA encoding CD147 protein.
2. To prepare phage expressing CD147 protein.
3. To study the kinetics of antibody response in mice after DNA and phage display immunizations.
4. To compare the efficacy of DNA and phage-display immunizations in antibody production.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved