HER2/neu-specific Breast Cancer Vaccine

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ABSTRACT

Breast cancer is the most common cancer among women. Of all breast cancers cases, approximately 30 percent have amplification of the self-antigen HER2/neu. Later studies demonstrated that HER2/neu-specific antibody and T cell responses were found in HER2/neu-positive breast cancer patients, indicating HER2/neu is a good target for active immunotherapy. A humanized anti HER2/neu antibody trastuzumab, was developed and found to be an effective therapy for HER2/neu. However, acquired antibody resistance occurs sooner or later in antibody treated patients. Such limitations of antibody therapy provoked scientists into the search for other therapeutic strategies. HER2/neu-targeted immunotherapeutic strategies, including vaccines using tumor lysates, protein/peptide, DNA, adenoviral vectors (AdV) and dendritic cells (DCs) pulsed with the above reagents, have been shown to be effective in experimental models. However, they have also been proven to be incapable of breaking tolerance towards HER2/neu in clinical trials and eliciting then have not elicited adequate antitumor immunity in curing HER2/neu positive breast cancer in transgenic mice with HER2/neu-specific immune tolerance, although both humoral and cellular immune responses could be detected.

$CD4^+$ helper T (Th) cells play crucial roles in priming, expansion and memory of both humoral and $CD8^+$ cytotoxic T lymphocyte (CTL) responses. Therefore, they are essential in antitumor immunity. The tetanus toxoid Th epitope 947-967 P30, FNNFTVSFWLRVPKVSASHLE, has been found to be a universal epitope in sensitizing and proliferating $CD4^+$ T cells ex vivo. OVA-P30 peptide vaccine could break $CD8^+$ and $CD4^+$ T cell tolerances against the neo-self-antigen OVA; it was able to protect transgenic rat insulin promoter (RIP)-mOVA mice from tumor growth.

Adenovirus-based vaccines are able to induce high frequencies of transgene product-specific $CD8^+$ T cell responses. In this study, we immunized C57BL/6 mice with OVA-expressing AdV_{OVA}. We found that AdV_{OVA} induced sustained OVA-specific CTL responses, leading to preventive antitumor immunity against OVA-expressing BL6-10_{OVA} tumor cell challenge in wild-type C57BL/6 mice. In addition, we also immunized transgenic FVBneuN mice with neu-expressing AdVneu. We found that AdVneu vaccination induced neu-specific CTL responses, leading to partial reduction of breast carcinogenesis in FVBneuN mice.

To assess whether the foreign Th epitope P30 enhances $CD4^+$ and $CD8^+$ T cell responses, we constructed another two recombinant AdVs ($AdV_{OVA\text{-}P30}$ and $AdV_{neu\text{-}P30}$), expressing OVA-P30 and HER2/neu-P30 gene, respectively. We transfected C57BL/6 mouse bone marrow dendritic cells (DCs) with AdV_{OVA} and $AdV_{OVA\text{-}P30}$ for preparation of DC_{OVA} and $DC_{OVA\text{-}P30}$ vaccines. We immunized C57BL/6 mice with DC_{OVA} and $DC_{OVA\text{-}P30}$ and then assessed $CD4^+$ and $CD8^+$ T cell responses and antitumor immunity subsequent to immunization. We demonstrated that both DC_{OVA} and $DC_{OVA\text{-}P30}$ were capable of stimulating both enhanced $CD4^+$ and $CD8^+$ T cell responses, leading to preventive antitumor immunity against challenge of OVA-expressing BL6-10_{OVA} tumor in 100% (8/8) of the immunized mice. However, $DC_{OVA\text{-}P30}$ induced more efficient $CD4^+$ and $CD8^+$ T cell responses than DC_{OVA}, leading to significant reduction of growth of 3 day-established lung tumor metastasis in C57BL/6 mice, indicating that the foreign $CD4^+$ Th epitope P30 can enhance both $CD4^+$ and $CD8^+$ T cell responses. In this study, we also transfected transgenic FVBneuN mouse bone marrow DCs with AdV_{neu} and $AdV_{neu\text{-}P30}$ for preparation of DC_{neu} and $DC_{neu\text{-}P30}$ vaccines. We immunized transgenic FVBneuN mice with DC_{neu} and $DC_{neu\text{-}P30}$ and then assessed $CD4^+$ and $CD8^+$ T cell responses and antitumor immunity subsequent to immunization. We demonstrated that $DC_{neu\text{-}P30}$ but not DC_{neu} was capable of stimulating both enhanced $CD4^+$ and $CD8^+$ T cell responses, leading to preventive antitumor immunity against challenge with 0.3×10^6 neu-expressing Tg1-1 tumor cells in 100% (8/8) immunized transgenic FVBneuN mice; this significantly reduced lung metastasis tumor colonies in immunized transgenic FVBneuN mice challenged with 1×10^6 Tg1-1 tumor cells, confirming that incooperation of foreign $CD4^+$ Th epitope P30 into DC-based vaccines can at least partially break self-immune tolerance, leading to enhanced CTL responses and antitumor immunity in transgenic FVBneuN mice.

Taken together, our data demonstrate that the $CD4^+$ Th epitope P30 can enhance both $CD4^+$ and $CD8^+$ T cell responses, leading to enhanced DC-stimulated antitumor immunity. This may have impact in designing new DC-based vaccines for treatment of breast cancer and other types of human malignancies.

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DEDICATION

This thesis is dedicated to my family, who gave me unconditional love, financial support and encouragements for the pursuit of a higher education at University of Saskatchewan in Canada. I thank my father who has taught me the virtues of life and the value of education and hard work. With his love and encouragement, I will always be able to achieve my dreams.

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	Antibody
ADCC	Antibody dependent cell-mediated cytotoxicity
AdV	Adenovirus
AFP	Alpha-fetoprotein
Ag	Antigen
Akt	Protein Kinase B
APC	Antigen presenting cell
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guerin
BCR	B cell receptors
BGH	Bovine growth hormone
BM	Bone marrow
bp	base pair
BSA	Bovine serum albumin
CAR	Coxsackie-adenovirus receptor
Cbl	Casitas B-lineage Lymphoma
CD	Cluster of differentiation
CD40L	Co-stimulatory molecule 40 ligand
CDC	Complement dependent cytotoxicity
cDC	Classical DCs
CEA	carcinoembryonic antigen
CFSE	Carboxyfluoroscein diacetate succinimidyl ester
CHM	Cholesteryl group-bearing mannan

CHP	Cholesteryl group-bearing pullulan
CISH	Chromogenic in situ hybridization
CMF	Cyclophosphamide methotrexate and 5-fluorouracil
CMV	Cytomegalovirus
CPE	Cytopathic effects
CpG	Cytosine phosphate guanosine
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T-lymphocyte antigen
Cy	Cyclophosphamide
dATP	2’deoxyadenosine 5’-triphosphate
DC	Dendritic cell
dCTP	2’deoxycytosine 5’-phosphate
DFS	Disease-free survival
dGTP	2’ deoxyguanine 5’-phosphate
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethylsulfoxide
dNTP	deoxynucleotide triphosphate
ds	double stranded
DTT	Dithiothreitol
dTTP	2’deoxythymidine 5’-phosphate
EBV	Epstein–Barr virus
ECD	Extracellular domain
EDTA	Ethylenediamine tetracetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay

EMEM	Minimal essential medium with Earle’s salts
ER	Endoplasmic reticulum
FasL	Fas ligand
FBS	Fetal bovine serum
FDA	Food and drug administration
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
Flt-3L	Fms-like tyrosine kinase 3-ligand
FoxP3	Fork-head/winged helix transcription factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granuloctye-colony stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp100	glycoprotein-100
Grb	Growth factor receptor-bound protein
HA	Hemagglutinin
HAdV	Human adenoviruse
HB-EGF	Heparin-binding EGF-like growth factor
HCl	Hydrochloric acid
HDI	HER dimerization inhibitors
HEK	Human embryonic kidney
HER	Human epidermal growth factor receptor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HRP	Horseradish peroxidase
id	intradermal

il	intralymphatic
im	intramuscular
in	intranodal
ip	intraperitoneal
it	intratumoral
iv	intravenous
ICD	Intracellular domain
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-15	Interleukin 15
IL-18	Interleukin 18
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-7	Interleukin 7
IMO	Immuno-modulatory oligonucleotide
ITR	Inverted terminal repeat
JUNK	c-jun-N terminal kinase
kb	kilobase pair
kD	Kilodalton
LB	Lauria-Bertani
LC	Langerhans cell(s)
LN	Lymph node

LPS	Lipopolysaccharide
LTR	Long terminal repeat
MAb	monoclonal antibody
MAC	Molecule attacking complex
MAGE	Melanoma antigen
MAPK	Mitogen-activated protein kinase
MART-1	Melan-A/melanoma antigen recognized by T cell
MCS	Multiple cloning site
MDC	Macrophage derived chemokine
MF	Macrophages
MHC	Major histocompatibility complex
MIC	MHC-related protein
MIP	Macrophage inflammatory protein
MLV	Murine leukemia virus
MLP	Major late promoter
MMR	Macrophage mannose receptor
MMTV	Mouse mammary tumor virus
MOI	Multiplicity of infection
MoMLV	Moloney murine leukemia virus
MSC	Myeloid suppressor cell
MUC	Mucin
MVA	Modified vaccine virus
NDV	Newcastle disease virus
neu	Neuroblastoma
NGF	Neuregulin
NIH/3T3	Mouse embryonic fibroblast cell
NK	Natural killer

NRG	Ligand like neuregulin-1 (-1)
ori	origin of replication
OVA	Ovalbumin
P30	Tetanus toxin epitope 947-967
PAMP	Pathogen associated molecular patterns
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
PDZ	protein-interaction domain
PE	Phycoerythrin
PEG	Polyethylene glycol
PFS	Progression-free survival
PFU	Plaque forming unit
PI3K	Phosphatidylinositol 3-kinase(s)
PLC	Phospholipase C
pMHC	peptide-MHC complex
PSA	Prostate specific antigen
PSD	postsynaptic density protein
PSMA	Prostate specific membrane antigen
PTB	Phosphotyrosine binding
PTD	Protein transduction domain
RANTES	Regulated and normal T cell expressed and secreted
RGD	Arginine-Glycine-Aspartic
RIP	Rat insulin promoter
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase

RT-PCR	Reverse transcription-polymerase chain reaction
sc	subcutaneous
SARS	Severe acute respiratory syndrome
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SH2	Src homology 2
SH3	Src homology 3
Shc	Src homology 2 domain-containing
SV40	Simian virus 40
TAA	Tumor associated antigen
TAE	Tris-acetate EDTA
TAP	Transporters associated with antigen processing
TARC	Thymus and activation regulated chemokine
TAT	Transactivating
TB	Terrific broth
TCR	T cell receptor
TE	Tris EDTA
Tg	Transgenic
TGF	Transforming growth factor
Th	T helper
Th1	T helper type I
Th2	T helper type II
TLR	Toll-like receptor
TM	Transmembrane
TMB	3,3’,5,5’-tetramethylbenzidine
TNF-α	Tumor necrosis factor α
Treg	Regulatory T cell

TSA	Tumor specific antigen
β-ME	β-mercaptoethanol

1 Introduction

1.1 Basic immunology

1.1.1 Innate immunity

The non-specific innate immune response is the first line of defense against pathogens. Usually, it takes place on the first day of pathogen infection. Innate immunity includes the prevention and control of infection, and the elimination of infected cells. It also contributes to the elimination of stressed host cells and provides warning signals to stimulate adaptive immunity. These functions are achieved by its components including physical and chemical barriers (i.e., skin, mucosa, blood-brain barriers; antimicrobial chemicals), effector cells like phagocytic cells (i.e., neutrophils, mononuclear phagocytes and dendritic cells), natural killer cells (NK), as well as blood proteins (i.e. complement) and cytokines.

Innate immune responses can be triggered by the recognitions of the pathogen associated molecular patterns (PAMPs) on pathogens by cell associated pattern recognition receptors (i.e. Toll-like receptors (TLRs) or C-type lectin), which send transduction signals or take up the pathogen (i.e. pentraxins or collectins).

Phagocytes are the most numerous effector cells in the innate immune system. They function during the identification, ingestion and destruction of microbes. Mononuclear phagocytes play a central role in both innate and adaptive immunities. They are derived from bone marrow (BM), and enter the peripheral blood as monocytes, and mature as macrophages. Phagocytosis follows the recognition of microbes with the binding of cell receptors to PAMPs, or the opsonization of microbes by protein coating. Activated phagocytes can destroy phagocytosed microbes by releasing proteolytic enzymes into phagolysomes or by releasing reactive oxygen species (ROS). Phagocytes can also play a role in antitumor immunity. Macrophages may recognize tumor surface antigens via the activating receptor NKG2D, which is also found on natural killer cells (NKs) (Diefenbach et al. 2001). Macrophages can be activated by interferon-γ (IFN-γ) secreting tumor-specific T cells or NK cells. The mechanisms that kill tumor cells are the same as those used to kill infectious microbes.

Natural killer cells are another important effector cell type in innate immunity. After recognition of microbes, NKs mediate cytotoxicity of infected cells by producing and releasing granule proteins (i.e., perforin or granuzyme) to adjacent target cells and initiating apoptosis of infected cells. NK cells can also release IFN-γ and activate macrophages to kill phagocytosed microbes. NK can also kill tumor cells (Bauer et al. 1999; Groh et al. 1999) that lack major histocompatibility complexes (MHC) but express NK2D ligands, i.e. MHC-related protein A and B (MICA, MICB). In addition, the inhibitory NK ligand has been detected in breast cancer cell lines. Soluble HLA-G is also related to apoptosis of T cells. Altogether, PAMPs, toll-like receptors (TLRs) and cells in the innate immune system are potential candidates to be used as adjuncts in antitumor immunotherapeutics.

1.1.2. Adaptive immunity

Adaptive immunity happens later than the innate immune responses. It has memories and is more specific. It consists of cellular immunity and humoral immunity. Adaptive immune responses need a series of steps to develop. In each step, different molecules, immune cells and specialized lymphoid tissues are involved. The immune cells include antigen-presenting cells (APCs, such as dendritic cells, mononuclear phagocytes; B cells; folicular dendritic cells), lymphocytes (such as CD4+ T, CD8+ T and B lymphocytes). Lymphoid tissues include primary (generative) lymphoid organs and secondary (peripheral) lymphoid organs. Primary lymphoid organs consist of bone marrow, where progenitors of lymphocytes and blood cells reside, and thymus, where central tolerance is induced through positive and negative selection of T cells. Secondary lymphoid organs include the spleen, lymph nodes, mucosa (Peyer's patches), respiratory airways and the skin; in these organs the naïve lymphocytes meet foreign antigens presented by APCs, become activated to undergo clonal expansion, and induce peripheral (foreign antigen) tolerance. Molecules involved in adaptive immunity include cytokines, complement, immunoglobulin super-families (such as MHC molecules, antibodies/immunoglobulins, T cell receptors (TCR) and B cell receptors (BCR)). These molecules are critical for specificity, signal transduction, and cell homing. For example, immunoglobulins are encoded by genes that can be re-arranged and play an essential role in B lymphocyte diversification.

1.1.2.1 Humoral immunity

Humoral immunity is mediated by antibodies, produced by B cells, which function to defend against extracellular microbes and microbial toxins. B cells derived from bone marrow stem cells meet pathogens in peripheral organs and become differentiated into plasma cells. Short-lived, antibody secreting plasma cells can travel around the body to perform their effector functions. Long-lived antibody secreting plasma cells can travel back to bone marrow or reside at the port of infection as memory cells; here they can magnify immune responses to fight against infection caused by the same antigen. The main effector function of humoral immunity, conveyed by antibodies, is neutralization. It blocks the binding of microbe/microbial toxins to receptors in host cells, therefore inhibiting infections by the microbe. It can also facilitate phagocytosis by binding of IgG Fc to Fc receptors on phagocytes after opsonization of microbes. Alternatively, it can induce antibody dependent cellular cytotoxicity (ADCC) by coating infectious cells. Another important function of antibodies is the activation of complement by the classical and alternative pathways, which induce the killing of microbes through the molecule attacking complex (MAC).

Humoral immunity plays a critical role in mucosal immunity and especially neonatal immunity. The dominant antibody in the mucosal system is IgA. Its main function is the neutralization of microbes by blocking their entry into host cells. Since neonates lack an effective immune response against microbes, passive humoral immunity by placental transfer of maternal antibodies, IgA and IgG, is critical for the prevention of infection in new bone infants. In antitumor immunity, antibodies can be utilized to fight cancers either by activating MACs, or by ADCC.

1.1.2.2 Cellular immunity

In contrast to humoral immune response, the cellular immune response is triggered mainly by the recognition of a peptide-major histocompatibility complex (pMHC), which is a MHC molecule and an epitope of a foreign antigen on antigen presenting cells. The major histocompatibility complex was discovered as the determinant of tissue transplant and later found to be involved in the presentation of antigens to lymphocytes in the immune responses. In humans, it is called the human leukocyte antigen (HLA). It is encoded by a polymorphic gene located on chromosome 17, which determines class I molecules, class II molecules, and several proteins, such as

transporters associated with antigen processing (TAP), all play critical roles in antigen presenting.

MHC molecules are required for antigen presenting to T lymphocytes but not in the discrimination of a foreign and self-antigens. Both MHC-I and MHC-II molecules are similar in molecular structure, but different in the components that determine the types of T lymphocytes they activate. They both consist of two non-covalently associated polypeptide chains. The MHC-I molecule consists of a 44-47 kD α-chain (α1, α2, α3) and a 12 kD β_2-microglubulin; α1 and α2 form a peptide-binding cleft of 8-11amino acid (aa) peptides. The MHC-II molecule consists of a 32-34 kD α-chain (α1, α2, α3) and a 29-32 kD β-chain; α1 and β2 form a peptide-binding cleft for peptides of approximately 25aa. The MHC-I molecule is expressed constitutively on all nucleated cells, while the MHC-II molecule is expressed only on antigen presenting cells, such as dendritic cells, B lymphocytes, macrophages and others. Both MHC-I and MHC-II lead to different antigen presentation through either the exogenous pathway or endogenous pathway, and stimulate different types of lymphocytes leading to different immune responses.

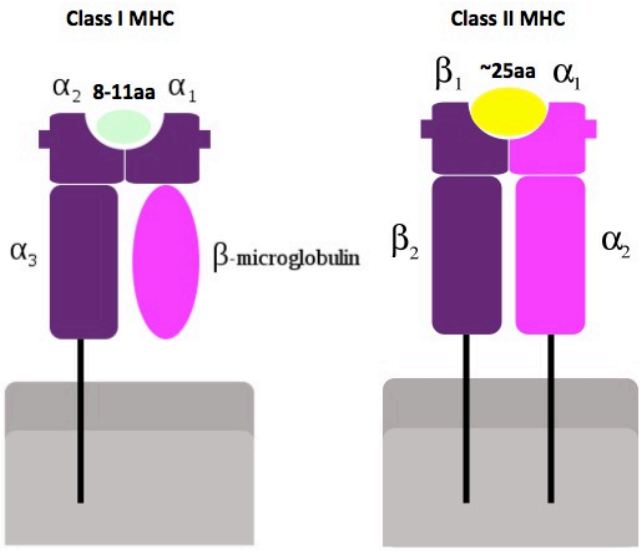


Fig. 1-1 The structure of class I and class II, MHC molecules. Each consists of an extracellular peptide-binding groove, immunoglobulin-like domains and transmembrane and cytoplasmic domains; Class I MHC molecule consist of two noncovalently linked polypeptide chains, α chain (including α_1 domain, α_2 domain, α_3 domain) and β_2-microglobulin; Class II MHC molecule consist of two noncovalently associated polypeptide chains, α chain (including α_1 domain, α_2 domain) and β chain ((including β_1 domain, β_2 domain). Class I and II molecules can bind to 8~11 aa peptide and ~25 aa peptide respectively.

In the endogenous pathway, with the aid of TAP, foreign antigens and self-peptides in APCs can be transported into the endoplasmic reticulum (ER), processed by the proteasome and then cleaved into 8-11 aa peptide. These peptides are then loaded into the cleft of MHC-I molecule to form pMHC-I complex, the complex is then transported to the cell surface, where it activates $CD8^+$ T cells by the interaction of pMHC-I and TCR on $CD8^+$ T cells (Germain 1995; Abbas et al. 2000).

In the exogenous pathway, antigens from the extracellular environment are taken up by APCs through endocytosis or phagocytosis. Then, they are degraded by enzymatic proteins in endosomes or lysosomes, generating ~25aa peptides. These peptides and newly synthesized MHC-II complexes combine as pMHC-II molecules, which are transported to cell surfaces of APCs to interact with TCR on $CD4^+$ T helper cells (Germain 1995; Watts 2004). It is important to note that cross-presentation to MHC-I can occur in the exogenous pathway.

In addition to antigen processing and presentation, an effective cellular immune response requires at least two signals (Bretscher and Cohn 1970). One signal is from the interaction of costimulatory molecules such as B7-1 (CD80), B7-2 (CD86) on APCs with receptors (CD28) on responding T cells. Another signal is provided by cytokines that are secreted from either APCs or activated T cells, which activate and stimulate proliferation and differentiation of effector T lymphocytes and B-lymphocytes, as well as other cells in innate immunity (Heath and Carbone 2001; Ackerman and Cresswell 2004). A lack of antigen presention or a lack of provision of the second signal, mediated by co-stimulators, was found to lead to immune tolerance or anergy to that specific antigen (Hicklin et al. 1998; Wang et al. 1999). Following antigen presentation, naïve lymphocytes become active, and undergo a series of steps for specific T lymphocyte clonal expansion, differentiation, and migration. The two types of T cells, activated by pMHC-I and pMHC-II, are (1) the effector $CD8^+$ T lymphocytes or cytotoxic T lymphocytes (CTLs) and (2) the effector $CD4^+$ T lymphocytes, mediating two different cellular immune responses.

CTLs are effective in eliminating intracellular microbes by killing infected cells. The mechanism of CTL mediated cytotoxicity (CTL response) is antigen specific and contact dependent. CTLs are activated by the binding of antigen specific T-cell receptors as well as CD8 co-receptors to pHMC-I on target cells. With the aid of the interaction of adhesion molecules on each cell, CTLs

and target cells form stable contacts. At the site of the contacts, CTLs kill the target cells by delivering cytotoxic proteins to the target cells.

The immune reactions mediated by effector $CD4^+$ T lymphocytes are diverse. Different subsets of effector $CD4^+$ T lymphocytes that produce different cytokines may be induced. The immune responses against microbes within phagocytes are modulated by $CD4^+$ T helper-1, also called Th-1 cells. Phagocytes can act as APCs and present microbe antigens to CD4+ T cells. Receiving the 2^{nd} signal from the interaction between the co-stimulator (CD40) and the T cell receptor on T cells, naïve $CD4^+$ T cells become mature and secrete IFN-γ, which stimulates the cytotoxicity of phagocytes as well as $CD4^+$ T helper dependant CTL responses. APCs also activate $CD4^+$ T helper-1 (Th-1) cells to secrete cytokines that stimulate B cells to produce antibodies, which can lead to opsonization and phagocytosis of microbes. $CD4^+$ T helper-2, also called Th-2 cells, can mediate humoral immune responses against helminthic infection by secreting cytokines that induce the secretion of coating antibody IgE and eosinophil/mast cell-mediated immune responses.

1.2 Tumor immunology

1.2.1 Immune tolerance

In antitumor immunity, tumor antigen-specific $CD8^+$ CTLs are effective in the killing of tumor cells. As tumor cells do not express MHC-II molecules or co-stimulators, professional APCs such as dendritic cells are needed. Tumor-associated antigens (TAAs) can be cross-presented by APCs to $CD4^+$ T cells to promote the differentiation and proliferation of cytotoxic $CD8^+$ T lymphocytes.

Immunological tolerance is important in the prevention of autoimmunity but it can lead to tumor development. The mechanism for induction of immune tolerance provides information on how immune responses are generated in eliminating tumor cells or how immune responses are down-regulated in organ/tissue transplantation.

During T cell development, lymphocytes undergo central tolerance, such that lymphocytes that recognize self-antigens with high affinity are induced to undergo apoptosis through death receptor or mitochondrial pathways. In central tolerance for T lymphocytes, $CD4^+CD8^+$ double

positive T cells go through positive selection in the thymus cortex; they meet self-antigens and are either eliminated if no reaction take place or develop into two main types of signal positive T cells ($CD4^+$ T cells or $CD8^+$ T cells), depending on the type of MHC molecule. The newly generated signal-positive T cells then go through negative selection in the thymus medulla; T cells, recognizing self-antigens with high affinity are eliminated; the remaining T cells survive as a naïve/unresponsive T cell for immune surveiliance. Mature lymphocytes in peripheral tissues also develop peripheral tolerance if they are capable of responding to tissue specific antigens. Most of the knowledge about peripheral tolerance is based on $CD4^+$ T cells. Active $CD4^+$ T cells can be induced to become functionally unresponsive via the lack of a 2^{nd} signal provided by co-stimulators or the lack of potent innate immune responses. $CD4^+CD25^+$ T cells, when they are exposed to self-antigens, can convert to regulatory T cells (Sakaguchi et al. 2010).

1.2.2 Immuno-surveillance, immuno-editing and tumor antigens

Early in the 1950s, Burnet and Thomas proposed a concept called “immuno-surveillance”, wherein lymphocytes patrolling the body can recognize and destroy clones of transformed cells before they grow into tumors (Burnet 1957). When knockout mice were available two decades later, tumors were found in these mice because of a lack of necessary immune elements (Kaplan et al. 1998; Street et al. 2001; Shankaran et al. 2001). The basic concept was renamed “cancer immunoediting” (Dunn et al. 2004), which included three phases: elimination, equilibrium, and escape. The elimination phase was the classic “immuno-surveillance”. The equilibrium phase was the attempt to maintain the tumor, by further genetic mutation or accumulation, making the tumor more resistant to immune attack. Tumor antigens were first established in transplantation. Mice, transplanted with tumor cells from other species, were identified with antibodies to antigens that were shared by the same type of tumors (Gross 1943; Prehn and Main 1957; Klein et al. 1960). Various types of tumor-associated antigens (TAAs) were discovered and classified as follows: mutated tumor suppressor gene product (p53); abnormal expression of oncogene (HER2/neu); antigens of oncogenic viruses (EBV, HPV); antigen of oncofetal gene production (CEA, AFP); differentiation antigens (gp100, MART-1) and others (Tuting et al. 1997; Abbas et al. 2000; Renkvist et al. 2001; Davis et al. 2003; Novellino et al. 2005).

There are various mechanisms that might induce tumor tolerance, leading to the growth, and spreading of the tumor, and eventually the death of the host.

i. Loss of antigen

Recent studies on the effect of DNA vaccines, encoding a suicide gene demonstrated that the DNA vaccine could reject most melanoma cells; later the tumor broke through the therapy and expressed a different phenotype by losing the expression of tumor associated antigen (Sanchez-Perez et al. 2005). Losses of melanoma-associated antigens, i.e. MART-1, MAGE-3, gp100, was also reported in clinical trials (Khong et al. 2004; Riker et al. 1999; Gajewski et al. 2001).

ii. Loss of MHC

Weak presentation of antigens by MHC loss in tumors is another mechanism to avoid immune destruction. Minimal presentation of antigens can be induced by: a mutation or defect in the expression of β_2-microglobin of the MHC-I molecule or a defect in TAP-1 or TAP-2 (Hicklin et al. 1998; Wang et al. 1999; Seliger et al. 1996; Vitale M et al. 1998).

iii. Lack of co-stimulatory signal and induction of regulatory T cell

Lack of MHC-II molecules or co-stimulatory molecules on tumor cells may induce suppressive regulatory $CD4^+$ T cells which play an important role in inhibiting autotumor immunity (Sakaguchi et al. 1995; Shimizu et al. 1999; Sakaguchi et al. 2001).

Natural Treg cell in peripheral tissues may suppress immune responses to tumors in a non-antigen-specific manner (Bluestone and Abbas 2003; Cozzo et al. 2003). Exprimental evidence from mouse models and cancer patients has demonstrated that deletion of Tr can enhance antitumor immunity (Liyanage et al. 2002; Curiel et al. 2004).

iv. Suppressor products from tumors

Transforming growth factor (TGF-β) is secreted in large quantities by tumor cells. It inhibits the proliferation of effector lymphocytes and macrophages, thereafter, enhancing tumor growth. Antigen masking by secretion of glycocalyx molecules by tumor cells may also depress antigen-presentation (Alroy et al. 1982).

1.3 HER2/neu positive breast cancer

1.3.1 HER2/neu biology

1.3.1.1 HER2 and HER family

Human Epidermal Growth Factor Receptor 2 (HER2), also known as c-erbB-2 and CD340 (cluster of differentiation 340), is a cell membrane surface-bound receptor tyrosine kinase, which belongs to the epidermal growth gactor receptor family (HER/EGFR family). The other three members of the HER family (HER1/ EGFR-1, HER3/ EGFR-3, HER4/ EGFR-4), have also been identified and function as receptor tyrosine kinases (RTKs). All HER/EGFR families contain an extracellular ligand-binding domain (ECD), a trans-membrane domain (TM), and an intracellular domain (ICD), which consists of a highly conserved tyrosine kinase domain and a cytoplasmic tail. The cytoplasmic tail contains a specific binding motif for a src-homology domain (SH2) (Albanell et al. 1999; Slichenmyer et al. 2001), which interacts with many signaling molecules and result in changes of gene expression, and triggering of biological functions (Gullick et al. 2001).

The first receptor tyrosine kinase identified was the 170 kDa protein HER1. It is the cellular homologue of the retroviral avian erythroblastosis tumor gene, v-erbB and is commonly over-expressed in human cancers (Cohen and Taylor 1974; Downward et al. 1984; Kim et al. 2001; Harari 2004; ROSS et al. 2003). The known ligands that bind to it are transforming growth factor-α (TGF-α), amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin and epiregulin (Rubin and Yarden 2001).

HER-3, also known as c-erbB3, is a 160 kDa receptor. It is the only family member that lacks tyrosine kinase activity (Guy et al. 1994) and is therefore unable to direct cell signaling (Kim et al. 1998). However, it can be a partner of other EGF receptor family members to form heterodimers that lead to activation of pathways in cell proliferation or differentiation. The known ligands which bind to it are heregulins and neu-differentiation factors, which are also known as neuregulins (Hellyer et al. 2001; Carraway et al.1997; Horan et al1995; Singer et al. 2001; Rubin and Yarden 2001).

HER-4, also known as c-erbB4, is a 180 kDa protein that has four different isoforms depending on how it is spliced (Plowman et al. 1993; Zimonjic et al. 1995; Junttila et al. 2000). Ligands include neuregulins, heparin-binding EGF-like growth factor and betacellulin. Bindings of ligands activate a variety of signals leading to mitogenesis and differentiation. Mutations and/or over-expression of HER-4 are associated with various tumors and linked to schizophrenia (Sardi et al. 2006; Garcia et al. 2000; Huang et al. 2000).

1.3.1.2 HER2 and neu

HER2 is a self-glycoprotein, with the molecular weight of 185 kDa, consisting of 1255 aa, and encoded by a proto-oncogene, *her2;* it is a located on the long arm of human chromosome 17q21 (Coussens et al. 1985). Like the other three members of the EGFR family, HER2 is composed of a cysteine rich extracellular domain (ECD) with four sub-domains consisting of two cysteine rich domains and the signal sequence, a single trans-membrane (TM) domain, followed by a cytoplasmic tyrosine kinase intracellular domain (ICD) (Yarden et al. 2001; Olayioye MA 2001; Slichenmyer, 2001). The extracellular domain is encoded by 632 aa, with 22 aa for the trans-membrane domain and the remaining 580 aa for the intracellular domain (ICD) (Yarden et al. 2001). HER2 is unique, and thought to be an orphan receptor as it has no known ligands. However, it is the preferential dimerisation partner of the other HER/ErbB family receptors, and involved with several signal transduction pathways: 1) the Ras/mitogen-activated protein kinase pathway, 2) the PI3K/Akt pathway; 3) the Janus kinase/signal transducer and activator of the transcription pathway; and, 4) the PLC-γ pathway, leading to proliferation, differentiation, migration and apoptosis (Yarden 2001; Yarden and Sliwkowski 2001; ROSS et al. 2003).

HER2 is associated with the rat neuroblastoma (neu), based on >85% similarities in nucleic acid and amino acid sequences (Yamamoto et al. 1986, King et al. 1985; Semba et al. 1985). The neu protein is also a trans-membrane molecule with a molecular weight of 185 kDa, consisting of 1260 aa. It was first found in rat neuroblastomas and glioblastomas, subsequently termed neu, and is involved in the transformatiion of tumorigenic cells (Padhy et al. 1982; Drebin et al. 1984; Schechter et al. 1984). Transfection of NIH/3T3 fibroblasts with mutant *neu,* where valine acid (V) is replaced with glutamic acid (E) at aa residue 664 (V664E), induced neoplastic transformation (Bargmann et al. 1986Padhy et al.1982; Drebin et al.1986; Yu et al. 1992). In vitro studies showed that down-regulation of this gene expression product by using specific

monoclonal antibodies can reduce the malignant phenotype of transfected NIH/3T3 cells and inhibit cell growth (Drebin et al.1985).

1.3.1.3 HER family and signaling pathway

The activation of HER to start cell signaling usually requires both its ligand and another member of HER family to form a more stable homodimer or heterodimer compared to a monomeric receptor complex (Akiyama et al. 1988; Kokai et al. 1989; Alroy and Yarden 1997;Connelly and Stern 1990). Ligand like neuregulin-1 (NRG-1) usually has two binding sites for HER receptors, a high-affinity but narrow-specificity site (N-terminal) and a low-affinity but broad-specificity site (C-terminal) (Tzahar et al. 1997). The high-affinity site usually binds to HER1, HER3 and HER4, while the low-affinity site binds to HER2 (Yarden 2001). With different ligand binding, ten different receptor dimers, either homodimers or heterodimers, can be formed by two of the four different receptors. Thereafter, the dimers can interact with intracellular substrates, leading to signal transduction pathways involved in cell growth, proliferation, differentiation, migration and apoptosis.

After ligand-binding and dimerization, auto-phosphorylations of tyrosine residues in the kinase domains occurs, the result is recruitment of signal transducers and activators with binding motifs. The binding motifs recognize phosphorylated tyrosines at carboxy-terminal and phosphotyrosine binding domains (PTBs), such as the adaptor proteins Grb2, Grb7, Shc, Crk, and Gab1, and lipid kinases, which interact with intracellular substrates (Sudol et al.1998; Biscardi et al. 1999; Stover et al.1995). Consequently, the various downstream signal transduction molecules, such as Src and phosphatidylinositol 3-kinase, phospholipase Cγ, and protein phosphatases, are activated which then activate the Ras- activated, Shc-activated, mitogen-activated protein kinase (MAPK), the PI(3)K-activated Akt and c-Jun N-terminal kinases (JUNK) pathways for cell proliferation (Tzahar and Yarden 1998; Yarden and Sliwkowski 2001; Prenzel et al. 2001; Baxevanis et al. 2004). It is interesting to note that HER1 is the only member to which the ubiquitin ligase Cbl binds (Levkowitz et al. 1996). HER3 is the one member out of the four, which efficiently induces phosphatidylinositol 3-kinase due to its multiple coupling sites for its regulatory subunit p85 (Jones, J. et al. 1999). In addition, a PDZ domain-containing protein PSD-95 (Postsynaptic Density Protein), known to function in receptor scaffolding, can interact with ErbB4 at neuronal

synapses, leading to the enhancement of neuregulin (NRG)-induced kinase activity (Garcia et al. 2000; Huang et al. 2000).

The hierarchical network formed by heterodimers, allows for cross-talk regarding intracellular signaling to initiate developmental events (Yu and Hung 2000). According to this network, the absence of HER2-specific ligands makes HER2 not only work for both ligand-dependent and ligand-independent dimers but may also make it a possible coordinator of the entire HER signaling network (Yarden 2001). A common partner for HER2 is HER3 that has high affinity for its ligands but lacks kinase activity. When cells grow normally, HER2 containing heterodimers are at a minimal level and the subsequent signaling activation is weak (Baxevanis et al. 2004). When a heterodimer is formed containing HER2, the dimer would possess a higher potent signal transduction capability compared to one without HER2 (Graus-Porta et al. 1997; Rubin and Yarden 2001). Therefore, over-expression of HER2 may be important in cancers.

1.3.1.4 HER2 in development

The HER family plays an important role in cell growth and differentiation, and is expressed throughout embryonic development. HER2 is the most widely expressed but at low levels: expression is detected in the central nervous system, developing bone, gastrointestinal tract, lung, breast, pancreas, ovary, skin, and genitourinary tracts in human fetuses, as well as adult tissues (Coussens et al.1985; Quirke et al. 1989; Press et al. 1990; Olayioye et al. 2000). The importance of HER2 in embryonic development was discovered in HER2 knockout mice. If HER2 is absent during mouse embryonic development, fetus die in the uterus by E11 (embryonic day 11). Death resulted from lack of HER2/HER4 binding to the ligand neuregulin-1 which is required for formation of myocardium; this in turn, causes trabeculae malformation in heart, and nervous system deficiencies as well (Lee et al. 1995; Morris et al. 1999; Meyer and Birchmeyer 1995). HER2/neu after birth is involved in normal breast development and growth (Di Augustineand Richards 1997; Jones et al. 1996; Normanno and Ciardiello 1997; Yang et al. 1995).

1.3.2 HER2 and breast cancer

1.3.2.1 The epidemic of breast cancer

Breast cancer is currently the most common cancer in women comprising 22.9% of invasive cancers in women (World Cancer Report. International Agency for Research on Cancer 2008); it

accounts for 16% of all female cancers. In Western countries it is the leading cause of cancer-related deaths. In Canada, 21,800 women are diagnosed with the disease each year, and 1 in 27 women will die from it (Canadian Cancer Society, 2006). Breast cancer is strongly age-related. With 5% of all breast cancers in women under 40 years old, and it is considerably more aggressive when occurring in young women (Sariego 2010). Risk factors of breast cancer include hormone levels (Yager and Davidson 2006), race, hereditary history, economic status and dietary iodine deficiency (Stoddard et al. 2008). Breast cancer can also occur in men, usually in an invasive form (Giordano. 2004).

1.3.2.2 Treatment of breast cancer

Treatments for breast cancer include surgery, radiation, hormone therapy, chemotherapy and anti-HER2/neu targeted therapy. Surgery is the physical removal of the tumor. Drugs can be used afterwards as an adjuvant therapy or can be used prior to surgery. Hormone blocking therapy can be a long-term treatment in hormone-positive cancer patients, after testing the levels of estrogen and progesterone receptor (ASCO 1998). In many cases, especially in advanced diseases like metastatic breast cancer, cancer does not respond to the conventional treatments listed above. Hormone therapy, chemotherapy and antibody therapy are combined in the treatment of metastatic breast cancer, which is advanced disease that remains incurable with a limited time of survival. There are toxic effects, such as drug-induced apoptosis in normal tissues when treatments are combined, with limited outcomes (Li et al.1994; Mesner et al.1997; Ozoren et al.2003; Kaufmann and Earnshaw. 2000). Therefore, there is urgent need for novel therapies that can be more specific and effective but less toxic for this devastating condition.

1.3.2.3 HER2 in Breast cancer

The HER2 gene is expressed only at low levels in normal adult tissues (Press 1990) but at high levels in malignances. Of all breast cancers cases, approximately 25~30% have an amplification of the HER2 gene or over-expression of its protein product (McCann et al. 1991; van de Vijver et al. 1987) and is associated with a high recurrence rate (Slamon et al.1987; Slamon et al. 1989; Gusterson et al.1992; Hynes and Stern 1994). Methods to detect and measure the HER2/neu status in clinical breast cancer samples can be either morphology-based or molecular-based, including matrix-blotting techniques, such as Southern, Northern or Western to detect the DNA, RNA and protein status.

The chromogenic in situ hybridization (CISH) technique and fluorescence in situ hybridization (FISH) are the predominant methods utilized and have been approved by the US Food and Drug Administration (Ventana INFORM™, Abbott-Vysis Path Vysis™) (Ross et al. 2003; Ross et al.1998; Press et al. 1997). Since most CISH assays are semiquantitative due to the broad distribution of HER2/neu in all epithelial cells, it is necessary to establish a meaningful relationship between the number of HER2 receptors and the interpretation of the HER2/neu immunostain. The scoring system from studies on cell lines showed that when the receptor number was under 20,000 per cell, no stain would show; partial stain (~10% of the cells) occurred with 100,000 receptor; light to moderate stain ocurred with 500,000; and strongly stained appeared with 2,000,000 receptors (Ross et al. 2003). On the surface of normal breast cells, there are only 20-50,000 HER2/neu receptors, whereas, on tumor cells, there were two million receptors (Lohrisch and Piccart 2001).

Gene amplification and the overexpression of HER2 also occurred frequently in other malignances, including ovarian cancer (Berchuck et al. 1990; Slamon et al.1989), endometrial cancer (Berchuck et al.1991), non-small-cell lung cancer (Kern et al. 1990), gastric cancer (Park et al.1989;Yokota et al.1988; Yonemura et al.1991), bladder cancer (Zhau et al.1990), prostate cancer (Zhau et al.1992), renal cancer (Herrera 1991) and pancreas cancer (Yamanaka et al. 1993; Lei et al. Piccart 2001).

1.3.2.4 HER2 for prognosis, predictive factor and immunotherapeutic target

A prognostic factor “is one that provides information regarding patient outcome at the time of diagnosis”. A predictive factor “is one that provides information regarding the likelihood of response to a given therapeutic modality” (Schnitt 2001). The outcome of a prognosis is the prediction of the probability of progression-free survival (PFS) or disease-free survival (DFS), as associated with the potential of the growth of a tumor and/or its metastases. The outcome of a predictive factor is the sensitivity expected to a certain therapy (Kaptain et al. 2001).

Slamon’s group examined the relativity of overexpression of HER2 to the survival of patients compared to those with normal HER2 expression. The accepted results are that HER2 overexpression has a poor prognosis (Slamon et al. 1987; Ross and Fletcher 1999; Ross et al. 2003). The utility of HER2 overexpression as a predictive factor for response to theraphy

suggests that HER2 results in resistance to hormonal therapy and some types of cytotoxic chemotherapy (Ross and Fletcher 1999; Ross et al. 2003), which is beneficial in predicting the therapeutic response to breast cancer treatment. No definitive conclusion should be drawn because the interpretations of the data available are complex.

Beyond the clinical utility of HER2 to guide the selection and management of treatment, HER2 is also an ideal target for specific-anticancer therapy, since it is amplified and overexpressed in many human malignancies and it serves as a tumor maker. There are three reasons for HER2/neu to be an ideal therapeutic target in breast cancer:

1. HER2 is expressed at a high level in 30% of invasive breast cancer, but at low levels of normal tissues.

2. The role of HER2 in the signaling network indicates that it is important in the growth of breast cancer and in the development of new anticancer therapies, targeting the HER2 receptor (Yarden 2001; Yarden and Sliwkowski 2001).

3. Both humoral and cellular immune responses against the self-protein HER2/neu have been observed without any sign or indications of autoimmunity in breast cancer patients (Disis et al. 1994; Disis et al. 1997; Disis et al. 2000). The anti- HER2 monoclonal antibody (MAb) has been developed, approved (Herceptin$^{(R)}$ (trastuzumab) by the US Food and Drug Administration (FDA) and used in clinical breast cancer cases. The MAb can improve disease-free survival of median stage HER2 positive breast cancers (Jahanzeb 2008; Clifford 2007).

Although clinical results show that the immune responses are not strong enough to eliminate the tumors, HER2/neu specific T-cells have been identified in HER2/neu positive patients, indicating a portion of the T cell repertoire is able to recognize the self Ag (Fisk et al. 1995; Peoples et al. 1995), with the possibility of finding a novel approach to circumventing the tolerance problem. These factors combined may make HER2/neu a suitable target in developing novel immunotherapies, whether passive or active, for HER2/neu positive breast cancer.

1.3.3 Investigations into HER2-targeted immunotherapies

1.3.3.1 Monoclonal antibody targeting HER2

There are two classes of therapeutic agents targeting HER2 in clinical practice today (Mendelsohn 2003); one is monoclonal antibodies (MAbs) that bind the ECD of HER2, thereby affecting the dimerization of HER2; the others class tyrosine kinase inhibitors (TKI), which block phosphorylation and activation, and thus prevent subsequent downstream signaling events.

Trastuzumab, or hercepin, is a recombinant, humanized MAb, developed by Genentech Inc. (San Francisco, CA) that contains 95% human sequences with only 5% remaining murine sequences (Carter et al. 1992). It was approved by the Federal Drug Administration (FDA) in 1998 and used in clinical treatments for metastatic and early breast cancers. Preclinical studies in vitro and in animal models found that such MAbs bind to the HER2 in a way that does not harm the homodimerization or heterodimerization with other members of the HER family. The anti-proliferative effects are associated with blockage of the G1 phase of the cell cycle which is associated with antiangiogenic effect and antibody-dependent cellular cytotoxicity (ADCC) (Nahta et al. 2003; Clynes et al. 2000). Other antibody studies targeting the HER2 signaling pathway are ongoing, with some already in clinical trials, such as pertuzumab, a fully humanized MAb targeting a different epitope of the ECD of HER2, also known as HER dimerization inhibitor (Baselga et al. 2002).

Clinical studies found MAbs favorable in treatment of the early stage of breast cancer, but with a low response rate (Vogel et al. 2002; Cobleigh et al. 1999). However, there were also serious adverse events, especially cardiotoxicity and hypersensitivity reactions, in patients with such treatment. Therefore, it should be administrated as a combination therapy with other treatments, such as chemotherapy, to increase response rate and survival length for advanced breast cancer patients with less side effects (Esteva et al. 2002; Fountzilas et al. 2001; Burstein et al. 2003; Robert et al. 2006; Chia et al. 2006; Perez et al. 2005; Pegram et al. 2007). There are also, other drawbacks. First, MAb resistance eventually develops in cancer patients under MAb treatment, which might be due to a lack of trastuzumab-binding extracellular domain. An example is MAb resistance occurs if truncated HER2 lack the ECD but retaining its kinase activity (Molina et al.2002; Nagy et al. 2005). It was also found that MUC4, a membrane-associated mucin, was

correlated with resistance and indirectly correlated with the binding of trastuzumab; it did so by masking membrane proteins, such as HER2. Secondly, the strategy and schedule of trastuzumab administration is in question, due to its short half-life in patients and the risk factors in the patient population, such as advanced age, preexisting cardiac disease or hypertension (Suter et al. 2004).

Combining MAb treatment with chemokines, peptide vaccines, or DC vaccines may have potential clinical utilities. Trastuzumab plus taxanes, produced by plants of the genus Taxus (yews), were found to induce recruitment of natural killer cells to tumor sites (Arnould et al. 2006). Furthermore, trastuzumab plus paclitaxel were found to enhance endogenous humoral and cellular anti-HER2 responses, which were associated with favorable clinical outcomes in patients with advanced breast cancer (Taylor et al. 2007). Also, HER2 peptide-vaccinated patients became sensitive to the effects of trastuzumab on autologous breast tumor cells (Mittendorf et al. 2006). Recently anti- HER2 antibody treatment combined with an AdV_{HER-2} vaccine was capable of curing 4 out of 10 mice bearing well-established Tg1-1 breast tumors, and significantly delayed the death of the remaining 4 tumor-bearing mice (Chen et al. 2011). Taken together, a combination MAb treatment with other therapies may be used as a new therapeutic strategy for advanced HER2 -positive breast cancer.

1.3.3.2 HER2/neu-targeted immunotherapeutic strategies

1.3.3.2.1 Tumor cell based vaccine

Autologous or allogeneic whole-cell based tumor cell vaccines have been studied for decades. The hypothesis is that tumor cells express many tumor-associated antigens (TAAs) and thus present multiple tumor Ags, which may induce sufficient innate immunity as well as adaptive immunity to generate antitumor immunity. However, the tumor cell based vaccines tend to be less immunogenic.

The induction of antigen-specific immune responses depends on the presence of co-stimulatory signals provided by APCs. Therefore, most solid tumors are unable to provide all the signals necessary for T-cell activation since they do not express the co-stimulatory molecules. Autologous or allogeneic tumor cell vaccines need to be used in combination with strong adjuvants (i.e. BCG (Bacillus Calmette-Guérin); influenza virus) (Wiseman et al. 1995; Ahlert et

al. 1997), cytokines (i.e.: GM-CSF, IL-12) (Mach and Dranoff, 2000) or fused dendritic cells (Avigan et al. 2003) to improve their immunogenicities (Nawrocki et al. 2001). Thus, co-stimulatory factors, such as CD80 or cytokines, have been introduced in these vaccines to confer an immuno-stimulant potential to tumor cell-based vaccines (Dols et al. 2003a, Dols et al. 2003b). GM-CSF was the most potent cytokine adjuvant for vaccination (Dranoff et al. 1993). Tumor cells, engineered to secrete GM-CSF, reportedly reduce spontaneous tumor development in Tg mice if used with cyclophosphamide (Cy) or HER2/neu monoclonal antibody, which enhance the induction of neu-specific $CD8^+$ T cells through Fc-mediated activation of dendritic cells (Machiels et al. 2001; Wolpoe et al. 2003; Kim et al. 2008).

Both autologous and allogeneic HER2-positive tumor cells have been used as vaccines to induce antitumor immune responses in clinical trials of breast cancer (Jiang et al. 2000). For example, clinical trials using SVBR-1 or SKBR-3, stage VI breast cancer cells transfected with GM-CSF plus IFN-α or plus cyclophosphamide/ trastuzumab, have been conducted. In these styudies, HER2-specific delayed-type hypersensitivity developed in most patients who received vaccine alone or with Cy. HER2-specific antibody responses were enhanced by Cy but higher Cy doses suppressed immunity (Emens et al. 2009).

1.3.3.2.2 Protein-based vaccine

Protein-based vaccines, where entire epitopes of immunogenic proteins are presented with MHC class II molecules on APCs through the exogenous pathway, have been studied for decades. Such a therapy may stimulate humoral immune responses to an infectious disease. An example of a protein-based vaccine that has been developed for clinical use is the protein subunit vaccine against Hepatitis B virus.

With regard to HER2/neu positive breast cancer, whole HER2 protein or the ECD of HER2 may induce anti-HER2 immune response although the response is too weak to protect mice after tumor challenge (Taylor et al. 1996; Dela Cruz et al. 2003); others have demonstrated that they may induce tolerance (Bernards et al. 1987; Disis et al. 1998). Whole HER2 protein or ICD or ECD of HER2 protein together with adjuvants or cytokines may induce stronger immune responses (Disis et al. 2004b). Nevertheless, the disadvantage of a protein vaccine is the lack of CTL responses, which is essential in eliminating of tumor cells.

Phase I clinical studies on stage II/III/VI breast cancer patients using HER2 proteins (HER2 ICD, aa 676–1255), observed T-cell responses specific to HER2 ICD in 89% of immunized patients and 82% developed anti-HER2 IgGs. More than 50% of patients maintained cellular immunity for 9–12 months after completion of immunizationsin combination with GM-CSF injected intradermally once a month for 6 months. Patients who received the highest dose more rapidly developed anti- HER2 immunity (Disis et al. 2004b). Another phase I clinical study on stage II/III, using dHER2 (HER2 ECD and ICD) showed that Abs specific to HER2 ECD and ICD developed after four immunizations (Limentani 2005). However, some years later, another group used a protein-based vaccine composed of a truncated HER2 protein (aa 1–146), complexed to a delivery system consisting of cholesteryl pullulan nanogels (CHP) to vaccinate breast and ovarian cancer patients. Kitano found that the vaccine was well-tolerated and induced $CD4^+$ T and/or $CD8^+$ T-cellular responses specific to the truncated HER2 protein (Kitano et al. 2006). A later study of this vaccine in combination with GM-CSF in patients with HER2 positive cancer found that none of them developed Abs which recognized the HER2 antigen expressed in its native form at the surface of tumor cells (Kageyama et al. 2008).

1.3.3.2.3 Peptide-based vaccine

Compared to a protein-based vaccine, a peptide vaccine is more specific and able to elicit both humoral and cellular immune responses according to the MHC type restriction of the epitope identified. Peptide vaccines are also small and easy to produce. However, the drawbacks are: 1) the requirement of epitope identification; 2) the short half-life *in vivo*, and 3) the possibility of tolerance induction.

Among the HER2 peptides described, E75 (p369, aa 369-377) has been entered into several clinical trials. Completed clinical studies on E75 vaccines, combined with incomplete Freund's adjuvant (IFA) or GM-CSF showed that vaccination induced a specific anti-peptide immune response with no associated toxicity (Zaks et al. 1998; Knutson et al.2002; Murray et al.2002); this result was correlated with higher disease-free survival and significantly lower recurrence rates in node-positive breast cancer patients (Peoples et al. 2008). Some clinical trials with E75 vaccine, combined with trastuzumab have been completed or are ongoing (Mittendorf et al. 2006; Benavides et al. 2009; Peoples 2008). Results showed an increase in the specific and stronger cytotoxicity of CTLs in patients, which could be explained by a higher internalization

and faster recycling rates of HER2 following trastuzumab binding (Mittendorf et al. 2006).

It is important to note that HER2 expression could down-regulate expression of MHC class I antigens, which, in turn, impaires the ability to produce and display MHC class I peptide ligands to specific CTLs (Choudhury et al. 2004; Herrmann et al. 2004; Vertuani et al. 2009). Thus, designing HER2 targeted vaccines that induce both HER2-specific CTL and antibody responses.

Preclinical and clinical studies on HER2 multiple-peptide vaccines demonstrated that these vaccines induced both HER2/neu specific humoral and cellular immune responses (Keith et al. 2002; Zaks and Rosenberg 1998; Disis et al. 1999). The humoral response was less important than the anti-HER2 cellular response (Disis et al. 2000; Disis et al. 2004a), due to the fact that the vaccines were derived from HLA-restricted T epitopes and/or that the vaccine adjuvant (GM-CSF) promotes predominantly a cellular response of Th1 type, characterized by IFN-γ secretion (Knutson et al. 2001). Clinical studies also showed concomitant epitope spreading, which may be general for immunotherapy in oncology and/or a benefit in vaccination trials (Butterfield et al. 2003; Disis et al. 2008). As for clinical studies on MHC-Class II peptide vaccine, phase I clinical trial showed that vaccines, composed of the MHC-II peptide of HER2 (aa 776–779) alone (AE36) or fused on the C-terminal part with a sequence of 4 aa (LRMK) (AE37) with or without GM-CSF, could interact with MHC-II molecules and increase dose-dependent $CD4^+$ T cell responses (Holmes et al. 2008).

1.3.3.2.4 DNA based Vaccines

The immune responses against transgene products expressed by DNA plasmids were first discovered by Wolff and colleagues (Wolff et al. 1990). They found that direct intramuscular (i.m.) inoculation of naked plasmid DNA in mice could induce both transgene specific cellular and humoral immune responses. This finding caused a tremendous amount of research interest in the use of DNA vaccination in preventing infectious diseases.

1.3.3.2.4.1 The design of DNA vaccine and its advantage

Several key elements need to be taken into account in design of DNA vaccine. First, for safety reasons, the plasmid DNA should persist in the eukaryotic cell nuclei as a circular non-replicating episome that does not integrate its DNA into the host's genome. Secondly, plasmid DNA should contain a replication of origin (ori) that allows the plasmid to replicate within

bacterial cells, as well as an antibiotic resistance gene to serve as a selective drug marker. Thirdly, multiple cloning sites (MCSs) are needed for gene insertion, with an appropriate transcription terminator segment. Finally, the promoter, (or a eukaryotic transcription regulatory element) located upstream from the gene is needed to allow for gene transcription. Cytomegalovirus (CMV) and the simian virus 40 (SV40) promoters are common promoters. Elements that stabilize the mRNA transcript, such as polyadenylation sequences, can be used downstream of the promoter (Gurunathan et al. 2000).

Plasmid DNA as a vaccine has many advantages. DNA vaccine is inexpensive, extremely stable, easy to produce and easy to purify and store. It can be easily modified to enhance Ag immunogenicity by removing or inserting special sequences, such as cytokine genes and co-stimulatory molecules (Gurunathan et al. 2000; Reyes-Sandoval and Ertl 2001; Haupt et al. 2002; Donnelly et al. 2005). DNA for vaccines, which persists in host cells, may result in long-term expression (Wolff et al. 1990, Wolff et al.1992), thus, providing constant Ag for long lasting immunity (Reyes-Sandoval and Ertl 2001). The inherent unmethylated CpG motifs within DNA plasmid from bacteria can act as an adjuvant or a danger signal (Pardoll 2002), as these motifs are members of the pathogen-associated molecular pattern molecules (PAMPs). The antigen expressed within the host cell with the appropriate post-translational modifications makes it escape from clearance by host neutralizing antibody. Most importantly, the nature of DNA vaccines allows for unrestricted MHC presentation of class I and class II epitopes to CD4+ Th and CD8+ CTLs, leading to the induction of both cellular and humoral immune responses specific for the target Ag. One major disadvantage of DNA vaccines is the potential risk that DNA may integrate into the host genome, which could result in disruption of the host genes, leading to tumor growth or autoimmunity (Mahon et al. 1998).

1.3.3.2.4.2 Delivery system and administration route

The dose of DNA, as well as the efficiency and efficacy of immune responses induced by DNA vaccines, depends largely upon the administration route and delivery method.

High doses of plasmid DNA have the potential to induce high cellular and relatively low humoral immune responses to numerous antigens in mice, even in the absence of adjuvant (Ulmer et al. 1993; Wang et al. 1993; Tang et al. 1992; Fynan et al. 1993; Cox et al. 1993; Davis

et al. 1993; Lu et al. 1995; Robinson et al. 1993; Yankauckas et al. 1993). To generate adequate immune responses, DNA for vaccines needs to be transfected into antigen presenting cells (APCs) or somatic cells to enhance presentation of transgene-encoded antigens to the responding T lymphocytes (Gurunathan et al. 2000; Wolff et al.1990; Lu et al. 1995).

There are many different delivery systems and immunization routes available for DNA vaccines. Viruses, such as modified vaccine virus (MVA), fowlpox, and adenovirus have innate transfection capabilities (Bejon et al. 2006). These viruses have been tested for potential use as DNA vaccine delivery vehicles to increase DNA transfection. Non-viral methods have also been investigated, such as the gene gun (particle bombardment) (Yoshida et al. 2000), liposomes (Khatri et al. 2008; Reimer et al. 1999; Ropert et al. 1999), nanoparticle-based approaches (Cohen et al. 2000; Mansouri et al. 2004; Roy et al. 1999; Vijayanathan et al. 2002; Torchilin et al. 2006) and high-pressure liquid delivery methods (Trimble et al. 2003; O'Hagan et al. 2004).

Direct intramuscular (i.m.) or intradermal (i.d.) injections of DNA vectors are common routes for DNA vaccine delivery. For i.m injection, muscle cells (myocytes) are the major cells that take up DNA vectors through phagocytosis or pinocytosis. However, the uptake efficiency is rather low; nearly 90% of the DNA degrades, and less than 1% of the remaining DNA enters the nuclei (Babiuk et al. 2003). To increase transfection efficiency, small pores in cells are induced by electroporation (Banga and Prausnitz 1998; Widera et al. 2000). However, since myocytes express low level of MHCI molecules and no MHCII and co-stimulatory molecules, DNA vaccination do not efficiently prime T cells (Hohlfeld et al. 1994) and may induce immune ignorance.

Dendritic cells (DCs) play a key role in inducing Ag-specific T cells and not the host myocytes when one is vaccinated with DNA (Corr et al. 1996; Iwasaki et al. 1997). For i.d DNA, DNA vectors have been found to preferentially localize in DC areas of the draining lymph node and in Langerhan's cells in the skin (Casares et al. 1997). This was confirmed by utilizing DNA labeling technique such as green fluorescent protein (GFP) and fluorescein isothiocyanate (FITC) (Condon et al. 1996; Chattergoon et al. 1998; Dupuis et al. 2000).

1.3.3.2.4.3 Application of DNA vaccine to HER2 breast cancer study

The first DNA vaccine in mice resulted in cellular immunity against influenza nucleoproteins,

and the discovery of immune responses against the gene expression product of the DNA plasmid (Ulmer et al. 1993). Thus, the DNA vaccine became attractive for antiviral immunization strategies and cancer immunotherapy (Donnelly et al. 1995; Lu et al. 2008).

A variety of research groups have shown that DNA vaccines expressing full length HER2 or partial length (ECD or ICD) of HER2 induces HER2-specific humoral and T cell immune responses (Amici et al. 1998, Chen et al. 1998, Piechocki et al. 2001, Pilon et al. 2001). However, in general, antitumor immune responses induced by DNA vaccines were not able to convey any beneficial therapeutic effects in HER2/neu transgenic (Tg) mice (Amici et al. 1998, Foy et al. 2001; Quaglino et al. 2004a; Radkevich-Brown and Jacob et al. 2009).

To improve immune responses, DNA vaccines were constructed to include GM-CSF sequence and performed by i.m. injection via electroporation to enhance transgene expression. These vaccines were found to elicit stronger HER2/neu-specific antitumor immunity in mouse models (Wei et al. 2005; Jacob et al. 2006; Jacob et al. 2007). A recent study showed that HER2 DNA-based vaccine in combination with a novel agonist of the Toll-like receptor 9, called immuno-modulatory oligonucleotide (IMO), stimulated a stronger anti-tumor antibody-dependent cellular cytotoxicity (ADCC) (Aurisicchio et al. 2009). The same anti-HER2 DNA-based vaccine was investigated by Jacob et al in three different mouse strains. They found that both the amplitude of the induced immune response and the vaccine efficacy depended on the genetic background of the mice. They also found that depletion of T-regulatory cells (Treg) could result in better anti-tumor immunity, although it may exacerbate autoimmunity (Radkevich-Brown et al. 2009).

Some anti-HER2/neu DNA-based vaccination strategies have entered the clinical phase and now are active, such as phase I clinical study on V930 DNA vaccine which encodes HER2 and carcinoembryonic antigen (CEA) (Merck & Co., Inc 2006); pNGVL3 plasmid DNA vaccines encoding HER2 intracellular domain (Salazar L.G. 2006); both studies will be finished by the end of 2012.

1.3.3.2.5 Viral vector vaccine

Compared to non-viral DNA vectors, viral vectors possese higher efficiency in transgene delivery (Arthur et al. 1997; Dietz and Vuk-Pavlovic 1998; Zhong et al. 1999). There are now many viral vectors available for DNA tranfection, including retroviruses, lentiviruses,

adenoviruses (AdV), pox virus, herpes virus, influenza virus, and adeno-associated viruses (Humrich and Jenne 2003). The most frequently used vectors are the retroviruses, and the adenoviruses (Dyer and Herrling 2000).

1.3.3.2.5.1 Retroviruses

Retroviruses are RNA viruse, whoes replication requires the enzyme reverse transcripatase. The insertion of double stranded DNA may increase the risk of mutagenesis to the host genome. The advantages of using retroviral vectors is their transfection efficiency, and the ability to present TAA to both MHC class I and II molecules and thus induce both humoral and cellular immunity. As autologous units, they may also induce fewer neutralizing antibodies (Reeves et al. 1996; Specht et al. 1997).

There are three types of retroviruses that are used as viral vectors: oncoviruses, such as murine leukemia virus (MLV), lentivirus, such as human immunodeficiency virus (HIV), and the foamy viruses (Meyer zum Buschenfelde et al. 2000; zum Buschenfelde et al. 2001; Cui et al. 2003; Breckpot et al. 2004; He et al. 2005; Kim et al. 2005b; Robbins and Ghivizzani 1998; Humrich and Jenne 2003). The moloney murine leukemia viruses (MMLV) have been engineered to be replication defective and will induce HER2/neu specific $CD8^+$ CTL and $CD4^+$ Th1 cells (Meyer zum Buschenfelde et al. 2000; zum Buschenfelde et al. 2001). The lentiviruses, such as the human immunodeficiency virus (HIV), are used to transduce DC; they easily transduce both actively dividing and non-dividing cells and induce strong and long lasting effective protective and therapeutic immunities in various model systems (Robbins and Ghivizzani 1998; Humrich and Jenne 2003; Cui et al. 2003; Breckpot et al. 2004; He et al. 2005; Kim et al. 2005).

Although those viral vectors may induce immune responses, there are safety issues, such as virus infections and DNA integration.

1.3.3.2.5.2 Adenovirus vaccine

1.3.3.2.5.2.1 Adenovirus and its life cycle

Adenoviral vectors, unlike retroviruses, are a safe vector for *in vivo* gene delivery since they do not integrate into the host genome. They can also efficiently infect and transfer genes into a variety of cell types, including dividing and non-divided cells.

Adenoviruses were first isolated in 1953 from human tonsils and adenoidal tissue (Rowe et al. 1953; Hilleman and Werner, 1954), from which the name was derived. Adenoviruses infect various species of vertebrates, including humans. Although adenovirus has a tropisom to the respiratory epithelium, it can also infect and transfer genes in a variety of cell types: dividing and non-dividing cells, macrophages, dendritic cells, myoblasts, endothelial cells, smooth muscle cells, fibroblasts and chondrocytes.

Based on neutralization assays with animal antisera and the biophysical and biochemical criteria, there have been 51 serotypes of human adenoviruses described and grouped into six groups to date. Among human adenoviruses (HAdV), serotype 5 (HAdV5) and serotype 2 (HAdV2) are well-studied, with HAdV5 used as a prototype for generating HAdV vectors because of its low pathogenecity. Studies on the structure and the genome of adenovirus are mostly based on HAd5.

1.3.3.2.5.2.2 Structure and its genome

Adenoviruses are medium-sized (90–100 nm), nonenveloped (naked) icosahedral viruses composed of a nucleo-capsid and a double-stranded linear DNA genome. The viral capsid is composed of three structural proteins: hexon, fibre, and pentose base (Bergelson et al. 1997). Hexon is the major component that forms the 20 facets of the icosahedrons, whereas pentose forms the 12vertices together with the fibre. Hexon residues are responsible for type specificity. The unique "spike" or fiber, associated with each penton base of the capsid, is a trimeric protein that allows for virus attachment to the host cell via the binding to coxsackie-adenovirus receptors (CARs) located on the surface of the host cell (Bergelson et al. 1997). The head domain sequences may explain differences in receptor specificity between serotypes. Although hexons and fibres contain most of the epitopes of adenovirus that can be recognized by neutralizing antibodies, there are also epitopes on pentoses recognized by some neutralizing antibodies and integrins on host cells.

There are three immunoreactive regions in the pentoses, one is located in the N-terminal domain; one overlaps with the fibre-site, and; the other is on both sides of a conserved symmetrical arginyl-glycine-aspartic acid (RGD) motif; the RGD motif is involved in internalization of virus and helps escape from neutralization via the fibre protein steric hindrance that prevents IgG binding to all RGDs.

The double-stranded linear DNA genome is around 37 Kbp with a terminal 55 kDa protein attached to each of the 5' ends of the linear dsDNA. Each of these featured proteins used as a primer in viral replication and ensures the linear genome is adequately replicated (Rekosh et al, 1977). Its DNA can be transcribed into at least 30 mRNA, which can be classified as early genes and late genes. The early genes include E1A, E1B, E2, E3, and E4 involved in cell cycle, viral DNA replication, and delayed early transcripts (IX and IVa2), for invasion of adenovirus into host cells. Late genes (L1 to L5) express the structural proteins (Shenk, 1996; Shenk 2001). Although this virus has a larger genome than other viruses, it is still very simple with its replication and survival reliant on the host cell.

1.3.3.2.5.2.3 Life cycle and immune response to adenovirus

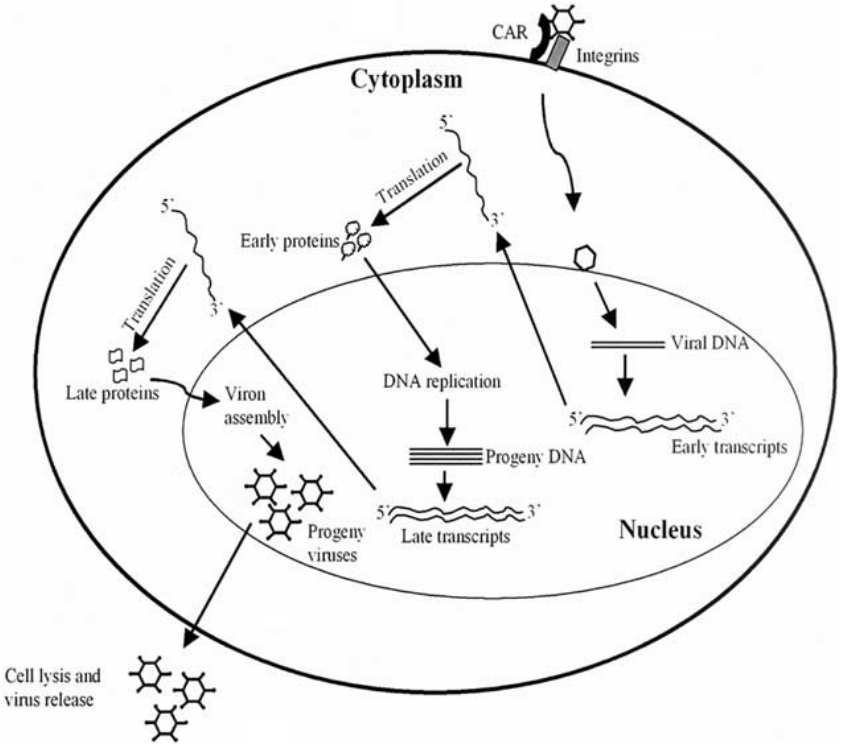


Fig. 1-2 Replication of adenovirus. The replication cycle of adenovirus begins with its infection into host cell. The viral fiber protein attaches to a cellular receptor, such as coxsackievirus group B and adenovirus receptor (CAR). The adenovirus is endocytosed and internalized through the interaction between its penton base and integrins. It is then transported to the nuclear pore complex and DNA released into the nucleus to initiate a cascade of viral gene expressions.

Early and late proteins are produced. These proteins and newly synthesized viral DNA are encapsidated into capsid in nucleus to form progeny viruses. Through cell lysis these virons can be released and further infect host cells nearby.

Infection of adenoviruses into host cells is initiated with the attachment of viral fiber proteins to cellular receptors, such as coxsackievirus group B and adenovirus receptor (CAR) (Bergelson et al. 1997). The adenoviral particle enters the host cell through endocytosis, which is mediated by the interaction between the penton bases and integrins. Subsequently, the capsid proteins start to disassemble sequentially, allowing the viruses to escape from neutralizationl clearance by the host. The virions are subsequently transported into the nuclear pore complex and release their DNA into the nucleus to initiate a cascade of viral gene expressions. Proteins are packaged at 20-24 hour after infection (Greber et al. 1993). After the viral DNA and capsid proteins are assembled and progeny viruses are generated. At 2-3 days post infection, around 10,000 virues per cell can be released for infection of new cells.

Accompanying the infection and replication of the adenovirus are the host immune responses against the virus. The first stage of clearance by the innate immune system, independent of the transgene, occurs within 24 hours. Internalization and degradation by phagocytes, natural killer cells and neutrophils removes 90% of adenovirus (Cartmell et al., 1999; Worgall et al.1997; Michou et al. 1997). The remainder is cleared more slowly over the following weeks by the second stage of clearance, which creates memory toward the virus. The second phase is important for inducing immune responses against the transient transgene expressed product, since during this time, the remaining 10% of adenoviruses with transgenes enter host cells to express the transgene and present the transgene products to APCs (Yang et al, 1996; Yang et al., 1994a&b, Yang et al., 1995). In this second phase, the viral structural proteins/transgene products are processed and bound to MHC molecules and then presented to helper T cells through interactions between protein/peptide-MHCII complexes and T cell receptors (TCRs). With the third signals provided by B7-1/B7-2 and CD28/CTLA4, humoral and cellular immunity to virion and transgene products is generated. Therefore, the lifetimes of adenoviruses must be considered for administration methods so as to overcome the innate and humeral immunity against viral vector and increase the expression of the transgene when utilizing adenovirus as a

vector. These requirements have led to the development of three generations of adenovirus vector systems.

1.3.3.2.5.2.4 Adenovirus as vaccine vector

As a vaccine vector, normally, adenovirus can carry up to 1.2 kb of foreign DNA (Bett et al.1993). However, insertion of large amount of foreign DNA requires deletion of part of the viral genome.

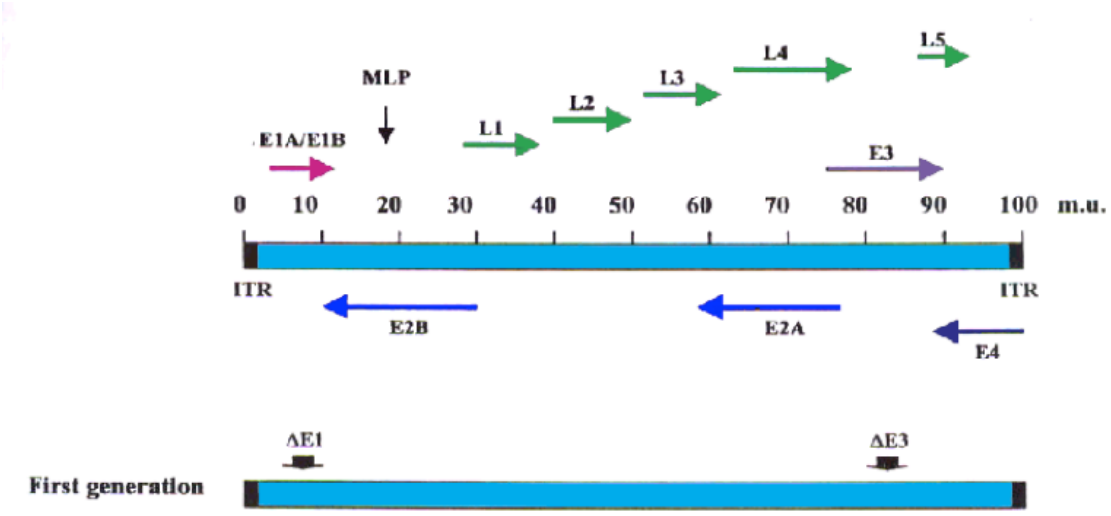


Fig. 1-3 Schematic diagram of adenovirus genome and the first generation of adenoviral vector. The genome length is about 36 kb and is divided into 100 m.u.; E1A/E1B, E2A, E2B, E3, E4 represent early transcriptional genes; L1-L5 represent the late gene; black boxes at the ends of the genome represent the inverted terminal repeats (ITR); arrow above and below the central open box indicate the direction of transcription; the deletion locations (E1 and E3) for the first generation of vector are indicated in black arrows on the bottom figure.

For insertion of DNA larger than 2 kb but smaller than 7.5 kb, the adenoviral vectors can be used. In recombinant HAdVs, the early gene E1/E3, involved in replication of the virus is deleted (Saito 1985). Application of this generation requires complement cell lines like 293 to provide in trans the E1protein, which is more essential in viral replication (Graham et al. 1977). The main drawback with this vector vaccine is that the immune responses induced are, first, against viral capsid antigens and later against the transiently expressed transgene product. The peak responses occur at 3-4 days, and disappear in 1-2 weeks (Cartmell et al. 1999; Worgall et al.1997; Michou et al. 1997; Yang et al, 1996; Yang et al. 1994a, b). Long-term expression of HAdV5 vector-encoded product was found in lungs or livers of immunodeficient mice. The immunogenicity of E1-deleted HAdV5 vectors, as well as its ability to activate immune cells in

the innate immune system, indicate that they could be used for vaccine to induce antitumor immunity (Zhang 2001).

The E1-deleted AdHu5 vectors have been used as a vaccine carrier for rabies virus glycoprotein termed Adrab.gp or HuAd5rab.gp (Xiang et al.1996). These vectors induce neutralizing antibodies, as well as rabies virus-specific $CD8^+$ and $CD4^+$ T cells against rabies virus challenge (Xiang et al. 1995; Xiang et al.1996]. E1-deleted HuAd5 vectors have also been studied in rodents for protecting against Dengue virus (Jaiswal and Swaminathan 2003), Ebola virus (Sullivan et al. 2000; Sullivan 2003), SARS–coronavirus (Gao 2003) and human papillomavirus (He et al. 2000; Tobery et al. 2003; Liu et al. 2000). E1-deleted AdHu5 vaccine vectors, expressing antigens of HIV-1, have also been developed and some entered clinical trials (Fitzgerald et al. 2003; Pinto et al. 2004; Casimiro et al. 2003; Shiver et al. 2002; Lubeck et al. 1997; Pinto et al. 2003; Cohen et al. 2001). Most of these vaccines protect the host through induction of neutralizing antibodies of IgG2a isotype and small amounts of IgG1 against transgene product. Since adenoviral vector interventions in humans critically depend on the selectivity and efficiency of gene transfer to target tissues, receptor-binding Ad protein, the fiber, can be redesigned to enhance the selectiveness and transduction rate (Campbell et al. 2003; Belousova et al. 2008).

Adenoviral vectors, encoding the extracellular domain (ECD) and the transmembrane domain (TM) of HER2 (Ad-HER2-ECD-TM) and the full-length of HER2 with mutation to inactivate its kinase function (Ad-HER2-ki), have been studied for the assessment of their immunogenicity for inducing antitumor immunity, as well as their oncogenicity in both wild-type (WT) and HER2-tolerant transgenic mice. Among these adenoviral vaccines, Ad-HER2-ki was the most immunogenic, by exhibiting high immunogenicities in WT animals, retaining immunogenicity in HER2-transgenic tolerant animals, and showing strong therapeutic efficacy in treatment models (Hartman et al. 2010). For evaluation of the efficacy of AdV vaccine expressing rat ErbB2 antigen in a therapeutic setting, studies in transgenic BALB-neuT mouse model demonstrated that AdV vaccine induced efficient antibody and $CD8^+$ T cell responses against rat ErbB2 (Cipriani et al. 2008). Compared to DNA vaccine expressing rat p185 neu protein, the adenoviral vaccine induced greater Th1-skewed humoral and CD8 T cell-mediated responses than the DNA vaccine (Gallo et al. 2006). Numerous studies have demonstrated that adenovirus-human HER2

vaccines could induce fully protection in WT mice from breast tumor cell challenge; it prolonged lifespans of transgenic mice, but showed less therapeutic effect in both WT and especially transgenic mice (Park et al. 2005; Gallo et al. 2006; Triulzi et al. 2010; Hartman et al. 2010; Felizardo et al. 2011).

We have recently demonstrated that $AdV_{HER2/neu}$ vaccine stimulated HER-2/neu-specific $CD8^+$ CTL responses, leading to a significant reduction in breast carcinogenesis in transgenic FVBneuN mice; it had little therapeutic effect on pre-existing Tg1-1 tumor even at an early stage (Chen et al. 2011). In addition, the HER2/neu adenoviral vaccine when combined with trastuzumab treatment was capable of curing 4 of 10 mice bearing well-established Tg1-1 breast tumors.

1.3.3.2.6 Dendrtic cell (DC) vaccines

Cancer vaccines can now induce therapeutic immunity capable of rejecting existing malignancies, either well-established or spreading extensively. Conventional vaccines, designed to defend against infectious diseases, are known as protective vaccines; effective immunity can be generated before infection but not after diseases are established. Therapeutic immunity is not easy to generate against existing diseases. However, it is now appealing to develop even more powerful vaccine modalities to manipulate the nature of the host's immune system to overcome the difficulty of treating established diseases.

DCs are considered to be the most powerful and efficient antigen-presenting cells (APCs) and, to a large extent, responsible for priming T cell responses (Zinkernagel 1974; Linsley et al. 2002). DCs are termed "nature's adjuvant" in generating effective immune responses (Steinman and Dhodapkar 2001; Steinman and Pope 2002). Thus, it is logical to utilize DCs in vaccination schemes to achieve stronger antitumor immunity. An understanding of how DCs induce, regulate, and maintain T cell immunity is as important as understanding tumor immunology in the design of novel cancer vaccines with improved clinical efficacy (Banchereau and Steinman 1998; Steinman and Banchereau 2007; Melief 2008).

1.3.3.2.6.1 DC biology

DCs are found in the skin, spleen, liver and peripheral blood. The term is defined based upon its adherent cell type and stellate morphology (Steinman and Cohn 1973). DCs are characterized by

cytoplasmic extensions or membrane processes that form dendrites, pseudopods or veils. They express high levels of DC marker (CD11c), MHC class I and II molecules, costimulatory molecules (CD80, CD86 and CD40), adhesion molecules (CD11b, CD54), and receptors (FCrR, MMR) for efficient Ag capture.

DCs were first discovered by Paul Langerhans in the late 1800s and named Langerhans cells. In the following decades, different subsets and functions were studied. There are two major DC subsets: 1) the classical DCs (cDCs) or myeloid DCs, and; 2) the plasmacytoid DCs. In human skin, Langerhans cells (LCs) reside in the epidermis. $CD1a^+$ DCs and $CD14^+$ DCs, as well as macrophages, reside in dermis and belong to cDCs (Zaba et al. 2007; Klechevsky et al. 2008). LCs are potent inducers of antigen specific $CD8^+$ T cells (Celluzzi et al. 1997). Whereas, $CD14^+$ DCs are potent inducers of humoral immunity (Klechevsky et al. 2008, Caux et al. 1997). Plasmacytoid DCs (pDCs) are the key cells, linking innate and adaptive immunity. They can circulate in the blood of both adults and neonates (O'Doherty et al. 1994; Sorg et al. 1999) can also enter secondary lymphoid tissues including spleen, and Peyer's patches. pDCs are considered the front line a antiviral immunity (Siegal et al. 1999), as they accumulate at inflammatory sites (Eckert and Schmid 1989), and are capable of rapidly producing high amounts of type-I IFN in response to viruses (Siegal et al. 1999).

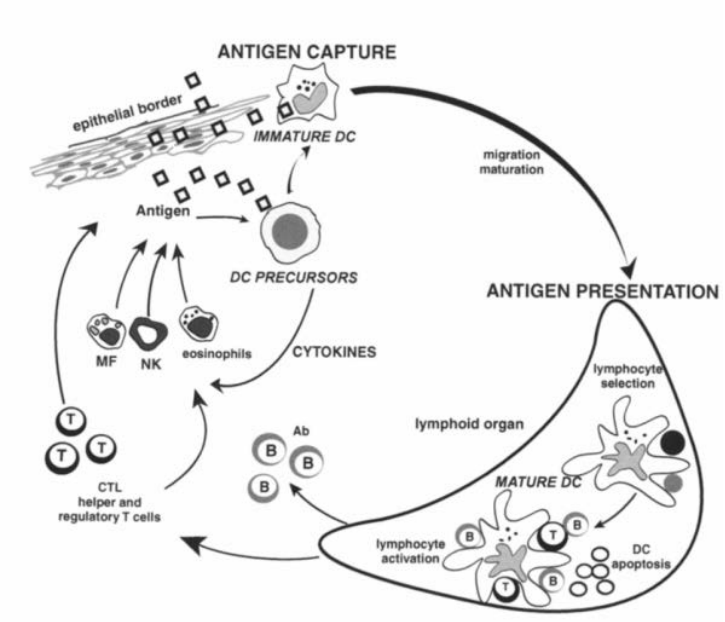


Fig. 1-4 The DC life cycle. The DC precursors, circulating in the blood or residing tissues as immature DCs, can be activated when encountering pathogens (e.g. viruses); they secret various cytokines, such as IFN-γ, which, in turn, activates eosinophils, macrophages (MF) and natural killer (NK) cells; such DCs then undergo a maturational process, including up-regulation of MHC molecules, co-stimulatory molecules, and adhesions; they then migrate to lymphoid organs after antigen capture; in the lymphoid organs, DCs display peptide-MHC, which selects for rare circulating antigen-specific lymphocytes, which induces terminal maturation of DCs to allow for lymphocyte expansion and differentiation; the activated T lymphocytes migrate to the injured tissue site, where cytotoxic T lymophocytes (CTLs) eventually lyse infected cells. B cells, activated by both T cells and DCs, mature to become plasma cells and produce antibodies; helper T cells function by secreting cytokines, which permit the activation of macrophages, NK cells, eosinophils, cytotoxic T cells and B cells.

DCs in both mice and humans, derive from a common proliferating progenitors found in bone marrow; they enter the blood stream before migration to other tissues (Steinman et al. 1999). At these sites, they exist as immature DCs, staying highly active in the uptake and processing of protein antigens and searching for signals that may trigger their maturation (Sallusto et al. 1995). They may receive maturation or “danger” signals, such as pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), bacterial DNA, double stranded RNA (Pardoll 2002), and toll-like receptors (TLRs) (Pasare and Medzhitov 2005). The DCs then undergo the programmed maturational process by lowering antigen uptake capability (Sallusto et al. 1995); they also upregulate their expression of MHC molecules to increase the density of MHC: peptide complexes (signal 1) (Zinkernagel1974), as well as costimulatory molecules (signal 2) and certain homing chemokine receptors for their migration (Sallusto et al. 1998). They then enter draining lymphatic vessels and migrate to secondary lymphoid tissues or regional lymph nodes (Bancherau, 1998). There, they present antigens and release so-called ’signal 3’ factors, such as interleukin-12 (IL-12), IFN-γ to lymphocytes. The type of factors released depend on how the DCs were activated, leading to induction of immune responses (Scott 1993; Ohshima et al. 1997).

As the most powerful antigen presenting cells, DCs can deliver tumor antigens to T cells and induce antitumor immune responses. In the first reported clinical study on follicular B-cell lymphoma patients treated with a DC vaccine, there was one complete tumor regression within the four treated

patients (Hsu et al. 1996). This finding sparked interesting in the utility of DCs as an attractive and novel tool for treatment of malignant diseases. In the following years, DC-based vaccines have been frequently reported to stimulate more efficient antitumor immunity compared to peptide and DNA-based vaccines (Toes et al. 1998; Yang et al. 1999; Bellone et al. 2000).

There are some limitations or handicaps in developing DC-based vaccines:

(i) DCs for clinical use are required to be autologous, although they comprise only less than 1% of circulating immune cells in the peripheral blood.

(ii) DCs may generate immune tolerance. Therefore, tumor antigens may be taken up by the "wrong" type of DCs in the periphery, which might lead to an "unwanted" type of immune response.

(iii) DCs from patients or tumor bearing mice are often impaired and may be anergic (Chaux et al. 1997; Gabrilovich et al. 1997; Troy et al. 1998).

Therefore, special efforts should be made to overcome these limitations.

1.3.3.2.6.2 In vitro DC culture and the DC maturation

To obtain sufficient numbers of DCs from *in vitro* DC culture (Pilon-Thomas et al. 2004), several methods have been developed to isolate and/or generate DCs: 1) leukapheresis of cells in circulating blood; 2) culture technique for murine BM-derived progenitors and human peripheral blood $CD14^+$ monocytes (Inaba et al. 1992, Romani et al. 1996, Lutz et al. 1999, Berger and Schultz 2003), and; 3) human $CD34^+$ DC progenitors isolated from BM or cord blood (Romani et al. 1994, strobl et al. 1997)

Immature DCs are efficient in antigens uptake. However, they could induce antigen-specific tolerance or immune silencing, which is bad for inducing antitumor immunity but good for utilization in transplantation, allergy, autoimmune and chronic inflammatory diseases (Jonuleit et al. 2000; Dhodapkar et al. 2001). DC maturation is as important as antigen loading. The details are described in the following sections.

The first reported DC culture system used monocytic DC precursors stimulated with the cytokines granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 for over a week (Romani et al. 1994). Addition of CD40L to the DC culture resulted in relative maturation and greater antitumor immunity in a squamous cell carcinoma mouse model (Labeur et al. 1999);

LPS was another stimuli for maturation of DCs in vitro DC cultures (Chen et al. 2001b; de Vries et al. 2003).

DC maturation can induce upregulation of MHC molecules and co-stimulatory molecules (CD40, CD54, CD80 and CD86) (Cella et al. 1997). Cytokines, such as Flt-3L, granuloctye-colony stimulating factor (G-CSF), IL-1α, IL-1β, IL-6, IL-12, MCP-1 (CCL-2), MIP-1α (CCL-3), MIP-1β (CCL-4), RANTES (CCL-5), TARC (CCL-17), macrophage derived chemokine (MDC; CCL-22), MIP-2 (CXCL2) may also be secreted (Chen et al. 2001b).

1.3.3.2.6.3 The administration route for DC vaccines

DCs migrate and relocate to lymphoid tissues, where they interact with T cells and induce T cell responses. However, only a small portion of vaccinated DCs migrate into regional lymph nodes after administration (De Vries et al. 2003). Therefore, a more appropriate administration routes are essential to facilitate successful DC migration into regional lymph nodes.

Different administration routes can influence different DC homing outcomes and induce different types of immune responses. For example, DC injected subcutaneously (s.c.) preferentially migrated to the draining lymph nodes 24 hours after injection and peaked at 48 hours but remained detectable for five days (Lappin et al. 1999; Eggert et al. 1999; Okada et al. 2001a). If DCs were injected intravenously (i.v.), they preferentially accumulated in the spleen, liver and lungs (Lappin et al. 1999). However, compared to i.v. injection, s.c. resulted in greater protective immunity against tumor challenge (Okada et al. 2001a). Bedrosian's group have observed that DCs could be guided by ultrasound and enter into lymph nodes distal from sites of malignant diseases; this would make DC's IL-12 directly exert its full effects on T cell activation in the lymph nodes, and enhance the vaccine efficiency (Bedrosian et al. 2003). More research on the effect of administration routes relative to outcomes needs to be studied for the optimal DC immunization.

1.3.3.2.6.4 DC vaccines loaded with different antigents

1.3.3.2.6.4.1 DCs loaded with tumor cell lysates

Tumor cell lysates are easy to obtain by repeated freeze/thaw or sonication of tumor cells. Autologous DCs, loaded with tumor cell lysates, avoid the needs for identification of TAAs

(Zhou et al. 2002) and individual HLA classification, given that tumors express a wide variety of TAAs, as well as undefined TAAs and rare mutations. Necrotic tumor cell lysates could induce DC maturation (Sauter et al. 2000). TAAs in lysates could be presented on MHC class I and class II molecules by autologous DC, resulting in a far more efficient cross-presentation (Li et al. 2001a). They could also activate $CD4^+$ helper T cells, which is important for effective antitumor immune responses (Toes et al. 1999) and the generation of long term $CD8^+$ T cell memory (Zajac et al. 1998). DCs loaded with tumor cell lysates have been utilizated for treatment of skin cancer (Nestle et al. 1998) and metastatic fibrosarcoma in children (Geiger et al. 2000).

However, some disadvantages, due to the nature of tumor lysates, hold back the development of tumor lysate-loaded DC vaccines. Thess include:

(i) There may be insufficient number of autologous tumor cells from some cancer patients.
(ii) There is the possibility for the induction of autoimmunitiy due to the presentation of non-tumor-Ags (Gilboa 2001)
(iii) The profiles of TAAs may have been changed so that they may not be applicable for advanced tumors with metastases

1.3.3.2.6.4.2 DCs loaded with TAA peptides

DCs, pulsed with various HER2/neu epiptops, have been used as antitumor vaccines in preclinical animal and human clinical trials. Human DCs, pulsed with HER2/neu peptides p63-71 and p780-788, or mouse DCs, pulsed with HER2 peptides p369-377 (E75), p435-443, p654-662, and p689-697, induced HER2 peptide-specific CD8+ T cell responses (Shiku et al. 2000. Rongcun et al. 1999; Anderson et al. 2000b; Seliger et al. 2000; Baxevanis et al. 2002; Kono et al. 2002; Morse et al. 2003; Sotiropoulou et al. 2003a; Sotiropoulou et al. 2003b; Baxevanis et al. 2006]. Vaccination using HLA-A2 restricted HER-2 peptide (p654-662)-pulsed DCs entered a phase I/II trial, where two patients had partial responses and another two patients also developed IFN-γ producing CD8+ T cells (Dees et al. 2004). However, DCs pulsed with a recently identified H-2Dq restricted-neu peptide showed no protective responses against neu-expressing breast cancer in Tg FVBneuN mice (Ercolini et al. 2003). However, specific tumor lysis activity was seen in immunized wild-type FVB/NJ mice. In addition, there was another preclinical study using a fusion protein, which consisted of HER2 ECD (p58-492) and HIV hemagglutinin (HA)

TAT domain. The fusion protein-loaded into DC vaccines induced both CD4+ and CD8+ T responses leading to a significantly reduced tumor growth (Viehl et al. 2005). A clinical trial with DCs, loaded with a mixture of 6 HLA class I- and II-restricted peptides (i.e., 3 HER2 ECD-derived peptides and 3 HER2 ICD-derived peptides) showed that immunized patients developed peptide-specific immune responses, leading to a significant reduction of tumor cells with HER2 expression in the biopsies of surgical patients (Czerniecki et al. 2007). Overall, HER2/neu peptide-pulsed DC vaccines can generate CTL responses, but are still not adequate for therapeutic effect in a clinical setting.

1.3.3.2.6.4.3 Adenovirus vector-transduced DC vaccine

Human adenovirus type 5 (HAdV5) vector has been proven to be safe and efficient in gene delivery because its genome does not integrate into the host cell's genome. Therefore, adenovirus is one of the most efficient vehicles for delivering transgenes into DCs. The disadvantages of using HAdV5 are its transient gene expression and its toxicity when used at high dose (Yang et al. 1994, Morral et al. 1997).

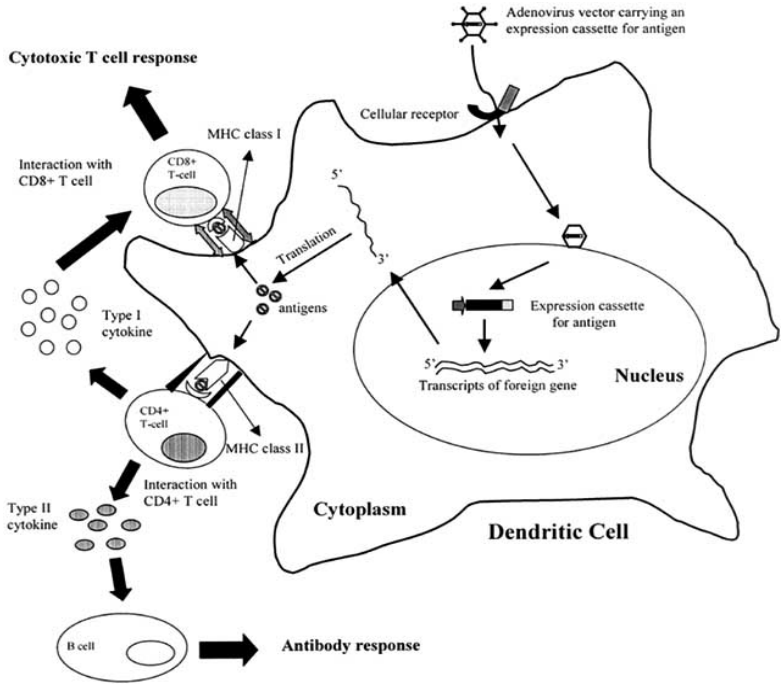


Fig. 1-5 Adenovirus-mediated gene transfer to dendritic cells. An adenoviral vector carries a gene expression cassette encoding for a tumor–associated antigen, infects a dendritic cell; the antigen can be expressed within the DC, which is processed and presented with MHC I to induce cytotoxic T cell responses or with MHC II to induce $CD4^+$ T cell and Ab responses.

Experimental tumor models have shown that DCs, transfected with HAd5 vector expressing tumor antigens, elicited strong antitumor CTL responses (Song et al., 1997; Song et al., 2000; Steitz et al., 2001; Tuting et al., 1999; Wan et al., 1997). DCs, which were transfected with adenoviral vector carrying full-length neu (AdNeuTK), induced protective antitumor immunity against neu-expressing tumor cell challenge in 60% of immunized mice with (Chen et al. 2001). DCs, transfected with adenovirus expressing full extracellular domain (ECD amino acids 22–652), and transmembrane domain (TM, amino acids 653–675) of HER-2, induced HER-2 specific CTL responses against neu expressing tumors (Ma et al. 2006). DCs, transfected with truncated neu expressing AdV, stimulated both neu-specific antibody and CTL responses in BALB-*neu*T mice (Sakai et al. 2004). Similar HER2/neu antitumor immunities, induced by $AdV_{HER2/neu}$- and/or modified $AdV_{HER2/neu}$-transduced DC vaccines, were found in other studies (Wu et al. 2005; Chong et al. 2005; Ma et al. 2006). For example, DCs transfected with $AdV_{HER2/neu\text{-}IL12}$ expressing HER2/neu and IL-12 or co-transfected with $AdV_{HER2/neu}$ and $AdV_{TNF\text{-}\alpha}$ expressing HER2/neu and TNF-α, respectively, demonstrated efficient HER2/neu-specific $CD4^+$ T cell and $CD8^+$ CTL responses (Chen et al 2001; Chen et al; 2002; Ma et al. 2006). Preclinical studies of HER2/neu-specific AdV-transfected DC vaccine showed that it could delay the onset of spontaneous HER2/neu over-expressing tumor growth in transgenic mice (Sakai et al. 2004).

To date, AdV-transfected DC-based vaccines can induce antitumor CTL responses and antitumor immunity in wild-type mice. However, the vaccine-induced CTL responses are not strong enough to induce efficient protective or therapeutic antitumor immunity against HER2/neu positive breast cancer in rat HER2/neu-gene transgenic (Tg) mice, because of immune tolerance towards HER2/neu. Based upon recent studies using adenovirus- and lentivirus-transfected DCs in a HER2/neu overexpressing tumor model, it has been demonstrated that not only the target antigen but also the virus system may determine the nature and magnitude of DC-stimulated antitumor immunity (Felizardo et al. 2011). Although infections of adenovirus and lentivirus did not affect DC maturation, both adenovirus- and lentivirus-transfected DCs expressing HER2/neu induced strong CTL responses, similarly leading to inhibition of tumor growth in mice. However, AdV-transduced DCs expressing HER2/neu elicited significant humoral responses, as well as local and systemical $CD4^+$ and $CD8^+$ T cell responses, whereas lentivirus-transfected DCs expressing HER2/neu predominantly stimulated IFN-γ-secreting $CD4^+$ T-cell responses,

leading to infiltration of $CD4^+$ T cells at the tumor sites (Felizardo et al. 2011). These results suggest that adenovirus-transfected DC vaccine may be a promising tool in inducing CTL responses against HER2/neu positive breast cancer.

1.3.3.2.7 Universal T help epitope from tetanus toxin and its potential function in breaking self-tolerance

$CD4^+$ T helper cells play a crucial role in $CD8^+$ CTL priming, expansion, and memory development (Ridge et al. 1998; Bennett et al. 1998; Schoenberger et al. 1998; Wang et al. 2003; Janssen et al. 2003; Kirberg et al. 1993; Cardin et al. 1996; Bourgeois et al. 2002; Shirai et al. 1994). It is generally believed that Th dependent CTL responses are essential in antitumor immunity. However, precursors of Th cells as well as CTLs with high affinity for self-antigens, including tumor Ags, are eliminated in the thymus, leading to immunological tolerance or poor tumor Ag-specific immunity derived from tumor Ag-specific vaccines.

The tetanus toxin epitope, P30 (FNNFTVSFWLRVPKVSASHLE) is a universally immunogenic epitope capable of stimulating $CD4^+$ T cell responses (Panina-Bordignon et al. 1989). To bypass self immune tolerance to tumor Ags, insertion of foreign helper epitopes into HER2/neu antigen was designed to overcome immune tolerance to HER2/neu by providing exogenous $CD4^+$ T cell help to tumor Ag-specific B and CTL responses (Dalum et al. 1997). This approach bypassed immune tolerance toward the highly conserved ubiquitin protein (Dalum et al. 1997) and the inflammatory cytokine TNF alpha, leading to endogenous production of therapeutic anti-TNF alpha Abs (Dalum et al. 1999; Dalum et al. 1996). It was later demonstrated that HER2/neu DNA vaccine containing the potent Th cell epitope P30 induced complete immune protection against tumor cell challenge in wild-type mice, but only a partial antitumor protection in Tg mice, whereas HER2/neu protein vaccine containing P30 epitope stimulated protective immune responses even in Tg mice (Renard et al. 2003). In an OVA model study, a DNA vaccine containing P30 could prevent the growth of OVA-expressing tumor in transgenic rat insulin promoter (RIP)-mOVA mice (Steinaa et al. 2005) which express a membrane bound truncated OVA sequence under the control of RIP in pancreatic islets as well as in the kidney proximal tubules and thymus and in the testis of male mice (Kurts et al. 1996), and exhibit deletional tolerance toward the $OVA_{265–280}$ (SIINFEKL, MHC class I epitope of OVA) epitope (Kurts et al.

1997). These results indicate that insertion of a single foreign Th epitope P30 in the self Ag could break the $CD8^+$ T cell tolerance, and that it is the tolerance in the $CD4^+$ Th cell compartment that helps maintaining the CTL tolerance against self Ag in these Tg mice.

2 Hypothesis

The tetanus toxoid Th P30 epitope has been found to be a universal epitope for sensitizing and inducing proliferation of $CD4^+$ T cells ex vivo. OVA-P30 peptide vaccine could break CD8 and CD4 T cell tolerances against neo-self-antigen OVA, and was able to protect RIPmOVA Tg C57BL6 mice from tumor growth, and that HER2/neu-P30 DNA vaccine could protect wt mice from tumor challenge, but induce only moderate protection in spontaneous HER2/neu positive tumor growth Tg mice. Therefore, we hypothesis OVA or HER2/neu linked with P30 in the form of AdV transduced DCs vaccine can stimulate more efficient antigen specific CTL responses.

3 Materials and methods

3.1 Materials

3.1.1 Reagents

All of the chemicals used and listed in Table 3-1 are molecular biology or research grade. Antibodies used and listed in Table 3-2 were either FITC, phycoerythrin (PE) or biotin labeled.

Table 3-1 List of chemicals

Chemicals	Supplier Name
Acrylamide:bisacrylamide	Bio-Rad
Agar	Invitrogen
Agarose	Invitrogen
Alkaline phosphatase	New England Biolab
Ammonium chloride	EM Sciences
Ammonium persulfate	Gibco
Ampicillin	Sigma
Bacto-tryptone	BD
Bovine insulin	Sigma
Calcium chloride	Sigma
Cesium chloride	Sigma
CFSE	Molecular Probes
Chloroform	EM Sciences
Coomassie Blue	Bio-Rad
DMEM	Gibco
DMSO	Sigma
dNTP mix (dATP, dCTP, dGTP, dTTP)	Invitrogen
EcoRV	NEB
Electroporation cuvette (0.2cm, 0.4 cm gap)	Bio-Rad
EMEM	Gibco
Ethanol	EM Sciences

Table 3-1 List of chemicals (Continued.)

Chemicals	Supplier Name
Ethidium bromide	Sigma
FBS	Cyclone
Ficoll-Paque	Amersham Biosciences
Formalin	EM Sciences
Gentamicin Reagent Solution	Gibco
Glutaraldehyde	Sigma
Glutamin	Sigma
Glycerol	BDH Inc
Glycine	EM Sciences
GM-CSF	R&D Systems
HindIII	NEB
High-Fidelity DNA Polymerase	Pharmingen
Hydrochloric acid	EM Sciences
Isopropanol	EM Sciences
GM-CSF	R&D Systems
HindIII	NEB
High-Fidelity DNA Polymerase	Pharmingen
Hydrochloric acid	EM Sciences
Isopropanol	EM Sciences
Kanamycin	Invitrogen
KpnI	NEB
Ligase	NEB
Lysozyme	Sigma
Methanol	EM Sciences
Non-fat dry milk	Carnation
NeuI peptide (PDSLRDLSVF)	San Diego, CA
OVAI peptide (SIINFEKL)	University of Calgary
PEG-8000	Sigma

Table 3-1 List of chemicals (Continued.)

Chemicals	Supplier Name
Pfx DNA polymerase	Invitrogen
Phenol	EM Sciences
Prestained molecular weight protein marker	Bio-Rad
Proteinase K	Invitrogen
RNAse	Amersham Biosciences
RPMI 1640	Gibco
SDS	Sigma/Bio-Rad
Sodium acetate	BDH Inc
Sodium azide	Sigma
Sodium hydroxide	EM Science
Streptomycin	Sigma
Sucrose	BDH Inc
Sulfuric Acid (H_2SO_4)	BDH Inc
T4 DNA ligase	USB
Taq DNA polymerase	Invitrogen
TEMED	Gibco
TRIS	EM Science
Tween 20	Bio-Rad
Reagent Supplier Name Triton X-100	Sigma
Trypan Blue Stain	Gibco
Trypsin/EDTA	Gibco
Yeast Extract	Difco
B-mercaptoethanol	Bio-Rad
λDNA/HindIII marker	Invitrogen
φX174/HaeIII fragment marker	Invitrogen
XbaI	NEB

Table 3-2 List of Kits

Commercial Kit	Supplier Name
Elutip-D columns	Scheicher & Schuell
Endo-free Giga Kit	Qiagen
Endo-free Mega Kit	Qiagen
GenElute Agarose Spin column	Sigma
Plasmid Mini prep Kit	Qiagen
Qiaprep Spin Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Quikchange site directed mutagenesis kit	Stratagene
RNeasy Mini Kit	Qiagent
SuperScript III First-Strand Synthesis for RT-PCR	Invitrogen

Table 3-3 List of Antibodies

Antibody	Supplier
Anti-H-2K^{b}/OVAI (pMHC I)	Dr. Germain (NIH, Bethesda, MD)
Anti-OVA	Dr. Xiang (Saskatoon Cancer Centre)
Anti-H-2D^{q}/neuI (pMHC I)	NIH Tetramer Facility
Anti-neu	Dr. Yang (University of Michigan)
Anti-IFN-γ	Pharmingen
Anti-mouse CD11c-FITC	Pharmingen
Anti-mouse CD4-FITC	Pharmingen
Anti-mouse CD4-PE	Pharmingen
Anti-mouse CD40	Pharmingen
Anti- mouse CD44-Biotin	BD Biosciences
Anti-mouse CD54	Pharmingen
Anti-mouse CD8-FITC	Beckman-Coulter

Table 3-3 List of Antibodies (Continued.)

Antibody	Supplier
Anti-mouse CD80	Pharmingen
Anti- mouse CD8-FITC	Pharmingen
Anti- mouse CD8-PE	Pharmingen
Anti-mouse CD86	Pharmingen
Anti-mouse H-2K^{b}	Pharmingen
Anti-mouse Ia^{b}	Pharmingen
Goat anti-mouse IgG1	Caltag
Goat anti-mouse IgG2a	Caltag
Goat anti-mouse IgG-FITC	Jackson Immuno Research
Rabbit anti-mouse IgG-FITC	Jackson Immuno Research
Streptavidin-FITC conjugated	Pharmingen
Streptavidin-PE conjugated	Pharmingen
Streptavidin-HRP conjugated	Jackson Immuno Research

3.1.2 Cell lines

3.1.2.1 Bacterial cells

Bacterial cells used in this project were DH5α *Escherichia coli* (*E. coli*) for vector propagation of bacterial hosts, and *E. coli* BJ5183 for homologous recombination leading to formation of AdV vectors, respectively.

For cell growth, bacterial cells were cultured in flasks, containing Lauria-Bertani (LB) broth, which consists of 1.0% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Difco) and 1.0% (w/v) NaCl. Appropriate antibiotic were supplemented to media, such as ampicillin (100 μg/ml) or kanamycin (50 μg/ml). Cells were grown at 37ºC in a shaking incubator with a speed of 200 r/min.

For inoculation of transformed bacterial colonies, cells were plated onto selective LB-agar plates. The plates contained containing 1.5% (w/v) agar in LB broth with appropriate antibiotics,

depending on the vector. The plates were incubated upside down in 5% CO_2 incubator at 37ºC overnight.

3.1.2.2 Tumor cell lines

Cell lines, used in this project, were 293-26, 293-39, $BL610_{OVA}$ and Tg1-1. All cell lines were cultured in a humidified CO_2 incubator at 37°C with a 5% CO_2 atmosphere. For cell counting, Trypan-Blue (Gibco) was used to stain dead cells, and cells were counted with a haemocytometer.

The two 293 cell lines, 293-26 and 293-39, contain adenoviral E1 genes. They were purchased from Microbix (Toronto, ON). 293-39 was used to construct adenovirus DNA for the formation of recombinant adenoviruses; 293-26 was used to amplify recombinant adenoviruses. These cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco), supplemented with 10% FBS, 5% glutamin and 30 μg/ml gentamicin solution (Gibco). They were passaged, using 1X citric saline solution (10% (w/v) KCl and 4.4% (w/v) sodium citrate).

$BL6\text{-}10_{OVA}$ cell line was the OVA transgene-expressing BL6-10 melanoma cell line. It was developed in Dr. Xiang's lab. This cell line was grown in αMEM, supplemented with 10% FBS, 0.5mg/ml hygromicin and 30 μg/ml gentamicin solution (Gibco) for OVA model study.

Tg1-1 cell line is a mouse breast cancer cell line ($H\text{-}2K^q$), derived from a spontaneous breast tumor grown in a female FVBneuN transgenic (Tg) mouse (Dr. T. Kipps, University of California, San Diego, CA). This cell line was maintained in DMEM (Gibco), supplemented with 10% FBS, 5% glutamine and 30 μg/ml gentamicin solution (Gibco). Cells were passaged, using Trypsin/ethylenediamine tetracetic acid (EDTA; Gibco) and used for HER2/neu model study.

3.1.3 Animals

Wild-type C57BL/6, and Tg FVB/neuN mice were used in this project. All animal experiments were approved by the University Committee on Animal Care and Supply in accordance with the Canadian Council for Animal Care guidelines. C57BL/6 mice were used in the OVA model study, while Tg FVB/neuN mice were used in HER2/neu model study.

C57BL/6 were obtained from Jackson Laboratories. Tg FVBneuN (H-2K^q) [FVB/NTgN(MMTVneu)202Mul] mice were obtained from Du Pont (Wilmington, DE), and bred in the College of Health Science Animal Facility. All mice were housed in College of Health Science Animal Facility, University of Saskatchewan.

3 .2 Methods

3.2.1 Molecular biology techniques

3.2.1.1 DNA techniques

3.2.1.1.1 PCR

Amplification and determination of targeted genes were performed by typical polymerase chain reaction (PCR) reactions. Before and after construction of recombinant vectors, PCR reactions were performed, using 1 μg of template DNA/cDNA in a final volume of 50 μl, containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 1.5 mM $MgCl_2$, 1 mM dNTP mix, 10 ng of each primer and 2.5 units of high-fidelity Taq polymerase (Invitrogen). A typical cycle consisted of initial DNA denaturation at 94ºC for 5 min, followed by 30~35 amplification cycles at 94ºC for 45 sec~3 min, 56-58ºC for 1 min and 72ºC for 1 minute, followed by final termination for 10 minute at 72ºC. Water was used as the template to serve as the negative controls.

After PCR amplification, one-tenth of the reaction sample was used to perform standard agarose gel electrophoresis to determine the length of amplified DNA.

3.2.1.1.2 Restriction enzyme digestion

All restriction enzymes were provided by New England Biolabs (NEB). Procedures were carried out according to the manufacturer's protocols.

One microgram of DNA fragments or the DNA vector was digested, using 1 unit of the specified enzymes in 1X, the final recommended buffer. The reactions were incubated at 37°C for 1~2 hours for complete restriction enzyme digestion. In the case of removal of the 5' terminal

phosphate group, calf intestinal alkaline phosphatase (New England Biolabs) was added to the reactions for DNA digestion.

All the reactions were subjected to standard agarose gel electrophoresis to separate different DNA fragments. DNA samples without any digestion were served as the negative controls. Targeted DNA bands were cut out for further purification by electrophoresis.

3.2.1.1.3 Ligation

Ligation of DNA fragments into DNA vectors was performed in a 20 μl reaction buffer, containing 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM adenosine triphosphate (ATP), 1-5 unit T4 DNA ligase (USB), 50 ng of vector DNA and at least 200 ng of purified DNA fragments. Ligations were performed in waterbath at 16°C overnight.

3.2.1.1.4 Purification of linear DNA fragments

PCR products were further purified using either PCR clean kits (QIAquick PCR Purification Kit from QIAGEN), or phenol: chloroform: isoamyl alcohol extraction. DNA fragments from agarose gels were purified using either gel purification kits (GenElute Agarose spin columns from Sigma) or phenol: chloroform: isoamyl alcohol extraction.

3.2.1.1.5 Agarose gel electrophoresis

Varying agarose concentrations ranging from 0.7% to 1% in TAE buffer [40 mM Tris-acetate, 1 mM EDTA containing 1 μg/ml ethidium bromide (EtBR; Sigma)], were cast in a GelHorizon 58 Apparatus; DNA samples were loaded onto these gels with gel loading buffer consiste of 0.042% (w/v) bromophenol blue, 6.67% (w/v) sucrose, and λDNA/Hind III and φX174/Hae III fragment DNA were used as markers (Invitrogen). Gel electrophoresis was performed in TAE buffer. To get gels visualized and documented, a gel documentation system (Bio-Rad) with UV illumination was applied.

3.2.1.1.6 Competent bacterial cell preparation

Competent bacterial cells were used in plasmid DNA transformation and amplification (Chan et al. 2006, Cohen et al. 1972, Sambrook et al. 1989). Bacterial cells (DH5α or BJ5183) in LB medium were grown until mid log-phase with an OD600 reading between 0.4-0.6. The cells were then spun down at 8000 rpm. The 5 mL cell pellet was resuspended in 200 µl ice-cold 50 mM calcium chloride (CaCl2) in 10 mM Tris-HCl (pH 8.0) for 5 mL cells. Competent bacterial cells were stored in 20% glycerol at -80°C in aliquots.

3.2.1.1.7 Heat shock transformation

In DNA transformation, 100 µl of competent cells were mixed with plasmid DNA or DNA ligation products and kept on ice for 30 min. The sample was heat shocked at 42°C for 90 sec by quickly placing the sample into a waterbath. It was then kept on ice for 10 min. Transformed bacteria cells were then cultured in 500 µl LB medium at 37°C in a 5% CO2 for 45 minutes. Cells were then added onto selective LB-agar plates by the plate streak method to get individual bacteria clone, and cultured at 37°C in 5% CO2 incubator for 12-16 hours.

3.2.1.1.8 Plasmid DNA purification

To screen positive recombinant colonies after transformation, single isolated colonies were selected; for recombinant BJ5183 colonies, a tiny single colony was selected. The colonies were further cultured to amplify copies of recombinant plasmid DNA in LB broth, containing selective antibiotics overnight. Cells were pelletted the next day and resuspended in 50 mM glucose, 10 mM EDTA, 25 mM Tris- HCl (pH 8.0), 2 mg/ml lysozyme (Sigma). The cells were then lysed with a 200 mM NaOH, 1% sodium dodecyl sulfate (SDS) solution, followed by addition of 3 M sodium acetate, pH 5.2-5.5. Chromosomal DNA, cellular protein and debris were precipitated, leaving the plasmid DNA in the supernatant. This fraction was extracted twice with phenol: chloroform/isoamyl alcohol, followed by DNA precipitation with ethanol precipitation. The DNA precipitation was resuspended in Tris EDTA (TE) buffer (pH 8.0), containing 40 µg/ml RNAse A. Its concentration was measured by spectrophotometry at standard A260/A280 readings. Plasmid DNA was visualized on agarose gels.

Positive bacterial colonies, cultured overnight in LB broth with appropriate antibiotics were kept in a shaking incubator at 37ºC. The sample of cells were treated, using the same solutions mentioned above, Plasmid Mini preps Kit (Qiagent) was used for DNA purification. The purified DNA was resuspended in sterile, distilled water. Its concentration was determined by spectrophotometry at standard A260/A280 readings. Purified DNA was stored at -20ºC and ready for transfections.

3.2.1.1.9 Sequencing

A 100 ng DNA template per round and 100 µl of 2 pmol/L per primer were sent to the sequencing facilities at the Plant Biotechnology Institute of the National Research Council of Canada in Saskatoon, Saskatchewan. Sequences were then analyzed through Blast on NCBI.

3.2.1.2 RNA methods

RNA was extracted from mammary cells with RNeasy Mini Kit (Qiagent), following the procedures provided by the manufacturer. SuperScript III First-Strand Synthesis for RT-PCR (Invitrogent) was used to synthesize cDNA, following the procedures provided by the manufacturer. Briefly, 5 ng of RNA was firstly mixed with: 1 µl Primer; 1 µl 50 µM oligo(dT)20; 1 µl 50 ng/µl random hexamers; 1 µl 10 mM dNTP mix; appropriate volume of DEPC-treated water to make a 10 µl reaction system. The reaction samples were incubated at 65°C for 5 min. The reaction samples were then transferred onto ice for 1 min. Later, they were mixed with cDNA Synthesis Mix: 10× RT buffer; 25 mM MgCl2, 0.1 M DTT, RNaseOUT (40 U/µl). After incubation for 2 min at 42 °C, 1 µl of reverse transcriptase: SuperScript III RT (200 U/µl), was added and the reaction was continued for 50 min at 42 °C followed by 15 min at 70 °C. The reaction was stopped by addition of 1 µl of RNase H and incubation at 37 °C for 20 min. A control reaction sample for RT-PCR using the forward primer, 5’-CAGGT TGTCT CCTGC GACTT-3’, and the reverse primer, 5’-CTTGC TCAGT GTCCT TGCTG-3’, was used to detect the level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.2.1.3 Western blot

Protein expression in mammary cells can be assessed by using Western blots. In general, cell proteins were firstly extracted using extraction buffer containing 125 mM Tris, 0.05% SDS and

10% β–ME. After centrifugation at 1000 x g for 5 min, the supernatants containing the protein samples were collected, and boiled with protein loading buffer, which contains 4% SDS, 20% glycerol, 0.12 M Tris pH 6.8, and 10% β-mercaptoethanol (BME), 2% bromophenol blue (BPB) (669.96 g/mol).

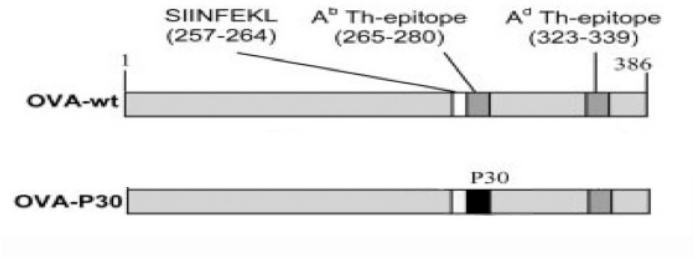
The protein samples were then separated on 12% SDS-polyacrylamide gel electrophoresis (PAGE). The gel was then cut and transferred onto polyvinylidene difluoride membrane (Millipore) and blocked with OYSSEY blocking buffer (LI-COR Biosciences). The blots were incubated with a panel of antibodies specific for OVA and β-actin, followed by futher incubation with goat-anti-mouse-IRDye™ 680CW. The blot membrane was scaned using ODYSSEY densitometer (LI-COR Biosciences).

3.2.2 Construction of recombinant AdVs

To construct recombinant adenoviruses, an AdEasy system (He et al. 1998) using the first generation of replication-deficient adenoviral vector was used in combination with a 293 cell line that expresses the early E1/E3 proteins. The targeted gene was first inserted into the vector pShuttle-CMV. The later was then transformed into bacterial cells BJ5183, containing the backbone gene of the first generation of adenovirus vector pAdEasy-1. The vectors pShuttle-CMV and pAdEasy-1 were obtained from AdEasy XL Adenoviral Vector System (Stratagene), respectively. AdVpLpA, containing no transgene, was previously constructed in Dr. Xiang's lab (Wright et al. 1999), and used as a control.

3.2.2.1 Construction of $AdV_{OVA\text{-}P30}$ and $AdV_{HER2/neu\text{-}P30}$

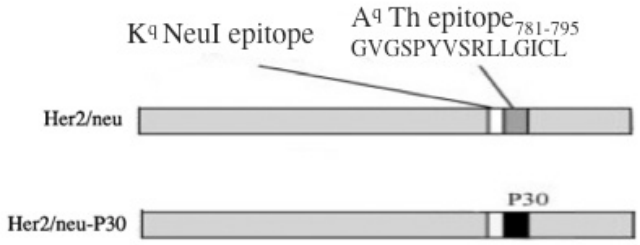
P30 was inserted into OVA to form a mutant gene OVA-P30 by replacing I_A^b epitope $OVA_{265–280}$ (TEWTSSNVMEERKIKV) with P30 using Splice Overlap Extension (SOE)-PCR method (refer to Fig. 3-1).



MGSIGAASME FCFDVFKELK VHHANENIFY CPIAIMSALA MVYLGAKDST RTQINKVVRF
DKLPGFGDSI EAQCGTSVNV HSSLRDILNQ ITKPNDVYSF SLASRLYAEE RYPILPEYLQ
CVKELYRGGL EPINFQTAAD QARELINSWV ESQTNGIIRN VLQPSSVDSQ TAMVLVNAIV
FKGLWEKTFK DEDTQAMPFR VTEQESKPVQ MMYQIGLFRV ASMASEKMKI LELPFASGTM
SMLVLLPDEV SGLEQLE**SII** NFEKLTEWTS SNVMEERKIK VYLPRMKMEE KYNLTSVLMA
(aa site 265-280 Replaced by P30 AA: FNNFTVSFWLRVPKVSASHLE)
MGITDVFSSS ANLSGISSAE SLKISQAVHA AHAEINEAGR EVVGSAEAGV DAASVSEEFR
ADHPFLFCIK HIATNAVLFG RCVSP

Fig. 3-1 P30 insertion site on OVA. The P30 sequence is used to replace the I_A^b epitope that locates at 265–280 aa of OVA protein.

P30 sequence was inserted into HER2/neu gene to form mutant HER2/neu-P30 by replacing the I_A^q epitope HER2/neu$_{781–195}$ (GVGSPYVSRL LGICL) with P30 using PCR plus digestion/ligation method.



MIIMELAAWC RWGFLLALLP PGIAGTQVCT GTDMKLRLPA SPETHLDMLR HLYQGCQVVQ
GNLELTYVPA NASLSFLQDI QEVQGYMLIA HNQVKRVPLQ RLRIVRGTQL FEDKYALAVL
DNRDPQDNVA ASTPGRTPEG LRELQLRSLT EILKGGVLIR GNPQLCYQDM VLWKDVFRKN
NQLAPVDIDT NRSRACPPCA PACKDNHCWG ESPEDCQILT GTICTSGCAR CKGRLPTDCC
HEQCAAGCTG PKHSDCLACL HFNHSGICEL HCPALVTYNT DTFESMHNPE GRYTFGASCV
TTCPYNYLST EVGSCTLVCP PNNQEVTAED GTQRCEKCSK PCARVCYGLG MEHLRGARAI
TSDNVQEFDG CKKIFGSLAF LPESFDGDPS SGIAPLRPEQ LQVFETLEEI TGYLYISAWP

DSLRDLSVFQ NLRIIRGRIL HDGAYSLTLQ GLGIHSLGLR SLRELGSGLA LIHRNAHLCF
VHTVPWDQLF RNPHQALLHS GNRPEEDLCV SSGLVCNSLC AHGHCWGPGP TQCVNCSHFL
RGQECVEECR VWKGLPREYV SDKRCLPCHP ECQPQNSSET CFGSEADQCA ACAHYKDSSS
CVARCPSGVK PDLSYMPIWK YPDEEGICQP CPINCTHSCV DLDERGCPAE QRASPVTFII
ATVEGVLLFL ILVVVVGILI KRRRQKIRKY TMRRLLQETE LVEPLTPSGA MPNQAQMRIL
KETELRKVKV LGSGAFGTVY KGIWIPDGEN VKIPVAIKVL RENTSPKANK EILDEAYVMA
GVGSPYVSRL LGICLTSTVQ LVTQLMPYGC LLDHVREHRG RLGSQDLLNW CVQIAKGMSY
(aa sequence site 781-795 replaced by P30 aa: FNNFTVSFWLRVPKVSASHLE)
LEDVRLVHRD LAARNVLVKS PNHVKITDFG LARLLDIDET EYHADGGKVP IKWMALESIL
RRRFTHQSDV WSYGVTVWEL MTFGAKPYDG IPAREIPDLL EKGERLPQPP ICTIDVYMIM
VKCWMIDSEC RPRFRELVSE FSRMARDPQR FVVIQNEDLG PSSPMDSTFY RSLLEDDDMG
DLVDAEEYLV PQQGFFSPDP TPGTGSTAHR RHRSSSTRSG GGELTLGLEP SEEGPPRSPL
APSEGAGSDV FDGDLAMGVT KGLQSLSPHD LSPLQRYSED PTLPLPPETD GYVAPLACSP
QPEYVNQSEV QPQPPLTPEG PLPPVRPAGA TLERPKTLSP GKNGVVKDVF AFGGAVENPE
YLVPREGTAS PPHPSPAFSP AFDNLYYWDQ NSSEQGPPPS NFEGTPTAEN PEYLGLDVPV

Fig. 3-2 P30 insertion site on HER2/neu. ThePP30 sequence is used to replace the I_A^b epitope that locates at 265–280 aa of HER2/neu protein.

3.2.2.3 Targeted gene insertion into pShuttle-CMV vector

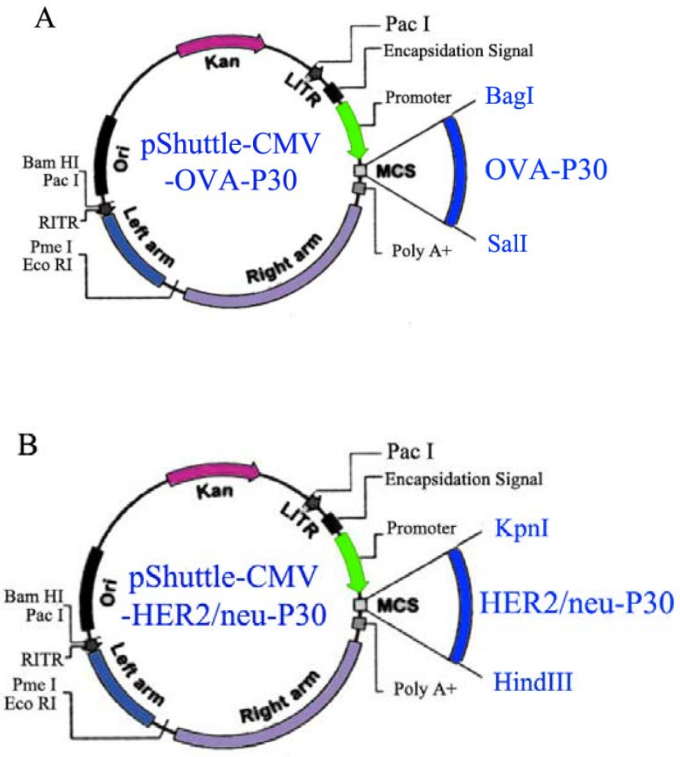


Fig. 3-3 Schemtic map of the insertion of target gene. (A) OVA-P30 gene was inserted into the pShuttle-CMV vector at the MCS site between BagI and SalI, and (B) HER2/neu-P30 was inserted into pShuttle-CMV vector at the MCS site between KpnI and HindIII.

The OVA-P30 fragment (BagI/SalI digested) was inserted into the pShuttle-CMV vector previously digested with BagI/SalI. Ligation products were then transformed into DH5α host bacterial cells for positive selection on LB broth agar plate with 100 μg/ml kanamycin overnight. The pShuttle-CMV-OVA-P30 DNA was further amplified from DH5α host bacterial cells grown in LB broth with 100 μg/ml kanamycin, using Plasmid Mini prep Kit (Qiagen). Similar procedures were applied to construction of pShuttle-CMV-HER2/neu-P30.

3.2.2.4 Overview of scheme of recombinant adenovirus construction

The formation of recombinant adenoviral vector was achieved in pAdEasy-1-containing BJ5183 E. coli competent cells by homologous recombination. After the insertion of OVA-P30 into pShuttle-CMV, a total of 1 μg of pShuttle-CMV-OVA-P30 was linearized by PmeI digestion. This linrized vector was then transformed into BJ5183 E. coli competent cells via heat shock transformation (as outlined in Section 3.2.1.1.7) for homologous recombination.

The transformed cells were then cultured on selective LB-agar plates containing 100 μg/ml kanamycin, and inverted overnight at 37°C in a CO_2 incubator. To enhance positive selection ratio of recombinant adenoviral plasmids, several small colonies were selected and grown in LB broth with 100 μg/ml kanamycin overnight at 37°C in a shaking incubator. The plasmid DNA was isolated from bacterial cells, using a Mini-preps kit (Qiagen). The plasmid DNA was screened for positive selection of the recombinant vector, using restriction enzyme analysis and then analyzed on a 0.7% agarose gel. The copy of the resulting positive pAdEasy-OVA-P30 plasmid was further amplified in DH5α cells. Large amounts of plasmid DNA from DH5α cells were then purified using Plasmid Mini kits (Qiagen), and stored at -20°C until use for the generation of recombinant adenoviruses. Purified plasmid pAdEasy-OVA-P30 was then linearized by PacI digestion, and then transfected into 293 cells to produce the replication-deficient recombinant adenovirus $AdV_{OVA\text{-}P30}$ (refer to Fig.3-4). A similar procedure was applied for creating $AdV_{HER2/neu\text{-}P30}$.

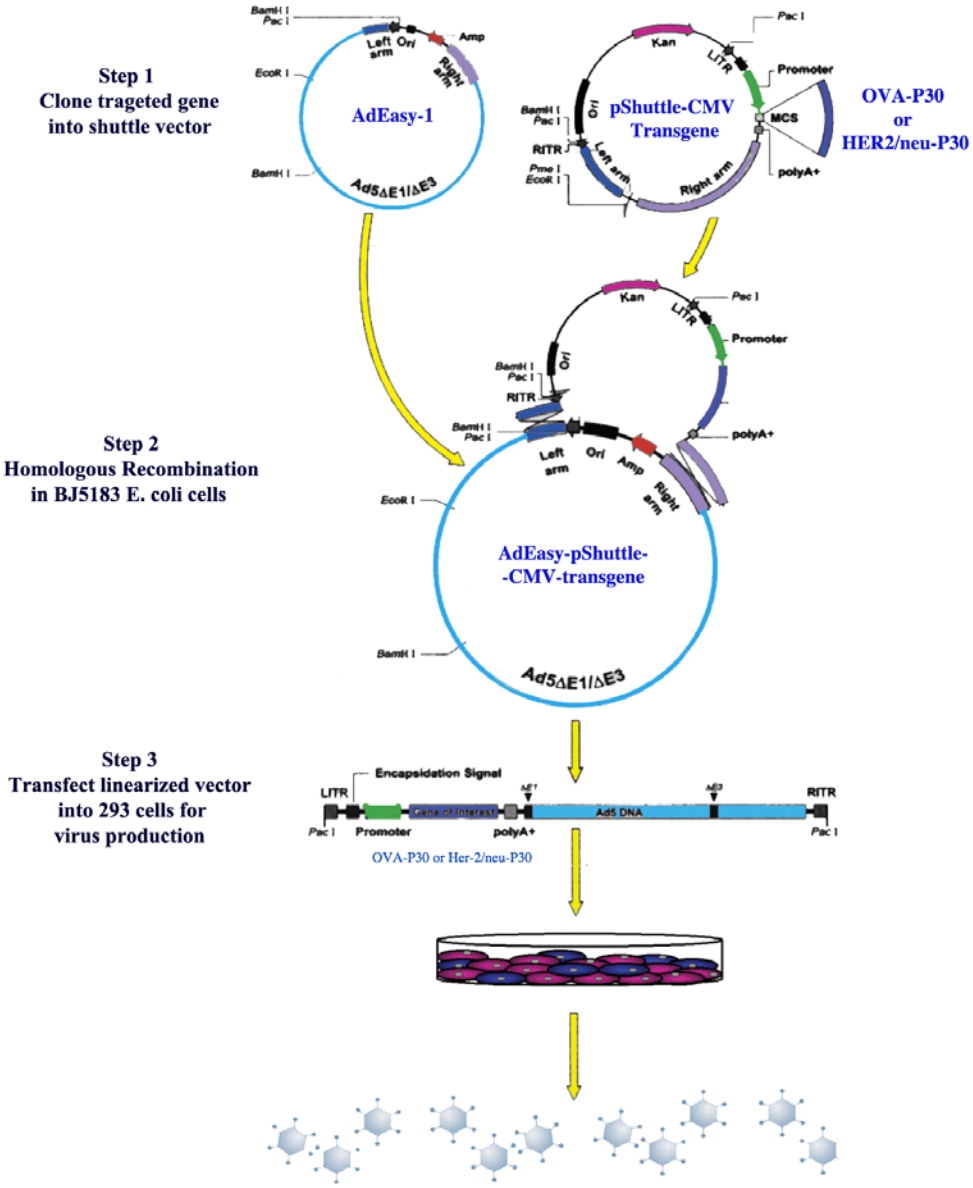


Fig. 3-4 Schematic overview of recombinant adenovirus construction, using the AdEasy system. The target gene was inserted into the pShuttle-CMV vector before homologous recombination with the HuAd5 backbone gene. The vector containing the target gene (pShuttle-CMV-target gene) was linearized with Pme I restriction enzyme, and then transformed into BJ5183 *E. coli* cells containing the backbone vector, pAdEasy-1. Homologous recombination between the two vectors in BJ5183 *E. coli* cells resulted in adenoviral vector pAdEasy-target gene. The recombinant vector was linearized with PacI and then tranfected into 293 cells. Adenovirus particals will be generated within host cells and released.

3.2.2.5 Generation of recombinant adenoviruses

To generate recombinant adenoviral plasmid vectors, pAdEasy-target-gene was linearized with PacI enzyme. It was tranfected into 293 cells using a liposome-based transfection method. The linearized recombinant adenoviral DNA vector (4 μg) was added into 500 μL OPTI-MEM medium. Lipofectamine (16 μl) was added into another 500 μL OPTI-MEM medium (Invitrogen). Each mixture was incubated at room temperature for 5 min. These two mixtures were then further mixed and kept at room temperature for 20 min. The DNA:liposome mixture solution was added to 293 cells plated at a cell density of 1x106 cells per 36 cm2 plate. The cells were incubated in 2 mL OPTI-MEM medium at 37°C in a CO2 incubator. The medium was replaced by DMEM with 10% FCS and 5% Glutamine after 4 hours, and continually monitored for 7-10 days by microscopy for formation of plaques. The cell culture medium was refreshed as required. When cytopathic effects (CPE) were apparent and extensive, cells with the medium, in case of any adenovirus releasing into the cell culture medium, underwent five rounds of freeze/thaw cycles performed at -80°C and 37°C, respectively, resulting in generation of the initial crude viral lysates. To amplify the crude viral lysates, more T75cm2 flasks of 293 cells were infected by the initial crude viral lysates. Infected cells went through further freeze/thaw cycles. The cell lysates were then spun down. The supernatants were used as the source of the virus for further infection of additional 293 cells. The final amplification step was performed, using a total of 36 T175 cm2 flasks. When CPE were apparent and extensive, cells were harvested and pelleted. Cell pellets from every 6 flasks were resuspended in 3 mL serum free EMEM medium, and were then subjected to five rounds of freeze/thaw cycles for preparation of cell lysate samples.

3.2.2.6 Purification of recombinant adenoviruses

The cell lysate samples were centrifuged at 10,000 ×g in a JA-17 rotor for one hour. The supernatant was gently collected and further purified by density gradient centrifugation. The density gradient centrifugation was performed by laying the sample onto the top of a discontinuous cesium chloride (CsCl) gradient. The gradient consisted of a layer of 1.25 g/ml CsCl, layered gently over the 1.40 g/ml CsCl layer in a Quick-Seal Centrifuge tube for ultracentrifugation. Samples were spun in a Beckman ultracentrifuge at 150,000 ×g (~50,000

rpm) for two hours, using a Type 80 Ti rotor at 20°C in brake-off mode. The opalescent band close to the bottom was collected with a 25G needle on a 5mL syringe. The band was layed onto a 1.34 gm/ml CsCl continuous gradient and spun at 150,000 ×g (~50,000 rpm) for 18 hours at 20°C. The viral band was carefully aspirated with 25G needle on a 5mL syringe. The sample was futher dialyzed to remove CsCl. All CsCl solutions were prepared and autocleaved in 1× TD buffer consisting of 140 mM NaCl, 5 mM KCl, 25 mM Tris and 0.7 mM Na_2HPO_4.

The samples were injected into a Slide-A-lyzer dialyzing cassette (Pierce) for dialysis using a dialysis buffer, containing10 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$ for 4 hours at 4°C. The buffer was changed once every hour to remove CsCl. Viruses were collected and the viral concentration was measured by a spectrophotometer at A_{260} with an optical density (O.D.) unit of 1, equivalent to10^{10} plaque forming unit (PFU)/ml (Xiang and Wu 2003). For viral storage, glycerol was added to the purified AdV samples at a final concentration of 10% (v/v) glycerol, then stored at -80°C.

3.2.3 Construction of the engineered dendritic cell vaccine

3.2.3.1 DC culture

Dendritic cells were generated from bone marrow (BM) culture (Lutz et al. 1999). BM cells were taken from femurs and tibias of mice; red blood cells were then depleted with 0.84% ammonium chloride. BM cells were plated in Dulbecco's modified Eagle's medium (DMEM) plus 10% FCS, GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) on the first day. Non-adherent granulocytes, B and T lymphocytes were gently removed on the third day, and fresh media were added. Two days later, loosely adherent proliferating DC aggregates were dislodged and replated. On the seventh day, non-adherent mature DC cells with typical morphologic features of THE DCs were harvested for AdV transfection and phenotypic analysis (refer to Fig.3-5).

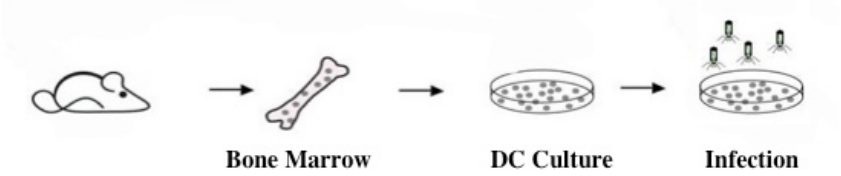


Fig. 3-5 A schematic map of DC vaccine production. Bone marrow was taken out of mice and cultured in plates with medium containing cytokines to stimulat DC maturation before introduction into DC vaccine via recombinant adenovirus tranfection.

3.2.3.2 AdV transfection

On the seventh day, mature DCs were harvested for transfection by recombinant adnenoviruses. For optimal transfection, a multiplicity of infection (MOI) of 200 was used per DC, as previously described (Chen et al. 2002). To enhance viral adsorption, DCs with virus were cultured in 1 mL DMEM in a 6-well culture plate for 1h at 37°C in a 5% CO2 incubator. More DC culture medium (4 mL) was added to each well for an additional 24-36 hours. DCs were then harvested for phenotypic analysis by flow cytometry. DCs, transfected with AdV_{OVA}, $AdV_{OVA\text{-}P30}$, $AdV_{HER2/neu}$, $AdV_{HER2/neu\text{-}P30}$, were termed DC_{OVA}, $DC_{OVA\text{-}P30}$, $DC_{HER2/neu}$, and $DC_{HER2/neu\text{-}P30}$, respectively. Engineered DC vaccines were stored at -196°C in liquid nitrogen tank for future use.

3.2.3.3 Flow cytometry analysis

Transfected DCs were phenotypicaly analysized by flow cytometry. Cells were firstly harvested and spun down by centrifugation. After washing twice with PBS, cells (5×10^6) were then incubated with 5 μg/ml biotin-labeled primary Ab for at least 30 mins on ice. Following three washes, cells were stained with FITC-avidin for another 30 min. Isotype-matched mAbs were used as negative controls. Cells (1×10^6) were analyzed by flow cytometry (Beckman-Coulter) after washing.

3.2.4 Animal study

3.2.4.1 Vaccination of Mice

3.2.4.1.1 Recombinant adenovirus vaccine

To evaluate CTL responses induced by recombinant adenovirus, AdV_{OVA}, or AdV_{neu} were injected (i.v.) into C57BL/6 mice or FVBneuN mice, repectively. In antitumor studies, both the (s.c.) and the (i.v.) administration were used, for anti-primary tumor study and anti-metastasic tumor studies, respectively.

3.2.4.1.2 Engineered DC vaccine

The same administration routes used for recombinant adenovirus vaccine were also used for the engineered DC vaccines when studing induced immune response as well as vaccine effectiveness in antitumor immunity studies. However, different doses of the freshly engineered DC vaccines were also used.

3.2.4.2 Assessment of CTL responses

3.2.4.2.1 Tetramer staining

C57BL/6 mice were immunized by i.v. injection with either AdV_{OVA} (1×10^7 PFU), $AdV_{OVA\text{-}P30}$ (1×10^7 PFU), DC_{OVA} (5×10^5), or $DC_{OVA\text{-}P30}$ (5×10^5). At 11 days after the immunization, 100 μL of mouse peripheral blood was stained with FITC-conjugated anti-CD8 Ab and PE-conjugated H-$2K^b$/$OVA_{257–264}$ tetramer for 30 min at room temperature and analyzed by flow cytometry.

FVBneuN Tg mice were immunized by i.v. injection with $AdV_{HER2/neu}$ (1×10^7 PFU) per mouse, Eleven days after AdV immunization, 100 μL of mouse peripheral blood was incubated with FITC-conjugated anti-CD8 Ab and PE-labeled H-$2D^q$/HER-2 peptide tetramer for 30 min at room temperature and analyzed by flow cytometry.

3.2.4.2.2 IFN-γ secreting $CD8^+$ T cells

Tg FVBneuN mice were immunized by i.v. injection of either DCHER2/neu-P30 (5×10^5), or DCHER2/neu (5×10^5). Six days after DC immunization, 150 μL of mouse peripheral blood was collected in heparin solution and lysed with RBC Lysis/Fixation Solution without fixation buffer. Cells were then pulsed with 0.5 μL 1.25 mM peptide in 250 μL DMEM. Five hours later, cells were permeated for intra cellular staining using Cytofix/Cytoperm solution following the manufacture procedure. Cells were harvested and stained with PE-conjugated-anti-IFN-γ mAb for 30 min on ice. After 2× washing, cells were stained with FITC-conjugated-anti-CD8 mAb for another 30 min and analyzed by flow cytometry.

3.2.4.3 Activated T cell detection

Activated $CD4^+$ and $CD8^+$ T cells were detected by evaluating $CD4^+CD44^+$ and $CD8^+CD44^+$ T cell with flow cytometry.

C57BL/6 mice were immunized by i.v. injection of either $DC_{OVA,}$ or $DC_{OVA\text{-}P30}$. It has been demonstrated that CD4+ T cells responses reach to the peak amount at day 1 or 2 after DC immunization (Ahmed et al. 2012). To detect $CD4^+$ $CD44^+$, two days after immunization, spleen cells were collected and stained with anti-CD4-FITC-conjugated antibody and anti-CD44-biotin-conjugated antibody. After washing 3×, cells were stained with Streptavidin-PE. Cells were analyzed by flow cytometry after washing 3×.

Tg FVBneuN mice were immunized by i.v. injection with either $DC_{HER2/neu\text{-}P30,}$ or $DC_{HER2/neu}$. To detect $CD44^+CD4^+$ T cells, two days after DC immunization, spleen cells were collected and stained with anti-CD4-PE- conjugated antibody and anti-CD44-biotin-conjugated. After 3× washing, cells were stained with Strevidin-FITC. Cells were harvested and analyzed by flow cytometry after washing 3×.

To detect $CD44^+CD8^+$T cells, six days after DC immunization, 100 µL of mouse peripheral blood were collected and stained with anti-CD8-PE- conjugated antibody and anti-CD44-biotin-conjugated. Cells were further stained with Strevidin-FITC after washing 3×. Cells were analyzed by flow cytometry.

3.2.4.4 Cytotoxicity assay

An *in vivo* cytotoxicity assay was performed in mice 10 days after recombinant adenovirus vaccination or 7 days after engineered DC vaccination. Briefly, naïve C57BL6 mouse spleens were removed and dispensed into single cells; red blood cells were then lysed with 0.84% ammonium chloride.

Splenocytes pulsed with OVAI peptide were labeled with carboxyl-fluorescein succinimidyl ester (CFSE) (3.0 mM, $CFSE^{high}$) and served as OVA-specific target cells, whereas spleen cells pulsed with irrelevant Mut1 peptide were weakly labeled with CFSE (0.6 mM, $CFSE^{low}$) and served as nonspecific control target cells. Immunized mice were then i.v. injected with a 1:1 ($CFSE^{high}$: $CFSE^{low}$) mixture of splenocyte targets. Sixteen hours after target cell delivery, spleens of the recipient mice were removed, and the relative proportions of $CFSE^{high}$ and

$CFSE^{low}$ target cells remaining in the spleens were analyzed by flow cytometry.

3.2.4.5 Antitumor immunity study

Two types of animal studies were conducted. The first type of animal study was performed for evaluation of preventative antitumor immunity.

C57BL/6 mice were s.c. vaccinated with AdV_{OVA} (1×10^7 PFU per mouse, 10 mice per group). Eleven days after the immunization, C57BL/6 mice were s.c. injected in the right thigh with BL6-10_{OVA} tumor cells (3×10^5 cells per mouse). Each mouse was monitored weekly for tumor growth measured in two perpendicular diameters using a caliper. Tumor volume (mm^3) was calculated using the formula $V=a\times b^2/2$, where a is the largest and b is the smallest diameter, and represented as mean±s.d. Mice with a volume >100 mm^3 (or 12 mm in diameters) were euthanized for humanitarian reasons. C57BL/6 mice (8 mice per group) were i.v. vaccinated with either DC_{OVA}, or $DC_{OVA\text{-}P30}$, and on the seventh day post immunization, BL6-10_{OVA} tumor cells (0.3×10^6 cells per mouse) were injected i.v. into each mouse. Each mouse was monitored weekly for 21 days and lungs were then collected for tumor colony counting.

Tg FVBneuN (10 mice per group) mice at 2 months old were vaccinated s.c. with $AdV_{HER2/neu}$ (1×10^7 PFU per mouse) at 1-month intervals for a total of five vaccinations. Spontaneous breast tumor development was monitored weekly for up to 12 months. Tg FVBneuN mice (8 mice per group), which are Tg1-1 tumor bearing, were vaccinated i.v. with either $DC_{HER2/neu}$, or $DC_{HER2/neu\text{-}P30}$, and seven days post immunization, Tg1-1 tumor cells (3×10^5 cells or 1×10^6 per mouse) were injected i.v. into each mouse. Each mouse was monitored weekly for 21 days and lungs were collected for analysis for tumor colony counting.

The second type of animal study was performed to evaluate of the therapeutic antitumor immunity. C57BL/6 mice (8 mice per group) were i.v. injected with BL6-10_{OVA} tumor cells (0.3×10^6 cells per mouse). Three days after tumor challenge, each mouse was i.v. vaccinated with either DC_{OVA}, or $DC_{OVA\text{-}P30}$. Mice were monitored weekly for 21 days and lungs were then collected for analysis for tumor colony counting.

3.2.5 Statistics analysis

Prism software (GraphPad Software, Inc., San Diego, CA) was used for statistical analyses. Kaplan-Meier survival chart and Log-rank test were performed to compare mouse survival rates and tumor development between different treatment groups. Student T tests were performed to determine the significance of differences between groups, and P values less than 0.05 ($P<0.05$) were considered statistically significant.

4 Results

4.1 Part A. Study on adenovirus vaccine in animal models

4.1.1 Immune response induced by AdV_{OVA}

To assess $CD8^+$ T cell immune responses, wild-type C57BL/6 mice were immunized i.v. with recombinant OVA-expressing adenovirus (AdV_{OVA}). Mouse peripheral blood (100 μL) obtained from immunized mice at different days after immunization were stained with FITC-anti-CD8 antibody and PE-H-$2K^b$/$OVA_{257-264}$ tetramer, and analyzed by flow cytometry. C57BL/6 mice immunized with PBS or AdV_{null} were used as negative controls. We found that AdV_{OVA} vaccine stimulated sustained OVA-specific $CD8^+$ T-cell responses, accounting for 18.6% of the total $CD8^+$ T-cell population (Fig 4-1); this is significantly larger than 0.07% OVA-specific $CD8^+$ T-cell responses in mice immunized with the control AdV_{Null} ($P<0.01$). The OVA-specific $CD8^+$ T-cell responses peaked on day 11 after the immunization, and then declined slowly (refer to Fig.4-2).

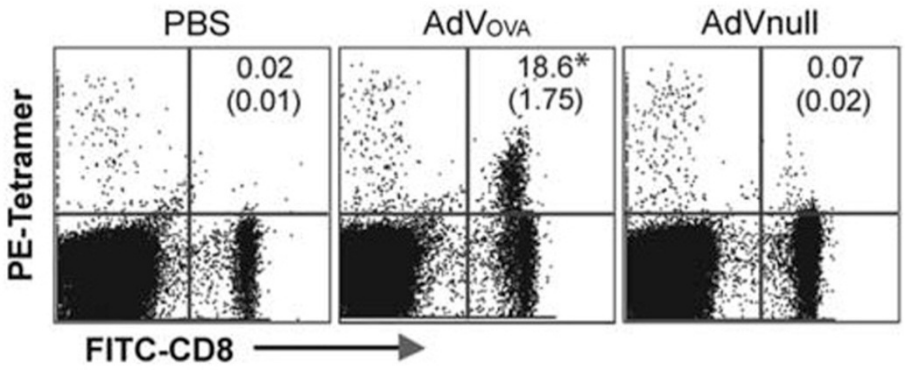


Fig.4-1 AdV_{OVA} stimulation of ovalbumin (OVA)-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses. Tail blood samples of AdVova-immunized C57BL/6 mice were harvested on day 11 after the immunization, stained with PE-H-$2K^b$/OVAI peptide tetramer and FITC-anti-CD8 Ab and then analyzed by flow cytometry; values in each panel represent the percentage of OVA-specific (tetramer-positive) $CD8^+$ T cells vs the total $CD8^+$ T-cell population; values in parenthesis represent standard deviation; student's t-test ($P<0.05$) result showed AdVova induced higher level of $CD8^+$ CTL than that of AdV_{null}. One representative experiment of two is shown.

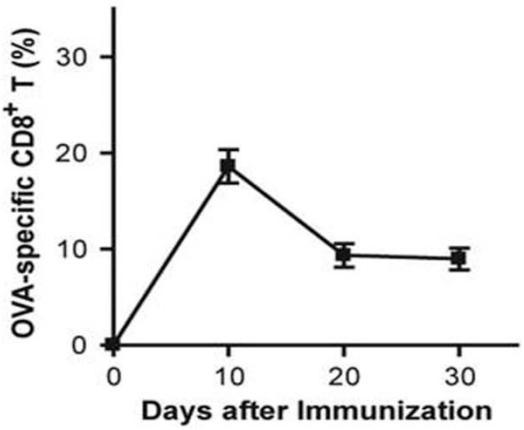


Fig.4-2 Quantification of OVA-specific CD8+ T cell population at different times. OVA-specific $CD8^+$ T cell response was induced by AdV_{OVA} after vaccination; mouse tail blood samples were harvested on different days after immunization and lymphocytes were stained with PE-H-$2K^b$/ OVAI peptide tetramer and fluorescein isothiocyanate (FITC)-anti-CD8 antibody (Ab) and then analyzed by flow cytometry. One representative experiment of two is shown.

4.1.2 OVA-specific *in vivo* cytotoxicity induced by AdV_{OVA}

To assess the functional effect of $CD8^+$ T cells, we performed an *in vivo* cytotoxicity assay. We used OVAI peptide-pulsed splenocytes which had been strongly labeled with CFSE ($CFSE^{high}$); and the control peptide Mut1-pulsed splenocytes which had been weakly labeled with CFSE ($CFSE^{low}$) as OVA-specific and control target cells. These target cells at 1: 1 ratio were introduced into recipient mice that had been vaccinated with AdV_{OVA}. As expected, there was a substantial loss (85%) of the $CFSE^{high}$ (OVAI peptide pulsed) cells in the AdV_{OVA}-immunized mice, whereas little cytotoxicity (8%) was induced in mice immunized with the control AdV_{null} (Fig. 4-3, $P<0.05$). Our data indicate that AdV_{OVA} vaccine efficiently stimulates $CD8^+$ T-cell differentiation into functional OVA-specific CTL effectors.

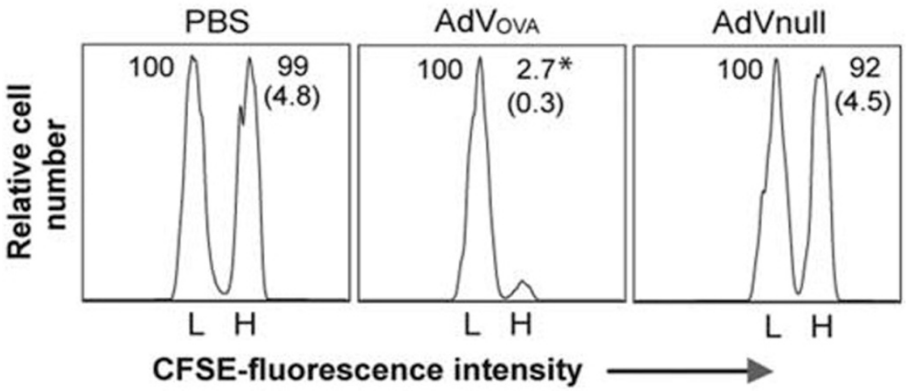


Fig. 4-3 *In vivo* cytotoxicity induced by AdV_{OVA}. Six days after the immunization, the immunized mice were injected i.v. with 2×10^6 cells, containing a 1:1 mixture of $CFSE^{high}$- and $CFSE^{low}$-labeled splenocytes that had been pulsed with OVAI or Mut1 peptides, respectively; after 16 h, the spleens of immunized mice were removed and the percentages of the residual $CFSE^{high}$ (H) and $CFSE^{low}$ (L) target cells remaining were analyzed by flow cytometry; the value in each panel represents the percentage of $CFSE^{high}$ vs $CFSE^{low}$ target cells remaining in the spleen; values in parenthese represent the standard deviation; student's t-test ($P<0.05$) showed AdVova induced higher cytotoxic responses than that of AdV_{null}; one representative experiment of three is shown.

4.1.3 Preventative antitumor immunity induced by AdV_{OVA}

To assess preventative antitumor immunity, AdV_{OVA}-immunized mice were challenged s.c. with OVA-expressing B16 melanoma BL6-10_{OVA} on day 60 after immunization. We found that none of the immunized mice (0/10) grew visible tumors (Fig. 4-4), indicating that AdV_{OVA} vaccination can induce long-term antitumor immunity.

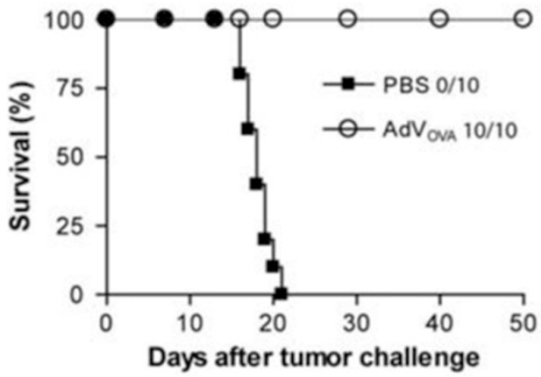


Fig. 4-4 AdV_{OVA} stimulation of preventaive ovalbumin (OVA)-specific antitumor immunity in wild-type C57BL/6 mice. AdV_{OVA}-immunized C57BL/6 mice were challenged s.c. with

BL6-10ova tumor cells on day 60 after immunization. Tumor growth was monitored; one representative experiment of two repeats is shown.

4.1.4 Immune responses induced by AdV_{neu}

Transgenic (Tg) FVBneuN mice that have neu-specific immune tolerance spontaneously develop multiple HER2/neu-expressing breast cancers (Reilly et al.2000; Miller et al. 2004; Czerniecki et al. 2007). These Tg mice were extensively used to evaluate HER2/neu-specific immunotherapeutics (Ambrosino et al. 2006; Wall et al. 2007; Whittington et al. 2008). To assess $CD8^+$ T cell responses, peripheral blood samples from the mice immunized with AdV_{neu}, were harvested on day 11 after immunization, stained with FITC-anti-CD8 Ab and PEanti-H-$2K^q$/HER2/neu peptide tetramer, and then analyzed by flow cytometry. We found that AdV_{neu}-stimulated HER2/neu-specific $CD8^+$ T-cell responses, accounting for 0.63% of the total $CD8^+$ T-cell population; this was a stronger response than the control mice immunized with AdV_{null} (0.18%, $P<0.05$, Fig. 4-5). Our data indicate that AdV_{neu} stimulated HER-2/neu-specific $CD8^+$ T-cell responses in Tg FVBneuN mice with HER2/neu-specific immune tolerance.

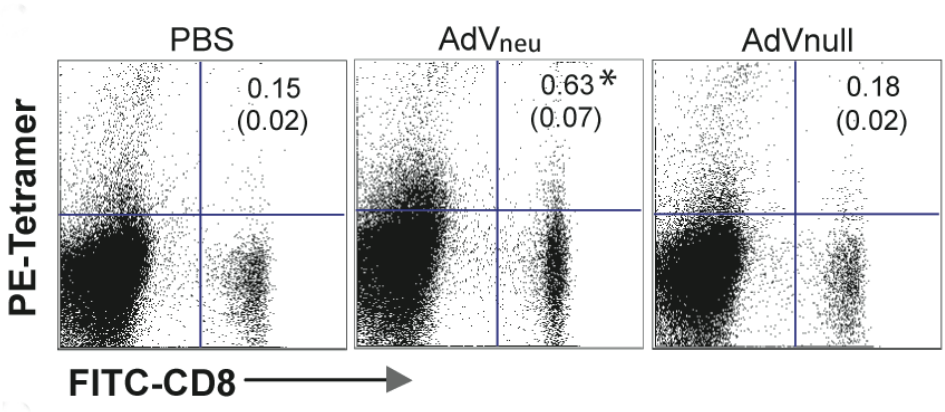


Fig. 4-5 Neu-specific $CD8^+$ T cell responses induced by AdV_{neu}. The tail blood samples from Tg FVBneuN mice immunized with AdV_{neu}, AdV_{null}, and PBS as a control, were harvested at day 11 after the immunization, stained with PE-H-$2K^q$/neuI peptide tetramer and FITC-anti-CD8 Ab and then analyzed by flow cytometry. Values in each panel represent the percentage of OVA-specific (tetramer-positive) $CD8^+$ T cells in the total $CD8^+$ T cell population; values in parenthese represent standard deviation; student's t-test ($P<0.05$) result showed AdVova induced higher level of $CD8^+$ T cell responses than that of AdV_{null}; one representative experiment of two is shown.

4.1.5 Preventive antitumor immunity induced by AdV_{neu}.

To determine whether AdV_{neu}-induced cellular immune responses could reduce breast carcinogenesis, Tg FVBneuN mice at the age of 2 months were vaccinated s.c. with AdV_{neu} at 1-month intervals for a total of four vaccinations. As shown in Fig. 4-6, AdV_{neu} vaccination protected 3/10 of the mice from breast carcinogenesis; it induced a significant delay in tumor formation in 7/10 of the mice compared with the control AdV_{Null} vaccination ($P<0.05$). Our data indicate that AdV_{neu} vaccination can partly overcome self-HER2/neu-specific immune tolerance and reduce breast carcinogenesis in Tg FVBneuN mice.

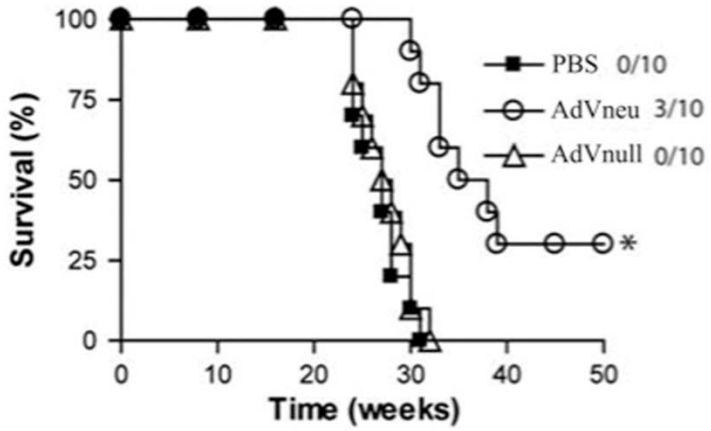


Fig. 4-6 Preventative antitumor immunity induced by AdV_{neu}. Transgenic FVBneuN mice at 2 months old were vaccinated s.c. with AdV_{neu} and at 1-month intervals for a total of four vaccinations; spontaneous formation of breast tumors was monitored weekly; significant differences ($P<0.05$) were found vs cohorts of the control groups (phosphate-buffered saline (PBS) and AdV_{null}) (log-rank test). One representative experiment of two is shown.

4.2 Part B. Study on $AdV_{OVA-P30}$-transfected DC vaccine in OVA animal model

4.2.1 Construction of $AdV_{OVA-P30}$

The construction of $AdV_{OVA-P30}$ consists of three steps:

(i) Insertion of P30 epitope into OVA gene (named OVA-P30).

(ii) OVA-P30 gene was then inserted into plasmid pShuttle-CMV (name pShuttle-CMV-OVA-P30).

(iii) pShuttle-CMV-OVA-P30 was transformed into BJ5183 cell to form AdV vector pAdEasy-OVA-P30 (Fig. 4-7) by homologous recombination.

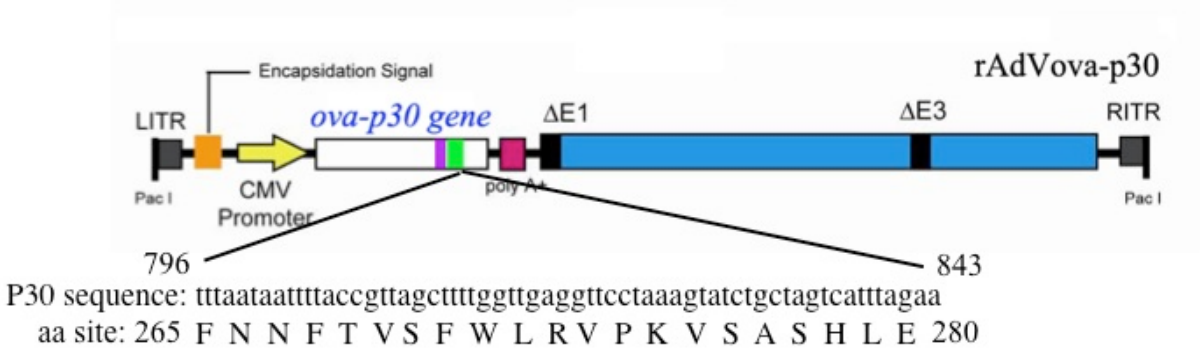


Fig. 4-7 Constructed plasmid DNA for $AdV_{OVA-P30}$. The E1/E3-deleted replication-deficient AdV vectors are under the regulation of the CMV early/immediate promoter/enhancer from pShuttle-CMV-OVA-P30; the diagram shows the linearized plasmid DNA pAdEasy-OVA-P30.

4.2.2 Expression of OVA-P30

Expression of OVA-P30 was determined by Western blot. Linear pAdEasy-OVA-P30 was firstly transfected into a 293 cell line (293-26 a cell expressing adenovirus type 5E1/E3 gene). Ten days later, proteins were obtained from cell lysates, and analyzed by Western blotting. Lysates from cells transfected with linear pAdEasy-pshuttle-CMV vector, as well as cells with no transfection were used as control. Results showed that OVA-P30 was highly expressed within the 293 cell line that had been transfected with linear $AdV_{OVA-P30}$ (Fig. 4-8).

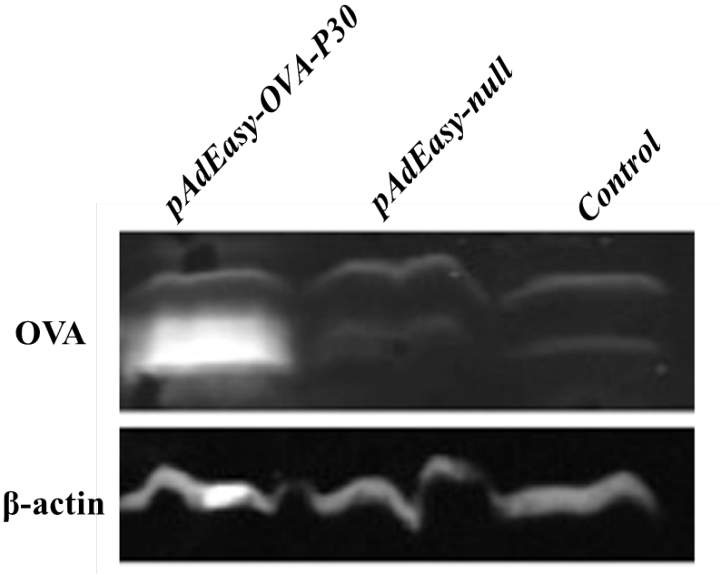


Fig. 4-8 Expression of OVA-P30 in 293 cells. Transfected 293 cells, as well as control cells were analyzed by western blot, using antibodies for expression of OVA and β-actin. Results were scanned by LI-COR Odyssey-CLx machine.

4.2.4 Assessment of OVA-specific CTL responses induced by the engineered $DC_{OVA-P30}$ vaccine

4.2.4.1 Generation of AdV_{OVA} and $AdV_{OVA-P30}$-engineered DC cell vaccines

Mature dendritic cells (DCs) were prepared by culturing bone-marrow cells for six days with DCI and DCII medium containing GM-CSF and IL4 (for detailed procedure, refer to 3.2.3.1). DCs were then transfected with AdV_{OVA} or $AdV_{OVA-P30}$, at the ratio of DC: adenovirus = 1:200 for 18 hrs in culture medium (for detailed procedure, refer to 3.2.3.2), to form DC_{OVA} and $DC_{OVA-P30}$. Transfected DC_{OVA} and $DC_{OVA-P30}$ were phenotypically analyzed by flow cytometry (for detailed procedure, refer to 3.2.3.3). We found that, similar to the mature DCs, transfected DC_{OVA} and $DC_{OVA-P30}$ expressed DC marker CD11c, a high amount of MHC class II molecule I_A^b and the costimulatory molecule CD80, indicating that they are mature DCs (Fig. 4-9).

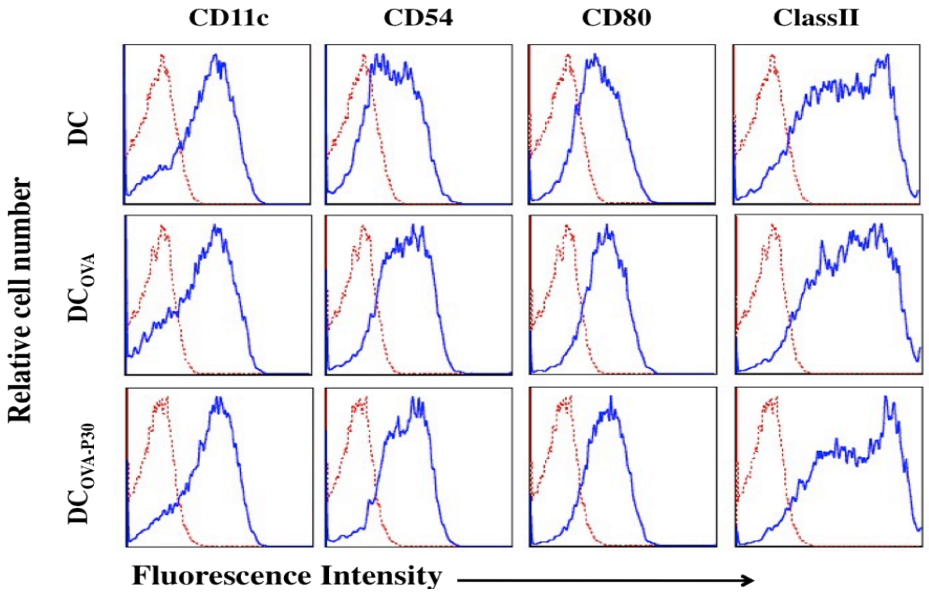


Fig. 4-9 Expressions of surface molecules on mature DC cells. Phenotypic changes of DCs, $AdV_{OVA-P30}$ transduced DC ($DC_{OVA-P30}$) and AdV_{neu} transduced DC (DC_{OVA}) were analyzed by flow cytometry, using anti-MHC class II (I_A^b), CD11c, CD54, CD80 Abs and FITC-labeled goat anti-mouse Ab (solid lines); isotype-matched mAbs (dotted lines) were used as controls. Data from one experiment of replicate experiments is shown.

4.2.4.2 T cell immune responses induced by engineered $DC_{OVA-P30}$ vaccine

C57/BL6 mice were injected i.v. with either DC_{OVA} or $DC_{OVA-P30}$. Splenocytes from each treatment group were stained with PE-anti-CD44 Ab and FITC-anti-CD4 Ab for assessment of $CD4^+$ T cell responses at day 1 or 2 after the immunization. At day 6, 50 μl of peripheral blood from immunized mice was stained with PE-H-$2K^b$/OVAI tetramer and FITC-anti-CD8 Ab for assessment of OVA-specific $CD8^+$ T cell responses.

We found that both DC_{OVA} and $DC_{OVA-P30}$ vaccines stimulated $CD4^+$ T cell responses (see Fig.4-10). However, the $CD4^+$ T cell response, induced by $DC_{OVA-P30,}$ (6.14%) were significantly higher than those of DC_{OVA} (4.34%) (Fig. 4-10, $P<0.05$), indicating that the universal foreign Th epitope P30 could enhance $CD4^+$ T cell responses *in vivo*. Both DC_{OVA} and $DC_{OVA-P30}$ vaccines also stimulated efficient OVA-specific $CD8^+$ T cell responses (Fig. 4-11, $P<0.05$). CTL responses induced by $DC_{OVA-P30}$ (8.52%) were also significantly higher than those of DC_{OVA} (4.66%) (Fig. 4-11, $P<0.05$), indicating that the universal foreign Th epitope P30 also enhanced $CD8^+$ T cell responses.

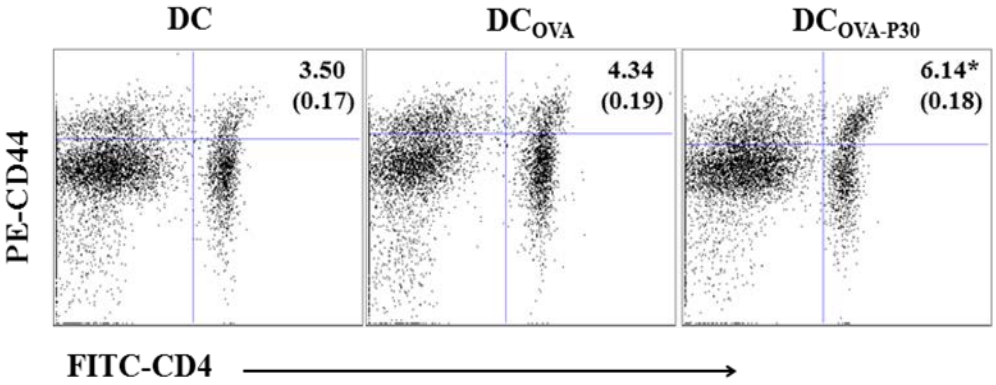


Fig. 4-10 CD4$^+$ T cell responses induced by DC$_{OVA}$ and DC$_{OVA-P30}$. Splenocytes of C57BL/6 mice immunized with 0.5×10^6 DC$_{OVA-P30}$ or DC$_{OVA}$ or DC as a control were harvested at day 1 after the immunization, stained with PE-anti-CD44 Ab and FITC-anti-CD4 Ab and then analyzed by flow cytometry. Values in each panel represent the percentage of activated CD4$^+$ T cells in the total CD4$^+$ T cell population; values in parenthese represent standard deviation amd student's t-test showed that the responses derived from DC$_{OVA-P30}$ were significant higher than those of DC$_{OVA}$ ($P<0.05$). Data from one experiment of three experiments is shown.

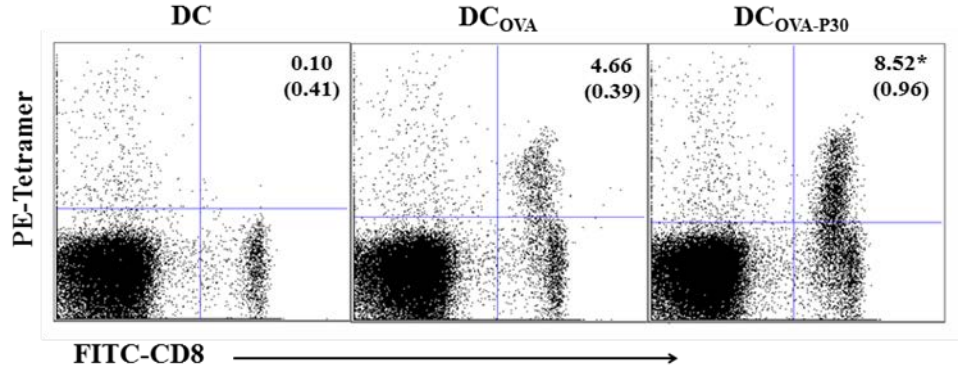


Fig. 4-11 OVA-specific CD8$^+$ T cell responses induced by DC$_{OVA}$ and DC$_{OVA-P30}$. Tail blood samples from C57BL/6 mice immunized with DC$_{OVA-P30}$ vs. control with DC$_{OVA}$ or DCs were harvested one day 6 after immunization, stained with PE-H-2K^b/OVAI peptide tetramer and FITC-anti-CD8 Ab and then analyzed by flow cytometry. Values in each panel represents the percentage of OVA-specific (tetramer-positive) CD8$^+$ T cells in the total CD8$^+$ T cell population. Values in parentheses represent standard deviation; student's t-test showed that the responses derived from DC$_{OVA-P30}$ were significant ($P<0.05$) higher than those of DC$_{OVA}$. Data from one experiment of three experiments is shown.

4.2.5 Vaccine studies

4.2.5.1 Assessment of *in vivo* cytotoxicity

To assess the cytotoxicity of activated $CD8^+$ T cells, *in vivo* cytotoxicity assays were performed. C57BL/6 mouse spleen cells, pulsed with OVAI peptide, were strongly labeled with carboxyl-fluorescein succinimidyl ester (CFSE) (3.0 mM, $CFSE^{High}$) and served as OVA-specific target cells; spleen cells pulsed with irrelevant peptide Mut1 were weakly labeled with CFSE (0.6 mM, $CFSE^{Low}$) and served as nonspecific control target cells. Eleven days following the immunization with AdV_{OVA}, the immunized mice were then injected i.v. with a 1:1 ($CFSE^{High}$: $CFSE^{Low}$) mixture of splenocyte targets. Sixteen hours after target cell delivery, spleens of the recipient mice were removed and the relative proportions of $CFSE^{high}$ and $CFSE^{low}$ target cells, remaining in the splenocytes, were analyzed by flow cytometry.

There was a substantial loss of the $CFSE^{High}$-labeled OVA-specific target spleenocytes in mice, immunized with either DC_{OVA} or $DC_{OVA\text{-}P30}$, indicating that both DC_{OVA}- and $DC_{OVA\text{-}P30}$-stimulated $CD8^+$ T cells, differentiated into effector CTLs (Fig. 4-12). However, the loss of the $CFSE^{High}$-labeled OVA-specific target spleenocytes in mice immunized with $DC_{OVA\text{-}P30}$ (89%) was significantly more than the loss of the $CFSE^{High}$-labeled OVA-specific target splenocytes in mice immunized with DC_{OVA} (71%) ($P<0.05$); thus $DC_{OVA\text{-}P30}$ vaccines triggered more efficient CTL responses than DC_{OVA}.

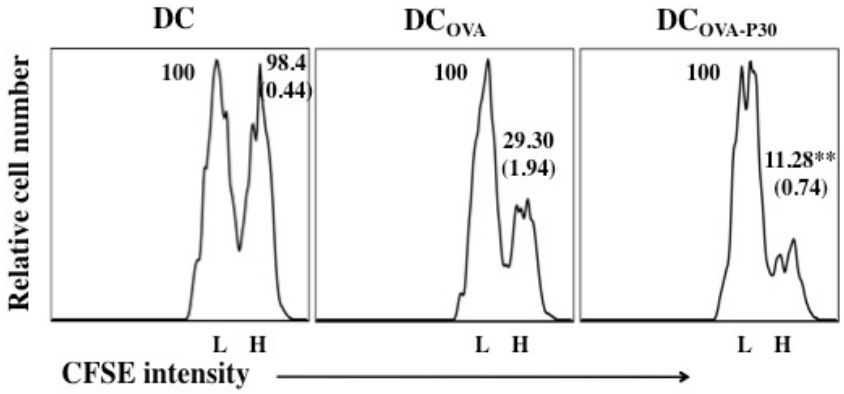


Fig. 4-12 *In vivo* cytotoxicity induced by DC_{OVA} and $DC_{OVA\text{-}P30}$. Splenocytes from mice immunized were analyzed for the percentage of $CFSE^{High}$ vs $CFSE^{Low}$ target cells remaining in the spleens. Values in each panel represents the percentage of $CFSE^{High}$ vs $CFSE^{Low}$ target cells remaining in the spleen; values in parentheses represent standard deviation; analysis with a

student's t-test showed that the responses induced by $DC_{OVA-P30}$ were significant ($P<0.05$) higher than those of DC_{OVA}; one representative experiment of three is shown.

4.2.5.2 Protective antitumor immunity induced by $DC_{OVA-P30}$ vaccine

To assess protective antitumor immunity, mice were vaccinated with either DC_{OVA}, $DC_{OVA-P30}$, or control DCs. OVA-expressing melanoma BL6-10_{OVA} cells (3×10^5/mouse) were injected i.v. six days later, and 21 one days after tumor injection, mice were killed and the lungs examined. Our data showed that both DC_{OVA} and $DC_{OVA-P30}$ vaccines protected all mice from BL6-10_{OVA} lung metastasis (Fig. 4-13 and Table 4-1).

Fig. 4-13 Preventaive effect of DC immunization on BL6-10_{OVA} melanoma metastasis in lungs of mice. C57BL6 mice were immunized either with DC, DC_{OVA} or $DC_{OVA-P30}$ vaccines, and were then challenged with BL6-10_{OVA} melanoma six days post-immunization. Mice were monitored for the next 21 days and photographs of representative lung samples from each group were shown.

Table 4-1 BL6-10_{OVA} melanoma colonies in lungs from mice immunized with DC_{OVA} and $DC_{OVA-P30}$

Vaccine	Tumor Bearing Mice (%)	Mean # of metastatic tumor colonies
$DC_{OVA-P30}$	0/8 (0%)	0
DC_{OVA}	0/8 (0%)	0
DC	8/8 (100%)	>300

Three different C57BL6 mice groups, immunized either with DC, DC_{OVA} or $DC_{OVA-P30}$ vaccines were challenged with BL6-10_{OVA} melanoma six days post-immunization; mice were monitored for the next 21 day afterwards. One representative experiment of two is shown.

4.2.5.3 Therapeutic antitumor immunity induced by $DC_{OVA-P30}$ vaccine

To assess therapeutic antitumor immunity, BL6-10_{OVA} cells (3×10^5) were injected i.v. into mice. Three days after tumor challenge, mice (n=8/group) were immunized i.v. with either with DCs, DC_{OVA} or $DC_{OVA-P30}$. Mice were killed 21 days after vaccination and the lungs examed.

The average number of tumor metastasis colonies, found in the lungs of mice immunized with $DC_{OVA-P30,}$ was 25, much fewer than the average number of 137 tumor metastasis colonies observed in the lungs of mice immunized with DC_{OVA} ($P<0.05$). Thus, $DC_{OVA-P30}$ induced more efficient therapeutic antitumor immunity against 3-day established tumors than DC_{OVA} (Fig. 4-14 and Table 4-2).

Fig. 4-14 Therapeutic effect of DC_{OVA} and $DC_{OVA-P30}$ vaccines agaist BL6-10_{OVA} melanoma metastasis in lungs of mice. C57BL6 mice (n=8) were challenged with BL6-10_{OVA} melanoma and then were immunized with DC, DC_{OVA} and $DC_{OVA-P30}$ three days later. Lungs were collected after 21 days. Photographs of representative lung samples from each group are shown; small black nodes in the lung represent lung melanoma metastasis colonies.

Table 4-2 Metastatic tumor colonies in lungs of mice immunized with DC_{OVA} and $DC_{OVA-P30}$

Vaccine	Tumor Bearing Mice (%)	Mean # of metastatic tumor colonies
$DC_{OVA-P30}$	8/8 (100%)	25 (16-35)*
DC_{OVA}	8/8 (100%)	137 (118-156)
DC	8/8 (100%)	>300

C57BL6 mice were challenged with BL6-10_{OVA} melanoma; and 3 days later immunized with either DC, DC_{OVA} or $DC_{OVA-P30}$. Lungs were collected after 21 days. Values in parenthese represent range of values; student's t-test showed that the responses derived from $DC_{OVA-P30}$ were significant($P<0.05$) higher than those of DC_{OVA}. One representative experiment of two is shown.

4.3 Part C. Study on $AdV_{neu-P30}$-transfected DC vaccine in the neu animal model

4.3.1 Construction of $AdV_{neu-P30}$

The construction of $AdV_{neu-p30}$ consisted of three steps:

(i) Insertion of P30 gene into the neu gene (named neu-p30).

(ii) *neu-p30* gene was then inserted into the plasmid pShuttle-CMV (name pShuttle-CMV-neu-P30).

(iii) pShuttle-CMV-neu-P30 was transformed into the BJ5183 cell to form AdV vector pAdEasy-neu-P30 by homologous recombination (Fig. 4-15).

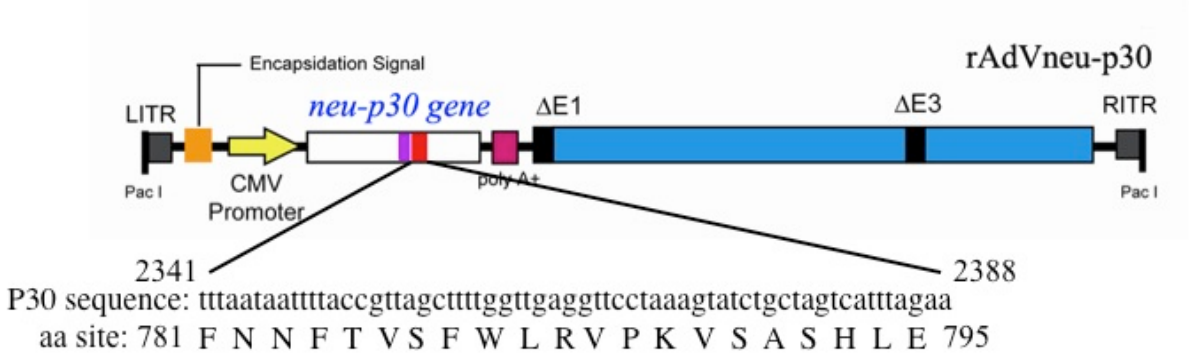


Fig. 4-15 Constructed plasmid DNA for $AdV_{neu-P30}$. The E1/E3-deleted replication-deficient AdV vectors are under the regulation of the CMV early/immediate promoter/enhancer from pShuttle-CMV-neu-P30; the diagram shows the linearized plasmid DNA AdEasy-neu-P30.

4.3.2 Expression of neu-P30

After 293 cells were transfected with linear plasmid pAdEasy-pshuttle-CMV-neu-P30 (or pAdEasy-neu-P30), virions ($AdV_{neu-P30}$) were released into the culture media and purified by differential ultracentrifugation. The purified $AdV_{neu-P30}$ (5×10^{10} PFU/mL) was stored at -80°C for future use. *Neu-p30* gene expression in 293 cells was detected by flow cytometry, using anti-neu antibody 24 hr after $AdV_{neu-P30}$ infection. The 293 cells without transfection were used as a negative control. The 293 cells, infected with $AdV_{neu-P30,}$ expressed a much higher level of neu than the cells without infection, indicating $AdV_{neu-P30}$ was functional (Fig. 4-16).

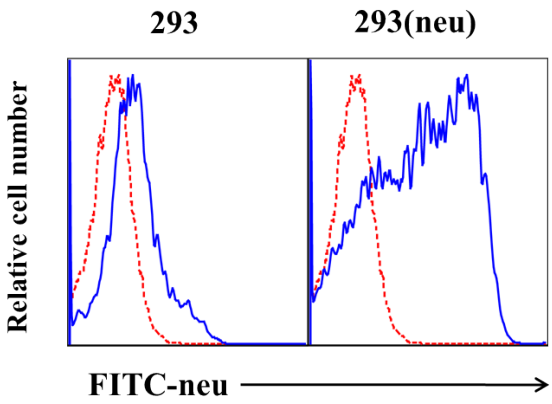


Fig. 4-16 Expression of neu. AdV_{neu}-p30 transfected 293 cells (293neu) as well as the control 293 cells were analyzed by flow cytometry, using the anti-neu antibody (solid lines) and an irrelevant isotype-matched control antibody (dotted lines). Data from a single analysis is shown.

4.3.3 Immune response derived from engineered $DC_{neu-P30}$ vaccine

4.3.3.1 Generation of AdV_{neu} and $AdV_{neu-P30}$-engineered DC cell vaccine

Mature DC cells were prepared by culturing bone marrow cells for six days with DCI and DCII medium containing GM-CSF and IL4 (see detailed procedure in section 3.2.3.1). DCs were then transfected with AdV_{neu} or $AdV_{neu-P30}$ using a DC: adenovirus ratio= 1:200 for 18 hrs to generate DC_{neu} and $DC_{neu-P30}$ (see detailed procedure in section 3.2.3.2). Transfected DC_{neu} and $DC_{neu-P30}$ were phenotypically analyzed by flow cytometry (detailed procedure refer to 3.2.3.3). Similar to mature DCs, transfected DC_{neu} and $DC_{neu-P30}$ also expressed the DC marker CD11c, a high level of MHC class II molecule I_A^b and the costimulatory molecule CD80, indicating that they are mature DCs (Fig. 4-17). DC cells infected with AdV_{neu} or $AdV_{neu-P30}$ express high level of neu.

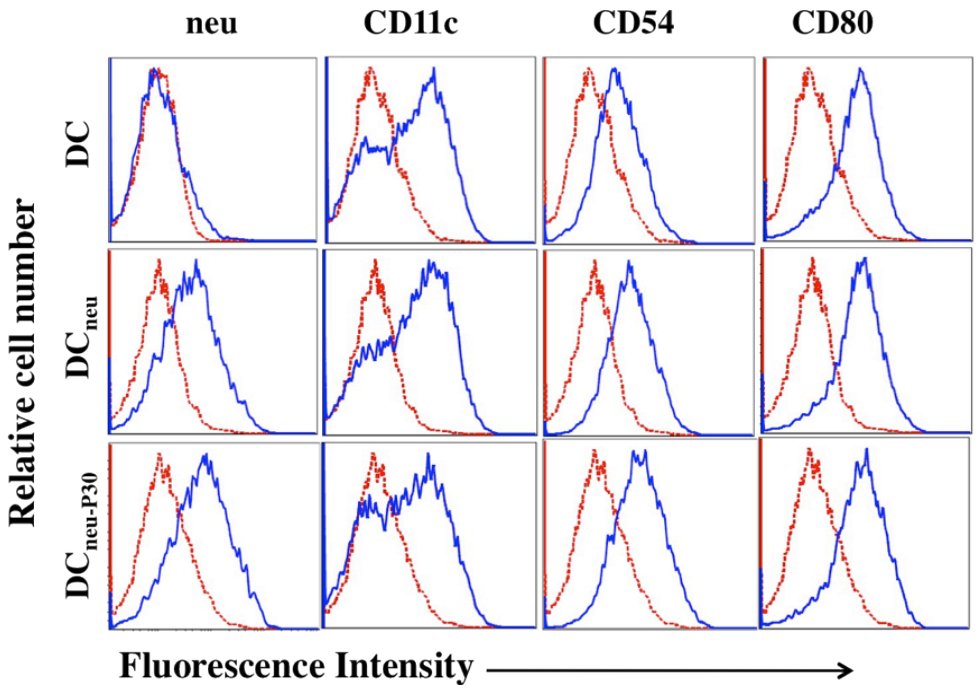


Fig. 4-17 Expressions of DC molecules and neu on mature DC cell. The phenotype of DCs, $DC_{neu-P30}$, and DC_{neu} were analyzed by flow cytometry, using anti-neu, anti-CD11c, anti-CD54 and anti-CD80 Abs dectected with FITC-labeled goat anti-mouse Ab (solid lines). Isotype-matched mAbs (dotted lines) were used as controls.

4.3.3.2 Immune response induced by engineered DC $_{neu-P30}$ cell vaccine

The transgenic FVBneuN mice were i.v. immunized with DC_{neu} or $DC_{neu-P30}$. Splenocytes from each mouse group were stained with PE-anti-CD44 Ab and FITC-anti-CD4 Ab for assessment of $CD4^+$ T cell responses at day 1 or 2 subsequent to DC immunization. On day 6, peripheral blood samples (50 μl) derived from immunized mice were stained with PE-anti-IFN-γ Ab and FITC-anti-CD8 Ab for assessment of $CD8^+$ T cell responses.

We found that both DC_{neu} and $DC_{neu-P30}$ vaccines stimulated $CD4^+$ T cell responses (Fig. 4-18). However, the $CD4^+$ T cell response (5.03%), induced by $DC_{neu-P30,}$ was similar to that of DC_{neu} (4.74%). Both DC_{neu} and $DC_{neu-P30}$ vaccines also stimulated $CD44^+$ and IFN-γ-secreting $CD8^+$ T cell responses (Fig. 4-19 and Fig. 4-20). However, the CTL response (3.54% $CD8^+44^+$ T; 0.99% IFN-γ-secreting $CD8^+$ T) induced by $DC_{neu-P30,}$ was significantly ($P<0.05$) higher than that DC_{neu} (2.55% for $CD8^+44^+$ T; 0.42% for IFN-γ-secreting $CD8^+$ T), indicating that the universal foreign Th epitope P30 enhanced $CD8^+$ T cell responses.

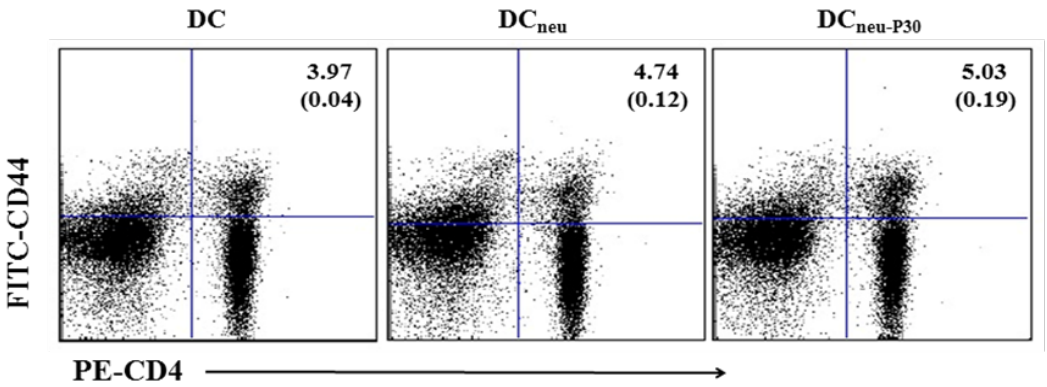


Fig. 4-18 Activated CD4$^+$ T cell responses induced by DC$_{neu}$ and DC$_{neu-P30}$. Splenocytes from Tg FVBneuN mice, immunized with DC$_{neu-P30,}$ DC$_{OVA}$, or DC, were harvested one day after immunization and were stained with PE-anti-CD4 Ab and FITC-anti-CD44 Ab before analyzing with flow cytometry. Values in each panel represent the percentage of activated CD4$^+$ T cells in the total CD4$^+$ T cell population. Values in parenthesis represent standard deviation. Data from one of two relicate experiment is shown.

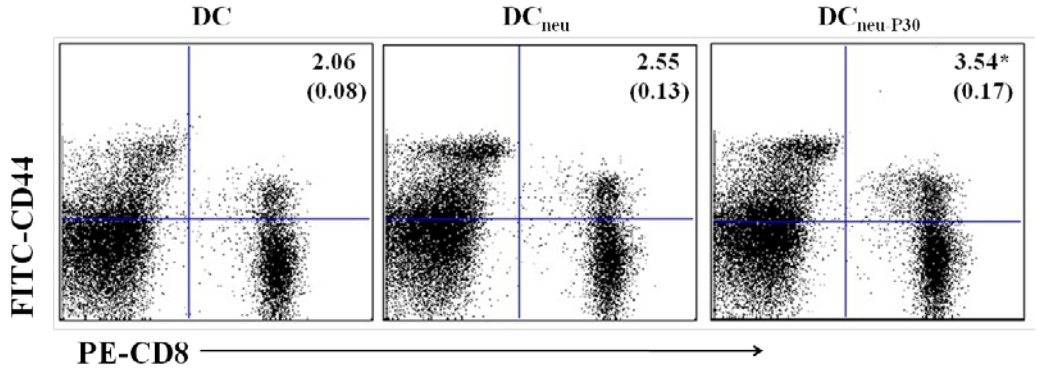


Fig. 4-19 Activated CD8$^+$ T cell responses induced by DC vaccination. Tail blood samples from Tg FVBneuN mice, immunized with DC$_{neu-P30}$, DC$_{OVA}$, or DC, were harvested on day 6 after immunization and stained with PE-anti-CD8 and FITC-anti-CD44 Ab and then analyzed by flow cytometry. Values in each panel represent the percent of activated CD8$^+$ T cells in the total CD8$^+$ T cell population. Values in parentheses represent standard deviation. Analysis with a student's t-test results showed that responses induced by DC$_{neu-P30}$ were significant ($P<0.05$) higher than those of DC$_{neu}$. Data from one of two relicate experiment is shown.

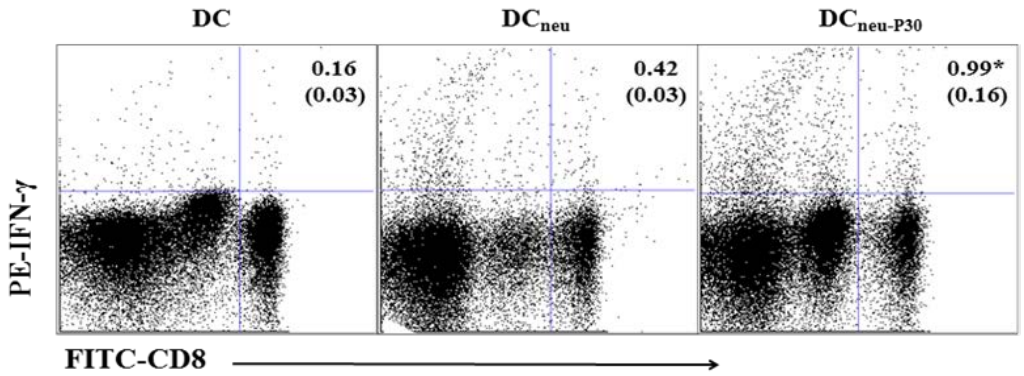


Fig. 4-20 IFN-γ secreting CD8$^+$ T cell responses induced by DC vaccination. Tail blood samples from Tg FVBneuN mice immunized with $DC_{neu\text{-}P30}$, DC_{OVA}, or DCs, were harvested on day 6 after immunization and were stained with PE-anti-CD8 and FITC-anti-IFN-γ Ab after permeabilized and then analyzed by flow cytometry. Values in each panel represent the percent of IFN-γ secreting CD8$^+$ T cells in the total CD8$^+$ T cell population. Values in parenthesis represent standard deviation. Analysis with a student's t-test results showed that the responses derived from $DC_{neu\text{-}P30}$ were significant ($P<0.05$) higher than those of DC_{neu}; Data from one of two relicate experiment is shown.

4.3.3.3 Protective immunity induced by $DC_{neu\text{-}P30}$ vaccine

To assess protective immunity, three groups of transgenic FVBneuN mice were vaccinated with DC_{neu}, $DC_{neu\text{-}P30}$, or DCs, respectively. Neu-expressing Tg1-1 cells (3×10^5 or 1×10^6) were injected i.v. into each group of mice six days later. Mice were killed 21 days after tumor injection and the lungs collected for observation.

Both DC_{neu}- and $DC_{neu\text{-}P30}$-vaccinated mice were protected against Tg1-1 tumor cell challenge (3×10^5) (see Fig. 4-21 and Table 4-3). However, when immunized mice were challenged with Tg1-1 tumor cells (1×10^6), the average number of lung tumor metastasis colonies in mice, immunized with $DC_{neu\text{-}P30,}$ was 16. The average number of lung tumor metastasis colonies was 144 in mice, immunized with DC_{neu} (Fig. 4-22 and Table 4-4), indicating that $DC_{neu\text{-}P30}$ vaccine induces more efficient antitumor immunity in Tg FVBneuN mice with neu-specific immune tolerance.

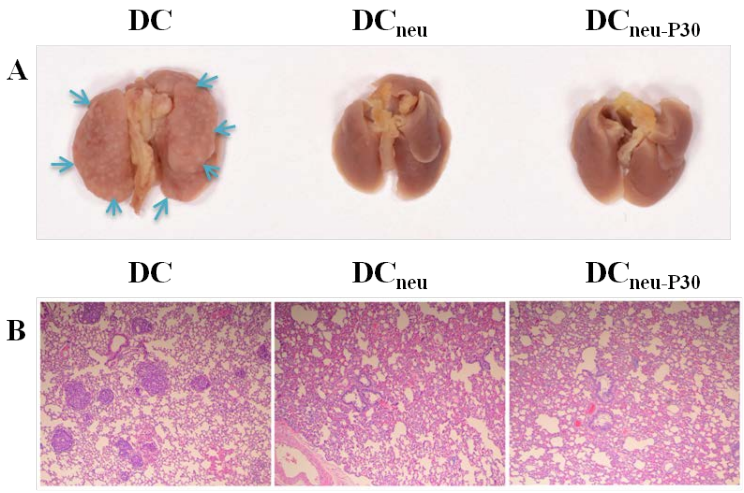


Fig. 4-21 Prevention of Tg1-1 (0.3 M) tumor metastasis in lungs of DC$_{neu}$ and DC$_{neu-P30}$ immunized mice. Tg FVBneuN mice immunized with DC, DC$_{neu}$ or DC$_{neu-P30}$, were challenged with Tg 1-1 tumor cells (3×10^5) six days post-immunization. Mice were killed 21 days after tumor cell challenge. Photographs of representative lung samples from each group are shown. (A) Tumor clonies (white) are shown and indicated by arrows. (B) Histology of of lungs (magnification=100×).

Table 4-3 Metastatic tumor colonies in lungs from FVBneuN mice immunized with DC$_{neu}$ and DC$_{neu-P30}$ (a)

Vaccine	Tumor Bearing Mice (%)	Mean # of metastatic tumor colonies
DC$_{neu-P30}$	0/8 (0%)	0
DC$_{neu}$	0/8 (0%)	0
DC	8/8 (100%)	123 (112-134)

Tg FVBneuN mice were immunized with either DCs, DC$_{neu}$ or DC$_{neu-P30}$. Immunized mice were then challenged i.v. with Tg1-1 cancer cells (3×10^5) six days after immunization. Mice were killed 21 days after tumor cell challenge and lungs were examined for white tumor colonies. Data form one experiment of two replicate experiments is shown.

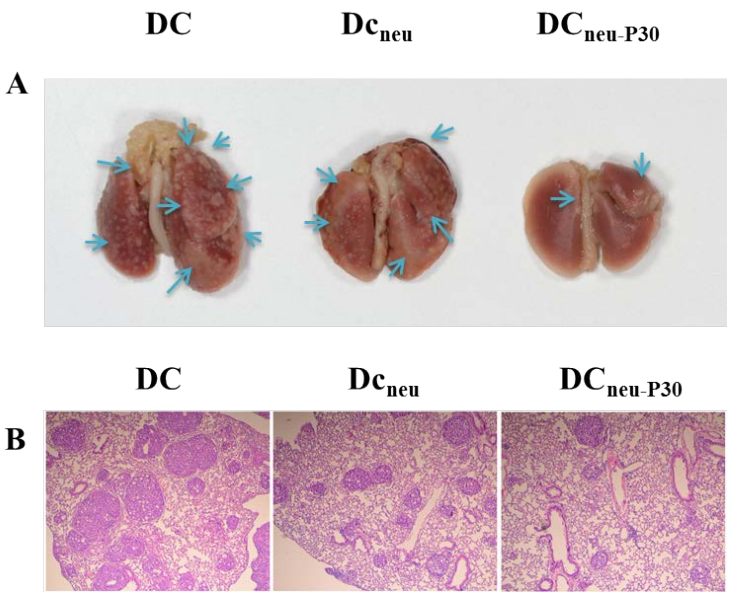


Fig. 4-22 Prevention of Tg1-1(1 M) tumor metastasis in lungs of DC_{neu} and $DC_{neu-P30}$ immunized mice. Tg FVBneuN mice, immunized with either DCs, DC_{neu} or $DC_{neu-P30}$, were then challenged with Tg 1-1 tumor cells (1×10^6) six days post-immunization. Mice were killed 21 days after tumor cell challenge. (A) Photographs of representative lung samples from each group are shown. Tumor clonies (white) were shown and indicated by arrows. (B) Histology of lungs (magnification=100×).

Table 4-4 Metastatic tumor colonies in lungs from FVBneuN mice immunized with DC_{neu} and $DC_{neu-P30}$ (b)

Vaccine	Tumor Bearing Mice (%)	Mean # of metastatic tumor colonies
$DC_{neu-P30}$	8/8 (100%)	16 (12-20)*
DC_{neu}	8/8 (100%)	144 (128-160)
DC	8/8 (100%)	>300

Tg FVBneuN mice were immunized with either DC, DC_{neu} or $DC_{neu-P30}$. Immunized mice were then challenged i.v. with Tg1-1 cancer cells (1×10^6) six days after immunization. Mice were killed 21 days after tumor cell challenge and lungs were examined for white tumor colonies. Student's t-test results showed that the CTL responses derived from $DC_{neu-P30}$ were significant ($P<0.05$) higher than those of DC_{neu}. Data form one experiment of two replicate experiments is shown.

The results derived from DCneu-P30 and DCneu vaccinations demonstrated that DCneu-P30 vaccine stimulated stronger CD8+ T cell responses and antitumor immunity than that of DCneu vaccine. Our data indicate that the universal Th epitope P30 enhances CD8+ T cell responses, leading to antitumor immunity in Tg FVBneuN mice by breaking self-immune tolerance.

5 Discussion

Conventional cancer therapies, including surgery, radiation therapy and chemotherapy have demonstrated considerable clinical success in the past. However, tumor-free survival is not always accomplished. For example, surgery and radiation therapy are quite effective in treatment of localized tumors but they often have only a palliative role in treatment of disseminated diseases. Chemotherapy remains the treatment modality of choice but severe toxic side-effects often limit its use.

The identification of tumor-associated Ags and tumor-specific T-cell responses in cancer patients led to the development of immunotherapies, aimed at augmenting antitumor immune responses. Antitumor immunotherapies include: 1) active immunotherapy, such as the use of various antitumor vaccines to stimulate the patients' antitumor $CD8^+$ CTL responses (Antonia et al. 2004), and 2) adoptive immunotherapy, such as infusion of the antitumor monoclonal Ab trastuzumab or tumor-specific tumor-infiltrating lymphocytes (Gonzalez-Angulo et.al, 2006; Knutson et al. 2005).

The original anti-HER-2/neu murine monoclonal antibodies inhibited HER-2/neu-positive tumor growth *in vivo* (Harwerth et al. 1993; Knutson et al. 2004; Hudziak et al. 1989) Trastuzumab is a humanized monoclonal Ab, directed against the extracellular domain of HER-2/neu. Its use, in combination with chemotherapy, was approved by the FDA in 1998 for metastatic HER-2/neu overexpressing breast cancer (Slamon et al. 2001). Preclinical studies demonstrated interesting properties of trastuzumab, including internalization and degradation of the HER-2 protein (Molina et al. 2001), inhibition of cell-cycle progression via inhibition of the mitogen-activated protein kinase pathway (Le et al. 2003; Jackson et al. 2004), suppression of the anti-apoptotic phosphatidylinositol 3-kinase and Akt pathway (Yakes et al. 2002; Mohsin et al. 2005) and Ab-dependent cellular cytotoxicity (Clynes et al. 2000; Gennari et al. 2004). Clinical studies showed that approximately one third of patients with advanced HER-2/neu-positive breast cancer will respond to trastuzumab monotherapy (Vogel et al. 2002; Cobleigh et al.1999). Trastuzumab-based therapy has also been shown to be effective in both adjuvant and neo-adjuvant setting in the management of early stage HER-2/neu-positive breast cancer (Baselga et al. 2004; Buzdar et al.2005). However, one of the major limitations of trastuzumab immunotherapy is the

development of Ab resistance, usually within one year from the beginning of treatment in the metastatic setting (Nahta et al. 2006; Berns et al.2007). Thus, there is a need to search for other therapeutic strategies.

One of the most remarkable features of AdV-based vaccines is their ability to induce exceptionally high and sustained frequencies of transgene product-specific $CD8^+$ T-cell responses; unlike those induced by other subunit vaccine carriers such as DNA vaccines or poxvirus vectors, these $CD8^+$ T cells do not contract after the initial CTL activation (Bruna-Romero et al. 2001; Hassett et al.2000). The replication-defective AdV vector genome is similar to those of wild-type AdV vectors (Tatsis et al. 2007; Yang et al. 2006). These replication-defective AdV vectors have been found in the muscle at the site of inoculation, in the liver and in the lymphatic tissues of experimental animals (Tatsis et al. 2007; Yang et al. 2006).

In this study, we also demonstrated that AdV_{OVA} vaccination induced sustained $CD8^+$ CTL responses due to persistent Ag stimulation, leading to 100% protection from tumor cell challenge, which is consistent with previous reports by others (Tatsis et al. 2007; Yang et al. 2006). The AdV_{neu} vaccination stimulated both HER-2/neu-specific Ab and $CD8^+$ CTL responses and preventive antitumor immunity in wild-type mice (Gallo et al. 2005; Park et al. 2005; Wang et al. 2005). However, it does not reduce breast carcinogenesis in Tg mice with self-immune tolerance though their survival was prolonged (Millar et al. 2007; Kianizad et al. 2007). In this study, we demonstrated that AdVneu induced neu-specific functional $CD8^+$ T cell responses and that AdVneu vaccination can reduce breast carcinogenesis in Tg FVBneuN mice with self-immune tolerance, consistent with another recent report by Berzofsky et al. (Grinshtein et al. 2009). The more effective reduction in breast carcinogenesis by our AdVneu vaccine in Tg FVBneuN mice may possibly be due to more efficient transfection by our AdVneu with fiber gene modification (Sas et al. 2008). Thus, more efficient transgene neu expression occurs. The AdVneu without fiber gene modification that was used by others (Millar et al. 2007; Kianizad et al. 2007).

It is generally believed that $CD4^+$ and $CD8^+$ T cells recognizing self-antigens, including many tumor Ags, with high affinity in the thymus, are deleted (Sprent et al. 2002; Sprent et al.2003). Therefore, vaccination attempts to break immunological tolerance may be hampered.

Understanding the mechanisms behind tolerance is paramount for understanding the fundamental concepts in immunology, as well as for vaccine development. $CD4^+$ T helper cells play crucial roles in priming, expansion, and memory development of both B cell and $CD8^+$ CTL responses. The critical role of Th cells is well documented and is found to be essential in antitumor immunity (Dalum et al. 1996; Dalum et al. 1999; Hertz et al. 2001; Renard et al. 2003).

The tetanus toxin epitope, P30 (FNNFTVSFWLRVPKVSASHLE) is a universally immunogenic epitope, capable of stimulating $CD4^+$ T cell responses (Panina-Bordignon et al. 1989). The HER2/neu DNA vaccine, containing the potent Th cell epitope P30, induced complete immune protection against tumor cell challenge in wild-type mice but only partial antitumor protection in Tg mice. The HER2/neu protein vaccine, containing P30 epitope, stimulated protective immune responses even in Tg mice (Renard et al. 2003). In an OVA model study, vaccination with DNA, containing P30, prevented the growth of OVA-expressing tumor in transgenic rat insulin promoter (RIP)-mOVA mice (Steinaa et al. 2005). A membrane bound truncated OVA sequence under the control of RIP was found in pancreatic islets as well as in the kidney proximal tubules, thymus and the testis of male mice (Kurts et al. 1996) and also exhibited deletional tolerance toward the $OVA_{265–280}$ (SIINFEKL, MHC class I epitope of OVA) epitope (Kurts et al. 1997). Thus, the foreign Th epitope P30 broke $CD8^+$ T cell tolerance against self Ag in these Tg mice. In this study, we constructed recombinant $AdV_{OVA\text{-}P30,}$ expressing ovalbumin (OVA)-P30 and AdV-transfected DC ($DC_{OVA\text{-}P30}$) vaccine by transfection of BM-derived DCs with $AdV_{OVA\text{-}P30}$. We immunized C57BL/6 mice with $DC_{OVA\text{-}P30}$ and then assessed $CD4^+$ and $CD8^+$ T cell responses and antitumor immunity, after immunization. We demonstrated that $DC_{OVA\text{-}P30}$ was capable of stimulating both enhanced OVA-specific $CD4^+$ and $CD8^+$ T cell responses, leading to preventive antitumor immunity against OVA-expressing BL6-10_{OVA} tumor cell challenge in immunized C57BL/6 mice (100%); and significant therapeutic antitumor immunity against OVA-expressing BL6-10_{OVA} tumors established 3 days prior to immunization in C57BL/6 mice.

HER2/neu has proven to be an attractive Ag for immunotherapeutic targeting (Yarden 2001; Yarden and Sliwkowski 2001). DNA vaccines, expressing full length HER2 or partial length (ECD or ICD) of HER2, induced HER2-specific humoral and T cell immune responses (Amici et al. 1998, Chen et al. 1998, Piechocki et al. 2001, Pilon et al. 2001). However, they were not able to convey any beneficial therapeutic effects in HER2/neu transgenic (Tg) mice (Amici et al.

1998, Foy et al. 2001; Quaglino et al. 2004a; Radkevich-Brown and Jacob et al. 2009). DCs are the most powerful and efficient antigen-presenting cells (APCs). They are largely responsible for priming T cell responses (Zinkernagel 1974; Linsley et al. 2002). DCs, engineered to express tumor antigens, have been widely studied and utilized as cancer vaccines that generate an effective antitumor immune response both in murine models and in human clinical studies (Koski et al. 2008).

We previously showed that AdVneu-transfected DCneu vaccine induced more efficient preventive antitumor immunity against neu-expressing Tg1-1 tumor challenge s.c. than OVA-expressing DNA vaccine in wild-type FVB/NJ mice (Chan et al. 2006). Later, we constructed DCneu vaccine by transfection of DCs with fibre-modified $_{RGD}$AdVneu and showed that recombinant $_{RGD}$AdVneu with fibre gene, modified by RGD insertion to the viral knob's H1 loop, had enhanced transfection of DCs, lacking coxsackievirus and AdV receptor (CAR), leading to enhanced transgene neu expression in DCneu (Sas et al. 2008). We demonstrated that $_{RGD}$AdVneu-transfected DCneu stimulated more efficient antitumor immunity than AdVneu-transfected DCneu in wild-type FVB/NJ mice; however, it still failed to protect transgenic FVBneuN mice from Tg1-1 tumor challenge (Sas et al. 2008). In this study, we constructed recombinant $AdV_{neu\text{-}P30,}$ expressing HER2/neu-P30, and AdV-transfected DC ($DC_{neu\text{-}P30}$) vaccine by transfection of BM-derived DCs with $AdV_{neu\text{-}P30}$. We immunized transgenic FVBneuN mice with $DC_{neu\text{-}P30}$ and then assessed $CD4^+$ and $CD8^+$ T cell responses and antitumor immunity subsequent to immunization. We demonstrated that $DC_{neu\text{-}P30}$ were capable of stimulating enhanced $CD8^+$ T cell responses, leading to preventive antitumor immunity against the challenge of neu-expressing Tg1-1 tumor cells (3×10^5) in 100% (8/8) immunized transgenic FVBneuN mice; this treatment also significantly reduced lung metastasis tumor colonies in immunized transgenic FVBneuN mice, challenged with Tg1-1 tumor cells (1×10^6). Thus, incorporation of the foreign $CD4^+$ Th epitope P30 into a DC-based vaccine at least partially broke self-immune tolerance, leading to enhanced CTL responses and antitumor immunity in transgenic FVBneuN mice.

6 Conclusion and future direction

Our data demonstrated that $CD4^+$ Th epitope P30 enhanced both $CD4^+$ and $CD8^+$ T cell responses, leading to enhanced DC-stimulated antitumor immunity. This may impact the design of new DC-based vaccines for the treatment of breast cancer and other types of human malignancies.

Conventional anti-cancer therapies, surgery, radiationtherapy and/or chemotherapy, have gained considerable clinical success in the past. Because of the limitations, imposed by current treatments, tumor-free survival is not always accomplished. For instance, surgery and radiationtherapy are quite effective in the treatment of localized tumors but they can only play a palliative role in the treatment of disseminated diseases. Chemotherapy in these cases remains the treatment modality of choice but severe toxic effects toward normal tissues often limit its use.

The identification of tumor antigens and tumor specific T cell responses in cancer patients led to the development of immunotherapies, aimed at augmenting antitumor immune responses. Many preclinical studies indicated that both active and adoptive immunotherapies were quite effective against small tumor burdens but incapable of controlling large tumor masses.

The major limitation for combining chemotherapy and immunotherapy is that the chemotherapeutic drugs are generally immunosuppressive because the drugs are toxic to the dividing immune stem cells in the bone marrow.

In our study, $CD4^+$ Th epitope P30 enhanced both $CD4^+$ and $CD8^+$ T cell responses, leading to enhanced DC-stimulated antitumor immunity. However, our engineered $DC_{neu\text{-}P30}$ vaccine induced only partial immune protection against challenge of a small (3×10^5) and large (1×10^6) amounts of Tg1-1 tumor cells, in transgenic FVBneuN mice. Therefore, a therapeutic effect against established tumors may require a combination of chemotherapy to eradicate of the tumor masses along with active specific immunotherapy such as DC vaccine to eliminate potential tumor metastasis. Such a protocal may have great potential for the treatment of cancer patients, but needs to be further confirmed in larger controlled and randomized trials in the future.

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