DENDRITIC CELL BASED CANCER VACCINES USING ADENOVIRALLY MEDIATED EXPRESSION OF THE HER-2/NEU GENE AND APOPTOTIC TUMOR CELLS EXPRESSING HEAT SHOCK PROTEIN 70

A Thesis Submitted to the College of Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy
In the Department of Pathology and Laboratory Medicine
University of Saskatchewan
Saskatoon

By

Tim Chan

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ABSTRACT

Human Epidermal Growth Factor Receptor 2 (HER-2/neu) is a breast tumor antigen (Ag) commonly overexpressed in 30% of breast cancer cases. Both HER-2/neu-targeted DNA-based and transgene modified dendritic cell (DC)-based vaccines are potent elements in eliciting HER-2/neu specific antitumor immune responses; however, there has been no side-by-side comparison of these two different immunization methods. We utilized an in vivo murine tumor model expressing the rat neu Ag to compare the immunization efficacy between DC transduced with replication-deficient adenovirus containing neu (AdV_neu), to form DC_neu, and plasmid DNA (pcDNA) vaccine. DC_neu displayed an upregulation of immunologically important molecules and inflammatory cytokines expression such as IL-6 that partially mediated conversion of the regulatory T (Tr) cell suppression. Wildtype FVB/N mice immunized with DC_neu induced stronger HER-2/neu-specific humoral and cellular immune responses compared to plasmid DNA immunized mice. Furthermore, mice immunized with DC_neu remained completely protected from tumor challenge compared to partial or no protection observed in DNA immunized mice in two tumor animal models. In FVBneuN transgenic mice, which develop spontaneous breast tumors at 4-8 months of age, DC_neu significantly delayed tumor onset when immunization conducted in mice at a younger age. Taken together, we demonstrated that a HER-2/neu-gene modified DC vaccine is more potent than a plasmid DNA vaccine in inducing neu specific immune responses resulting in greater protective and preventative effects in the tumor models examined.

In another study, we examined the use of a DC-based cancer vaccine involving the phagocytosis of apoptotic tumor cells expressing heat shock protein 70 (HSP70). The dual role of HSP70, as an antigenic peptide chaperone and danger signal, makes it
especially important in DC-based vaccination. In this study, we investigated the impacts of apoptotic transgenic MCA/HSP tumor cells expressing HSP70 on DC maturation, T cell stimulation and overall vaccine efficacy. We found that DC with phagocytosis of MCA/HSP in the early phase of apoptosis expressed more peptide-major histocompatibility class (pMHC) I complexes, stimulated stronger cytotoxic T lymphocytes (CTL) responses and induced greater immune protection against MCA tumor cell challenge, compared to mice immunized with DC that phagocytosed MCA/HSP cells in the late phase of apoptosis. Taken together, our data demonstrated that HSP70 expression on apoptotic tumor cells stimulated DC maturation and DC with phagocytosis of apoptotic tumor cells expressing HSP70 in early phase of apoptosis more efficiently induced tumor-specific CTL responses and immunity than DC with phagocytosis of apoptotic tumor cells in late phase of apoptosis.

Overall, we have examined variations in designing DC-based cancer vaccines in two completely different model systems. Taken together, our results may have an important impact in designing DC-based antitumor vaccines.
First, I would like to give my sincere thanks to Dr. Jim Xiang, who without his support and mentorship, the work presented in this thesis would not have happened. I am appreciative of his knowledge and for allowing me to work independently. I am also grateful to him for providing me with various opportunities to pursue a dynamic and fascinating area of tumor immunology as well as explore opportunities outside of the lab. He has provided me with a solid foundation as I start laying down the building blocks of my research career. I will be forever thankful.

I would also like to thank the members of my Advisory Committee, Drs. Krahn, Sami, Saxena, Sharma, Professor Qureshi and the Late Dr. Massey for their continuous support and feedback throughout the progress of this project. A special thank you goes out to Dr. Bathe who kindly agreed to be the external examiner.

I am also thankful to all the current and past members of Dr. Xiang’s lab and of the Research Unit, who have made the research environment enjoyable, a great place to work and scientifically stimulating. A special thanks goes out to Sheena Sas, a graduate student in the lab, who was always willing to assist whenever I needed it.

Another thank you goes to the financial support obtained from the College of Medicine Graduate Student Scholarship, Arthur Smyth Memorial Scholarship and John Larson Cancer Research Trust Fund, University of Saskatchewan.

A countless and sincere thanks goes out to my family and friends for their unconditional love, continuous support and encouragements as I continued with my education in pursuit of this degree.
DEDICATION

To my dad,

The Late Kam C. Chan,

who instilled the virtues and values of hard work, perseverance and the pearl of an education. With his love and encouragement, I have always been able to achieve my dreams. I will never forget the challenges he faced and the opportunities he has provided for me.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>AdV (Ad)</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAR</td>
<td>coxsackie-adenovirus receptor</td>
</tr>
<tr>
<td>CDC</td>
<td>complement dependent cytotoxicity</td>
</tr>
<tr>
<td>CDR</td>
<td>complimentary determining regions</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluoroscein succinimidyl ester</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell mediated immunity</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effects</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine guanine dinucleotide pairs</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>Cy</td>
<td>cyclophosphamide</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>dATP</td>
<td>2’deoxyadenosine 5’-triphosphate</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>dCTP</td>
<td>2’deoxycytosine 5’-phosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>2’ deoxyguanine 5’-phosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
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<td>dithiothreitol</td>
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<tr>
<td>dTTP</td>
<td>2’deoxythymidine 5’-phosphate</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetracetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EMEM</td>
<td>Minimal Essential Medium with Earle’s Salts</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas Ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>Flt-3L</td>
<td>fms-like tyrosine ligand 3</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HAd</td>
<td>human adenovirus</td>
</tr>
<tr>
<td>HDI</td>
<td>HER dimerization inhibitors</td>
</tr>
<tr>
<td>HER</td>
<td>Human Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HSP70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>i.d.</td>
<td>intradermal</td>
</tr>
<tr>
<td>i.l.</td>
<td>intralymphnode</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.t.</td>
<td>intratumoral</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>ICAM</td>
<td>intracellular adhesion molecule</td>
</tr>
<tr>
<td>ICD</td>
<td>intracellular domain</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin 10</td>
</tr>
<tr>
<td>IL-12</td>
<td>interleukin 12</td>
</tr>
<tr>
<td>IL-18</td>
<td>interleukin 18</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin 2</td>
</tr>
<tr>
<td>IL-4</td>
<td>interleukin 4</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td>ITR</td>
<td>inverted terminal repeats</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo-dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Lauria-Bertani</td>
</tr>
<tr>
<td>LCM</td>
<td>Lymphocytic choriomenigitis</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MLR</td>
<td>mixed lymphocyte reaction</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney murine leukemia virus</td>
</tr>
<tr>
<td>MMTV</td>
<td>mouse mammary tumor virus</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MUC</td>
<td>mucin</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>ori</td>
<td>origin of replication</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>pMHC</td>
<td>peptide-MHC complex (SIINFEKL-MHC class I complex)</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T cell Expressed and Secreted</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-guanine-aspartate</td>
</tr>
<tr>
<td>RPA</td>
<td>RNase Protection Assay</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor associated antigen</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate ETDA</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific broth</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate ETDA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N-tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>Tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper type I</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper type II</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>Tr</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TSA</td>
<td>Tumor specific antigen</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine-5’-triphosphate</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
</tbody>
</table>
1.0 REVIEW OF THE LITERATURE

1.1. Introduction

Traditional forms of cancer treatments includes the use of surgical resection, chemotherapy and radiation therapy, with the latter two focusing directly on the ability to inhibit DNA replication and cell division (Atkins and Gershell 2002). Generally, these conventional forms of treatment do not specifically target the tumor itself directly. However, with the increasing knowledge in the fields of immunology and molecular biology and its applications, the area of tumor immunology, more specifically cancer immunotherapy, has emerged and newer forms of cancer treatments are being studied to address the issue of tumor specificity (Tuting et al. 1997b; Davis et al. 2003). The use of cancer vaccines allows one to target specific defined antigens expressed on tumor cells and to induce antitumor immune response. These developments in tumor immunology have stemmed from our increased understanding of how the immune response works and the identification of tumor Ag.

1.2. The antitumor immune response

The immune system is comprised of various players on a team, divided into both the innate and the adaptive arms of immunity. The innate immune response is non-specific and is involved in surveying the local milieu or microenvironment to provide
“danger” signals to work in collaboration with the adaptive immune response. The generation of effective Ag-specific adaptive immune responses requires presentation of the Ag, the selection and activation of T cells that differentiate to provide various effector mechanisms to eliminate tumor cells. It is also important to note that tumors have also evolved various active and passive immune escape mechanisms to evade the immune system (Ahmad et al. 2004; Campoli et al. 2005). An overview of the adaptive arm of the immune response is described in the following section.

1.2.1. Antigen presenting cells

There are three types of antigen presenting cells (APC) involved with the uptake of Ag and presentation of epitope peptides to naïve T cells for activation. Each of them is involved in generating different types of immune responses. Dendritic cells (DC) are one of the most powerful professional APC in the body. DC are characterized by their efficient uptake of Ag, constitutive expression of major histocompatibility complex (MHC) class II molecules and costimulatory molecules, such as B7-1 (CD80), and play a vital role in immune responses (Banchereau and Steinman 1998). B cells also constitutively express MHC class II molecules but only express costimulatory molecules following activation. On the other hand, macrophages only express MHC class II and costimulatory molecules upon activation. These three types of APC differentially express MHC class II molecules and costimulatory molecules and they are involved in different aspects of generating an immune response (Abbas et al. 2000).
1.2.2. Antigen presentation

Two main pathways for Ag presentation have been categorized for the loading of Ag peptides on MHC molecules to activate specific subsets of T cells. These pathways are (i) the endogenous pathway and (ii) the exogenous pathway for peptide presentation on MHC class I and II molecules, respectively.

1.2.2.1. Endogenous pathway

In the endogenous pathway, intracellular Ag and proteins are transported from the cytoplasm into the endoplasmic reticulum (ER) by first passing through the proteasome for proteolytic cleavage. Proteins are targeted to the proteasome by ubiquitination then unfolded and cleaved into peptides 8-12 amino acids (aa) in length. These peptides are transported into the ER with the aid of a membrane protein called transporter associated with Ag processing (TAP). The peptides in the ER are loaded into the binding grooves of MHC class I molecules, consisting of the class I α chains and β2 microglobulin, and the interaction stabilized. MHC class I-peptide complexes are transported from the ER to the cell surface where they interact with the T cell receptor (TCR) of CD8+ T cells (Germain 1995; Abbas et al. 2000). The expression of MHC class I molecules is not restricted, as all nucleated cells express it on their cell surfaces.

1.2.2.2. Exogenous pathway

The exogenous pathway is involved with the uptake of captured Ag from the extracellular environment and internalization through endocytosis or phagocytosis. The proteins are enzymatically degraded in the formed endosomes and lysosomes to generate peptides of 10 to 30 aa in length. These generated peptides then interact with the newly
synthesized MHC class II molecules, consisting of α and β chains, to form a stable MHC class II-peptide complex. The stabilized complex is then transported to the cell surface for expression and potential interaction with the TCR on CD4⁺ T cells (Germain 1995; Watts 2004). Expression of MHC class II molecules is limited exclusively to cells with Ag presentation capabilities such as DCs. It is also important to note that the fate of Ag presentation of exogenous Ag is not limited to MHC class II molecules but also has the ability to be cross-presented onto MHC class I molecules (Heath and Carbone 2001; Ackerman and Cresswell 2004). This is done through a process called cross-priming or cross-presentation, first described by Bevan (Bevan 1976), and can stimulate CD8⁺ T cells to mount an immune response. Some of the mechanisms involved with cross-priming include the regurgitation of peptides and loading onto MHC class I molecules via Ag in the phagolysosomes acquiring access to the cytosol through the use of heat shock proteins (HSPs), direct entry through macropinocytosis and high concentrations of the Ag (Heath and Carbone 2001; Ackerman and Cresswell 2004).

1.2.3. Generating the immune response

1.2.3.1. Cell activation

The activation of naïve T cells requires the presence of the necessary signals to ensure the appropriate immune response is generated, either a cellular or humoral response. The cellular response is characterized by the generation of CD8⁺ cytotoxic T lymphocytes (CTL) that are able to recognize and lyse tumor cells while the humoral response involves antibodies (Ab) generated from B cells. The activation of T cells requires a coordinated effort provided by two signals (Bretscher and Cohn 1970). The Ag-specific interaction between the TCR of naïve T cells with the peptide-MHC
molecule complex on APC consists of the first signal. The second signal stems from costimulatory molecules expressed on APC, such as B7-1 (CD80), B7-2 (CD86) and other costimulatory family members, and interacting with its cognate receptor on T cells, such as CD28 for B7-1 and B7-2. For both T or B cells that only interact with the peptide-MHC molecule, but fail to receive a costimulatory signal, the cells are not activated and become tolerant or anergic. Upon receiving the proper signals, the activated cells rapidly proliferate and are able to begin their effector functions. In addition, the activation of T helper (Th) cells is central to the development of an immune response by activating Ag-specific effector cells and the recruitment of cells in the innate immune response (Hung et al. 1998).

1.2.3.2. Cell mediated immunity

Cell mediated immunity (CMI), or cellular response, involves the generation of CTLs that effectively and specifically lyse their target cells. CMI is generally regarded as the most favorable effective antitumor immune response. The CTL response can be generated through a T helper (Th) cell-dependent and Th cell-independent process. The Th cell-dependent process requires the presence of CD4$^+$ Th1 cells, upon receiving the appropriate signals, for the interactions between the APC and both CD4$^+$ T-helper 1 (Th1) and CD8$^+$ T cells. This is important because tumor cells generally do not express MHC class II; however, this is not absolute as a significant proportion of melanoma cells in tumors have been shown to express one or more MHC class II genes (Altomonte et al. 2003). Activated Th1 cells also express CD40 ligand (CD40L) that interacts with CD40 expressed on APCs, including DCs, to further enhance their effector functions by up-regulating the expression of pro-inflammatory cytokines and important cell surface
molecules such as MHC and B7-1 molecules. Th1 cells are also able to produce interleukin-2 (IL-2) and interferon-γ (IFN-γ) to provide support for clonal expansion and differentiation of CD8⁺ T cells, either directly or indirectly (Knutson and Disis 2005). Generation of CD8⁺ T cell response via Th1 cell-independent methods can also be induced, as seen in virus-infected cells, whereby the APC directly stimulates the CD8⁺ T cell and bypasses the need for coordinated help from Th1 CD4⁺ T cells.

Once the activated T cells have proliferated and differentiated into effector T cells, CD8⁺ CTL cells are able to recognize cells displaying a specific epitope on MHC class I molecules and mediate cytolysis through the release of cytotoxic granule proteins, mainly perforin and granzymes. Another mechanism of cytolysis involves the interaction of Fas ligand (FasL), expressed on CTL, interacting with the target protein Fas to result in apoptosis of the target cell. Previously, the effector functions for Th1 cells were once thought to only provide the necessary help required in activating CTLs; however, Th1 cells are also able to recognize target cells. This results in direct cytolysis through FasL-Fas pathways, TNF-related apoptosis-inducing ligand (TRAIL) and the release of cytokines inducing cytotoxicity (Knutson and Disis 2005).

### 1.2.3.3. Humoral immunity

B cell activation resulting in the production of Ab is characteristic of humoral immunity that specifically recognizes the Ag. B cells have the ability to process Ag and present short-length peptides bound to MHC class II molecules. The CD4⁺ Th2 cells recognize the peptide-MHC class II complex on B cells and the cells become activated with costimulation. Activated Th2 cells express CD40L that is able to interact with constitutively expressed CD40 on B cells in germinal centers. Th2 cells also secrete
characteristic cytokines such as IL-4 and IL-10, and determine the immunoglobulin (Ig) subclass produced. The IgG1 subtype is normally associated with the humoral response, whereas the presence of IFN-γ induces class switching of the Ab to IgG2a. The IgG2a subtype is commonly associated with CMI response. B cells proliferate and undergo somatic mutation of the Ig genes, to end up with B cells producing Ab with the highest affinity for the Ag. Abs generated would be Ag specific and mediate their effects through a variety of effector mechanisms such as complement dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC involves macrophages and natural killer (NK) cells mediating the cell cytotoxicity effects through the recognition of a receptor on the constant Fc portion of the Ig.

1.2.4. Tolerance

T cells play a crucial role in the development of immune responses and initially undergo a maturation process in the thymus. The T cells first go through an intrathymic selection method to remove non-self restricted thymocytes and high affinity double-positive self-reactive thymocytes by deletion then undergo a negative selection method to remove high avidity binding self-reactive single-positive T cells prior to leaving the thymus (De Visser et al. 2003; Hogquist et al. 2005). This selection method provides the mechanism for central tolerance since high affinity T cells are removed by clonal deletion (Hogquist et al. 2005). However, self-reactive mature T cells may still exist in the periphery, as some self-Ags are not expressed in the thymus but the T cells become tolerant by peripheral tolerance mechanisms such as clonal elimination, ignorance or activation induced cell death. Remaining T cells may also be rendered unresponsive or
anergic due to the failure to provide the T cells with the proper costimulatory activation (Goodnow 1996).

Another component of tolerance arises from immunoregulation of T cells through suppression from a specialized subset of CD4$^+$ T cells, known as regulatory T cells (Tr). Tr cells play an important role in autoimmunity and tumor immunity (Sakaguchi et al. 1995; Shimizu et al. 1999; Sakaguchi et al. 2001). Tr represent only 5-10% of CD4$^+$ T cells in healthy mice and adults. These cells are identified based upon their expression of CD25 (α chain of IL-2 receptor), high expression levels of CTL associated protein-4 (CTLA-4), glucocorticoid-induced TNF-related receptor (GITR) and the forkhead transcription factor Foxp3 (O'Garra and Vieira 2004; Wei et al. 2004). Natural self-Ag-reactive Tr cells develop in the thymus and then enter into peripheral tissues, where they function to suppress the activation of other self-reactive T cells in a non-Ag-specific manner (Bluestone and Abbas 2003; Cozzo et al. 2003). The presence of CD4$^+$CD25$^+$ Tr cells has been investigated in cancers, with elevated numbers of CD4$^+$CD25$^+$ Tr cells detected in cancer patients compared to healthy individuals (Liyanage et al. 2002; Curiel et al. 2004). These CD4$^+$CD25$^+$ Tr cells were tumor Ag-specific and able to mediate immune suppression of antitumor immunity in both an Ag-specific and cell-dependent fashion (O'Garra and Vieira 2004; Wang et al. 2004a). There are also other suppressor T cells such as IL-10 and transforming growth factor β (TGF-β) CD4$^+$ subsets of Tr cell, referred to as T regulatory 1 (Tr1) and Th3 cells, respectively.
1.3. Tumor Ag

The ideal tumor Ag would be specifically expressed only on tumor cells while completely absent in normal cells and deemed critical for cancer cell survival. This is not the case, as early classification schemes grouped tumor Ags into two categories: (i) tumor specific Ags (TSA) and (ii) tumor associated Ags (TAA). The definition of TSAs was used to identify Ags that were uniquely and solely expressed in tumor cells. On the other hand, TAA was defined as being expressed in normal cells but aberrant or dysregulated expression observed in tumors. This suggested that TAAs were of lower immunogenicity and patients with tumors were already tolerized to the Ag. For the tumor Ags identified, very few TSA have been identified with a fair majority of the tumor Ag being TAA identified.

With newer methods available for molecularly identifying and characterizing tumor Ags, modifications to the classification scheme have been made over time; taking into consideration that majority of the tumor Ags were TAAs. The new classification scheme was devised based upon the function or the expression pattern of the Ag and classified into five different categories as follows: (Tuting et al. 1997b; Abbas et al. 2000; Renkvist et al. 2001; Davis et al. 2003; Novellino et al. 2005)

(i) Differentiation Ag, which includes the well-studied melanocyte Ags tyrosinase, glycoprotein-100 (gp100), Melan-A/melanoma antigen recognized by T cell (MART-1) Ag, prostate specific Ag (PSA), prostate-specific membrane Ag.

(ii) Cancer-testis Ag or oncofetal Ag, where the TAA expression is limited in normal tissues to gametes and trophoblasts but becomes
aberrantly expressed in a wide variety of cancers. Some examples include melanoma antigen-1 (MAGE-1), MAGE-3, carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP).

(iii) Overexpressed oncogenes or mutated tumor suppressor genes such as HER-2/neu and p21ras, bcr/abl rearrangement, p53

(iv) Oncogenic viral Ags commonly associated with cancer formation such as the E6 and E7 Ags of human papilloma virus serotypes 16 and 18 (HPV 16/18) in cervical cancers, Epstein-Barr Virus (EBV) in Burkitt lymphomas, hepatitis B and C

(v) Unique Ags that are products of mutations or rearrangements expressed from an individual patient such as a clonal idiotype Ab from B cell malignancy.

1.4. HER-2/neu

1.4.1. Gene identification

In the early 1980s, the rat neu gene was first identified in N-ethyl-N-nitrourea chemically induced neuroblastomas and glioblastomas in rats and seemed to be involved with the transforming capabilities into tumorigenic cells (Padhy et al. 1982; Drebin et al. 1984; Schechter et al. 1984). The resulting gene expressed a transmembrane protein with a relative molecular mass of 185,000 (p185) and subsequently termed neu. The human homologue was identified based upon its sequence similarity with the avian erythroblastosis viral gene, v-erb-B, and named as c-erb-B2 (King et al. 1985; Semba et
Meanwhile, epidermal growth factor receptors (EGFR) were being studied and a gene that localized to the same region of the chromosome similar to neu, but was completely different from EGFR or human EGFR-1 (HER-1), was given the name HER-2 (Coussens et al. 1985). The human gene was located on the long arm of chromosome 17 at q21 (Coussens et al. 1985). It was later confirmed by sequence analysis that the rat neu gene identified was exactly identical to the HER-2 gene (Yamamoto et al. 1986), resulting in the name HER-2/neu.

To provide some clarity throughout this dissertation, neu directly refers to the rat gene and its expressed protein whereas HER-2 refers to the human gene/protein. When referring to the gene and its gene product in general terms, HER-2/neu will be used.

The human HER-2 gene encodes for a 185 kilo-Dalton (kDa) protein that consists of 1255 aa. There are 653 aa that encodes for the extracellular domain (ECD) containing 4 subdomains including two cysteine rich domains and the signal sequence, 22 aa for the transmembrane domain (TM) and the remaining 580 aa encodes for the intracellular domain (ICD) containing the conserved tyrosine kinase domain. In a similar fashion, neu consists of a total 1260 aa, of which the first 657 aa from the amino end is the ECD, followed by the 22 aa TM and the remainder encoding for the ICD. Figure 1.1 shows a protein sequence alignment between the aa sequences of HER-2 and neu; notice there is a high degree of homology (~89%) observed between these two sequences.

1.5. Other Human Epidermal Growth Factor Receptor family members

There are currently four members of the human epidermal growth factor receptor (HER) family, including HER-2/neu, identified and functions as receptor tyrosine
Figure 1.1 Amino acid sequence alignment between human HER-2 (1255 amino acid) and rat neu (1260 amino acid) protein. Identical matching amino acids are indicated with an asterisk (*). A ClustalW alignment algorithm was performed.
kinases (RTKs), as described below. The main general characteristics for each of these members include an extracellular ligand-binding domain and two cysteine rich domains with a short hydrophobic transmembrane region. This is followed with an intracellular domain containing a highly conserved tyrosine kinase domain and a cytoplasmic tail containing specific binding motifs for src-homology domain (SH2) signaling proteins (Albanell and Baselga 1999; Slichenmyer and Fry 2001).

1.5.1. EGFR/HER-1

HER-1, also commonly referred to as epidermal growth factor receptor (EGFR) or c-erbB1, was the first RTK to be identified as the cellular homologue of the retroviral avian erythroblastosis tumor gene, v-erbB (Cohen and Taylor 1974; Downward et al. 1984). The known ligands that binds to this 170 kDa protein include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin and epiregulin (Rubin and Yarden 2001). HER-1 is one of the classical receptors studied in cancers and is commonly over-expressed in a wide variety of human tumors (Kim et al. 2001; Harari 2004).

1.5.2. HER-3

HER-3, also referred to as c-erbB3, is a unique member of the HER family as it is the only HER family member lacking tyrosine kinase activity (Guy et al. 1994). Known ligands that binds to the 160 kDa HER-3 receptor includes heregulins and neudifferentiation factors, which are also known as neuregulins (Rubin and Yarden 2001). Since HER-3 is unable to be involved directly in cell signaling, the ligand bound HER-3 forms heterodimers with its preferred partner, HER-2 (Kim et al. 1998).
1.5.3. HER-4

HER-4, also referred to as c-erbB4, is a 180 kDa protein (Plowman et al. 1993) that has similar ligand binding affinity with HER-3 but possesses RTK activity. Interestingly, this gene undergoes alternative splicing resulting in four different isoforms present (Junttila et al. 2000). Overexpression of HER-4 has also been observed in various tumors.

1.6. Functional properties of HER-2/neu

1.6.1. Normal functions of HER-2/neu

Cell signaling with the HER family RTK requires the formation of homodimers and heterodimers upon ligand binding. There are ten different dimer combination possibilities within the four family members, in which HER-2/neu is associated in three heterodimers (HER-1/HER-2, HER-2/HER-3 and HER-2/HER-4) and its homodimer. HER-2 is considered an orphan receptor in respect to that there are no known ligands that binds to it; however, it has the capability of inducing ligand-dependent and ligand-independent dimerizations (Brennan et al. 2000). The formation of heterodimers or even the cleavage of the ECD results in cell signaling (Egeblad et al. 2001). The formation of heterodimers results in more potent signal transduction compared to homodimers, in particular when HER-2 is a part of the heterodimer (Graus-Porta et al. 1997; Rubin and Yarden 2001). The most common partner for HER-2 is HER-3, which has a high binding affinity site for the ligand but lacks kinase activity.
After receptor dimerization, certain tyrosine residues located in the kinase domain are autophosphorylated. This allows for recruitment of signal transducers and activators of intracellular substrates that contains binding motifs such as the Src homology region 2 (Shc2) and phosphotyrosine binding domain (PTB) to interact with phosphatidylinositol-3-kinase (PI3K), Src, ras-GAP, phospholipase C-γ (PLCγ), Shc, Grb-2, Grb-7, Crk, c-Cbl, c-Abl and Shp-2 (Prenzel et al. 2001; Baxevanis et al. 2004). This in turn allows for the activation of the mitogen-activated protein kinases (MAPK) and c-Jun N-terminal kinases (JUNK) and Akt pathway (Tzahar and Yarden 1998; Yarden and Sliwkowski 2001) for cell proliferation. Receptor endocytosis occurs at a drastically delayed rate for HER-2 hetero/homodimers in comparison to the fast rate of endocytosis commonly seen with EGFR (HER-1) endocytosis, resulting in important biological consequences as a result of the prolonged signaling (Karunagaran et al. 1996; Prenzel et al. 2001; Baxevanis et al. 2004). The formation of heterodimers also forms a hierarchical network to allow for various ligands to bind and cross-talk for intracellular signaling to initiate a series of events involved with gene transcription, cell growth, differentiation, survival and migration (Yu and Hung 2000). Under normal conditions, with minimal formation of HER-2 heterodimers and subsequent weak signaling activation, cells grow normally (Baxevanis et al. 2004). Figure 1.2 shows the signaling network created by the HER hetero/homodimers formed.

1.6.2. HER-2/neu involvement in development

HER family proteins are expressed throughout development and at low levels in adult epithelial tissues. These proteins play an important role in cell growth and differentiation. HER-2 is the most widely expressed receptor with expression detected
Figure 1.2  HER Family members and its signaling network. (a) The input layer is comprised of the various ligands and the ten dimeric receptor combinations (b) For clarity, the signaling involved with the signal-processing layer is shown only for two receptor dimers: ErbB1 homodimer and ErbB2–ErbB3 heterodimer. Only some of the pathways and transcription factors are represented in this layer. (c) The effects on cell apoptosis, migration, growth, adhesion and differentiation are seen in the output layer. (Adapted from Yarden and Sliwkowski 2001).
in the gastrointestinal tract, lung, breast, pancreas, ovary, skin, central nervous system and genitourinary tracts of fetal and adult tissues (Press et al. 1990; Olayioye et al. 2000). The absolute importance of the HER-2/neu gene has been demonstrated in HER-2/neu knockout mice. The absence of HER-2/neu during development causes embryonic lethality by E11 (embryonic day 11) due to trabeculae malformation resulting in severe heart malformation and nervous system deficiencies (Lee et al. 1995; Morris et al. 1999).

1.6.3. HER-2/neu involvement in malignancies

In a normal cell, there are two copies of the HER-2 gene (one on each chromosome) with as many as 20,000-50,000 receptors expressed on the cell surface of normal cells. However, gene amplification/overexpression of HER-2 increases the number up to 2 million receptors (Pegram et al. 2000; Lohrisch and Piccart 2001) that may contribute to disease initiation and progression of cancer (Yarden 2001). The overexpression of HER-2 results in increased cell proliferation, anchorage-independent cell growth, tumorigenicity and metastatic potential compared to cells expressing basal levels of HER-2 (Pegram et al. 2000).

In clinical cases, gene amplification and overexpression of HER-2 is seen in a variety of cancers such as carcinomas of the bladder (Sato et al. 1992; Sauter et al. 1993; Tetu et al. 1996), pancreas (Yamanaka et al. 1993; Lei et al. 1995), non-small-cell lung cancer (Kern et al. 1990; Weiner et al. 1990; Shi et al. 1992), ovary (Slamon et al. 1989; Meden and Kuhn 1997), endometrium (Saffari et al. 1995; Rolitsky et al. 1999), colon (D'Emilia et al. 1989), renal (Herrera 1991), stomach (Jaehne et al. 1992; Kono et al. 2002a), prostate (Arai et al. 1997; Mark et al. 1999) and breast cancer (Slamon et al.
1987; Slamon et al. 1989; Ross and Fletcher 1999). The commonly used methods to detect gene amplifications and protein overexpressions are fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC), respectively.

1.7. Role of HER-2/neu in breast cancer

The overexpression of HER-2/neu has been implicated in the transformation of breast cells resulting in a malignant phenotype. Gene amplification or overexpression is commonly seen in 30% of invasive breast cancers, 50% of inflammatory breast cancer and 60%-70% of ductal carcinoma in situ (DCIS) (Slamon et al. 1987; Slamon et al. 1989; Ross and Fletcher 1999). The main mechanism for increased HER-2 expression in more than 90% of these cases is gene amplification, whereby a cell may have as many as 50 to 100 gene copies found in transformed cells (Pauletti et al. 1996; Pegram et al. 2000). Transcriptional and post-transcriptional dysregulation account for the remainder of the receptor overexpression (Slamon et al. 1989).

The transforming capabilities were first suggested when the neu gene was transfected into mouse fibroblasts 3T3 cells, resulting in cellular transformation and tumorigenesis (Padhy et al. 1982; Drebin et al. 1986; Bargmann et al. 1986a). Similar results were seen when HER-2 was transfected into mouse 3T3 cells (Hudziak et al. 1987). As well, the presence of anti-neu Abs resulted in inhibition of cell growth and seemed to reverse the transformed phenotype observed (Drebin et al. 1986).

Further sequence analysis revealed the presence of a mutation in neu whereby the valine (Val; V) found at aa residue 664 is replaced with a glutamic acid (Glu; E), V664E, (Bargmann et al. 1986b). This mutation occurred within the transmembrane domain and caused constitutive homodimerization in a ligand independent fashion
maintaining a permanently active signaling complex (Weiner et al. 1989a; Weiner et al. 1989b). Other somatic mutations in neu have been observed including deletions, insertions, point mutations and splice variants within the ECD that induces mammary tumorigenesis in neu-transgenic (Tg) mice (Siegel and Muller 1996; Siegel et al. 1999). In contrast to HER-2, gene amplification/overexpression is not observed as one of the main mechanisms of neu leading to a tumorigenic phenotype.

Meanwhile, no mutations to date have been identified in HER-2 that are identical to neu that results in transformation (Lemoine et al. 1990; Zoll et al. 1992). However, a single nucleotide polymorphism at codon 655 involving an adenine-to-guanine substitution, converting the codon from isoleucine (Ile; I) to valine (Val; V), within the TM region of HER-2, has been identified (Papewalis et al. 1991). This mutation has been linked to an increased risk of breast cancer (Xie et al. 2000; Millikan et al. 2005). Younger women with the Ile/Val or Val/Val genotype are associated with an increased risk; however, this remains controversial as numerous other studies have found no additional risk associated for individuals in developing breast cancers with the same polymorphism (Hishida et al. 2002; Nelson et al. 2005).

The role of HER-2/neu involvement in breast cancer has also been demonstrated in transgenic (Tg) mouse animal models. The expression of both wildtype and activating forms of neu, under the control of the mouse mammary tumor virus (MMTV) promoter long terminal repeat (LTR), in Tg mice leads to the formation of mammary tumors (Muller et al. 1988; Bouchard et al. 1989; Suda et al. 1990; Guy et al. 1992; Lucchini et al. 1992; Guy et al. 1996). Interestingly, Tg mice containing the activated forms of neu developed mammary tumors at an accelerated rate while wildtype neu Tg mice developed tumors in a stochastic fashion and lung metastasis after a long latency.
period (Guy et al. 1992; Guy et al. 1996). One of these mice, FVB/N-TgN(MMTVneu)202Mul Tg mice, herein referred to as FVBneuN Tg mice, develop spontaneous breast tumors after 6 months of age (Guy et al. 1992). The development of mammary tumors in these mice share similar morphological features with human breast adenocarcinomas as well as the progression from atypical hyperplasia to in situ carcinoma and then to invasive carcinoma (Munn et al. 1995; Cardiff and Wellings 1999; Nanni et al. 2003). Another Tg strain developed is the BALB-neuT mice, which carry the activated form of neu. This animal model is a more aggressive model of mammary carcinogenesis with neu overexpression beginning at 3 weeks of age, atypical hyperplasia by 6 weeks that leads to invasive, metastasizing tumors in all ten mammary glands by 25 weeks of age (Boggio et al. 1998; Di Carlo et al. 1999; Cifaldi et al. 2001). A variety of other Tg mouse models have been created using the endogenous neu promoter (Weinstein et al. 2000; Andrechek et al. 2003) and HER-2 under the control of the whey acidic protein (Piechocki et al. 2003).

1.8. Clinical significance in breast cancer

1.8.1. Prognostic factor

Breast cancer is one of the most common cancers affecting women. An estimated 1 in 9 women will develop breast cancer and 21,800 female Canadians will be diagnosed with the disease this year (Canadian Cancer Society, 2005). HER-2 overexpression is commonly used as both a prognostic and predictive factor in breast cancer. Prognostic factors are associated with the potential growth of the primary tumor and metastases while predictive factors are associated with the relative sensitivity to specific therapies
Slamon’s group initially correlated overexpression of HER-2 in patients with shortened relapse-free interval and reduced survival compared to patients with normal HER-2 expression (Slamon et al. 1987) and has been extensively examined by various groups as reviewed by Ross and Fletcher (Ross and Fletcher 1999). It is now commonly accepted that patients with node-positive and HER-2 overexpressing tumor have an overall worse prognosis (Kaptain et al. 2001). As well, HER-2 expression in tumors has been associated with the absence of estrogen receptor and progesterone receptor staining, DNA aneuploidy, high proliferation rates, high nuclear grade and invasive disease (Ross and Fletcher 1999; Ross et al. 2003).

1.8.2. Predictive factor

The use of HER-2 as a predictive factor would be beneficial in predicting the potential therapeutic response in treating breast cancers. A common association is the resistance of HER-2 overexpressing tumor to anti-hormone therapy treatment with tumors undergoing ER-independent tumor growth and resisting tamoxifen therapy (Ross and Fletcher 1999; Lohrisch and Piccart 2001). The predictive response rates for chemotherapy treatment have been contradictory as the majority of reports are based upon retrospective studies and involve small sample sizes (Lohrisch and Piccart 2001; Ross et al. 2004).

1.9. HER-2/neu as an immunotherapy target

HER-2/neu serves as an attractive tumor marker, because of its amplified/overexpressed pattern seen in a wide variety of human malignancies. It is also an ideal therapeutic target in the case of breast cancer because (i) its functional
important in breast cancer growth, (ii) its high levels expression in breast tumors but low levels of expression in normal tissue, (iii) HER-2/neu is a transmembrane protein and amenable to both humoral and cellular immune effector mechanisms responses (Ross et al. 2003). Immunological intervention using monoclonal Ab (mAb) inhibits neoplastic changes and shows tumor selectivity with human breast tumor cells expressing the highest levels of HER-2 obtaining the highest levels of cell growth inhibition (Hudziak et al. 1989).

As well, the presence of pre-existing T and B-cell immune responses (both cellular and Ab) against HER-2 has been observed in breast cancer patients as reported by Disis’ group (Disis et al. 1994; Disis et al. 1997; Disis et al. 2000). Despite the generation of an immune response against a self-protein, patients expressing natural Ab and T-cell immunity towards HER-2 do not seem to develop classical signs or indications of autoimmunity. However, these generated immune responses are often not strong enough to counteract the tumors as the tumors continue to grow. Despite this, the existence of pre-existing immune responses suggested tolerance towards HER-2/neu is circumventable. Also, HER-2 specific T-cells have been identified in patients with HER-2 overexpressing tumors, which indicated a portion of the T cell repertoire is able to recognize a self Ag (Fisk et al. 1995; Peoples et al. 1995). All of these factors combined make HER-2/neu suitable for immunotherapeutic targeting with passive or active methods of immune vaccination.
1.10. Passive immunotherapeutic strategies targeting HER-2/neu

1.10.1. Monoclonal antibodies

1.10.1.1. Herceptin

Herceptin (trastuzumab) is a recombinant humanized mAb developed by Genentech Inc. (San Francisco, CA) that was approved by the Federal Drug Administration (FDA) of the United States in 1998 as a single agent or in combination with chemotherapy for the treatment of metastatic breast cancers. The Ab directly targets the ECD between aa 529 and 627 in a cysteine rich domain of HER-2/neu (Frankel 2002). The Ab was humanized through a series of modifications by taking the complimentary determining regions (CDR) of the mouse mAb, 4D5 (Hudziak et al. 1989) and placing it into the framework of the human IgG\textsubscript{1} and various aa substitutions resulting in a chimeric antibody that contains 95% human sequences with only 5% murine sequences remaining (Carter et al. 1992). The resulting Ab also has a higher binding affinity to HER-2 ($K_{d}$=0.1 nM) compared to the original mouse mAb, 4D5 ($K_{d}$=0.3 nM). It also maintains similar \textit{in vitro} properties to the parent antibody such as HER-2 specific suppression of HER-2 positive tumor cell growth and prevents the growth of human breast tumor xenografts \textit{in vivo} (Harries and Smith 2002).

The exact mechanisms by which Herceptin acts upon HER-2 positive cells are unknown, but various mechanisms have been proposed supported by \textit{in vitro} studies. Herceptin may induce HER-2 receptor downregulation whereby the internalized receptor inhibits signal transduction due to decreased formation of homo- and hetero-dimers and antagonizes cell division and growth. Other mechanisms of actions may include reduced
production of vascular endothelial growth factor (VEGF), which is required in angiogenesis, preventing the cleavage of HER-2 ECD and involving the host immune response via induction of ADCC and CDC (Sliwkowski et al. 1999; Baselga and Albanell 2001; Adams and Weiner 2005). As well, improved cell lysis from HER-2 specific CTL clones were observed when target cells were treated with Herceptin (zum Buschenfelde et al. 2002). Preclinical studies using combinations of Herceptin with chemotherapy drugs such as paclitaxel, doxorubicin and cisplatin, which are common chemotherapeutic drugs used for breast cancer treatment, has shown synergistic effects compared to modest effects when the chemotherapeutic agents were used alone (Colomer et al. 2001; Harries and Smith 2002).

Herceptin has a favorable pharmacological profile with minimal toxicity; however, cardiotoxicity has been observed when combined with anthracycline chemotherapy (Harries and Smith 2002). One of the pivotal studies showing the effectiveness of Herceptin in a monotherapy treatment regimen was in a phase II clinical trial involving 222 women previously treated with chemotherapy. The overall response rate (including both complete responses rate and partial response rates) was 15% with a median response duration of 9.1 months (Cobleigh et al. 1999). In another Phase II study by Vogel and colleagues, patients were given Herceptin at an earlier stage and the response rate (including complete and partial response rates) was 26% with a median duration of 18.8 months (Vogel et al. 2002). Phase II clinical trials from Slamon’s research group incorporated the use of chemotherapy with Herceptin showed dramatic increase in response rate, prolonged duration of responses and lengthened survival in metastatic breast cancer patients (Slamon et al. 2001). Similar clinical trials involving
patients given Herceptin with various chemotherapy drugs has also shown improved response rates and survival (Horton 2002).

The use of Herceptin as a form of adjuvant therapy has recently been evaluated in various international clinical trials, based upon the impressive results seen when Herceptin was used in treating metastatic breast cancers. In 2005, the phase III HERceptin Adjuvant (HERA) study reported women with HER2 positive early-stage invasive breast cancer given one year Herceptin treatment after adjuvant chemotherapy had a significantly improved disease survival (Piccart-Gebhart et al. 2005). Similar results were also reported by the National Surgical Adjuvant Breast and Bowel Project and the North Central Cancer Group Trial N9831, that differed only slightly in trial design compared to the HERA trial (Romond et al. 2005).

Although there is much interest and success in the use of Herceptin in the treatment of breast cancers, there are a few drawbacks. One of them is that it does not completely resolve the cancer as Herceptin acts in a cytostatic fashion, which requires constant infusion of the Ab. This passive mechanism of immunity is also short-lived and the Ab is eventually cleared from the body. As well, a large number of patients still fail to respond to treatment or even if they do respond, patients often will relapse (Foy et al. 2002).

1.10.1.2. Omnitarg (Pertuzumab)

Omnitarg (Pertuzumab) is another humanized mAb recently developed by Genentech Inc. that is HER-2 specific and derived from the mouse mAb, 2C4. This novel mAb, Omnitarg, functions differently from its counterpart, Herceptin, in that its epitope is located in the ECD region that is completely different from that of Herceptin.
and functions by inhibiting the formation of heterodimers between HER-2 and its other family members (Spicer 2004; Adams et al. 2005). Omnitarg is the first member of a newer class of agents referred to as HER dimerization inhibitors (HDIs). HDI gets its name based upon the mechanism of action mediated through inhibition of heterodimerization of HER-2/EGFR and HER-2/HER-3 in both normal and overexpressing cells, which downregulate receptor phosphorylation and intracellular signaling pathways (Agus et al. 2002); however immune mediated responses may also occur. In preclinical animal models, administration of Omnitarg alone resulted in growth inhibition of tumor xenografts while the combination of Omnitarg with chemotherapy showed either additive or synergistic effects (Spicer 2004). The combination of Herceptin with Omnitarg has also shown synergistic inhibition in tumor cell growth in vitro (Nahta et al. 2004). Currently, Omnitarg, is being examined for its effectiveness in several phase II clinical trials in a wide variety of cancers but results from phase I trials indicate it is well tolerated, has a favorable pharmacologic profile and clinical activity (Agus et al. 2005).

1.11. Active immunotherapeutic strategies targeting HER-2/neu

1.11.1. Protein based vaccines

An advantage of using a protein-based vaccine is the ability to display the entire array of peptide epitopes that would be able to stimulate an immune response. However, one important drawback is the inefficient generation of CTL responses, since the proteins taken up by APCs are generally presented through the exogenous pathway onto MHC class II molecules and not onto MHC class I molecules.
Disis and several other groups have shown that immunization with the whole HER-2 protein or the ECD induces tolerance (Bernards et al. 1987; Disis et al. 1996b). However, other groups demonstrated an anti-HER-2 immune response after immunization with recombinant ECD, though it was too weak to protect mice against s.c. tumor challenge (Taylor et al. 1996; Dela Cruz et al. 2003). Interestingly, immunization of with the HER-2 ICD protein induced an immune response that circumvents the tolerance observed in rats (Disis et al. 1998) or in combination with heat shock protein (HSP) complexes (Manjili et al. 2002) and partially protected mice from tumor challenge (Foy et al. 2001). In most of these reports, the proteins were mixed with adjuvants, such as complete Freund’s adjuvant and Montanide 720, to induce stronger immune responses against the protein. In one report, Disis’ group immunized patients with the HER-2 ICD protein with granulocyte/macrophage-colony stimulating factor (GM-CSF) intradermally (i.d.) and found both HER-2 specific-T and B cells were elicited (Disis et al. 2004b), suggesting that the ICD is more immunogenic than the ECD. Several surprising results were also obtained in studies using Tg mice showing induction of anti-neu humoral and cellular immunity, prevention of tumor development in 50% of mice immunized with ECD protein and delayed the formation of spontaneous tumors (Esserman et al. 1999).

1.11.2. Peptide based vaccines

The use of synthetic peptides offers an alternative vaccine strategy to the use of proteins and has several advantages. Peptides are simple, economical to produce, lack infectious and oncogenic potential and can induce a very epitope specific response. However, one major drawback to the use of peptides is the requirement for identification
of epitopes that would be able to bind to MHC class I and class II molecules. Another drawback is haplotype restriction based upon the individual. This process is both tedious and time-consuming, resulting in the lack of epitopes identified for all TAA. Often times, tumors also down-regulate their expression of MHC class molecules as a mechanism to evade the host immune system. As well, direct injections of peptides themselves are considered to be weakly immunogenic (Disis et al. 1996a). There is also the potential that targeting a single epitope would become ineffective when Ag-negative tumors develop.

In the case of HER-2/neu, a variety of different approaches have been utilized to identify class I human leukocyte Ag-A2 (HLA-A2) restricted peptide epitopes (Fisk et al. 1995; Peoples et al. 1995; Kawashima et al. 1998; Kono et al. 1998; Rongcun et al. 1999; Baxevanis et al. 2006), HLA-A24 (Nagata et al. 1997), HLA-A3 (Kawashima et al. 1999), murine H-2K\textsuperscript{d} (Nagata et al. 1997) and H-2K\textsuperscript{q} (Ercolini et al. 2003). Both human and murine MHC class II HER-2/neu epitopes have also been identified that would be able to provide T helper responses (Disis et al. 1999; Kobayashi et al. 2000; Perez et al. 2002; Baxevanis et al. 2006). The identification of these epitopes have allowed groups to employ them for use in various methods such as direct peptide vaccination with GM-CSF as an adjuvant injected intradermally (i.d.) (Disis et al. 1996a; Knutson et al. 2001; Murray et al. 2002) and polysaccharide-peptide conjugates (Gu et al. 1998). Disis et al have also previously shown that although the whole HER-2 protein is not immunogenic itself, immunization of both rats and humans with multiple peptides derived from HER-2/neu elicited strong humoral and T cell responses (Disis et al. 1996b; Disis et al. 1999; Salazar et al. 2003). Although clinical studies using HER-2 peptide vaccine induced HER-2/neu-specific CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and humoral
response in breast cancer patients, it has not resulted in tumor rejection (Zaks and Rosenberg 1998; Disis et al. 1999; Baxevanis et al. 2006). One possibility for this may be due to the short-lived peptide specific immunity derived from HER-2/neu peptide immunization (Knutson et al. 2002). One interesting feature that has been observed in some peptide vaccination regimens is epitope spreading (Lehmann et al. 1992), whereby an immune response, both cellular and humoral, is generated to other regions of the Ag not originally included in the vaccine (Disis et al. 1999; Anderson et al. 2000a; Disis et al. 2002; Disis et al. 2004a).

1.11.3. Cell based vaccines

Tumor cell based vaccines offer the potential for multiple tumor Ag presentation; however, they tend to be less immunogenic as they do not express MHC class II molecules or costimulatory molecules. Another disadvantage of this type of vaccine is the lack of specific tumor Ag targeting and the limited availability of patient samples for an autologous cancer vaccine. The immunogenicity of tumor cells may be further improved by engineering them to express various cytokines or even the use of allogeneic tumor cells (Nawrocki et al. 2001).

HER-2/neu-positive tumor cells have been used as vaccines to induce preventive antitumor immune responses (Cefai et al. 1999; Reilly et al. 2000; Nanni et al. 2001; Reilly et al. 2001). Recently, it has been reported that combinatorial vaccination of mice with tumor cells engineered to secrete GM-CSF combined with the use of cyclophosphamide (Cy) or with an antitumor antibody reduced the development of spontaneous tumors in Tg mice (Machiels et al. 2001; Wolpoe et al. 2003). More recently, it has also been shown that IL-12 engineered allogeneic tumor cell vaccines
greatly enhanced antitumor immune responses (De Giovanni et al. 2004; Nanni et al. 2004).

1.11.4. DNA based vaccines

In the early 1980’s, reports emerged detailing the induction of immune responses against transgene products expressed from DNA plasmids. The first published report using direct intramuscular (i.m.) inoculation of naked plasmid DNA for gene expression in mice appeared in 1990 (Wolff et al. 1990) followed by the first reported application of a DNA vaccine demonstrating cellular immunity and protective efficacy against the influenza nucleoprotein (Ulmer et al. 1993). This sparked a tremendous amount of interest resulting in the use of DNA vaccination in a wide variety of model systems for protective immunity and potential use in cancer immunotherapy.

1.11.4.1. Advantages and disadvantages of using DNA vaccines

DNA vaccines offer several advantages for its use such as DNA is economically feasible to produce and relatively easy to purify in large quantities with high purity. DNA also does not require specialized handling or storage conditions. It has the capability of expressing the Ag in its natural form with the appropriate post-translational modifications in the host while not inducing neutralizing Abs, commonly associated with the use of viral vectors, to allow for repeated boosters. There is also a constant and persistent source of the Ag from the prolonged expression of the Ag from the plasmid vector. DNA vaccines are capable of inducing both CD4⁺ Th and CD8⁺ CTL and humoral immune responses specific for the target Ag. This method allows for unrestricted MHC presentation of a broad range of epitopes on MHC class I and II
molecules. More importantly, DNA sequences can easily be modified to remove or insert special sequences to enhance Ag immunogenicity (Gurunathan et al. 2000; Reyes-Sandoval and Ertl 2001; Haupt et al. 2002; Donnelly et al. 2005).

A disadvantage of using DNA vaccines is the potential risk of the plasmid DNA integrating into the host genome, which may disrupt the functions of proto-oncogenes and tumor suppressor genes. There may also be the ability for the host to develop autoreactive Abs and T cells against DNA that may lead to autoimmunity. Another disadvantage is the potential for the host to become tolerant to the Ag with the persistent source of Ag expression. In addition, the possibility of mutagenic integration and subsequent tumor development with the use of plasmid DNA has not been fully evaluated (Mahon et al. 1998).

1.11.4.2. Design of plasmid vectors

DNA vaccines require the use of a plasmid vector that contains several key elements along with the gene encoding the Ag. Key elements of a plasmid vector include a replication of origin (ori) to allow the plasmid to replicate within cells, a selective marker such as an antibiotic resistance gene, and eukaryotic transcription regulatory elements such as promoters/enhancers located upstream from the gene. Some common promoters utilized in plasmids are the strong cytomegalovirus (CMV) immediate early region and the simian virus 40 (SV40) promoter. Another key element is a method to stabilize the mRNA transcript, which is accomplished with the addition of bovine growth hormone or SV40 polyadenylation sequences (Gurunathan et al. 2000).

Another feature inherent with DNA plasmids isolated from bacteria is the presence of CpG motifs, composed of cytosine guanine dinucleotide pairs (CpG) flanked
by two 5’ purines and two 3’ pyrimidines. CpG motifs are generally methylated in eukaryotes but remain unmethylated in bacteria. These unmethylated CpG motifs act in an immunostimulatory fashion and considered a member of the pathogen-associated molecular pattern molecules (PAMPs) to act as a danger signal (Pardoll 2002). PAMPs interact with the Toll-like receptors (TLRs) expressed on APCs and activates the APCs to produce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferons, IL-6, IL-12 and IL-18 (Yamamoto et al. 1992; Krieg 1996). Figure 1.3 shows a schematic representation of a basic plasmid vector.

1.11.4.3. **Formulation and delivery route**

DNA vaccines are versatile in the fact that different formulations and routes of immunization are available. The plasmid DNA may be resuspended in an aqueous solution, such as saline, and commonly used for injectable delivery via intramuscular (i.m.) or i.d. or even coated with gold particles for gene gun-mediated delivery (Fynan et al. 1993). For i.m. injections, doses of 10 to 100 µg of DNA is injected into skeletal muscle cells (myocytes) whereas gene gun-mediated uses a smaller fraction (<1 µg) to delivery the DNA with a helium-driven biolistic bombardment device. Gene gun-mediated delivery results in higher levels of transgene expression compared to i.m. injection using comparable doses (Yoshida et al. 2000). An alternative approach to improve gene expression with i.m. injection has been through the use of electroporation to induce small pores in cells and effectively increasing the transfer of plasmid DNA into the cell (Banga and Prausnitz 1998; Widera et al. 2000).
Figure 1.3 Schematic view of a simple DNA plasmid vector. There are a few basic elements found in all plasmid vectors used in DNA vaccines. There is a promoter such as the CMV promoter followed by a multiple cloning site (MCS) to insert your gene of interest for expression, and a poly A signal. There is also a resistance gene, such as ampicillin, used for culturing and selecting the plasmid in bacteria and an origin of replication (ori), to increase number of plasmid vector copies. Unmethylated CpG motifs are located at various all throughout the plasmid. Figure created using Discovery Studio Gene program (Accelrys San Diego, CA).
1.11.4.4. Development of immune responses using DNA vaccines

The DNA injected i.m. is taken up by myocytes from the extracellular spaces either through phagocytosis or pinocytosis; meanwhile, gene gun-mediated methods propels the DNA-coated gold particles directly into the cell cytoplasm. The uptake of DNA into myocytes is inefficient as deoxyribonucleases (DNases) will degrade 90% of the DNA, and less than 1% of the remaining 10% enters into the nucleus for gene expression (Babiuk et al. 2003). These targeted cells act as a source for Ag expression, which in myocytes can last between one month to beyond 1.5 years (Wolff et al. 1992; Robinson 1999). However, myocytes are not directly involved with Ag presentation and stimulating an immune response. It is known that myocytes express low levels of MHC class I molecules and lack the expression of costimulatory molecules, which would induce immune tolerance or ignorance. Myocytes also lack MHC class II expression. Further proof that myocytes are not directly involved is shown with surgical excision of muscle tissues immediately after injection did not abrogate the immune response (Torres et al. 1997) and suggests another key player is involved. This finding was also supported in experiments using mouse bone marrow chimeras that upon immunization, revealed Ag-specific T cells that were restricted to the donor’s bone marrow and not to the host myocytes (Corr et al. 1996; Iwasaki et al. 1997). This suggested the involvement of bone marrow derived APC, such as DC, play a key role in DNA vaccination.

The actual mechanisms involved with DNA vaccine have not been truly defined; however, there is experimental evidence to support that DC can be directly transfected at the inoculation site, the draining lymph nodes or through indirect methods. It has been shown that a small proportion of DC (~0.4%) in the draining lymph node and
Langerhans’ cell in the skin carries the plasmid DNA for Ag presentation to T cells (Casares et al. 1997). This was reconfirmed using green fluorescent protein (GFP) reporter plasmid (Condon et al. 1996; Chattergoon et al. 1998) or fluorescein isothiocyanate (FITC) labeled DNA (Dupuis et al. 2000). Direct transfer of plasmid DNA allows for endogenous synthesis of the encoded Ag in DC with peptides directly loaded onto MHC class I molecules. There is also evidence that cross-priming occurs, whereby DC can present peptide epitopes on MHC class I molecules without de novo synthesis. This is accomplished by DC acquiring secreted proteins/peptides from transfected cells or even phagocytosis of apoptotic or necrotic bodies and presenting peptides on both MHC class I and class II molecules (Albert et al. 1998b; Reyes-Sandoval and Ertl 2001). The combination of the two proposed methods may aid in explaining the mechanisms involved in generating immune responses using DNA vaccines (Srivastava and Liu 2003; Donnelly et al. 2005), as outlined in Figure 1.4.

Immune responses generated from DNA vaccines generally results in a balanced Th1/Th2 response but varies depending upon type, dose and form of the encoded Ag, the genetic background of experimental animals, the presence of immunostimulatory cytokines and adjuvants as well as the route of delivery. Studies have also shown that i.m. injections tend to preferentially favor induction of a Th1 biased immune response with expansion of IFN-γ-producing CD4+ T cells and production of IgG2a isotype antibodies. Gene gun targeting, on the other hand, induced a Th2 biased immune response with increased IgG1 Ab production and IL-4 release. (Pertmer et al. 1996; Feltquate et al. 1997; Gurunathan et al. 2000).
Figure 1.4 Mechanisms involved with DNA vaccination. (A) A previous mechanism, but no longer believed, involved DNA transfection of myocytes which in turn would interact directly with T cell. (B) Current mechanisms include direct transfection of APCs. This allows for transgene expression and direct Ag presentation to mediate T cell interaction. (C) Another possible mechanism involves the injected DNA plasmid entering into the myocyte. The expressed transgene from the DNA plasmid is then secreted from the myocyte and subsequently taken up by APCs for presentation and T cell stimulation. (Adapted from Srivastava and Liu 2003).
1.11.4.5. **HER-2/neu targeted DNA vaccines**

DNA vaccines have previously been used in several preclinical models involving mice. Genetic DNA vaccines using plasmid DNA expressing various forms of the ECD and complete HER-2/neu have been reported to induce both HER-2/neu-specific humoral and T-cell immune responses (Amici et al. 1998; Chen et al. 1998; Esserman et al. 1999; Piechocki et al. 2001; Pilon et al. 2001). DNA vaccine expressing the ICD induced stronger anti-neu immunity than the ECD (Foy et al. 2001); whereas, the full-length HER-2 DNA vaccine induced stronger antitumor immune responses (Wei et al. 1999). In general, however, these antitumor immune responses could only partially protect mice from rechallenge of HER-2/neu positive tumor cells and only partially reduced tumor development in various rat neu gene Tg mice (Amici et al. 1998; Esserman et al. 1999; Foy et al. 2001; Renard et al. 2003). Recent reports using electroporated DNA and xenogeneic DNA vaccines have shown enhanced induction of antitumor immunity (Quaglino et al. 2004a; Gallo et al. 2005; Pupa et al. 2005). In addition, the inclusion of weekly cytokine injections such as IL-12 along with DNA immunization elicited stronger antitumor immune responses that protected 63% of BALB-neuT mice after two DNA electroporations or complete protection after four electroporations (Spadaro et al. 2005). However, most of these DNA vaccines were not able to induce any beneficial therapeutic effects (Amici et al. 1998; Esserman et al. 1999; Foy et al. 2001; Quaglino et al. 2004a). Although promising, the effectiveness of DNA-based vaccine might not be able to efficiently break CTL tolerance (Rovero et al. 2000).
1.11.5. Dendritic cells

DC represent the most powerful professional APC of the immune system and quintessential for not only the initiation but also the regulation of immune responses (Banchereau and Steinman 1998). DCs comprises only less than 1% of circulating cells and are a heterogeneous population that serve as sentinel cells found in tissues such as the skin, spleen and liver. The first DC described were Langerhans cells, discovered by Paul Langerhans in the late 1800s. DCs were later “rediscovered” by Steinman and Cohn in 1973 as an adherent cell type in mouse splenocytes with stellate morphology (Steinman and Cohn 1973). This cell was later termed DC based upon morphological features such as the numerous cytoplasmic extensions or membrane processes that form dendrites, pseudopods or veils. DCs characteristically express high levels of MHC class I and II molecules, accessory/costimulatory molecules B7-1 (CD80), B7-2 (CD86), CD40, adhesion molecules CD11b, CD11c and intracellular adhesion molecules (ICAMs)–ICAM-1 (CD54). They also express receptors for efficient Ag capture and secrete chemokines, as outlined in Figure 1.5. DC are characterized by the lack of lineage specific expression markers such as CD3 (T cells), CD19 (B cells), CD14 (monocytes/macrophages), CD56 (NK cells) and CD66b (granulocytes) (Timmerman and Levy 1999; Brossart et al. 2001; Steinman and Dhodapkar 2001). DC have often been termed as “nature’s adjuvant” for their ability to activate naïve T cells and to generate an effective immune response (Steinman and Dhodapkar 2001; Steinman and Pope 2002). In the last few decades, the functions of DC have been extensively studied and their immunotherapeutic potential sought.
1.11.5.1. Sources of DC

Circulating DC comprise only a small amount of circulating cells in the blood and require tremendous leukapheresis efforts to obtain sufficient numbers. The administration of cytokines such as GM-CSF and fms-like tyrosine ligand 3 (Flt-3L) allows for expansion of DC \textit{in vivo}. Examining the ability to generate vast quantities of DC using \textit{ex vivo} methods have allowed for a better understanding of DC biology and its use in immunotherapy. DC, in both mice and humans, is derived from a bone marrow common progenitor cell (Ardavin 2003). In mice, bone marrow cells are cultured in the presence of GM-CSF to stimulate the proliferation of myeloid cells, which includes granulocytes, macrophages and monocytes (Inaba et al. 1992; Inaba et al. 1993a). Large quantities of BM-derived DC can be obtained by culturing BM cells in the presence of...
GM-CSF and IL-4 for 5-7 days (Lutz et al. 1999). In humans, CD34+ hematopoietic stem cells, found either in the bone marrow or in the blood, differentiate to become DC. CD34+ stem cells are cultured in vitro with cytokine cocktails containing GM-CSF with TNF-α, stem cell factor (SCF), IL-4, IL-13 and Flt-3L (Santiago-Schwarz et al. 1992; Romani et al. 1994; Caux et al. 1996; Cella et al. 1997b). Another common source of DC in humans is CD14+ blood monocytes. CD14+ monocyte cells are isolated directly from the blood, either through phlebotomy or apheresis, and differentiated simply using GM-CSF and IL-4 culture media (Sallusto and Lanzavecchia 1994; Berger and Schultz 2003). There are also numerous subsets of DCs identified to date and involved in the generation of immune responses and immune tolerance (Reid et al. 2000; Shortman and Liu 2002).

1.11.5.2. Immunobiology of DC

The biological cycle of DC involves maximal capture of Ag from the microenvironment and efficient presentation to lymphocytes. This cycle is divided into two distinct phases, based upon maturational status: immature and mature DCs. Characteristics of immature DC include highly efficient capture of Ag with low T cell stimulatory activity and commonly found in peripheral tissues. On the other hand, characteristics of mature DC include low Ag capture capability, high T cell stimulatory activity and migratory activity to secondary lymphoid tissues or regional lymph nodes (Bancherau, 1998). Figure 1.6 illustrates the overall life cycle of DC between the two phases.
Figure 1.6 The life cycle of dendritic cells (DC). Precursor DC circulating in the blood enter into tissues as immature DCs. Upon encountering pathogens (e.g. viruses), various cytokines such as IFN are secreted, which in turn activated eosinophils, macrophages (MF) and natural killer (NK) cells. After antigen capture, immature DCs undergo a maturational process and migrate to lymphoid organs. This maturational process induces the DC to display peptide-MHC, which allow for the selection of rare circulating antigen-specific lymphocytes and act in a concerted fashion to induce terminal maturation of DC and allow for lymphocyte expansion and differentiation. The activated T lymphocytes migrate to the injured tissue site to cross the epithelial border. Helper T cells function by secreting cytokines, which permit the activation of macrophages, NK cells and eosinophils. Generation of cytotoxic T cells (CTL) eventually lyse infected cells and B cells, activated by both T cells and DCs, mature to become plasma cells and produce antibodies. (Adapted from Banchereau et al. 2000).
1.11.5.2.1. Immature DC

Immature DC, derived from the bone marrow, enter into the blood and reside in a steady state in tissues as resident cells. At these sites, immature DCs efficiently capture Ags through various mechanisms. This includes extracellular fluid macropinocytosis (Sallusto et al. 1995; Steinman and Swanson 1995), phagocytosis (Inaba et al. 1993b), receptor mediated endocytosis using C-type lectins such as the mannose receptor (Sallusto et al. 1995), Fcγ receptor I (CD64) and II (CD32) mediated endocytosis [uptake of immune complexes or opsonized particles] (Sallusto and Lanzavecchia 1994) and DEC-205 (Jiang et al. 1995; Guermonprez et al. 2002). The integrins αvβ3 and αvβ5 and CD36 may also be involved in the uptake of dying and apoptotic cells (Albert et al. 1998a). Immature DC also internalize peptides complexed with chaperone proteins such as heat shock protein (HSP) gp96 and HSP70 as well as bacteria, viruses and intracellular parasites (Banchereau et al. 2000). Captured Ags are processed intracellularly through the exogenous pathway and the DC undergoes a maturation process, which eventually loads the peptide onto MHC class II molecules. Meanwhile, cross-priming does occur whereby captured Ag accesses the cytoplasm and peptide epitopes are presented in complex with MHC class I molecules. The processing capabilities and environmental stimuli will determine if immature DCs undergo the maturational process to become a mature DC or remain as immature DC to induce T-cell hyporesponsiveness leading to inhibition of immunoreactivity (Banchereau and Steinman 1998; Lutz et al. 2000). In this case, immature DC lacking expression of costimulatory molecules travel to the regional LN where it attempts to present the Ag to T cells but fails to activate the T cell. This induces T cell anergy and possibly deletion.
Immature DC also express CD40, CD54, CD58, CD80, CD86 and CCR7, a chemokine receptor, at relatively low levels. On the other hand, immature DC secrete proinflammatory chemokines including macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), monocyte chemoattractant protein (MCP)-1 (CCL2), MCP-2 (CCL8), MCP-4 (CCL13), Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES; CCL5), thymus-expressed chemokine (TECK; CCL25) and stromal derived factor (SDF-1; CXCL12) (Brossart et al. 2001; Whiteside and Odoux 2004). Proinflammatory chemokines produced upon DC activation signal the migration of the DC and recruitment of naïve T cells to the regional LN. There is also abundant expression of the chemokine receptors CCR1, CCR2, CCR5, CCR6, CXCR1 and CXCR4 to allow for DC recruitment to sites of inflammation for Ag uptake (Caux et al. 2000; Chen et al. 2001b; Berger and Schultz 2003).

1.11.5.2.2. Mature DCs

After immature DC capture the Ag and the encountered local inflammatory mediators or “danger signals” induces a programmed coordinated maturational process to enhance DC capabilities of peptide presentation to T cells to generate an immune response. Some of the mediators of DC maturation include (i) PAMPs such as lipopolysaccharide (LPS), bacterial DNA and double stranded RNA (Pardoll 2002), (ii) proinflammatory cytokines including TNF-α, IL-1β, prostaglandin E2, IL-6, (iii) T cell derived signals such as CD40L, (iv) apoptotic body uptake, which in turn can stimulate a variety of other cells to produce potent mediators. On the other hand, presence of IL-10 in the microenvironment impedes DC maturation.
Maturation mediators are controlled by a series of cell surface receptors such as TLR, cytokine receptors, TNF-receptor (TNF-R), Fc receptors and sensors of cell death (Guermonprez et al. 2002; Steinman and Pope 2002). Upon receiving the maturation stimuli, a coordinated series of events results in down-regulation of Ag uptake machinery, loss of endocytic activity, upregulated expression of adhesion molecules, costimulatory molecules and stable peptide-MHC molecules on the cell surface of DC. This up-regulation can achieve 10-100 fold greater density of molecules compared to monocytes or B cells (Timmerman, 1999). The costimulatory molecules, CD80, CD86 and CD40 are expressed at high levels along with adhesion molecules, CD54, CD11b and CD11c. MHC class II expression is upregulated with majority of the peptide-MHC complexes expressed on the cell surface instead of remaining intracellularly localized in the lysosomes. Also, the MHC class II complexes remains stable for longer periods of time in comparison to the short half-life of MHC class II molecules seen in immature DC (Cella et al. 1997a; Pierre et al. 1997; Banchereau et al. 2000). This increased expression and stability allows for enhanced interaction with CD4\(^+\) Th cells.

There are also distinctive changes in the morphology with the loss of cell adhesiveness, cytoskeleton reorganization allowing the numerous veils to be longer, and high cell motility. Mature DCs, in humans, have easily been identified with the expression of CD83; however, mouse DC do not have a true maturation marker identified. During the maturational process, the expression of proinflammatory chemokines previously expressed by immature DC and chemokine receptors are decreased or down-regulated, while the DC becomes responsive to MIP-3\(\beta\) (ELC; CCL19) and secondary lymphoid tissue chemokine (SLC; 6Ckine; CCL21). Mature DC
are also able to express cytokines such as IL-1, IL-6, IL-12 and IL-18 (Liu 2001). In conjunction, expression of the CCR7 chemokine receptor is upregulated in mature DC, to respond to MIP-3β and SLC chemokines, while down-regulating the CCR1, CCR2, CXCR-4 and CCR5 receptors (Caux et al. 2000; Chen et al. 2001b).

1.11.6. DC vaccines

The unique immunostimulatory activities of DCs and their inherent involvement in previous vaccine strategies have made them an attractive tool for immunotherapeutic use in the treatment of malignant diseases. Previous cancer vaccine strategies (protein, peptide, cell and DNA-based vaccines) required the host APC for priming T cell responses. However, APC from patients and tumor bearing mice are often functionally impaired (Chaux et al. 1997; Gabrilovich et al. 1997; Troy et al. 1998). An approach to correct this problem was to use DC-based vaccines, which would directly allow for interaction with T cells instead of going through various non-efficient intermediate steps. The first clinical study involving a DC vaccine was reported in 1996 resulting in one patient with complete tumor regression and further sparked the utility of DC cancer vaccines (Hsu et al. 1996). DC are able to deliver tumor Ag derived from various sources that would allow efficient presentation to engage an antitumor immune response. DC-based vaccines have been reported to be superior compared to peptide and DNA-based vaccines by stimulating a more efficient antitumor immunity (Toes et al. 1998; Yang et al. 1999; Bellone et al. 2000). Certain aspects of DC-based vaccine require a brief examination such as maturation, methods of administration and Ag loading, as discussed in the following sections below.
1.11.6.1. The importance of the maturation status

Various studies have shown the importance of DC maturational status on the induction of antitumor immune responses. In comparing DC cultured under various conditions, ranging from GM-CSF alone to GM-CSF and IL-4, and the addition of various maturation stimuli, Labeur et al. analyzed both the phenotypic and functional characteristics of the generated DC (Labeur et al. 1999). They showed DC cultures supplemented with IL-4 induced relative maturation compared to culture with GM-CSF alone. Moreover, the addition of CD40L increased the maturation of DC and resulted in greater antitumor immunity and immunotherapy in mice with established tumors in a squamous cell carcinoma model (Labeur et al. 1999). Similar results have also been confirmed by other groups in preclinical and clinical studies (Chen et al. 2001b; de Vries et al. 2003). In a systematic analysis on the relative impact of maturation on the surface phenotype, cytokine and cytokine/chemokine receptors and antitumor immunity was performed. DC cultured in GM-CSF and IL-4 with the addition of LPS exhibited significantly greater expression of MHC class II, CD40, CD54, CD80 and CD86 on the cell surface. Increased expression of cytokines, such as Flt-3L, granulocyte-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) and the chemokine receptor CCR7 was detected in comparison to intermediate mature and immature DC (Chen et al. 2001b). On the other hand, immature DCs are useful in inducing Ag-specific tolerance or immune silencing for potential applications in managing transplantation, allergy, autoimmune and chronic inflammatory diseases (Jonuleit et al. 2000; Dhodapkar et al. 2003).
Therefore, the maturation status of DC is an important factor to consider in the utilization of DC vaccines.

### 1.11.6.2. Route of DC vaccination

The route of administration plays an influential role in the migration of DC to lymphoid tissues and its overall efficacy in a DC-based vaccine. The various methods of administration include subcutaneous (s.c.), i.d., intravenous (i.v.), intranodal, intralymphatic (i.l.), intraperitoneal (i.p.) and intratumoral (i.t.) injections (Nencioni and Brossart 2004). Studies in mice have shown direct evidence that DC injected s.c. preferentially migrated to the draining LN compared to i.v. injected (Eggert et al. 1999; Okada et al. 2001a). DC injected i.v. accumulated largely in the spleen, liver and lungs, whereas s.c. injected DC were able to home to the T cell areas of the draining LN via the afferent system. DC injected s.c. were found in the popliteal LN 24 hours after injection and peaked at 48 hours but remained detectable for five days (Lappin et al. 1999). In another animal study, a single vaccination given either i.d. and s.c. resulted in greater protective immunity from tumor challenge compared to i.p. and i.v. injection (Okada et al. 2001a). A clinical study involving three cohorts of prostate cancer patients immunized with Ag-loaded DC via i.v., i.d. and i.l. routes resulted in the development of specific T cell responses irregardless of the route of immunization; however, greater levels of IFN-γ were observed with i.d. and i.l. routes of immunization (Fong et al. 2001b). This suggested that the route of immunization affects the quality of the T cell response generated. Alternatively, intranodal injection, which substantially increases the number of DC in the LN to interact with T cells, produced more potent responses but
destroyed the LN architecture (Barratt-Boytes and Figdor 2004). However, more studies are required to determine the optimal route for DC immunization.

1.11.6.3. **Methods for Ag loading**

A wide variety of methods are available for *ex vivo* loading of tumor Ag into DC that have been developed and used in both preclinical and clinical studies (Markiewicz and Kast 2004). Table 1.1 describes the multitude of *ex vivo* methods available for loading tumor Ag and only some of the common methods are discussed further in Sections 1.11.6.3.1 to 1.11.6.3.6.

<table>
<thead>
<tr>
<th>Methods for <em>ex vivo</em> loading of tumor Ag onto DC</th>
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<tbody>
<tr>
<td>• Synthetic or eluted peptides</td>
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<tr>
<td>• Soluble protein</td>
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<tr>
<td>• Transfection with cDNA or RNA encoding tumor Ag</td>
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<tr>
<td>• Recombinant viral vectors</td>
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<tr>
<td>• DC-tumor cell fusions (Hybrid cells)</td>
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<td>• DC derived exosomes</td>
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<tr>
<td>• Heat shock proteins</td>
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<td>• Tumor-derived</td>
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<td>• Transfection via electroporation, liposomes</td>
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<td>• DNA</td>
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<td>• Immunomodulatory molecules/tumor Ag</td>
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<td>• Transfection</td>
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<tr>
<td>• Electroporation, Naked, liposomes</td>
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<tr>
<td>• Viral Transduction</td>
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1. Adapted from Brossart et al. 2001; Markiewicz and Kast 2004
1.11.6.3.1. Tumor lysates

Vaccination strategies targeting a single peptide epitope may be defined too narrowly with the immune system devoting enormous effector resources into a single response. This increases the chances of failing upon antigenic changes within the tumor. As tumors are heterogeneous in nature, tumors may express a wide variety of TAAs. The use of tumor lysates as a source of Ag offers the potential advantage of inducing a response against multiple known and unknown TAA expressed in tumors. Tumor lysates are obtained by repeated freezing/thawing or by sonicating tumor cells. This method decreases the time consuming and substantial efforts of identifying and synthesizing individual immunodominant peptide epitopes by allowing the DC to naturally process the tumor-derived Ags (Zhou et al. 2002). The drawback to this method is the limited availability of tumor cells obtained from patients. However, tumor Ag laden DC have been shown to induce tumor Ag specific CTL responses and provide some beneficial responses for patients (Zhang et al. 2002).

1.11.6.3.2. Exosomes

Exosomes are late-endosome derived small membrane vesicles ranging from 60-90 nm in diameter and secreted by both DC and tumor cells. Exosomes contain numerous MHC class I and II molecules, costimulatory molecules such as CD86 and various cell derived products such as HSP (Steinman and Dhodapkar 2001; Zhou et al. 2002). Mice immunized using DC loaded with tumor-derived exosomes or even DC derived exosomes resulted in tumor protection and therapeutic effects leading to regression of established tumors (Zitvogel et al. 1998; Wolfers et al. 2001). This method
can be further used for pulsing *in vitro* generated DC or for cell-free based vaccines (Andre et al. 2004).

### 1.11.6.3.3. Proteins

Immature DC allows for increased Ag loading by allowing the protein to be captured, processed then presented by DC as immunogenic peptides in complex with MHC molecules. By directly co-culturing the protein with DC, it bypasses the MHC/HLA haplotype restriction issue, circumventing the need to identify epitopes. Both Ag-specific CTL responses *in vitro* and antitumor immunity *in vivo* have been detected in both animal models and humans. This method has been successfully used in clinical studies for follicular lymphoma and multiple myeloma using idiotype immunoglobulins (Markiewicz and Kast 2004). However, it should also be noted that the incorporation of exogenous soluble Ags into DC results in inefficient processing and loading onto MHC class I molecules.

In addition to using native proteins, modifications to incorporate specific sequences, thus creating fusion proteins, can facilitate the processing more effectively or even enhance the immunogenicity of the protein. Fusion proteins containing the human immunodeficiency virus (HIV) trans-activating (TAT) protein transduction domain allow for proficient protein entry into cells. In preclinical models, ovalbumin (OVA)-TAT fusion protein were efficiently taken up by murine DC and processed the Ag to stimulate both CD8$^+$ and CD4$^+$ T cells that prevented tumor engraftment of OVA-expressing tumor cells (Shibagaki and Udey 2002). TAT-based fusion proteins have also been applied to HER-2/neu in both preclinical *in vitro* and *in vivo* animal studies with encouraging results (Tanaka et al. 2003; Viehl et al. 2005). Moreover, proteins
complexed with hydrophobized polysaccharide nanoparticles such as cholesteryl group bearing mannan or pullulan, induced both cellular and humoral immune responses effectively preventing tumor growth upon tumor challenge (Gu et al. 1998; Wang et al. 1999).

1.11.6.3.4. Peptides

Peptides are easily adaptable for use in DC based cancer vaccines simply by pulsing the peptides to DC. This route allows for direct peptide loading onto MHC class I and II molecules, depending upon the peptide epitope, to induce epitope-specific T cells responses. Sources of peptides include chemically derived synthesis, acid elution of peptides from tumor cells or affinity purification of MHC-peptide complexes (Zhou et al. 2002). Peptide pulsed DC have been shown to induce both peptide specific CD8$^+$ and CD4$^+$ T cell responses in preclinical animal models, healthy volunteers and patients in a wide assortment of cancers (Mayordomo et al. 1995; Zitvogel et al. 1996; Meidenbauer et al. 2001; Zhang et al. 2002; Allan et al. 2004). Peptide pulsed DC remains one of the most widely used methods to load Ag onto DC to date.

In the case of HER-2/neu, peptide pulsed DC have been examined as potential candidates in both preclinical animal and human clinical trials. Using the HER-2 peptides p63-71 and p780-788, BALB/c (H-2K$^d$) mice immunized weekly with peptide pulsed DC suppressed the growth of a previously transplanted HER-2 expressing syngeneic tumor and efficiently stimulated both CD4$^+$ and CD8$^+$ T cell responses (Shiku et al. 2000). These results were encouraging as the peptide motif for murine H-2K$^d$ shares a similar binding motif with the human HLA-A24 and in vitro stimulated CTL were detected in both healthy individuals and ovarian cancer patients using peripheral
blood lymphocytes (PBL) (Ikuta et al. 2000; Okugawa et al. 2000; Ikuta et al. 2002). Similar studies with human PBL have examined in vitro generated HLA-A2 restricted CTLs in both cancer patients and healthy individuals using the HER-2 peptides p369-377 [E75], p435-443 and p689-697 (Rongcun et al. 1999; Anderson et al. 2000b; Seliger et al. 2000; Baxevanis et al. 2002; Kono et al. 2002b; Morse et al. 2003; Sotiropoulou et al. 2003a; Sotiropoulou et al. 2003b; Baxevanis et al. 2006). In one study, Muller et al. reported the generation of HER-2 specific CTL with cytolytic activity in vitro using the PBL from acute lymphoblastic leukemia patients, which indicated the wide scale applicability of peptide-pulsed DC vaccines for a variety of cancers (Muller et al. 2003).

Besides using peptide pulsed DC to generate CTL in vitro, they have also been used to mount a specific immune response in vivo. A clinical study by Brossart et al. utilized monocyte-derived DC with two different HLA-A2-restricted HER-2 peptides or MUC-1 peptides to generate CTLs in patients with advanced breast cancer or ovarian cancers. Ag specific CTL were detected in 50% of the patients examined, as assessed by IFN-γ and cytotoxicity assays (Brossart et al. 2000a). More recently, a phase I/II trial utilized CD34+ expanded DC pulsed with the HLA-A2 restricted HER-2 peptide, p654-662 or a modified, more stable form of the peptide. This resulted in two patients with partial response to therapy while an additional two patients developed IFN-γ producing CD8+ T cells (Dees et al. 2004).

In a mouse animal model, DC pulsed with a recently identified H-2D^q restricted-neu peptide induced specific lysis activity and completely protected wild-type FVB/N mice from challenge with NT2 neu expressing tumor cells; however, even after two immunizations with the vaccine, no protective response was observed in FVBneuN Tg mice, which are already tolerized to neu (Ercolini et al. 2003). In addition, recent work
has shown that multiple vaccinations were required with a HLA-A2-restricted peptide-pulsed DC in A2 x neu crossed Tg mice (A2/Kb Tg mice crossed with FVBneuN mice). The addition of IL-12 and anti-OX40 mAb also induced a more robust immune response. This resulted in a greater degree of tumor growth inhibition compared to unmodified peptide-pulsed DC (45% inhibition versus 12-25% inhibition) (Lustgarten et al. 2004). The T cells within these crossed Tg mice were of low avidity and clinically represented the tolerance situation observed in patients involving the deletion of high avidity T cells. Overall, these studies have shown that HER-2/neu peptide pulsed DC can generate CTL but still does not provide adequate protection or therapeutic response in a clinical setting.

1.11.6.3.5. Necrotic and apoptotic Cells

DC can easily phagocytose dying (both apoptotic and necrotic) tumor cells to induce protective immunity (Albert et al. 1998b; Fonteneau et al. 2001). Advantages of using dying tumor cells for ex vivo loading of DCs include the ability for DC to present and to cross present the entire spectrum of Ag from the tumor cell on both MHC class I and II molecules. This would be independent of the MHC/HLA haplotype restriction allowing for applicability in all patients, unlike the restriction imposed for peptide-pulsed DC. There should also be a reduction in the probability of the tumor escaping from the generation of Ag-negative variants (Zhang et al. 2002; Berger and Schultz 2003). On the other hand, several disadvantages have arisen with the use of this method. One of them is the potential risk of inducing autoimmunity (Ludewig et al. 2000) even though immature DC can induce tolerance to self-Ag from apoptotic cells (Steinman et al. 2000). Another pitfall is the lack of sufficient quantities of patient tumor samples but
this too may be addressed using allogeneic tumor cell lines as an alternative. Some other disadvantages include the lack of monitoring since no defined tumor Ag is identified and an inherent limitation of DC to present a subset of epitopes from tumor cells instead of the complete set of epitopes (Berger and Schultz 2003).

Apoptotic cell death is a common way for a cell to die and characterized by cell shrinkage, chromatin condensation, DNA cleavage and laddering and membrane blebbing. This process is also important in self and non-self discrimination, as failure would result with dying cells triggering an immune response against themselves (Matzinger 1994). Cells that undergo apoptosis are readily engulfed by DC (Albert et al. 1998b) via αβ5, CD36 (Albert et al. 1998a) and phosphatidylserine (PS) receptor (Fadok et al. 2000). Subsequently, DC efficiently present the Ags of apoptotic cells to class I-restricted CTLs (Albert et al. 1998b). Apoptosis occurs at two different stages with the first stage considered as early apoptosis, phenotypically characterized by the expression of Annexin V and lack of propidium iodide staining (PI) staining. The second stage of apoptosis, called late apoptosis or secondary necrosis, is a result when the plasma membrane and intracellular organelles breakdown. Characteristics of late phase apoptosis are cells staining for both Annexin V and PI. On the other hand, cells dying of necrosis are phagocytosed through the interaction of CD91 (Basu et al. 2001) and c-type lectin LOX1 (Delneste et al. 2002) receptors with HSP expressed on necrotic tumor cells. The uptake of dying tumor cells has been shown to impact both DC maturation and DC immunogenicity.

Controversy still exists with respect to the optimal form of killed tumor cells for stimulating effective immune priming and antitumor activity in DC-based vaccine strategies. The contradictory and discordant findings stem from earlier reports showing
DC that phagocytosed apoptotic cells could exclusively cross-present Ags to CTLs (Albert et al. 1998b). DC pulsed with apoptotic tumor cells induced a strong Ag-specific T cell response in vitro, and when injected into mice, such pulsed DC induced tumor-specific CTL responses and long-term protection from parental tumor challenge (Henry et al. 1999). It was later shown that necrotic, but not apoptotic cells could induce DC maturation to elicit immunity (Basu et al. 2000; Sauter et al. 2000). In another report, however, both apoptotic and necrotic tumor cells could equivalently induce DC-mediated immune priming and antitumor therapeutic efficacy (Kotera et al. 2001). More recently, it has further been shown that DC charged with apoptotic tumor cells induced long-lived protective CD4⁺ and CD8⁺ T cell immunity (Scheffer et al. 2003) and apoptotic but not necrotic tumor cells are efficient vaccines in vivo (Goldszmid et al. 2003). Apparently contradicting these results, it has also been reported that the presence of apoptotic cells during macrophage activation increases their secretion of anti-inflammatory mediators (Fadok et al. 1998) and decreases their secretion of proinflammatory cytokines (Voll et al. 1997), resulting in inflammatory response suppression and immune tolerance (Steinman et al. 2000; Liu et al. 2002). In line with this notion, there is also evidence for the lack of immunogenicity (Ochsenbein et al. 1999) or reduced immunogenicity of apoptotic tumor cells as compared with their viable counterparts (Ponner et al. 1998). Given the predominance of apoptotic cell death in most common cancer treatment regimens (Thompson 1995), it is important to know whether the enhanced tumor Ag presentation associated with DC presenting these Ags will enhance the development of immunity, or of immune tolerance to the tumor.
1.11.6.3.5.1. **Role of HSP70**

HSPs are intracellular molecular chaperones located in the cytoplasm, ER or the mitochondria. HSPs are present at low levels and function at a steady state under normal conditions but easily induced to high levels upon encountering cellular stress to prevent protein aggregation and to protect cells from apoptosis (Todryk et al. 2003; Beere 2005; Calderwood et al. 2005). HSP70 is expressed in the cytoplasm and is one of the prototypes in the HSP family. The release of HSP70 from cells undergoing a necrotic cell death rather than apoptosis provides an enhanced immunogenic effort. Studies have shown that HSP70 has immunomodulatory effects in Ag processing and presentation in DC through a variety of mechanisms (Li et al. 2002; Noessner et al. 2002; Millar et al. 2003; Calderwood et al. 2005). Cleaved peptides derived from protein degradation can interact with HSP70 to form HSP-peptide complexes within the stressed cell, in turn allowing for presentation on MHC class I molecules. The HSP-peptide complexes exposed on the cell surface or released from stressed cells can also be taken up by APC, which in turn processes and presents the Ag peptides on MHC molecules. It has also been demonstrated that receptor-mediated internalization of HSP70 induced DC maturation (Kuppner et al. 2001; Noessner et al. 2002; Castelli et al. 2004). This suggested the discordant results observed may be a function of HSP70 expression. For example, recent reports showed early phase apoptotic cells inhibited DC maturation while late phase apoptotic cells increased the expression of HSP70 expression and induced DC maturation (Melcher et al. 1999; Pietra et al. 2001; Buttiglieri et al. 2003; Galetto et al. 2003; Masse et al. 2004).
1.11.6.3.6. Engineered DC

Modifying DC with DNA to express an array of cytokines, chemokines and tumor Ag (i.e. TAA) have provided a powerful advantage to manipulate the properties of DC for use in cancer therapies. Gene engineered DC offers several attractive advantages compared to \textit{ex vivo} Ag pulsing with peptides and whole tumor cell preparations (Arthur et al. 1997; Humrich and Jenne 2003; Nakamura et al. 2005). One advantage of using tumor Ag expression within DC is the constant source of Ag available for presentation and the prolonged duration of Ag presentation. This contrasts the use of lysate or peptide pulsed DC, where the duration of expression is restricted not only by the peptide’s affinity to bind to the MHC molecule but by the half-life of the peptide-MHC complex formed and the MHC turnover rate (Kaplan et al. 1999; Kirk and Mule 2000). Whole tumor cell preparations face the issues involved with standardization of the TAA and availability of tumor cells. As well, gene-engineered DC with the tumor Ag guarantees that the Ag is processed endogenously for presentation onto MHC class I molecules, which leads to effective generation of a CTL-mediated immune response. Another important advantage with engineered DC is the reduced requirement to identify specific epitopes as the Ag presents both naturally processed CTL and helper epitopes. It has also been found that DC expressing tumor Ag transgene are more potent primers of antitumor immune responses than their soluble Ag-pulsed counterparts in both \textit{in vitro} and \textit{in vivo} animal studies (Tuting et al. 1997a; Zhang et al. 2002). Several different methods, both non-viral and viral methods have been developed for \textit{ex vivo} loading of DC. It should also be noted that the aim of this form of DC-based immunotherapy is not necessarily the long-term expression of genes but rather the development of strong TAA-specific cellular and humoral immune responses.
1.11.6.3.6.1. Non-viral methods

A common method for tumor Ag transgene expression is the use of a plasmid encoding the cDNA for the Ag, similar to the expression vectors used in DNA vaccination. The direct uptake of plasmid DNA by DC is one of the mechanisms for how DNA vaccines function; however, only a small fraction of DC is directly transfected by the plasmid. Traditional methods for gene transfection such as liposomes-mediated transfection, calcium phosphate precipitation and electroporation have been used for transgene expression in DC (Alijagic et al. 1995; Arthur et al. 1997; Van Tendeloo et al. 1998; Lohmann et al. 2000; Rughetti et al. 2000; Lundqvist et al. 2002). In these studies, variable levels of gene expression have been observed with relatively low efficiency between 5-20%. Often times, expression levels were not repeatable or consistent, suggesting the vehicle used in gene transfer is important for transfection efficiency (Kirk and Mule 2000). Transfections using non-viral methods are generally inefficient, possibly due to the limited ability of the DNA to reach the nucleus for transcription to occur (Luo and Saltzman 2000). In addition, the physical methods perturbed and altered both the function and phenotype of DC or became highly toxic (Arthur et al. 1997; Rughetti et al. 2000; Lundqvist et al. 2002). On the other hand, electroporation of RNA has shown increased transfection efficiency and cell viability (Lundqvist et al. 2002; Grunebach et al. 2005).

There have also been some recent improvements made in the use of non-viral delivery systems. One recent development is the usage of a novel 35 aa cationic peptide CL22 complexed with plasmid DNA (Irvine et al. 2000). This stimulated stronger autologous T cell responses and CTL activation against the influenza A nucleoprotein
using human DC and generated stronger specific T cell responses that resulted in tumor rejection in a mouse animal model. Immunolipoplexes incorporating Ab targeting DC surface molecules such as anti-CD11c, anti-CD71 (transferrin receptor) and anti-CD205 (mouse DEC205 homologue), increased the transfection efficiency and induced strong antitumor immune responses with antitumor effects (Altin et al. 2004; Tan et al. 2005a). Furthermore, gene gun based methods for transfection has also shown dramatic increase in the transfection efficiency compared to electroporation and naked DNA delivery alone (Larregina et al. 2004).

1.11.6.3.6.2. Viral vectors

Viral vectors possesses the capabilities to dramatically increase the efficacy of transgene expression to levels as high as 90-100% compared to the 5-20% seen with traditional transfection methods (Arthur et al. 1997; Dietz and Vuk-Pavlovic 1998; Zhong et al. 1999). Recombinant viral vectors containing a transgene insert used for DC transduction have included retroviruses, lentiviruses, adenoviruses (AdV), pox virus, herpes virus, influenza virus, and adeno-associated viruses (Humrich and Jenne 2003). The use of viral vectors to transduce DC allow for (i) controlling the maturation status of transduced DC, (ii) the expression levels of the TAA and (iii) the site of injection to induce the optimal antitumor immune response. Two viral vectors most commonly used for genetic modification of DC are retrovirus and AdV vectors, as discussed below.
a) Retroviruses

Retroviruses are RNA viruses and a member of the *Retroviridae* family. Retroviruses replicate through a double stranded DNA intermediate, eventually reaching the nucleus and integrating into the host genome. An advantage of using retroviral vectors is the ability for the TAA to be presented on both MHC class I and II molecules to activate TAA-specific CD8$^+$ and CD4$^+$ T cells. Retroviruses are also autologous units, which do not lead to the development of neutralizing antibodies that would limit their ability to infect cells (Reeves et al. 1996; Specht et al. 1997). Reports have also indicated the requirement for cognate help from CD4$^+$ Th cells in eliciting protective and therapeutic antitumor immunity (De Veerman et al. 1999; Schnell et al. 2000). As well, retroviral vectors limits the alteration in DC viability, phenotype, maturation or functions while the transgene remains stably expressed. Retrovirus vectors, which are replication deficient and based upon the Moloney murine leukemia virus (MMLV) genome, have been used to express a wide variety of TAA ranging from mucin-1 (MUC-1) (Henderson et al. 1996), melanoma MART-1 (Reeves et al. 1996), gp100 (Lapointe et al. 2001) and HER-2/neu (Meyer zum Buschenfelde et al. 2000; zum Buschenfelde et al. 2001). Retrovirally transduced human CD34$^+$ derived DC expressing HER-2 from healthy normal individuals and patients with breast cancer induced HER2-specific CD8$^+$ CTL and CD4$^+$ Th1 cells that recognize different epitopes of HER-2 (Meyer zum Buschenfelde et al. 2000; zum Buschenfelde et al. 2001).

Despite the immune responses observed, there are some drawbacks associated with the use of retroviruses as a viral vector. One of them is the requirement of actively dividing cells, such as the CD34$^+$ hematopoietic stem cells and bone marrow cells, for
efficient transduction and integration into the cell. Transduction efficiency with both differentiated immature and mature DC resulted in reduced efficiency. To bypass this, progenitor cells are used for viral transduction and differentiated into mature DC afterwards (Aicher et al. 1997; Meyer zum Buschenfelde et al. 2000; Markiewicz and Kast 2004).

Lentiviruses, a member of the Retroviridae virus family, which includes the Human Immunodeficiency Virus (HIV) and simian immunodeficiency virus have also been used to transduce DC. Lentiviruses, in contrast to retroviral vectors, easily transduces both actively dividing and non-dividing cells, such as the differentiated immature and mature DC (Robbins and Ghivizzani 1998; Humrich and Jenne 2003). Lentiviral vectors or lentivectors have also shown the ability to induce strong long lasting T cell responses that translate into effective protective and therapeutic responses in various model systems (Cui et al. 2003; Breckpot et al. 2004; He et al. 2005; Kim et al. 2005b). One of the main drawbacks that remain in the use of these vectors is the safety issues such as DNA integration, which needs to be continually addressed.

b) Adenovirus

The other most commonly utilized viral vector is AdV. More specifically, first generation AdV vectors, used traditionally in gene therapy applications but extended for immunotherapy application, allows for efficient transgene expression in DC. An overview of AdV and its vectors are discussed below and in the following section. AdV is a favorite vehicle for gene delivery to DC for several reasons such as its safety. AdV vectors transiently express the transgene and does not involve viral DNA integration into the host genome; therefore, avoiding the possibility of disrupting vital cellular genes or
inducing cancer as seen for retroviral vectors (Dave et al. 2004). AdV vectors also have a large gene carrying capacity allowing insertion up to 37 kb in size, limited pathogenicity with the deletion of E1 and E3 viral genes and are relatively easy to construct and to propagate with large quantities produced (Zhang 1999; Basak et al. 2004). Ex vivo transduced DC provide an advantage to overcoming pre-existing anti-AdV immunity and does not involve injection of “free” AdV viral particles, thus allowing for induction of effective anti-tumor immune responses (Brossart et al. 1997; Kaplan et al. 1999; Wan et al. 1999; Basak et al. 2004).

A vast improvement in the transduction efficiency using AdV is seen compared to other physical methods. Rates greater than 90% are seen in transduced immature DC and the efficiency is reduced (~50%) in transduced mature DC (Dietz and Vuk-Pavlovic 1998; Zhong et al. 1999). Reports have shown varying transduction efficiencies ranging between 20%-83% in murine and human DC when using multiplicities of infection (MOI) between 50-1000 are used (Song et al. 1997; Dietz and Vuk-Pavlovic 1998; Miller et al. 2002; Nakamura et al. 2002; Tuettenberg et al. 2003). Discrepancies may be attributed to the culture conditions used as well as the number of viral particles present in prepared AdV stocks (Hirschowitz et al. 2000). Interestingly, high MOI levels are often required to obtain these transduction rates, and are dose-dependent. The requirement of high MOI is based upon DC lacking expression of the coxsackie-adenovirus receptor (CAR), one of the surface receptors needed for AdV attachment to the cell (Rea et al. 1999; Zhong et al. 1999; Okada et al. 2001b). It is important to note that the high MOI required for transduction also creates cytotoxic effects to DC in a dose dependent manner. This has recently spurred interests in determining methods to improve the transduction efficiency while decreasing the amount of AdV required for
transduction. One approach has been complexing the AdV with liposomes, resulting in a five-fold increase in transduction efficiency in human DC (Dietz and Vuk-Pavlovic 1998). Other methods include altering the viral tropism by adding adapter molecules such as bispecific antibodies targeting CD40 (Tillman et al. 1999) or creating genetic fusions between the fiber and receptor-specific ligand (Kashentseva et al. 2002; Belousova et al. 2003). Another method includes the incorporation of an arginine-glycine-aspartic acid (Arg-Gly-Asp, RGD) motif into the HI loop of the fiber protein thereby allowing for CAR-independent attachment and entry into the cell via the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Okada et al. 2001b; Okada et al. 2003b; Worgall et al. 2004). The RGD-fiber modified AdV allowed for a 10-fold increase in transduction efficiency and 5-10 fold increase in transgene expression compared to unmodified AdV, leading to enhanced antitumor activities.

One issue that has shown conflicting results is whether AdV induces maturation of DC. Zhong et al. reported that AdV transduction does not perturb the DC maturational status (Zhong et al. 1999). However, additional studies by other groups have shown that AdV vectors can indeed modulate DC maturation by increasing the expression of MHC, costimulatory molecules and adhesion molecules such as CD40, CD54, CD80 and CD86 (Rea et al. 1999; Hirschowitz et al. 2000; Morelli et al. 2000; Miller et al. 2002; Molinier-Frenkel et al. 2003). Maturation of DC may be mediated by the viral fiber protein independent of viral gene or transgene expression (Hirschowitz et al. 2000; Miller et al. 2003; Molinier-Frenkel et al. 2003; Okada et al. 2003a). This in turn allows for signaling through PI3K and induces production of TNF-\(\alpha\) and type I-IFNs (both $\alpha$ and $\beta$) for maturation (Philpott et al. 2004; Hensley et al. 2005). Increased expression of IL-1$\beta$, IL-6, IL-12 p40/IL-12 p70, IL-15 and IFN-$\gamma$ has also been observed.
in AdV transduced DC, in a dose-dependent manner (Hirschowitz et al. 2000; Morelli et al. 2000; Tan et al. 2005b). Decreased expression of CCR6 with increasing expression of CCR7 is also observed (Okada et al. 2003a).

AdV-mediated expression of TAA in DC results in endogenous synthesis of the Ag for processing through MHC class I and II presentation pathways. This in turn allows for interaction and activation of CD4$^+$ and CD8$^+$ T cells, along with B cells to induce an antibody response, as illustrated in Figure 1.7. Numerous studies have previously shown AdV-mediated expression of TAA elicited strong CTL and CD4$^+$ Th cellular antitumor responses (Song et al. 1997; Tuting et al. 1999; Song et al. 2000; Nakamura et al. 2002; Nakamura et al. 2005).

1.11.6.4. HER-2/neu targeted DC vaccines

Previous studies using HER-2/neu engineered DC vaccines have shown the ability to induce an antitumor immune response. DC derived from CD34$^+$ hematopoietic progenitor cells were transduced with a retrovirus vector containing the full length HER-2 gene resulting in a low efficiency transduction rate but led to the generation of both CTL and Th1 T cell clones in vitro in both normal and breast cancer patients (Meyer zum Buschenfelde et al. 2000; zum Buschenfelde et al. 2001). Chen et al utilized an AdV expressing the full length neu (AdNeuTK) to transduce DC that elicited protective immunity in 60% of mice against a lethal challenge against neu expressing NDL tumor cells (Chen et al. 2001a). CD4$^+$ T cells were required during the initiation phase while the main effector cells were CD8$^+$ T cells for rejection of tumors. In a different study by Chen et al, DC transduced with AdVHER expressing only the ECD and TM of HER-2 induced HER-2 specific CTL responses but only resulted in 25% of mice protected from
Figure 1.7 Adenovirus-mediated gene transfer to dendritic cells. An adenoviral vector carrying a gene expression cassette encoding for a tumor associated antigen such as HER-2/neu into DC. The expressed antigen can be processed and presented with MHC class I to induce a CTL response or with MHC class II to induce a CD4+ Th response, which, in turn, can further stimulate the CTL or the Ab response.
a challenge of $3 \times 10^5$ MCA26/HER-2 tumor cells (Chen et al. 2002c). To enhance the efficacy of the $\text{DC}_{\text{HER}-2}$ vaccine, DC were cotransduced with both AdVHER-2 and AdVTNF-α, forming $\text{DC}_{\text{HER}-2/\text{TNF-}\alpha}$, that further increased the expression of CD40, CD86 and ICAM-1 (CD54) and mounted a 36% stronger HER-2 specific T cell response. More importantly, $\text{DC}_{\text{HER}-2/\text{TNF-}\alpha}$, immunized mice were completely protected upon lethal challenge of HER-2 expressing tumor cells in this animal model. In a more recent report, transduction of DC with an AdV expressing truncated neu (Ad.neu) stimulated the production of both neu-specific antibodies and T cells in Tg mice (Sakai et al. 2004). Moreover, immunization of BALB-neuT mice, which generally develops mammary tumors in all ten glands by 25 weeks of age, resulted in 65% of the mice remaining tumor free at 28 weeks of age using the engineered DC vaccine compared to the control groups that all developed tumors by this same period. These studies indicated that DC-based vaccines targeting HER-2/neu is an attractive strategy.

1.12. Overview of AdV vectors

1.12.1. AdV family

Human adenoviruses (HAd, and referred to as AdV) are non-enveloped, double stranded DNA viruses. AdV were first isolated in the early 1950s from human tonsils and adenoid tissue as well as respiratory secretions by two different groups (Rowe et al. 1953; Hilleman and Werner 1954). The viruses isolated by both groups were related and given a variety of names until a mutually agreed upon name, adenoviruses, was given. The name was based upon the original tissue (adenoid) the virus isolates were detected in (Enders et al. 1956). HAd represents one group amongst the *Adenoviridae* family,
which also includes avian, bovine and simian adenoviruses. Currently, there are 51
different HAd serotypes, grouped from A to F based upon genome size, composition,
homology, organization and the ability to agglutinate red blood cells. To date, the most
well studied AdV is type 2 and type 5 of subgroup C.

1.12.2. Virus structure

AdV are non-enveloped, double stranded DNA viruses of ~36 kilobases (kb) in
size. The capsid is an icosahedral protein shell consisting of 20 triangular surfaces and
12 vertices that surrounds the linear, double-stranded DNA. The capsid structure of
AdV is composed of the hexon, which is the major protein component of the capsid
surface, the twelve-penton bases at each of the vertices and the twelve fiber
homotrimers, differing in length between the serotypes. Several minor proteins (VI,
VIII, IX and IVa2) also comprise the remainder of the viral capsid.

The AdV genome contains two identical short, inverted terminal repeats (ITR)
located at both the left and right ends of the genome. The ITR allows DNA replication
to occur at both ends. A cis-acting packaging sequence is located within several
hundred base pairs (bp) of the left end of the viral genome to allow for encapsidation of
viral DNA within the virus capsid.

1.12.3. AdV replication

The replication cycle of AdV has been well studied and first requires attachment
of the virus to the cell. This occurs via a high affinity interaction between the fiber knob
of AdV and CAR, the primary cellular receptor (Bergelson et al. 1997). Other primary
cellular receptors for attachment include MHC-class Iα2 subunit and sialic acid.
Following this, a secondary interaction with the RGD motif of the penton base with the secondary host cell receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins occurs to mediate virus internalization (Wickham et al. 1993). The binding to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins promotes receptor-mediated endocytosis mediated by clathrin-coated pits. The rate-limiting step for AdV replication is the ability of the virus to interact with CAR. CAR is expressed on a variety of cells such as epithelial cells and muscle cells but not on hematopoietic cells, including DC. CAR expression is also highly variable within tumors (Douglas et al. 2001; Kim et al. 2002; Okada et al. 2003a). Once the virus is inside the cell, a coordinated, sequential disassembly of the capsid proteins allows the virus to escape from the endosome and enter into the cytoplasm. Subsequently, the virion is transported to the nuclear pore complex and the viral DNA is released into the nucleus to initiate viral gene expressions (Greber et al. 1993). Transcription from both the left and right ITR produces at least 30 different mRNA species. Based upon the onset of viral DNA replication, the mRNA transcripts are divided into three classes: early transcripts (E1A, E1B, E2, E3 and E4), delayed early transcripts (IX and IVa2) and late transcripts (L1 to L5) (Shenk 1996). An overview of the AdV life cycle is illustrated in Figure 1.8.

Of the early transcripts, the E1A and E1B proteins are first to be synthesized and deemed necessary for virus replication. These proteins engage in a multitude of regulatory functions including i) induction of DNA synthesis in quiescent cells, ii) immortalization of primary cells, iii) activation of other early and late genes, and iv) induction of apoptosis. Early proteins encoded by E3 transcripts are not necessarily required for virus replication; however, the E3 proteins are involved with evading the host immune system and related to AdV mediated pathogenesis. The E3 proteins (10.4K, 14.5K and 14.7K) inhibits TNF activity and ultimately prevents viral replication.
Figure 1.8  The replicative life-cycle of an adenovirus. The replication cycle of AdV begins with the attachment of the viral fiber protein to the CAR receptor. Subsequently, the interaction between the penton base and the integrins on the cell surface mediates internalization of the adenoviral particle through endocytosis. The virion (viron) is then transported to the nuclear pore complex and releases its DNA into the nucleus for initiation of a viral gene expression cascade, producing the early and late proteins. The newly synthesized viral DNA becomes encapsidated into the viral capsid in the nucleus and progeny viruses are released through cell lysis.
The E3-11.6K Ad “death protein” facilitates the release of viral particles and gp19K reduces MHC class I expression thus preventing the host immune response from eliminating the virus (Ginsberg et al. 1989). E2 transcripts encode for terminal protein, DNA polymerase and DNA-binding protein, all of which are involved in viral DNA replication. After the onset of viral DNA replication, the late genes are expressed by alternative splicing and polyadenylation of a large pre-mRNA generated at the single major late promoter. All late mRNA transcripts have a tripartite leader at the 5’ termini to enhance translation of viral structural proteins. The accumulation of viral structural proteins and newly synthesized viral DNA in the nucleus triggers virus assembly and virions are released from infected cells 2 to 3 days after infection.

1.12.4. AdV vectors

AdV vectors have provided an immense tool in gene transfer technology. Some of the advantages of using AdV include its high gene transfer efficiency, the ability to infect both dividing and non-dividing cells, transient expression with no concerns over viral DNA integration. AdV can package an additional 2 kb of their original size to a maximum of 38 kb without deleterious effects; however, issues arise in regards to gene insert fragment size and safety concerns with increasing gene insert sizes (Bett et al. 1993; Zhang 1999). Thus in order to increase the insert size, genes from within the Ad genome is deleted to necessitate the accommodation of large gene inserts. Different generations of vectors have been designed to allow for increased gene insert sizes, while at the same time reducing AdV antigenicity. One of the problems associated with Adv is the high prevalence of AdV within the normal population with majority of individuals already possessing neutralizing Abs and cellular responses. Figure 1.9 illustrates the
transcripts of the early and late proteins of AdV as well as depicting the differences between the AdV vector generations produced to date.

1.12.4.1. First generation vectors

First generation AdV have been the most widely studied and remains the most commonly used viral vector to date. These vectors are constructed by deleting E1 and/or E3 regions (Graham and Prevec 1992, 1995). This allows for an insert of 7.5 kb of foreign DNA up to a maximum of 8.2 kb size, based upon the capability of AdV to package 105% of its genome size (Bett et al. 1993; Bett et al. 1994). The deletion of E1 gene also abolishes the oncogenic potential and inhibits viral replication, resulting in a replication-deficient virus. In order for the replication deficient virus to replicate, complementing cell lines are required to provide the E1 proteins in trans. Some of the complementing cell lines developed include human embryonic kidney (HEK) 293 (Graham et al. 1977), 911 (Fallaux et al. 1996) and PER.C6 (Fallaux et al. 1998) cell lines. The latter two cell lines have shown the distinct advantage of reducing the probability of creating replication-competent Ad (RCA). RCA are generated when recombination occurs between the endogenous E1 gene from the complementing cell line and the E1 deficient vector (Zhang 1999).

AdV vectors are produced through three different methods: (i) the Stow method involving in vitro ligation to join AdV genome fragments; (ii) intracellular homologous recombination between partial viral genomes (Zhang 1999) and (iii) intracellular homologous recombination using plasmids. The cloning systems used include cotransfecting into 293 cells (Miyake et al. 1996; Shenk 1996), yeast artificial cloning systems (Ketner et al. 1994) and Escherichia coli (E. coli) bacterial cloning systems.
Figure 1.9 The transcripts from AdV and the different types of AdV vector. The arrowhead depicts the promoter region for the transcript while the arrows depict the transcripts produced. Early transcripts (E1-E4) are shown using thinner arrows and the late transcripts (L1-L5) are shown with thick arrowheads. The ITR are located at both the left and right ends of the genome (not shown).
(Chartier et al. 1996; Shenk 1996; Crouzet et al. 1997). Both the first and second methods mentioned are not commonly used due to the scarcity of unique restriction sites in the AdV genome, low efficiency of homologous recombination, cumbersome process of generating an Ad and increased contamination by RCA. The latter method is the most preferred method commonly used and involves the use of highly efficient homologous recombination systems such as Cre-lox recombination based methods and/or bacterial cloning systems (Danthinne and Imperiale 2000).

In general, recombination system utilizes two vectors to produce the AdV vector. The first vector contains a fair majority of the right side of the AdV genome and the right ITR, but lacks the E1 and E3 genes. This cosmid or “backbone” vector contains its own ori and selection marker but lacks the viral packaging signal rendering the expressed gene products unable to package itself. The second vector, referred to as the shuttle vector, contains the gene of interest flanked by two homology arms and a small portion of the left side of the AdV genome that contains the packaging signal and the left ITR. When the homologous recombination event occurs between the backbone vector and the shuttle vector, the resulting vector will contain the transgene as well as both ends of the ITR. The resulting vector is then transfected into the packaging cell line that eventually produce the replication deficient/defective AdV.

Resulting E1 and/or E3 deleted AdV vectors provide transient transgene expression in vivo, reaching expression peak levels at 3-4 days and lasting up to 2 weeks. These first generation AdV vectors have been utilized in various applications for cancer gene therapy encoding genes for cytokines, thymidine kinase (HSV-TK) and p53.

It was initially believed that the replication-deficient nature of the E1/E3 deleted virus would not allow transcription of any of the viral gene products; however, low
levels of viral transcripts are still produced and viral gene products can further stimulate an immune response (Imperiale and Kochanek 2004). Therefore, one of the major drawbacks or possibly an advantage to the use of first generation AdV vectors is its immunogenicity factor. These Ad have the tendency to elicit strong immune responses against viral proteins, by inducing neutralizing Ab against the vector capsid proteins and CTL against the transgene products and viral Ag (Yang et al. 1994; Yang et al. 1995b; Worgall et al. 1997). In addition, during the inflammatory phase, cytokines such as TNF-α are secreted that further limit the transgene expression from AdV. Development of these AdV responses potentially limits the number of applications the AdV could be given since the presence of both neutralizing Ab and CTL would limit entry of the AdV into cells and ultimately its expression in targeted cells.

1.12.4.2. Second generation vectors

Second generation AdV vectors were created by further deleting additional early genes such as E2 and E4 genes and further preventing viral replication. The deletion of these genes provides a further increase to the insert gene size and improvement in the transgene persistence. This will result in reduced inflammation (Armentano et al. 1995; Gao et al. 1996; Amalfitano et al. 1998). However, in some studies, second generation AdV still remained immunogenic as evidenced by detection of T cell responses (O'Neal et al. 1998; Xu et al. 2005) and reduced transgene expression (Andrews et al. 2001). As well, the immunogenicity of AdV have been question following the death of an 18 year old patient in 1999 given gene therapy treatment with an E1/E4 deleted Ad to correct a partial ornithine transcarbamoylase (OTC) deficiency (Marshall 1999; Raper et al. 2002; Raper et al. 2003). The patient’s death was a result of direct infusion of an extremely
high dose of $3.8 \times 10^{13}$ Ad particles into the hepatic artery that led to disseminated intravascular coagulation and multi-organ failure.

### 1.12.4.3. Third generation (gutted) vectors

A third generation of AdV vector, dubbed the gutted, gutless or helper dependent AdV, was developed allowing for further increase in the size of transgene insert and a further reduction in the AdV immunogenicity. Gutted AdV vectors were created by the removal of all AdV genes except for cis-regulatory elements necessary for replication and packaging (i.e. the ITR and the packaging signal). Inserts up to 36-37 kb in size are achievable and this vector allows for a variety of constructs to be created by including tissue-specific promoters and numerous expression cassettes, with the remainder filled in with non coding stuffer DNA to achieve the appropriate packaging size (Kochanek 1999). For viral replication of the gutted vector, an E1-deleted helper virus is coinfectected into 293 cells to provide the necessary viral genes \textit{in trans} to allow for viral replication and packaging (Volpers and Kochanek 2004). Studies have shown the use of gutted vectors generated greatly reduced toxicity thus immunogenicity and improved transgene expression in comparison to the first generation Ad, while allowing for stable transgene expression, detected beyond one year (Muruve et al. 2004).
2.0 HYPOTHESIS AND SPECIFIC AIMS

This thesis presents research data from two projects, broken into Parts A and B, both of which involve the use of DC and the development of a cancer vaccine for their respective tumor animal models.

2.1. Part A: Adenovirus mediated transfer of the rat neu gene into DC as a breast cancer vaccine

RATIONALE: Breast cancer affects one in nine women and HER-2/neu is commonly overexpressed in 30% of breast cancer cases. Overexpression of HER-2/neu is correlated with a rather poor prognosis and overall an aggressive disease for patients. Developing a therapy to prevent/protect individuals from breast cancer would benefit a large section of the human population, given that one in nine women will develop breast cancer. HER-2/neu is a suitable target for immunotherapy especially since the FDA approved in 1998 the clinical use of Herceptin, a human mAb targeting HER-2/neu, for metastatic breast cancer patients. Despite the positive clinical signs observed with Herceptin, there are limitations to this passive form of immunotherapy used and more active forms are being sought. Active forms of immunotherapy attempt to use the body’s own immune system to mount an effective antitumor immune response against HER-2/neu expressing tumors (Emens et al. 2005). DC represent powerful professional APC involved in initiation of an immune response. DC are easily loaded with tumor Ag by
various methods. Taken into consideration that most cancer vaccines to date involve the use of DC, either indirectly or directly, DC-based vaccines make complete sense. DC vaccines are very popular and are a common entity in the various types of cancer vaccines utilized within our lab. Harnessing the powerful capabilities of DC and specific TAA targeting allows efficient generation of antitumor immune responses and effective protection and prevention of tumors from forming. Taken together, the main focus in Part A of this thesis dissertation seeks to determine if DC transduced with a replication defective AdV encoding the full length rat neu gene (DC\textsubscript{neu}) would induce an immune response that provides protective and preventive effects from tumors forming in mouse animal models of breast cancer. We also sought to compare, in a side-by-side study, the efficacies between our DC\textsubscript{neu} vaccine and plasmid DNA vaccine encoding the full-length rat neu gene.

**HYPOTHESIS:** By transducing DC with a replication deficient AdV, the rat neu gene would be expressed within DC\textsubscript{neu} and be privileged for efficient Ag processing. This would result in the neu Ag peptide loaded onto both MHC class I and class II molecules to stimulate both CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells, respectively. This, in turn, would stimulate strong immune responses, mainly T cell responses and provide protective immunity in mice challenged with a neu expressing tumor cell line. It would also provide sufficient levels of immunity to prevent spontaneous tumor development in a Tg mouse model.

**Specific Aim One:** We wanted to examine the ability of DC\textsubscript{neu} to stimulate an immune response that provides effective protection against challenge with neu expressing tumor cells in various mouse animal models and to compare in a side by side
study the mechanisms involved with the antitumor immune mechanisms and overall
effectiveness of our DC\textsubscript{neu} vaccine with plasmid DNA-based vaccine. (Section 4.1)

Specific Aim Two: We wanted to utilize a xenogeneic approach with a DC-based vaccine to improve the immunogenicity and assist in overcoming the tolerance of neu in FVB\textsubscript{neuN} Tg mice. (Section 4.2)

2.2. Part B: Enhancement of T cell immunity induced by DC phagocytosis of HSP70-transfected tumor cells on antitumor immunity

RATIONALE: DC are powerful APC that function in initiation of antitumor immune responses by presenting TAA and stimulating tumor specific T cell responses. DC can acquire soluble Ag from living cells and even take up cells that are dying through apoptotic or necrotic pathways. Currently, controversy exists in the literature as to the effects apoptotic cells have on the maturation status of DC, with some reports indicating that apoptotic cells are better than necrotic cells, vice versa or no difference observed.

In Dr. Xiang’s lab, we have previously demonstrated that apoptotic tumor cells with secondary necrosis induced DC maturation. Furthermore, vaccination of DC that phagocytosed apoptotic tumor cells in the late phase of apoptosis efficiently stimulated antitumor immunity (Chen et al. 2001c). However, the potential critical role of HSP70 expression in this model was not investigated at that time. There are some reports indicating necrotic tumor cells expressing HSP70 can promote DC maturation where as apoptotic cells are not able to (Basu et al. 2000; Sauter et al. 2000). On the other hand, it is possible that the effects of dying tumor cells on the maturation and function of DC
may depend not only on the type of cell death (apoptosis versus necrosis), but also on
the phase of tumor cell apoptosis (early apoptosis versus late apoptosis) in relation to the
expression of HSP70.

**HYPOTHESIS:** We hypothesized that the expression of HSP70 in apoptotic tumor
cells and subsequently used for phagocytosis by DC *in vitro* would affect DC maturation
and efficiently induce tumor-specific CTL responses. This will reveal that the phase of
apoptosis relative to the expression of HSP70 would have an important effect. This in
turn would induce stronger antitumor immunity by protecting immunized mice from
tumor challenge.

**Specific Aim One:** We wanted to investigate the impact of apoptotic tumor cells
at different phases of apoptosis, in respect to the presence of cellular HSP70 expression
or lack thereof, on functional stimulation of DC maturation, T cell stimulatory activity
and vaccine efficiency in a mouse tumor model of cancer. (Section 4.3)
3.0 MATERIALS AND METHODS

3.1. Reagents and suppliers

The reagents used in the experiments presented in this thesis are listed in Table 3.1. All of the reagents used were molecular biology or research grade. Commercially available kits directly used in this study are listed in Table 3.2. The addresses of the companies where the reagents were obtained are shown in Table 3.3.

Table 3.1: List of reagents and suppliers

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<th>Reagent</th>
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<td>$[^{51}\text{Cr}]-\text{sodium chromate}$</td>
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| Table 3.2: Commercially available kits used in this study |

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<tr>
<th>Commercial Kits</th>
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<td>Annexin V FITC Apoptosis Detection kit</td>
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<tr>
<td>CellTiter 96 Nonradioactive cell proliferation assay</td>
<td>Promega</td>
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<tr>
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<td>Scheicher &amp; Schuell</td>
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<td>Qiagen</td>
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<tr>
<td>Endo-free Mega Kit</td>
<td>Qiagen</td>
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<tr>
<td>GenElute Agarose Spin column</td>
<td>Sigma</td>
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<td>OptEIA Mouse IL-4 ELISA kit</td>
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<td>OptEIA Mouse TNF-α Kit</td>
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<td>Quickchange site directed mutagenesis kit</td>
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<td>Superscript first strand synthesis for RT-PCR kit</td>
<td>Invitrogen Life Technologies</td>
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<tr>
<td>TMB Substrate Kit</td>
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<td>Companies</td>
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<td>Caltag</td>
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<td>Interscience</td>
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<td>Invitrogen Life Technologies</td>
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<td>Jackson ImmunoResearch</td>
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<td>Microbix</td>
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<td>Molecular Probes</td>
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<td>NEB</td>
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<td>DuPont NEN Research Products, Boston, MA, USA</td>
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<tr>
<td>Nunc</td>
<td>Nalgene Nunc International, Rochester NY, USA</td>
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<td>Oncogene Research Products</td>
<td>Oncogene Research, San Diego, CA, USA</td>
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<td>Pharmingen</td>
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<td>Stratagene</td>
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<td>USB</td>
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3.2. Antibodies

A list of the various antibodies used throughout the study and their respective suppliers are listed in Table 3.4. Abs were either FITC, phycoerythrin (PE), horse radish peroxidase (HRP) or biotin labeled.

Table 3.4: List of antibodies and respective suppliers

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<td>Anti-FcγII/III/CD16/32</td>
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<td>Anti-human HER-2</td>
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3.3. Cell lines

HEK 293 cells, containing the adenoviral E1 genes transformed into the cells, were purchased from Microbix (Toronto, ON) and grown in Minimum Essential Medium containing Earle’s Salts (EMEM; Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone). All of the media used also included 30 µg/ml gentamicin solution (Gibco). The Tg1-1 mouse breast cancer cell line (H-2K\(^q\)) (Chen et al. 1998), derived from a spontaneous breast tumor from FVBneuN Tg mouse was obtained from Dr. T. Kipps, University of California, San Diego, CA. Another mouse mammary tumor cell line, CaD1, was obtained from Frederick Cancer Research and Development Center (Frederick, MD). MCA-26, a mouse colon cancer cell line, was obtained from American Type Culture Collection (ATCC). These cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco) supplemented with 10% FBS and gentamicin solution. The cell lines, EL4, a T cell lymphoma, and its ovalbumin (OVA) gene-transfected cell line, EG7, are from a C57BL/6 (H-2K\(^b\)) origin and obtained from ATCC. The B cell lymphoma cell line, A20, originating from BALB/c mice (H-2K\(^d\)), was also obtained from ATCC. These cells were maintained in DMEM, while EG7 was maintained in DMEM containing 0.5 mg/ml G418 (Gibco). The human breast cancer cell line, T47-D, was obtained from ATCC and grown in DMEM with 10% FBS and containing 8 µg/ml bovine insulin (Sigma).

In most cases, the cell lines were passaged using Trypsin/ethylenediamine tetracetic acid (EDTA; Gibco) whereas 293 cells were passaged using a 1X citric saline solution [10% (w/v) KCl and 4.4% (w/v) sodium citrate]. All cell lines were cultured in a humidified CO\(_2\) incubator with a 5 % CO\(_2\) atmosphere and at 37ºC. Cell counting was performed using Trypan-Blue stain (Gibco) and counted on a haemocytometer.
3.4. Animals

Wild-type female FVB/N (H-2Kq) and Tg FVBneuN (H-2Kq) [FVB/N-TgN(MMTVneu)202Mul mice] were obtained from Jackson Laboratories (Bar Harbor, ME). Female BALB/c (H-2Kd) and C57BL/6 (H-2Kb) mice were obtained from the University of Saskatchewan Animal Resource Centre and Charles River Laboratories (St. Laurent, Quebec, Canada), respectively. Ovalbumin (OVA)-specific T cell receptor (TCR)-Tg OT I mice developed on a C57BL/6 background were obtained from Jackson Laboratories. All mice were approximately 4-6 weeks old and housed in the animal facility at the Saskatoon Cancer Centre. All animal experiments were approved by the University Committee on Animal Care and Supply committee in accordance with the Canadian Council for Animal Care guidelines.

3.5. Bacterial cells

DH5α Escherichia coli (E. coli) cells were routinely used as the bacterial host for vector propagation. E. coli BJ5183 cells were obtained from Stratagene and used only for homologous recombination in the construction of AdV vectors. Both of these cells were grown at 37°C in a shaking incubator in a flask containing Lauria-Bertani (LB) broth consisting of 1.0% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Difco) and 1.0% (w/v) NaCl supplemented with the appropriate antibiotic such as ampicillin (100µg/ml) or kanamycin (50µg/ml), depending on the vector. For isolation of transformed bacterial cells, the cells were plated onto selective LB-agar plates, containing LB broth with 1.5% (w/v) agar supplemented with the appropriate antibiotic, and incubated inverted at 37°C overnight.
3.6. **General molecular biology techniques**

The routine molecular biology protocols used in this study were based upon the methods described in Molecular Cloning: A Laboratory Manual by Sambrook *et al* and Sambrook and Russel (Sambrook et al. 1989; Sambrook and Russell 2001).

3.6.1. **Restriction enzyme digest**

Restriction enzymes used in this study were obtained from either Amersham Biosciences or New England Biolabs. The restriction enzyme digests were performed on at least 1µg DNA in 1X the final recommended buffer suggested by the manufacturer and using at least 1 unit of the specified enzymes. For complete restriction enzyme digestion, the reactions were incubated at 37°C for 1 hour.

3.6.2. **Generating blunt ends from recessed or protruding 3’ overhangs**

Recessed 3’ overhangs produced by restriction enzyme digest were extended to produce blunt ends. Recessed ends were extended by adding 1µl of 10 mM dNTP mix and 8 units of DNA polymerase I Klenow fragment (Invitrogen) in 1X Nick Translation buffer [50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 1 mM dithiothreitol (DTT)]. The sample was incubated at room temperature for 30 min. The reaction was terminated by the addition of 3 µl 0.5 M EDTA and precipitated.

3.6.3. **Removal of 5’ terminal phosphate group**

Dephosphorylation of the 5’ phosphate group was accomplished using calf intestinal alkaline phosphatase (New England Biolabs) added to digested DNA
resuspended in 10 mM NaCl, 5 mM Tris-HCl, 1 mM MgCl₂ 0.1 mM DTT and incubated at 37°C for 1 hour. DNA was further purified with either gel purification kits or using phenol:chloroform:isoamyl alcohol extraction.

3.6.4. Agarose gel electrophoresis

Agarose gels were cast using a GelHorizon 58 Apparatus of varying agarose concentrations ranging from 0.7% to 2% in TAE buffer [40 mM Tris-acetate, 1 mM EDTA containing 1 µg/ml ethidium bromide (EtBR; Sigma)]. DNA samples were loaded onto the gels with gel loading buffer [0.042% (w/v) bromophenol blue, 6.67% (w/v) sucrose] along with the DNA markers, λDNA/Hind III and φX174/Hae III fragment markers (Invitrogen). Gel electrophoresis was performed between 90 to 110 V in TAE buffer for varying times to obtain optimal resolution. Gels were visualized by UV illumination on a gel documentation system (Bio-Rad).

3.6.5. Purifying linear DNA fragments

Linear DNA fragments for further downstream applications were isolated from DNA agarose gels and purified using the GenElute Agarose spin columns (Sigma). On certain occasions, based upon the fragment size or the amount of sample, electroelution was performed to elute the DNA from the gel. The electroeluted DNA sample was further purified and concentrated using Elutip-D columns (Schleicher & Schuell) according to manufacturer’s protocol, using 200 mM NaCl, 20 mM Tris-HCl and 1.0 mM EDTA, pH 7.4.
3.6.6. Site directed mutagenesis

Site directed mutagenesis was done accordingly to the protocol set out in the Quikchange Site Directed Mutagenesis kit (Stratagene) using complimentary primers that span the mutation region of interest and the thermostable, proof-reading enzyme, PfuTurbo polymerase (Stratagene).

3.6.7. Ligation

DNA ligation was performed using 50 ng of vector DNA and at least 200 ng of purified, insert DNA in a 10 µl volume containing 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM ATP and 1-5 unit T4 DNA ligase (USB). Ligations were performed overnight in a 14°C water bath.

3.6.8. Sequencing

Automated dideoxy sequencing was performed by Annette Kerviche at the Saskatchewan Cancer Agency or at the sequencing facilities at the Plant Biotechnology Institute of the National Research Council of Canada in Saskatoon, Canada.

3.6.9. Competent cells and transformation

3.6.9.1. Standard competent cells and transformation

Competent cells was prepared based upon a modified version of the original method described by Cohen et al 1972 (Cohen et al. 1972; Sambrook et al. 1989). Briefly, an overnight culture of bacterial cells were grown in LB medium until mid log-phase growth with an OD₆₀₀ reading between 0.4-0.6 then spun down. The cell pellet was washed twice, using 50 mM CaCl₂ 10 mM Tris-HCl (pH 8.0) then finally
resuspended in the same solution. Transformations were performed using 100 µl of competent cells with the DNA mixture and incubated on ice for 30 min. The sample was heat shocked by quickly placing the sample at 42°C for 50 sec then returned to ice for several minutes. SOC media [2% (w/v) bacto-tryptone, 0.5% (w/v) yeast-extract, 0.05% (w/v) NaCl, 20 mM glucose] was added to the sample then incubated at 37°C to allow the bacteria to recover before plating the cells on selective LB-agar plates. When blue-white colony screening was required, selective LB-agar plates containing 50 µg/ml X-gal (Gibco) and 0.1 mM isopropylthio-β-D-galactoside (IPTG; Gibco) were used.

3.6.9.2. Electrocompetent and electrotransformation

Electrocompetent cells were prepared using the method described previously by Dower et al (Dower et al. 1988; Sambrook and Russell 2001). The bacterial cells were grown overnight in selective LB broth then subcultured into fresh media the following morning. The fresh culture was grown until mid-log phase was reached, as evidenced when the culture reached the specified OD_{600} previously determined. Bacterial cells were chilled then centrifuged at 1,000 x g in a J10 rotor (Beckman). The sample was washed twice and resuspended in a 10% (v/v) glycerol in water solution then stored at -80°C until required. Bacterial cells were then placed into chilled electroporation cuvettes (0.2 cm gap; Bio-Rad) with the DNA and electroporated using the following conditions: 200 Ω (ohms), 2.5 kV and 25 µFD using a Gene Pulser with Pulse Controller (Bio-Rad). LB media was added to the sample and incubated at 37°C for one hour to allow bacteria to recover prior to plating the culture onto selective LB-agar plates with the appropriate antibiotic.
3.6.10. Isolation of plasmid DNA from host bacterial cells

3.6.10.1. Small scale (mini-preps)

To screen colonies after transformation for positive recombinants, small scale preparations using an alkali lysis method was performed. Briefly, single isolated colonies were selected and cultured overnight in LB containing the appropriate antibiotic. The overnight cultured cells were resuspended in 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), 2 mg/ml lysozyme (Sigma) then lysed with a 200 mM NaOH, 1% sodium dodecyl sulfate (SDS) solution followed by the addition of 3M sodium acetate, pH 5.2. This precipitated out the bacteria chromosomal DNA, cellular protein and debris. The supernatant was then extracted twice with phenol:chloroform:isoamyl alcohol followed by ethanol precipitation. The precipitated DNA was resuspended in TE (pH 8.0) containing 40µg/ml RNAse A. DNA concentration was measured by standard \(A_{260}/A_{280}\) spectrophotometric readings and visualizing on agarose gels.

3.6.10.2. Large scale (large-prep)

Bacterial cells were grown overnight in Terrific broth (TB) [1.2% (w/v) bacto-tryptone, 2.4% (w/v) yeast-extract, 0.4% (v/v) glycerol, 17 mM KH\(_2\)PO\(_4\) and 72 mM K\(_2\)HPO\(_4\)] in a shaking incubator at 37°C. The sample was treated using the same solutions used for small preps but also included a polyethylene glycol (PEG) purification step. The purified DNA was resuspended in TE (pH 8.0) or in sterile PBS for use in transfections and the concentration determined using methods mentioned above.
3.6.10.3. **Endotoxin free preparation**

Endotoxin free preparations of vector DNA, for use in plasmid DNA immunizations, were done using Endofree plasmid Mega and Giga kits (Qiagen). The manufacturer’s protocol was followed with the bacterial cells grown in LB broth, alkaline lysed, removal of endotoxin (LPS) and isopropanol precipitation of plasmid DNA. The concentration of isolated plasmid DNA was determined with standard $A_{260}/A_{280}$ spectrophotometric readings. Plasmid DNA was resuspended in sterile PBS at a final concentration of 1 mg/ml and stored at –20°C until use.

3.6.11. **RNA isolation**

RNA was isolated from freshly isolated tissue culture samples using the RNeasy mini kit (Qiagen), according to the manufacturer’s suggested protocol. Isolated RNA was resuspended in RNase free distilled water with the concentration and purity determined by standard $A_{260}/A_{280}$ spectrophotometric readings. Samples were stored at -80°C until needed.

3.6.12. **Reverse transcriptase PCR (RT-PCR) and cDNA synthesis**

Isolated RNA was used as a template for cDNA synthesis using the SuperScript First-strand Synthesis System for RT-PCR (Invitrogen), according to the instructions provided by the manufacturer. Briefly, 5µg of RNA was incubated in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl$_2$, 10 mM DTT, 0.5 mM of each dNTP, 0.5 µg Oligo(dT)$_{12-18}$, 40 units of RNase inhibitor and 50 units SuperScript II reverse transcriptase enzyme, for a final volume of 20 µl. The sample was incubated at 42°C for 50 min followed by 70°C for 15 min to terminate the reaction. The remaining RNA was
removed by further treating the sample with 1 unit of RNase H. Samples were used either immediately for PCR reaction or stored at -20°C until needed.

3.6.13. PCR reactions

Typical PCR reactions were performed using a predetermined amount of template DNA/cDNA in a final volume of 100 µl containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 1.5 mM MgCl₂, 1 mM dNTP mix, 10 ng of each primer and 2.5 units of Taq polymerase (Invitrogen). The typical cycles used in PCR reactions include the initial DNA denaturing step at 94°C for 5 min followed by 25-30 amplification cycles at 94°C for 1 min, 56-58°C for 1 min and 72°C for 1 min followed with a final termination step for 10 min at 72°C. After PCR amplification, one-tenth of the reaction volume was resolved by standard agarose gel electrophoresis. Water was used as the template to serve as the negative controls. For RT-PCR samples, a control reaction detecting the levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using the forward primer, 5’-CAGGTTGTCTCCTGCGACTT-3’, and the reverse primer, 5’-CTTGCTCAGTGTCCTTGCTG-3’.

3.7. Expression vector construction

3.7.1. Expression vectors

The psV2-neu vector, containing the full-length rat neu gene, was obtained from Dr. R. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). Vectors pUC18 and pcDNA3.1 (both + and -) were readily available in Dr. Xiang’s lab. The vectors, pCR2.1 and pCR-Blunt, from the TA cloning kit (Invitrogen) and Zero Blunt PCR cloning kit (Invitrogen), were also present in the lab.
3.7.1.1. Construction of the rat neu expression vector

The pSV2-neu vector was digested with Hind III and Sal I, liberating the 4.6 kb full length rat neu gene fragment and gel-purified. This fragment was ligated into the Hind III/Sal I cut pUC18 vector forming the vector, pUC18/neu. Next, pUC18/neu was digested with Hind III and Kpn I and the resulting 4.7 kb fragment was gel purified. This fragment was then ligated into the Hind III/Kpn I digested pCDNA3.1(+) vector, resulting in pcDNA-neu (Figure 3.1A). The vector construct was verified by restriction digest analysis.

3.7.1.2. Construction of the HER-2 expression vector

The cDNA for the full-length human HER-2 gene was cloned by RT-PCR using a cDNA library obtained from the human breast cancer cell line, T47-D, known to express HER-2. The primers used for this were the forward primer 5’-CAGTGAGCAACCATGGAGCTGG-3’ and the reverse primer 5’-TCCACAAAGACTCCCCAGTC-3’. To minimize the number of mutations incorporated based upon the polymerase used, the PCR amplification step was performed using a high fidelity polymerase, Platinum Pfx DNA Polymerase (Invitrogen), according to the manufacturer’s protocol. The resulting PCR product was resolved on an agarose gel and the 4 kb fragment was gel purified for ligation into the blunt ended pCR-Blunt vector. This ligation created the vector pCR-Blunt-HER2. Automated dideoxy nucleotide sequencing, using the M13 Reverse primer, T7 promoter primer and various primers spanning the full length HER-2 gene, was performed to ensure the product contained no mutations induced by the polymerase and to determine
Figure 3.1 Physical maps of expression vectors used. The expression vectors used include pcDNAneu (A), pcDNA-HER2mut (B) and pCDNA-HSP70 (C).
the gene orientation within the vector.

To address safety concerns with using HER-2, we mutated codon 753 from AAA to GCA, which changed the residue from a lysine to an alanine. This mutation occurred in the cytoplasmic domain in a critical area that prevents ATP from binding (Ben-Levy et al. 1994; Xu et al. 2001). The QuikChange Site Directed Mutagenesis kit (Stratagene) was used to perform site-directed mutagenesis, according to the manufacturer’s protocol using pCR-Blunt-HER2 as the template DNA. Complimentary primers 5’-CCAGTGCCATCGCAAGTGAGGAAGC-3’ and 5’-GGATGTGTTTTCCCTCAACACTGCGATGGCCACCTGG-3’, where the bolded underlined sequence represents the incorporation of the mutation, were used. Sequence analysis was done to verify the presence of the directed mutation within the gene. The resulting vector containing the mutation was renamed as pCR-Blunt-HER2mut. Next, pCR-Blunt-HER2mut was digested with the restriction enzymes Hind III and Xba I, resulting in the release of a 4.1 kb fragment that was gel purified and used in a subsequent ligation into Hind III/Xba I digested pcDNA3.1(-) vector creating pCDNA-HER2mut (Figure 3.1B).

3.7.1.3.Construction of the HSP70 expression vector.

Total RNA was extracted from EG7 tumor cells using RNeasy Mini Kit (Qiagen). A 2.0–kb cDNA fragment coding for the open reading frame of HSP70 molecule was cloned by RT-PCR from a cDNA library of EG7 cells using PFU polymerase and both the sense primer 5’- AGCCTTCCAGAAGCAGAGCG-3’ and the antisense primer 5’-GCAGCTATCAAGTGCAAAGAGTCTG-3’). The cloned cDNA fragment of HSP70 was ligated into the pCR2.1 vector, to form pCR2.1/HSP70. The HSP70 gene sequence
was verified by dideoxy nucleotide sequencing method. The HSP70 gene fragment
(\textit{Eco}R V/Bam H1) was digested and cloned into the plasmid pcDNA3.1, forming the
expression vector pcDNA-HSP70 (Figure 3.1C).

3.8. Adenovirus vectors construction

AdV\textsubscript{pLpA}, containing no transgene, was previously created and readily available
in Dr. Xiang’s lab (Wright et al. 1999). Both AdV\textsubscript{neu} and AdV\textsubscript{HER2mut} were created using
the AdEasy system, incorporating the use of efficient homologous recombination events
in bacterial cells (He et al. 1998). The vectors pShuttleCMV and pAdEasy-1 were
obtained from Dr. Lixin Zhang (John Hopkins/Sidney Kimmel) and Stratagene, respectively.

3.8.1. AdV\textsubscript{neu}

The vector, pcDNA-neu, was digested with \textit{Hind} III and \textit{Eco}R V releasing a 4.7
kb fragment containing the rat neu gene. This fragment was directionally cloned into
\textit{Hind} III/\textit{Eco}R V digested pShuttleCMV, creating pShuttleCMV-neu (Figure 3.2). The
DNA vectors was purified using Qiagen columns then subsequently used for digest with
\textit{Pme} I to linearize the vector. The linearized vector was treated with alkaline
phosphatase and gel purified followed by ethanol precipitation. The DNA was spun
down, dried and resuspended in distilled water. A total of 1 µg of the \textit{Pme} I-linearized
pShuttleCMV-neu and 100 ng of pAdEasy-1 (Figure 3.2) were used in the
cotransformation of BJ5183 \textit{E. coli} competent cells via electrotransformation (as
outlined in Section 3.6.9.2). The transformed cells were plated on selective LB-agar
plates containing 100 µg/ml kanamycin overnight inverted. Several small colonies were
Figure 3.2 Physical maps of vectors used in creating recombinant adenovirus. The vector, pShuttleCMV-neu (A) and pAdEasy-1 (B) underwent homologous recombination to create AdV<sub>neu</sub>. The vectors pShuttleCMV-HER2<sub>mut</sub> (C) was recombined with pAdEasy-1 (B) to create AdV<sub>HER2mut</sub>. 
selected and grown in LB broth with 100 µg/ml kanamycin overnight. Mini-preps were performed to isolate the vector DNA from bacteria and the DNA was screened using restriction enzyme analysis then analyzed on a 0.7% agarose gel. This allowed for positive selection of the recombinant vector. The recombinant vector, pAdEasy-neu, was further transformed into DH5α host bacterial cells using standard transformation methods. This step allowed for further plasmid amplification and purification of the viral vector of approximately 40 kb in size.

After purifying the plasmid DNA using plasmid mini kits (Qiagen), pAdEasy-neu was digested with Pac I, releasing a small fragment consisting of the kanamycin resistance gene and the ori. The complete Pac I digest was confirmed by electrophoreses on a 0.7% agarose gel electrophoresis and the reaction cleaned up using a phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by ethanol precipitation. A total of 5 µg of Pac I-digested pAdEasy-neu DNA was resuspended in sterile water for use in 293 cells transfection using Lipofectamine, according to manufacturer’s protocol, to produce AdV_neu. An outline of the steps involved in creating the adenovirus using the AdEasy system is illustrated in Figure 3.3. After transfection, the flasks were monitored for 7-10 days for the formation of plaques. Cells were harvested when cytopathic effects (CPE) were apparent and extensive in the flask. After the cells were harvested, the remaining cell pellet was resuspended in serum free EMEM with five rounds of freeze/thaws performed at -80°C and 37°C, respectively, to prepare a crude viral lysate.
**Figure 3.3** Schematic overview of the AdEasy system. The shuttle vector containing the neu gene, pShuttle-CMV-neu, is linearized with Pme I restriction enzyme and the backbone vector, pAdEasy-1, are co-transformed into BJ5183 *E. coli* cells to allow homologous recombination between the two vectors, resulting in pAdEasy-neu. The resulting pAdEasy-neu was then transfected into 293 cells for viral production of the replication-deficient recombinant adenovirus, AdV\textsubscript{neu}. 

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**Step 1** - Clone neu gene into shuttle vector

**Step 2** – Homologous Recombination in BJ5183 *E. coli* cells

**Step 3** - Transfect linearized vector into 293 cells for virus production
3.8.2. AdVHER2mut

A similar method used in the creation of AdV neu was used for the creation of AdVHER2mut. The gel purified Not I/Hind III full length, mutated HER-2 gene fragment was obtained from the digest of pCR-Blunt-HER2mut and ligated into the Not I/Hind III-digested pShuttleCMV vector, forming pShuttleCMV-HER2mut (Figure 3-2). This vector was then linearized with Pme I and gel purified. A modification was made to the bacterial cotransformation step, whereby the Pme I-linearized pShuttleCMV-HER2mut was used to transform electrocompetent BAD cells instead of BJ5183 E. coli cells. The only difference between BAD and BJ5183 cells was that BAD cells were simply BJ5183 cells already containing the pAdEasy-1 vector (Zeng et al. 2001). This method increased the recombinant efficiency between the backbone vector (pAdEasy-1) and the shuttle vector. Isolated recombinants were renamed as pAdEasy-HER2mut then transformed into DH5α cells for further plasmid amplification and purification. After plasmid purification, pAdEasy-HER2mut was subsequently digested with Pac I, purified and transfected into 293 cells, as described previously, to produce the adenovirus, AdVHER2mut.

3.9. Adenovirus amplification and purification

3.9.1. Amplification

Infected cell culture pellets were harvested when extensive CPE was observed. Cell pellets were resuspended in serum-free EMEM and subjected to five rounds of freeze-thaw cycles at -80°C and 37°C, respectively. The sample was spun down and the supernatant was used as the virus seed to further infect additional flasks of 293 cells.
During each amplification step, additional flasks of 293 cells were used, with the final amplification step using a total of 36 T175cm² flasks.

3.9.2. Purification

After harvesting the infected cells from the last amplification step, cells were subjected to five rounds of freeze-thaw cycles. The sample was then spun down at 9,500 x g in a JA-17 rotor for 10 min then the supernatant was gently layered on top of a cesium chloride (CsCl) discontinuous gradient using Quick-Seal Centrifuge tubes for ultracentrifugation. The discontinuous gradient consisted of a layer of 1.25 gm/ml CsCl layered gently over the 1.40 gm/ml CsCl layer. All of the CsCl solutions were prepared using 1X TD buffer consisting of 140 mM NaCl, 5 mM KCl, 25 mM Tris and 0.7 mM Na₂HPO₄. Samples were then spun in a Beckman ultracentrifuge for 2 hours at 142,000 x g (45,000 rpm) using a Type 80 Ti rotor at 20°C. The opalescent band was collected then placed onto a 1.34 gm/ml CsCl continuous gradient and spun at 142,000 x g for 18 hours at 20°C. After which, the viral band was carefully aspirated and placed into a Slide-A-lyzer dialyzing cassette (Pierce) and dialyzed in 10 mM Tris-HCl pH 7.4, 1mM MgCl₂, buffer solution for several hours at 4°C involving several buffer changes to remove the cesium chloride. Afterwards, glycerol was added for a final concentration of 10% (v/v) glycerol to the sample. The viral concentration was determined spectrophotometrically at A₂₆₀, with an optical density (O.D.) unit of 1 equivalent to 10¹⁰ plaque forming units (PFU)/ml (Xiang and Wu 2003). Purified AdVs were stored at -80°C until required.
3.10. Adenovirus PCR

To determine the presence of the gene insert within the purified AdV product, viral samples were first treated with Proteinase K (Invitrogen) for 1 hour at 56°C followed by heat inactivation at 95°C for 5 min. Sources of the DNA template included infected cell pellet, culture media or the purified AdV. The PCR reaction was performed using 4µl of the DNA template, 1X PCR buffer [20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 1 mM dNTP (0.25 mM each of dATP, dCTP, dGTP, dTTP), 1 µg/µl each primer and 2.5 units Taq DNA polymerase in a final volume of 100 µl. Water was used as a negative control and the respective pShuttleCMV vectors or pAdEasy-recombinants were used as positive controls.

The neu specific primers used include the forward primer 5’-AGATTGCCAAGGGGATGAGC-3’ and the reverse primer 5’CTGGATGACCACAAACGCTG-3’, resulting in a 488 bp product. The HER-2 specific primers were the forward primer 5’CTCAGTGACCTGTTTTGGACCG-3’ and reverse primer 5’-TCCACAAAAGACTCCCCAGTGC-3’, resulting in a 2.3 kb (2277 bp) product. The cycling conditions for PCR reactions included the initial denaturing step at 94°C for 5 min followed by two amplification cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 1 min. Another two additional amplification cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min followed by the remaining 30 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min then a final incubation at 72°C for 10 min. In the case of HER-2, the extension times at 72°C were performed for 2.5 min to obtain the expected fragment size, estimating Taq polymerase activity at 1 kb/min. Following the PCR amplification, 10µl of the PCR products were resolved by standard agarose gel electrophoresis.
3.11. Transfections

3.11.1. Electroporation

MCA-26 cell were transfected with the expression vector pcDNAneu, as previously described (Chen et al. 2002c). Briefly, twenty million MCA-26 cells were resuspended in 0.7 ml PBS and mixed with 0.3 ml PBS containing 10 µg pcDNA-neu or pcDNA-HSP70 DNA. Tumor cells were transfected via electroporation using a Gene Pulser (BioRad, Richmond, CA) with parameters of 300 V and 125 µFD capacitance in an electroporation cuvette with a 0.4 cm gap (BioRad). Transfected cells were selected for growth in medium containing 4 mg/ml G418. Selected clones, MCA-26neu and MCA-26-HSP70 (MCA-HSP70), were maintained in growth medium containing 0.5 mg/ml G418 and expression analyzed by flow cytometry and Western blotting.

3.11.2. Liposomes

The liposome-based method was used for transfection of recombinant adenoviral plasmid vectors created using the AdEasy system into 293 cells to produce the resulting AdV. The day before transfection, 293 cells were re-plated at a cell density at 2x10^6 cells per T25cm² flask. For transfections, 5 µg of the adenoviral vector plasmid (pAdEasy-neu and pAdEasy-HER2mut) was mixed with 20 µl of Lipofectamine (Invitrogen) in serum-free EMEM media and incubated at room temperature for 30 min. The DNA:liposomes complexes were added to the flasks and incubated for an additional 4 hours, prior to completely changing the media with EMEM with 10% FCS. These
flasks were continually monitored by microscopy for any signs of CPE. The growth medium was refreshed as required. Generally, the cells were harvested 7-10 days after transfection and used to prepare the initial primary viral stocks.

3.12. RNase protection assays

DC were subjected to RNase protection assays (RPA) (Liu et al. 2003) using the Riboquant Multi-Probe kit (Pharmingen), in accordance with manufacturer’s instructions. The RNA was extracted from the cells using RNeasy mini kits (Qiagen) and spectrophotometrically measured using standard $A_{260}/A_{280}$ readings. The cytokine/chemokine-related multi-probe template sets (Pharmingen) were radiolabeled using $[\alpha-^{33}P]$-uridine 5’ triphosphate (UTP; Amersham), generated through an *in vitro* transcription reaction using T7 RNA polymerase. Labeled probes were first purified by phenol-chloroform extraction and ethanol precipitated with their counts adjusted to $3 \times 10^5$ cpm/µl followed by hybridization to RNA samples (5 µg each). Reaction samples were subsequently digested with RNase, followed by Proteinase K treatment and phenol-chloroform extraction. Following ethanol precipitation, the protected samples were resuspended in 1x loading buffer and separated on 5.7% acrylamide-bisacrylamide TBE-urea gels (Invitrogen) with TBE (100 mM Tris, 90 mM boric acid, 1 mM EDTA) buffer. Gels were dried and exposed to a phosphorimager screen and by routine autoradiography. Results were visualized and the relative expressions of cytokine and chemokine encoding mRNA were quantified with Quantity One software (Bio-Rad, Hercules, CA).
3.13. Western blotting

Neu expression in Tg1-1, MCA-26neu and DC_{neu} cells was assessed using Western blots. Briefly, cell protein extracts were prepared from these cells using extraction buffer containing 125 mM Tris, 0.05% SDS and 10% β-ME and harvested from the supernatant after centrifugation at 1000 x g for 5 min. The supernatants containing the protein samples were boiled and electrophoresed through 7.5% polyacrylamide gels. The gel was transferred onto nitrocellulose papers (Schleicher & Schull) using 1X transfer buffer [25 mM Tris, 190 mM glycine, 0.05% SDS, 20% (v/v) methanol]. The blots were blocked using PBS containing 5% non-fat milk and incubated with the anti-neu Ab (Ab-3; Oncogene, 5 µg/ml) followed by horseradish peroxidase-conjugated goat anti-mouse IgG Ab (Jackson ImmunoRes), as previously described (Chen et al. 2002c). Detection of HER-2/neu was accomplished by using enhanced chemiluminescence reagent (New England Nuclear Life Science Products, Boston, Mass.) and exposed to Hyper-ECL film (Amersham).

3.14. Bone marrow-derived DC

Bone marrow (BM)-derived DC were prepared as described previously (Chen et al. 2002c), based upon a modified protocol described by Inaba et al. (Inaba et al. 1992; Lutz et al. 1999). Briefly, BM cells prepared from the femora and tibiae of mice were depleted of red blood cells with 0.84% Tris-ammonium chloride and plated in DC culture medium [DMEM plus 10% FCS, GM-CSF (20 ng/ml; R&D System) and IL-4 (20 ng/ml; R&D System)]. Nonadherent granulocytes, T and B cells were gently removed and fresh media were added on the third day. Two days later, loosely adherent
proliferating DC aggregates were dislodged and replated. Nonadherent cells were harvested on the sixth day. DCs generated in this manner displayed the typical morphologic features of DC (i.e., numerous dendritic processes) and used for phenotypic analysis, AdV transductions or co-cultivation with apoptotic cells.

3.15. DC transduction with AdV

DCs were transduced with the AdV, AdVpLpA, AdV_neu or AdV_HER2mut, at an optimal multiplicity of infection (MOI) of 100, as previously described (Chen et al. 2002c). Transduced DCs were termed DC_pLpA, DC_neu, and DC_HER2mut, respectively. To enact this, day 6 cultured DC were harvested and following viral adsorption for 1 h at 37°C in DMEM in 6-well culture plates, DC culture medium was replaced with DMEM containing 20% FCS. The transduced cells were incubated for an additional 24-36 h at 37°C then harvested for phenotypic analysis by flow cytometry, Western blot or used for immunizing mice.

3.16. Peptides

Peptides were synthesized, purified by high-performance liquid chromatography, resulting in >95% purity and validated by mass spectrometry. These peptides include the neu peptide PDSLRLDSLVF, that spans aa 420-429, and an irrelevant peptide, RPQASGVYM from LCM N protein (Ercolini et al. 2003). The OVA I peptide is SIINFEKL spanning aa 257-264 of OVA (Li et al. 2001). These peptides were synthesized and purified by Multiple Peptide Systems (San Diego, CA) or at the Peptide Core Synthesis Facility at the University of Calgary (Calgary, AB, Canada).
3.17. General immunology techniques

3.17.1. Enzyme linked absorbance assay (ELISA)

3.17.1.1. Cytokine ELISA

The cytokine ELISA were performed using mouse-cytokine specific ELISA kits (BD OptEIA-IL-4, IL-6, TNF-α and INF-γ) from Pharmingen, according to manufacturer’s instructions. Briefly, the capture antibody was diluted in coating buffer [0.1 M sodium carbonate] and added into Maxisorp wells (Nunc) for overnight incubation. The wells were washed using PBS with 0.05% (v/v) Tween-20 and plates were blocked using 3% BSA in PBS at room temperature for one hour followed by several washes. A volume of 100 µl of the serially diluted standards, prepared according to manufacturer’s protocol, and cell culture supernatants, from either DC or LN T cells, were added to the plate and incubated at room temperature for 2 hours. Next, the plate was washed several times then incubated with both the detection antibody and streptavidin-horseradish peroxidase (HRP) conjugate. After incubating for an hour, the plate was washed a total of 7 times then 3,3’,5,5’ tetramethylbenzidine (TMB) substrate (TMB substrate reagent set; Pharmingen) was added to each well. The reaction was stopped after a 30 min incubation period by the addition of 2N H₂SO₄. The plates were then read on a Bio-Rad microplate reader at a wavelength of 450 nm.

3.17.1.2. Indirect Cell-based ELISA

An indirect cell-based ELISA was performed to identify the presence and subtypes of tumor-specific antibodies, as described previously (Xiang et al. 1996).
Briefly, 1x10^5 Tg1-1 cells were plated into 96 well plates and incubated overnight to allow for cell attachment to the plate then fixed by adding 0.2% (v/v) glutaraldehyde solution (Sigma) to the wells and incubating the plate at 4°C for 15 min. Following this, the wells were washed three times with PBS with 0.05% Tween-20 then blocked with 3% BSA (w/v) in PBS. Serum samples from immunized mice diluted 1:20 and four-fold afterwards in PBS were added and incubated for 1 hour at 37°C. Following this, the plate was washed with PBS with 0.05% (v/v) Tween-20 and incubated with either biotin conjugated goat anti-mouse IgG1 and IgG2a antibody for another hour followed by additional washes and the addition of horseradish peroxidase (HRP) conjugated-streptavidin to the plate. TMB substrate was added to each well and incubated for 30 min before the addition of 2N H_2SO_4. Developed plates were read on a microplate reader at a wavelength of 450 nm.

3.17.2. Lymphocyte purification

3.17.2.1. Nylon wool columns

The spleen from naïve and immunized mice were harvested aseptically and a single cell suspension was prepared. The red blood cells were lysed using 0.84% Tris-ammonium chloride and resuspended in DMEM plus 10% FCS. The cell suspension was loaded onto pre-incubated nylon wool columns (Interscience) and further incubated for 1 hour at 37°C to allow the B cells to adhere to the nylon wool. Next, DMEM media was added to flush out the T cells from the column, resulting in an enriched population of T lymphocytes.
3.17.2.2. Magnetic bead separation

To further isolate a specific population of T lymphocytes, magnetic bead separation (Dynal) was employed, in accordance to the manufacturer’s protocol. Briefly, the isolated T cells were resuspended in 0.1% BSA in PBS and mixed with Dynabeads Mouse CD4 (L3T4) paramagnetic beads for negative selection or a positive selection of CD25⁺ T cells using anti-CD25 microbeads. The sample was incubated at 4°C for 30 min with gentle tilting and rotation on a nutator then placed in the magnet apparatus to separate the magnetically bound cells from unbound cells. In negative selection, the supernatant was removed containing the cell of interest whereas positive selection required further washing of the bound beads to release the cells.

3.17.3. Cytotoxicity assay

3.17.3.1. In vitro cytotoxicity assay

CTL assay were performed as previously described (Chen et al. 2002c). The spleens were removed from immunized mice for preparation of single cell suspensions by pressing the spleens against fine nylon mesh and lysing red blood cells with 0.84% Tris-ammonium chloride solution. The red blood cell-depleted splenic lymphocytes from vaccinated mice were co-cultured in 24 well plates with specific target cells. In DC_{neu} and DNA immunized mice, 5x10⁶ lymphocytes were co-cultured with 2x10⁵ irradiated Tg1-1 cells (20,000 rad) per well containing 2 ml of DMEM plus 10% FCS with 20 U/ml IL-2 (Peprotech). For mice vaccinated with DC with phagocytosis of apoptotic MCA, MCA/HSP or EG7 tumor cells, the lymphocytes were co-cultured with irradiated MCA or EG7 tumor cells (4,000 rad) at a 25:1 ratio (i.e. 5x10⁶ lymphocytes and 2X10⁵ irradiated MCA or EG7 cells) in 2 ml of DMEM plus 10% FCS with IL-2 in
each well of a 24-well plate, respectively. After four days, activated T cells were harvested and purified by Ficoll-Paque density gradient centrifugation, using Ficoll-Paque Plus (Amersham). These activated T cells were used as effector cells in the assay and diluted two-fold.

The target cells were radiolabeled using 50 µl of [⁵¹-Cr]-sodium chromate (36 mCi/ml; Amersham) for 1 hour at 37°C and washed extensively. A total of 10⁴ radiolabeled target cells/well were added to varying concentrations of effector cells plated in triplicates to 96 well plates and incubated for 8 hours at 37°C. The target cells used in the CTL assay with the effector cells from DC_{neu} and DNA immunized mice were Tg1-1 and the target control CaD1 cells. Radiolabeled target cells for effectors cells derived from mice immunized with DC with phagocytosis of apoptotic MCA, MCA/HSP or EG7 tumor cells are MCA and EG7 cells, whereas A20 and EL4 tumor cells served as target controls.

The amount of ⁵¹-Cr released into the culture supernatant upon cell killing was determined by transferring a 100µl of the supernatant to a biovial and the radioactivity measured using a Gamma 5500 B counter (Beckman). The percent specific lysis was calculated using the formula:

\[
\frac{(\text{experimental CPM} - \text{spontaneous CPM})}{(\text{maximal CPM} - \text{spontaneous CPM})} \times 100
\]

The spontaneous count per minute (CPM) release in the absence of effector cells was less than 10% of specific lysis; maximal CPM release was obtained by adding 1% (v/v) Triton X-100 to the wells with target cells.
3.17.3.2. *In vivo* cytotoxicity assay

*In vivo* cytotoxicity assay was also performed as previously described (Xiang et al. 2005). Briefly, FVB/N mice were immunized with DNA, DC_neu or PBS twice two weeks apart. To generate differentially labeled target cells, splenocytes from naïve mice were incubated with varying concentrations of carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) ranging from high (3.0 µM, CFSE\textsuperscript{high}) to low (0.6 µM, CFSE\textsuperscript{low}) concentrations. The CFSE\textsuperscript{high} cells were pulsed with the neu peptide, PDSLRDLSVF, and the CFSE\textsuperscript{low} cells were pulsed with the irrelevant peptide, RPQASGVYM, (Ercolini et al. 2003) and washed extensively. Both CFSE\textsuperscript{high} and CFSE\textsuperscript{low} were coinjected i.v. at a ratio of 1:1 in immunized mice, six days following the last immunization. Sixteen hours after injection, spleens were removed to analyze residual CFSE\textsuperscript{high} and CFSE\textsuperscript{low} target cells remaining in recipients’ spleens by flow cytometry.

3.17.4. Proliferation assays

3.17.4.1. Mixed lymphocyte reaction (MLR)

a) HSP70 Study

Spleens were removed from C57BL/6 mice for preparation of a splenocyte suspension with red blood cells lysed using 0.84% ammonium chloride. T cells were obtained from the splenic lymphocytes by nylon wool non-adherence to deplete residual APC. The primary MLRs were performed as previously described (Chen et al. 2001c). Briefly, irradiated BALB/c or C57BL/6 mouse BM-derived DC with phagocytosis of apoptotic MCA or EG7 tumor cells (4,000 rad, 0.4x10\(^5\) cells/well) were incubated in
graded doses with a constant amount (1x10^5 cells/well) of allogeneic T cells from a C57BL/6 or BALB/c mouse in each well of 96-well culture plates, respectively. After 3 days, T cell proliferation was measured by adding 1 µCi/well of [³H]-thymidine (1 mCi/ml, Amersham) to cultures followed by subsequent liquid scintillation counting after an overnight incubation period.

3.17.4.2. T cell proliferation

a) DC_neu study

Naïve T cells were prepared from FVB/N mouse splenocytes using nylon wool columns (Xiang and Moyana 2000). CD4^+25^+ Tr cells were further purified from these naive T cells by using anti-CD25 microbeads (Pasare and Medzhitov 2003). In all experiments, 90 to 95% of these isolated T cells were positive for both CD4 and CD25 markers. In the T cell proliferation assay, naïve T cells (1x10^5 cells per well) were incubated with DCs (0.2x10^5 cells per well) and its dilutions plus the anti-CD3 Ab (1 µg/ml) in the absence or presence of CD4^+25^+ Tr cells or anti-IL-6 Ab (10 µg/ml) in the round bottom 96-well plates for 2 days. T cell proliferation was measured using an overnight [³H]-thymidine (1 mCi/ml) uptake assay (1 µCi/well). The levels of [³H]-thymidine incorporation into the cellular DNA were determined by liquid scintillation counting after harvesting the cells onto glass fiber filters (Nunc) (Xiang and Moyana 2000).

b) HSP70 Study

Naïve OVA-specific CD8^+ T cells were isolated from OT I mouse spleens, and enriched by passage through nylon wool columns. CD8^+ cells were then purified by
negative selection using anti-mouse CD4 (L3T4) paramagnetic beads (Dynal, Inc) to yield populations that were >95% CD8⁺/Vα2Vβ5⁺. A constant number of naïve OT I CD8⁺ T cells (1x10⁵ cells/well) were cultured with irradiated (4,000 rad) DC with phagocytosis of apoptotic EG7 tumor cells (0.4x10⁵ cells/well), and their two-fold dilutions. After 3 days, all wells were pulsed for 12-16 hrs with 1 μCi of [³H]-thymidine and then harvested onto glass fiber filters, respectively. Harvesting cells in each well onto glass fiber filters followed by liquid scintillation counting determined thymidine incorporation. In addition, C57BL/6 mice were i.v. immunized with irradiated (4,000 rad) DC with phagocytosis of irradiated EG7 tumor cells (5x10⁵ cells).

To evaluate OVA-specific CD8⁺ T cell proliferation in vivo, we performed tetramer staining six days subsequent to immunizations. In the tetramer staining assay, tail blood samples collected were incubated with PE-H-2Kᵇ/OVA I (SIINFEKL) tetramer (Beckman Coulter) and FITC-anti-CD8 Ab. Erythrocytes were then lysed using the Lyse/Fixative Buffer Reagent (Beckman Coulter) and samples were analyzed by flow cytometry according to the company's protocol.

3.17.4.3. **MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay**

Tumor cell proliferation assays were determined using nonradioactive MTT assays. This was accomplished by using 2x10⁴ Tg1-1 or CaD1 tumor cells mixed with sera from immunized mice (1:50) and plated in a 96-well tissue culture plate incubated at 37°C (Yang et al. 1995a) in a total volume of 100 µl. Normal mouse sera and anti-neu Ab (Ab-4; clone 7.16.4) were used as negative and positive controls, respectively. After
the fifth day of culturing, cell proliferation was measured by MTT assays using CellTiter 96 Non-radioactive Cell Proliferation Assay kit (Promega, Madison, WI) by adding 15 µl of the Dye Solution to each sample well. After incubating for 4 hours at 37°C, the formazan crystals were solubilized using 100 µl of the Solubilization/Stop buffer and further incubated overnight before reading spectrophotometrically. Plates were read at 595 nm with background subtraction at 655 nm using a microplate reader. Cell growth inhibition was calculated by \[
\left[\frac{\text{blank absorbance} - \text{experimental absorbance}}{\text{blank absorbance} - \text{background absorbance}}\right] \times 100,
\]
where blank values refer to wells with no serum added and background values refer to wells with no cells added.

3.17.5. Flow cytometry

Cells were spun down using a Clay Adams SEROFUGE II centrifuge and incubated with 5 µg/ml of the respective primary antibody in 100 µl for at least 30 min on ice. Following three washes with PBS, cells were incubated with 50 µl of the appropriate secondary antibody, such as FITC-conjugated goat anti-rat IgG antibody (1:60), FITC-conjugated goat anti-mouse IgG antibody and FITC-conjugated streptavidin, on ice for an additional 30 min. After washing with PBS, cells were then analyzed by flow cytometry using an Epics XL flow cytometer (Beckman-Coulter). Isotype-matched monoclonal antibodies were used as controls.

3.17.6. Mouse serum collection

Mice were tail bled at specified time points and the blood collected into 1.5 ml tubes. The collected blood was then incubated at 37°C for 1 hour to allow the blood to clot followed by incubating the sample at 4°C overnight. The next day, the samples
were spun twice in a refrigerated centrifuge for 10 min at 10,000 x g. The sera were transferred into new tubes and stored at -20°C until further use.

3.17.7. Cell irradiation

Cells were irradiated using a gamma cell counter containing a ⁶⁰-Co Cobalt (Co) source (MDS Nordion; Ottawa, Canada) located at the University of Saskatchewan. The cells received differing doses of exposure ranging from 2,000 rad for DC, 20,000 rad for Tg1-1 cells; 1,000 rad for EG7 cells and 5,000 rad for MCA cells.

3.18. Analysis of DC phagocytosis

Apoptotic tumor cells were labeled with CFSE as previously described (Xiang et al. 2005). To evaluate DC phagocytosis of apoptotic tumor cells, DC were cultivated with CFSE-labeled apoptotic tumor cells at a ratio of 3:1 in the culture medium containing GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) at 37°C overnight. DCs were harvested, purified with Ficoll-Paque gradient, and then analyzed by flow cytometry and electron microscopy.

3.19. Generation and analysis of apoptotic tumor cells:

Apoptosis of EG7, MCA and MCA/HSP tumor cells were generated by in vitro irradiation with 1,000 and 5,000 rad, respectively, cultured in DMEM plus 10% FCS for 12 and 48 hr, and then harvested for electron microscopy and flow cytometric analysis or for DC phagocytosis. For flow cytometric analysis, the apoptotic tumor cells were analyzed using anti-HSP70 antibody and Annexin V-FITC (Pharmingen) by flow cytometry. The apoptotic tumor cells in early and late phase of apoptosis, which
expressed Annexin V (early apoptosis marker) and HSP70 (late apoptosis marker) (Chen et al. 2001c; Chen et al. 2005) were termed EG7-E, EG7-L, MCA-E, MCA-L, MCA/HSP-E and MCA/HSP-L, respectively.

3.20. Electron microscopy

Samples were sent to the Department of Pathology at the University of Saskatchewan to prepare the specimen for electron microscopy.

3.21. Vaccination of mice

3.21.1. DC_{neu} and DNA vaccine

We selected to immunize mice with s.c. and i.m. routes for administration of DCs and DNA, since they induced more efficient immune responses than intravenous (i.v.) or intraperitoneal (i.p.) administration of DC (Fong et al. 2001b; Okada et al. 2001a) and s.c. injection of DNA, (Davis et al. 1994; Gramzinski et al. 1998) respectively. To evaluate tumor immunity, FVB/N and BALB/c mice were vaccinated s.c. with 1x10^6 DC_{neu} cells and i.m. with 100 µg pcDNAneu DNA twice with a 14 day interval, respectively. Ten days subsequent to the last vaccination, the FVB/N and BALB/c mice (n = 4-10 per group) were challenged by s.c injection of 3x10^5 Tg1-1 and MCA-26neu tumor cells, respectively. Animal mortality and tumor growth were monitored daily for up to 60 days; mice with an average tumor diameters greater than 12 mm were euthanized for humanitarian reasons.
For evaluation of tumor prevention, FVBneuN Tg mice (n = 10 per group) at age of two and four months were vaccinated s.c. with $1 \times 10^6$ DC$_{neu}$ cells or i.m. with 100 µg pcDNAneu DNA at a one month interval, for a total of three vaccinations, respectively. Aged-matched FVBneuN left either untreated or injected with PBS were used as controls. Spontaneous breast tumor development was monitored weekly for up to 12 months. Tumor free time curves were recorded when the first tumor of each mouse reached a palpable size of greater than 3 mm in diameter.

### 3.21.2. DC$_{HER2mut}$ vaccine

FVBneuN Tg mice were immunized with DC$_{HER2mut}$ in a similar fashion as used in the immunization of these mice using DC$_{neu}$. Briefly, for the protection study, mice (n=8) were immunized twice with PBS, DC$_{neu}$ or DC$_{HER2mut}$ two weeks apart then challenged with $3 \times 10^5$ Tg1-1 cells injected s.c. ten days later. Tumor growth was monitored daily and mice were culled when tumors exceeded 12 mm in diameter for humane reasons.

In the prevention assay, FVBneuN mice (n=15) at 8 weeks of age were immunized with DC$_{HER2mut}$ a total of 3 times at 4-week intervals or treated with PBS to serve as the control group. Spontaneous breast tumor development was monitored weekly by palpitating the glands and the appearance of a tumor greater than 3 mm was recorded as a tumor.

### 3.21.3. DC with phagocytosis of apoptotic cells vaccine

For evaluation of antitumor immunity, mice were vaccinated twice s.c. with
5x10^5 DC with phagocytosis of apoptotic MCA, MCA/HSP or EG7 tumor cells (DC\textsubscript{MCA}, DC\textsubscript{MCA/HSP}, DC\textsubscript{EG7}, respectively). Ten days after the final immunization, mice (n = 10 per group) were challenged by s.c injection of 5x10^5 MCA or EG7 tumor cells. Animal mortality and tumor growth were monitored daily for up to 10 weeks; for humanitarian reasons, all mice with tumors that achieved a size of 15 mm in diameter were sacrificed.

3.22. **Lymph node cell culture**

To evaluate the type of immune response induced, mice were immunized either twice s.c. with 1x10^6 DC\textsubscript{neu} or i.m. with 100 µg DNA vector. The draining inguinal and popliteal LN were harvested 3 days after the last immunization and a single cell suspension prepared by pressing the LN through a mesh screen. A million LN T cells were cultured with 5x10^4 irradiated Tg1-1 tumor cells in DMEM plus 10% FCS with 20 U/ml interleukin-2 (IL-2) for 1 to 3 days. Both the LN T cells as well as the DC culture supernatants were assayed for the expression of IL-4, IL-6, TNF-α and IFN-γ using the cytokine ELISA kits, as mentioned in previous sections.

3.23. **Statistical Analysis**

Statistical analyses were done using Prism software (GraphPad Software, Inc., San Diego, CA) to form Kaplan-Meier survival chart and to perform Logrank test to compare mouse survival and tumor development between groups. Student t tests were performed to determine the significance of differences between groups. \( P \) values <0.05 were considered statistically significant.
4.0 RESULTS AND DISCUSSION

4.1 Part A – Adenovirus mediated transfer of the Rat neu gene into DC as a breast cancer vaccine

4.1.1 Adenovirus generation and verification

AdV vectors have proven to be beneficial and results in highly efficient method for gene transfer. We first began by creating a replication deficient AdV containing the full-length rat neu gene, AdV\textsubscript{neu}. This was accomplished using the AdEasy system and AdV stocks were purified and tested to ensure the rat neu gene was still present within the viral vector construct. Previous first hand experience in Dr. Xiang’s lab found some stability issues when inserting larger genes into the AdV. A PCR analysis was performed utilizing neu-specific PCR primer pairs to detect the presence of rat neu within the AdV construct during the amplification stages and in the final purified AdV. This method allows for rapid identification and guarantees the purified virus contains the transgene within the construct. From the analytical gel shown in Figure 4.1, we observed a 488 bp band, representing the presence of the neu gene within the AdV construct, from virus infected 293 cells (D), the culture media from infected cells (E) and most importantly, the purified virus (F). The positive control used was the shuttle vector, pShuttleCMVneu while both the negative controls resulted in no bands produced.
Figure 4.1  PCR analysis of the adenoviral vector, AdV$_{\text{neu}}$, from the AdEasy system to detect the presence of the rat neu gene within the construct. After 30 cycles, 10µl of the reaction was electrophoresed on a 1% agarose gel. The samples were loaded into each lane as follows: water control (A), the backbone vector, pAdEasy-1 (B); the shuttle vector, pShuttle-CMV/neu (C); crude viral lysate from infected 293 cells (D); infected culture media (E) and the purified AdV$_{\text{neu}}$ (F). M is the DNA marker loaded. The presence of a 488 bp band represents the neu gene.
This showed the specificity of the PCR reaction and confirmed the presence of the gene within our final purified product.

4.1.2 Examining neu expression in tumor and DC cells:

Tumor cell lines were chosen based upon their expression or lack thereof for neu, as detected by both flow cytometry and western blotting (Figure 4.2). This allowed us to select cell lines for developing our animal models. The mouse breast cancer cell line Tg1-1 was derived from a spontaneous breast tumor derived from FVBneuN Tg mice that overexpresses the neu Ag, as detected by flow cytometry using the anti-neu Ab. In contrast, another mammary cell line CaD1 and mouse colon cancer cell line, MCA26, did not to express neu. We also generated the transfected MCA-26neu tumor cell line, using the pCDNAneu vector. This clone was selected by employing positive screening methods using antibiotic resistance and gene expression selection, as detected by flow cytometry (Figure 4.2A). DC themselves did not express neu; however, 24-36 hours after AdV\textsubscript{neu} transduction, neu expression was detected on the cell surface of DC\textsubscript{neu}. Obviously, the expression of neu was mediated by DC transduction with AdV\textsubscript{neu}.

Furthermore, to confirm the expression of neu within the various cells, Western blot analysis using protein extracts from the above six types of cells were examined for neu expression. As shown in Figure 4.2B, Tg1-1, MCA-26neu and DC\textsubscript{neu} cells displayed significant amount of neu Ag expression, as detected by the presence of a 185 kD band, whereas CaD1, MCA-26 and DC did not express any neu molecule. These results are in accordance with the data obtained from flow cytometry.
**Figure 4.2** Analysis of neu expression. (A) The expression of cell surface neu on CaD1, MCA26, DC, Tg1-1, MCA26neu and DCneu (solid lines) as analyzed by flow cytometry using the mouse anti-neu (Ab-4) and FITC-conjugated goat anti-mouse IgG Abs. Isotype-matched mAbs (dashed lines) were used as controls. (B) Western blot analysis. Protein extracts were obtained from the above cell lines/preparations and loaded to each well of a polyacrylamide gel under reducing conditions. The transferred nitrocellulose blot was incubated with mouse anti-neu Ab (Ab-3) followed by peroxidase-conjugated goat anti-mouse IgG Ab. Enhanced chemiluminescence reagent was used to detect expression of neu.
4.1.3 DC_{neu} cells up-regulate expression of immunologically important molecules, cytokines and chemokines

Previous studies have shown following AdV transduction, DC often undergo changes in their maturational status (Chen et al. 2002c; Molinier-Frenkel et al. 2003). Increases in cell expression of MHC class II, CD40, CD80, CD86 are associated with DC maturation. We therefore used flow cytometry to examine levels of commonly expressed cell surface markers on DC using a panel of anti-MHC class II, CD40, CD54, CD80 and CD86 Abs against both nontransduced DC and DC_{neu}. As shown in Figure 4.3, we found that DC cultured under normal conditions were relatively mature as evidenced by expression of high levels of both MHC class II, CD54, CD80 and CD86. CD40 levels were very low in DC. These results are consistent with the protocol used in culturing BM-derived DC in our lab, requiring both GM-CSF and IL-4, to produce relatively mature DC (Chen et al. 2001b). However, after AdV transduction, we found DC_{neu} underwent a further increase in the maturation level. This was evidenced by increased expression, relative to DC alone; in MHC class II, CD40, CD54 and CD80. The expression of CD86, the B7-2 costimulatory molecule, did not result in significantly altered expression levels upon AdV transduction. Transduction with the control AdVpLpA also led to a mild to moderate up-regulation of MHC class II, CD40, CD54 and CD80 expression on DC_{pLpA} cells that were similar to DC_{neu}. This data indicated that the up-regulation of MHC class II, CD40, CD54 and CD80 expression was derived from AdV transduction of DC itself and phenotypically suggested enhanced DC maturation.

The maturational process regulates the expression of cytokines, chemokines and chemokine receptors, either increasing or decreasing them. Each different cytokine,
Figure 4.3 Phenotypic analysis of DC. A comparison of the phenotypic changes between non-transduced DC and AdVneu-mediated transgene expression in DC (DC_{neu}), as measured by flow cytometry. The expression of rat neu, MHC class II, CD40, CD54, CD80 and CD86 molecules were analyzed.
chemokine and the respective receptor can be analyzed and quantitated individually; however, this is time consuming especially when there is a large set to analyze. The use of RNAse protection assay (RPA) offers the advantage of using isolated RNA from cells hybridized with protection probes specific for characteristic genes then digested with RNAse enzyme. Form ed RNA-probe complexes are resistant to enzyme digestion and easily identified by running on an acrylamide gel, followed by densitometry to quantifying gene expression levels. This method allows for examining multiple sets of relevant genes at a single time.

As shown in Figure 4.4A, the results from the RPA showed nontransduced DC expressed minimal amounts of TNF-α, IL-1β, IL-6, MIP-1β while IP-10 was the most abundantly expressed cytokine. After AdV transduction, increased expression of all of the cytokines examined was seen in DC_{neu}. Both IL-6 and MIP-1β were the most dominantly expressed in DC_{neu} compared to DC. The increased levels of cytokine expression seen in DC_{neu} compared to DC ranged from a 5-fold increase for IP-10, to over 300-fold level for IL-6. The same banding intensity seen for the housekeeping gene, GAPDH, indicated that similar amounts of RNA were loaded and the differences seen in the cytokine expression levels are a result from AdV transduction.

In addition, to confirm the results observed in the RPA, cytokine-based ELISAs were performed for IL-6 and TNF-α only. In DC, both of these cytokines expressed minimal amounts and were either below or just above the level of detection. On the other hand, DC_{neu} was found to express ~650 pg/ml IL-6 and ~400 pg/ml TNF-α (Figure 4.4B). These results are in concordance with the expression pattern detected using RPA.
Figure 4.4 RNAse protection assay and cytokine ELISA from DC. A. DC_{neu} and DC were assessed by RNAse protection assay for their expression of various cytokines, as indicated. Autoradiograph of the RNAse protection assay gels is shown for each DC population and graphic presentation of the data showing the relative expression levels of each transcript for DC_{neu} and DC. B. The supernatant of DC cultured cells were measured for the expression of IL-6 and TNF-α using cytokine ELISA kits.
4.1.4 DC\textsubscript{neu} partially convert CD4\textsuperscript{+}25\textsuperscript{+} Tr-suppression by IL-6 secretion:

It has recently been reported that the inflammatory cytokine IL-6 is required for T cell activation \textit{in vivo} and can convert Tr-mediated immune tolerance (Pasare and Medzhitov 2003). Since we found that DC\textsubscript{neu} expressed abundant levels of IL-6, we next examined whether DC\textsubscript{neu} could also convert the CD4\textsuperscript{+}25\textsuperscript{+} Tr-mediated immune suppression. To examine this, we conducted a T cell proliferation assay by using DC\textsubscript{neu} in the presence of Tr cells. As shown in Figure 4.5, CD4\textsuperscript{+}25\textsuperscript{+} Tr cells resulted in almost complete inhibition of T cell proliferation using DC stimulation contrasting the high proliferation activity with naïve T cells (Figure 4.5 A and B), whereas the Tr inhibition of T cell proliferation was reduced to only 45% when DC\textsubscript{neu} was used as stimulators (Figure 4.5B). This indicated that DC\textsubscript{neu} could partially convert Tr-mediated suppression. Furthermore, this partial inhibition was abrogated by the addition of anti-IL-6 Ab, but not by an irrelevant control Ab, thus indicating that the partial conversion of Tr suppression by DC\textsubscript{neu} was mediated by IL-6 secretion.

4.1.5 DC\textsubscript{neu} mounts an effective protective antitumor immune response in wildtype but is ineffective in Tg mice

As a proof of principle study, we set out to determine if the DC\textsubscript{neu} vaccine provided protection in mice from challenge with Tg1-1 cells, derived from a spontaneous tumor in FVBneuN Tg mice and endogenously overexpresses neu (Chen et al. 1998). This cell line is highly tumorigenic as wildtype mice easily formed tumors with a minimal dose of 1x10\textsuperscript{5} tumor cells injected s.c. for tumor formation. We first started by immunizing wildtype parental FVB mice twice with a total of 1x10\textsuperscript{6} DC\textsubscript{neu} (n=5) s.c. into both legs two weeks apart or with PBS (n=4). Ten days later, mice were
Figure 4.5  T cell proliferation assay. (A) Irradiated (4,000 rad) DCs (0.2x10^5 cells/well) and its dilutions were co-cultured with a constant number of naïve T cells (0.1x10^6 cells/well) in the absence (●) or presence (▲) of CD4^+CD25^+ Tr cells (0.1x10^6 cells/well) in RPMI plus 10% FCS for 2 days. T cell proliferation was measured using an overnight ^3H-thymidine (1µCi/well) uptake assay. (B) Irradiated AdV-transfected DC (DC_neu and the control DC_pLpA) were used in a T cell proliferation assay in the presence of Tr cells and anti-IL-6 or control antibody. One representative experiment of three is shown.
challenged with $0.4 \times 10^6$ Tg1-1 cells in the right flank s.c then monitored for tumor growth. The Kaplan-Meier survival curve is shown in Figure 4.6, showing control mice treated with PBS alone failed to provide any protective antitumor effects as all of the mice developed tumors. On the other hand, mice immunized with DC$_{\text{neu}}$ remained completely protected from Tg1-1 tumor challenge. This protection lasted greater than two months and suggested that DC$_{\text{neu}}$ provided complete protection against a neu-expressing tumor.

These results were encouraging, however the tumor model employed above does not closely mimic the clinical situation seen in humans, in regards to tolerance. Wildtype FVB mice are not tolerized to rat neu protein whereas the FVBneuN Tg mice are tolerized to suggest that overcoming self-tolerance is required for effective protection (Reilly et al. 2000; Reilly et al. 2001). In previous studies by that group, immunization of wildtype FVB mice resulted in complete protection using an irradiated whole cell alone or combined with recombinant vaccinia virus vaccinations whereas FVBneuN Tg immunized mice provided only a delay in tumor growth. Therefore, we evaluated the ability of the DC$_{\text{neu}}$ vaccine to protect these Tg mice using the same vaccination protocol as previously used in our above protection study for the wildtype mice. Ten days after the second immunization, Tg mice were challenged with varying doses of Tg1-1 cells. The doses ranged from $3 \times 10^4$ cells to $3 \times 10^6$ cells and mice were continuously examined for tumor growth. In all of the three doses of Tg1-1 cells used for tumor challenge, tumors developed in all of the mice, albeit not at the same time. Mice challenged with the lower doses took longer to reach a similar tumor size with that formed using a higher tumor cell dose before killing. For DC$_{\text{neu}}$ immunized mice challenged at the high dose of Tg1-1 cells of $3 \times 10^6$ cells (Figure 4.7A), tumors
Figure 4.6 Vaccination of mice with DC$_{\text{neu}}$ induced protective antitumor immunity against tumor challenge. Wildtype FVB/N mice (n=5) were vaccinated twice with $1 \times 10^6$ DC$_{\text{neu}}$ cells s.c. then challenged with $4 \times 10^5$ Tg1-1 cells injected s.c. in the right flank. Control mice (n=4) were treated with PBS then challenged with Tg1-1 cells. Mice were monitored for tumor growth and survival.
Figure 4.7 Kaplan-Meier survival curves for DC_{neu} immunized FVBneuN transgenic mice challenged with Tg1-1 cells. The transgenic mice (n=4 for each group) were immunized twice with 1x10^6 DC_{neu} (■) s.c. or control mice (PBS; ▲) then challenged with Tg1-1 cells ranging from 3x10^6 cells (A), 0.3x10^6 cells (B) and 0.03x10^6 cells (C), given subcutaneously in the right thigh. Mice were continually monitored for tumor growth and mice survival recorded.
developed at a similar rate as the control, whereas a ten-fold decrease in tumor dose resulted in a slight delay with one mouse being protected (Figure 4.7B). On the other hand, a further 10-fold decrease in Tg1-1 cell challenge (3x10^4 cells) still failed to provide complete protection; however, a significant delay in tumor growth was observed (Figure 4.7C). These results reconfirmed data obtained from Reilly’s group in respect to wildtype FVB/N mice being able to mount a strong protective immunity that resisted tumor growth upon tumor cell challenge whereas FVBneuN Tg lacked the protective ability (Reilly et al. 2000).

4.1.6 DC_{neu} induced much stronger Th1 type immune response than pcDNA_{neu}

Tumor rejection and growth has been shown to be associated with IFN-γ and IgG2a dominant Th1 and IL-4 and IgG1 dominant Th2 type immune response, respectively (Xiang and Moyana 2000). To investigate the type of immune response derived from using DC_{neu} and DNA vaccines, the supernatants of LN T cells from immunized mice were examined for cytokine secretion using cytokine ELISA kits. In response to irradiated Tg1-1 cells, the regional LN T cells derived from mice immunized with DC_{neu} or pcDNA_{neu} predominantly secreted IFN-γ (Figure 4.8). This indicated the induction of mainly a Th1 type immune response. However, differing levels were seen with the former one secreting significantly higher levels of IFN-γ (1,050 pg/ml) than the latter one (200 pg/ml). This indicated that the DC_{neu} vaccine induced a much stronger Th1 type immune response than DNA vaccine. In contrast, the expressed levels of IL-4 were minimal, with the detected levels in both DC_{neu} and DNA immunized mice above or below the sensitivity of the assay used (<17 pg/ml), respectively. Based upon these levels, it seemed that DC_{neu} immunization favored a stronger Th1 response in
Figure 4.8 DC\textsubscript{neu} induces strong expression of interferon gamma (IFN-\textgamma) compared to DNA. FVB/N mice were immunized twice with the respective vaccine. Single cell suspensions of the inguinal and popliteal lymph nodes were removed from immunized mice three days after the last immunization and cocultured with irradiated Tg1-1 cells (20,000 rads) for 3 days. Supernatants were analyzed for the presence of IFN-\textgamma and IL-4, characteristic cytokines of Th1 and Th2 responses, respectively. For DC\textsubscript{neu} immunized mice, the level of IFN\textgamma is 1050 pg/ml and 17 pg/ml for IL-4. Meanwhile, DNA immunized mice had only 200 pg/ml of IFN\textgamma and undetectable amounts of IL-4 produced.
comparison to that of DNA vaccination.

4.1.7 DC\textsubscript{neu} vaccine stimulates stronger HER-2/neu-specific humoral response than DNA vaccine

Previous vaccines targeting HER-2/neu have shown the presence of anti-neu Abs, especially the induction of IgG, both IgG1 and IgG2a, Abs in the sera of immunized animals (Chen et al. 1998; Rovero et al. 2000; Cappello et al. 2003; Sakai et al. 2004). To examine whether these neu-targeted vaccines induced a neu-specific humoral response, FVB/N mice were vaccinated twice with DC\textsubscript{neu} and pcDNA\textsubscript{neu} DNA, and the sera collected two weeks after each vaccination. The presence of HER-2/neu-specific IgG1 and IgG2a Abs, indicative of a Th2 and Th1 response, respectively, was first assessed by flow cytometric analysis on Tg1-1 cells. Tg1-1 cells were incubated with mouse sera (1:50) from the respective groups, followed by FITC-conjugated goat anti-mouse IgG1 and IgG2a Abs, respectively. As shown in Figure 4.9A, neu-specific IgG2a but not IgG1 response was detected in mice vaccinated with DC\textsubscript{neu} after one immunization. This indicated induction of a neu-specific Th1 type response. After the second immunization, increasing levels of both IgG1 and IgG2a were detected in the sera of the same mice, with significantly higher levels of IgG2a detected. This indicated that both Th1 and Th2 type responses were stimulated after the boost and involved antibody isotype class switching (Coffman et al. 1989). In contrast, we were only able to detect a minimal increase of the IgG2a Ab in the sera of DNA immunized mice, confirming a mild induction of a Th1 type response. No neu-specific Abs were detected in the sera of unvaccinated mice and this served as our control for this analysis.
Figure 4.9 The presence of IgG1 and IgG2a anti-neu antibodies in immunized mice. Flow cytometric analysis (A) using serum from DC_{neu} and DNA immunized mice. Mice were immunized twice with serum collected 2 weeks after each immunization. Tg1-1 cells were stained with serum (1:50) followed by goat anti-mouse IgG1 and IgG2a-FITC conjugated antibodies to detect presence of IgG1 and IgG2a antibodies, respectively (solid line). Sera from naïve mice were used as controls (dashed line). (B) An ELISA based method of serum IgG subtype. Sera from immunized mice (1:80) were incubated with Tg1-1 cells fixed in 96-well plates followed by peroxidase-conjugated antibodies specific for IgG1 and IgG2a antibodies, respectively. Samples were developed with substrate and absorbances read at 450 nm. Each bar represents mean ± SEM.
To reconfirm the above results, an indirect cell-based ELISA using Tg1-1 cells grown in 96-well plates was used to assess the presence of neu-specific IgG1 and IgG2a Abs. Serum samples (1:80) were incubated on the plates, followed by peroxidase-conjugated goat anti-mouse IgG1 and IgG2a Abs, respectively. As shown in Figure 4-9B, mice immunized twice with DC_{neu} induced higher levels of neu-specific IgG2a and IgG1 Ab responses, whereas vaccination of mice with pcDNAneu twice only stimulated a mild HER-2/neu-specific IgG2a Ab response. We also confirmed that the second immunization aids in further boosting the presence of neu-Abs in the sera of immunized mice whereas Abs in the sera of control mice remained undetected. Taken together, both of these two methods confirmed the presence of anti-neu Abs with DC_{neu} significantly producing a vast amount compared to pcDNAneu DNA immunization. As well, DC_{neu} immunization generated a strong Th1/Th2 response while only a minor induction of a Th1 response was observed with DNA immunization.

4.1.8 DC_{neu}-derived neu-specific antibody induces down-regulation of HER-2/neu expression and inhibits growth of Tg1-1 tumor cells

Previous studies have shown that the Abs recognizing neu induced neu receptor down-regulation and resulted in growth inhibition of tumor cells (Rovero et al. 2000; Yip et al. 2001). As shown in Figure 4.10A, an overnight incubation of Tg1-1 cells with sera from DC_{neu} immunized mice resulted in an apparent down-regulation of HER-2/neu expression in Tg1-1 cells to almost 50%, when directly compared to the expression level of Tg1-1 cells not cultured in the presence of sera. The sera from DNA immunized mice also showed a slight but not a significant decrease in neu-receptor expression on the cell surface.
Figure 4.10 Analysis of anti-HER-2/neu IgG Ab response on tumor cells. (A) The anti-HER-2/neu IgG Ab down-regulated HER-2/neu expression. Tg1-1 tumor cells were incubated in the medium with or without sera (1:50) from mice immunized with DNA (a) or DC_{neu} (b) overnight. Cells were washed with PBS, stained with the anti-neu Ab and FITC-anti-mouse IgG Ab then analyzed by flow cytometry. Cells incubated with sera from immunized mice are seen as solid lines in the histogram and non-sera incubated cells are shown as a dashed line. (B) The presence of anti-neu Ab inhibits growth of Tg1-1, as determined by MTT assay after five days of culture. Sera from respective groups were diluted 1:50 and anti-neu Ab used as a positive control. Student’s t test, * P<0.005 when compared to sera from PBS immunized mice and ** P<0.005 when comparing sera from DC_{neu} immunized mice to DNA immunized mice. (C) On the other hand, the presence of these Abs caused CaD1 tumor cell proliferation, as determined by MTT assay. Each bar represents the mean ± SEM.
Since HER-2/neu expression is related to tumor cell growth, especially within breast cancer, we next examined whether the presence of anti-neu Abs affected cell proliferation of Tg1-1 cells in vitro using MTT assays. The use of the MTT assay provided a quantitative, non-radioactive based method for measuring cell viability based upon the ability of mitochondrial dehydrogenase enzyme activity to cleave the tetrazolium rings and form formazen crystals in viable cells only. As shown in Figure 4.10B, the sera from DC\textsubscript{neu} immunized mice clearly inhibited \~73% of TG1-1 cell growth in vitro after five days, compared to cells incubated with sera from PBS vaccinated mice ($P<0.0001$; Student’s $t$ test). In fact, proliferation and not inhibition of Tg1-1 cells cultured with the sera from PBS immunized mice was observed. Sera from DNA immunized mice also resulted in \~38% inhibition of Tg1-1 proliferation ($P<0.005$ in comparison to PBS; Student’s $t$ test) but the sera from DC\textsubscript{neu} immunized mice clearly had a greater growth inhibitory effect ($P<0.005$ between DC\textsubscript{neu} and DNA sera, Student’s $t$ test). In addition, culturing the tumor cells in the presence of the neu mAb (clone 7.16.4) (Drebin et al. 1985; Drebin et al. 1986) also resulted in a significant growth inhibition of Tg1-1 cells (\~58%). Meanwhile, sera from the respective groups of immunized mice did not contribute to any inhibited growth of neu-negative CaD1 tumor cells (Figure 4.10C). This indicated that the growth inhibition was neu-specific.

4.1.9 DC\textsubscript{neu} vaccine stimulates stronger HER-2/neu-specific CTL response than DNA vaccine:

Next, we addressed the specific anti-tumor effector function induced by vaccination of mice with DC\textsubscript{neu} and pcDNAneu DNA. Spleen lymphocytes from immunized mice were cocultivated with irradiated Tg1-1 tumor cells for 3 days followed
by harvesting activated T cells. To assess the cytotoxic activity, we conducted a chromium release assay using activated T cells as effector cells and $^{51}$Cr-labeled Tg1-1 tumor cells as target cells. As shown in Figure 4.11A, the activated T cells from mice vaccinated with DC$_{neu}$ showed significantly higher cell killing activity towards Tg1-1 cells. At an effector:target (E:T) ratio of 50, the specific killing activity for activated T cells from mice vaccinated with DC$_{neu}$ was 36%, which was significantly higher compared to the 12% killing activity using activated T cells derived from DNA vaccinated mice. The specificity of cell killing towards neu was also demonstrated by the inability of activated T cells derived from DC$_{neu}$ mice to lyse CaD1 cells.

Results from the \textit{in vitro} study were also confirmed using an \textit{in vivo} cytotoxicity assay (Figure 4.11B). This assay involved differentially CFSE-labeled peptide-pulsed splenocytes, using CFSE$^{\text{high}}$ (neu peptide pulsed; PDSLRDLSVF) and CFSE$^{\text{low}}$ (irrelevant peptide, LCM N protein peptide-pulsed) labeled cells, transferred i.v. into recipient DNA and DC$_{neu}$ immunized mice. These labeled splenocytes acted as target cells with the disappearance of the cells resulting mainly from cell killing mechanisms involving T cells. Flow cytometry analysis was used to examine the elimination of labeled cells by determining the amount of labeled cells remaining. Levels of CFSE$^{\text{low}}$ cells remained relatively unaffected; indicating the cell killing was specifically geared towards neu, whereas the CFSE$^{\text{high}}$ level in DNA immunized mice decreased minimally by 8%. However, a greater degree of loss of CFSE$^{\text{high}}$ cells (~38%) was observed in DC$_{neu}$ immunized mice. This indicated that DC$_{neu}$ immunization produced a stronger CTL response compared to DNA immunization.
Figure 4.11 DC\textsubscript{neu} immunizations induce a higher degree of neu-specific CTLs compared to DNA immunizations. (A) \textit{In vitro} cytotoxicity assays. Splenic lymphocytes were harvested from mice vaccinated with DC\textsubscript{neu} (■) and pcDNA\textsubscript{neu} (Δ) and co-cultured with irradiated Tg1-1 cells (20,000 rad) for 4 days, then used as effector cells in a chromium release assay with \textsuperscript{51}Cr-labeled Tg1-1 tumor cells. To confirm that T cell cytotoxicity was neu tumor specific, we also included neu-negative CaD1 tumor cells (▼) as a target control and activated T cells from DC\textsubscript{neu}-immunized mice as effector cells. Each point represents the mean of triplicates. (B) \textit{In vivo} cytotoxicity assay. Differentially labeled CFSE\textsuperscript{high} and CFSE\textsuperscript{low} naïve FVB/N splenocytes were pulsed with the neu peptide and an irrelevant peptide, respectively, then i.v. injected into PBS, DNA and DC\textsubscript{neu} immunized mice. Sixteen hours later, the spleens’ of mice were harvested to determine the remaining CFSE\textsuperscript{high} and CFSE\textsuperscript{low} cells remaining, as determined by flow cytometry. The value in each panel represents the percentage of CFSE\textsuperscript{low} (L) versus CFSE\textsuperscript{high} (H) cells remaining in the spleen.
4.1.10 DC\textsubscript{neu} vaccine induces more efficient protective tumor-specific immunity than DNA vaccine in wild-type mouse models:

To examine whether the neu-targeted vaccines were capable of inducing antitumor immunity \textit{in vivo}, we vaccinated wild-type FVB/N mice twice with DC\textsubscript{neu} and pcDNA\textsubscript{neu} DNA, then challenged 10 days later with $3 \times 10^5$ Tg1-1 tumor cells. As shown in Figure 4.12A, Tg1-1 tumor cell challenges were invariably lethal within 6 weeks post-implantation for PBS and DC\textsubscript{pLpA} vaccinated mice. Nevertheless, vaccination of mice with DC\textsubscript{neu} (n=8) twice induced stronger antitumor immunity, resulting in protection of all of the mice from challenge with $3 \times 10^5$ Tg1-1 tumor cells and remaining tumor free at the end of the study, whereas only half of the mice (4/8) mice given two DNA vaccinations remained tumor free ($P<0.05$; log-rank test). This data indicated that DC\textsubscript{neu} vaccination induced a more efficient protective tumor-specific immunity \textit{in vivo} in comparison to DNA vaccination.

To confirm the above finding, we repeated the study using a MCA26-neu animal model. We vaccinated BALB/c mice (n=10) twice with DC\textsubscript{neu} s.c. and pcDNA\textsubscript{neu} DNA i.m. then challenged the mice 10 days later with $3 \times 10^5$ MCA-26neu tumor cells expressing the neu Ag. As shown in Figure 4.12B, DC\textsubscript{neu} vaccination showed stronger antitumor immunity resulting in 100% complete protection in mice when challenged against MCA-26neu tumor cells, whereas pcDNA\textsubscript{neu} DNA immunization failed to protect mice from tumor growth ($P<0.05$; log-rank test). This confirmed that DC\textsubscript{neu} vaccination induced more efficient and effective protective tumor-specific immunity \textit{in vivo} than DNA vaccination. On the other hand, immunization of mice with DC\textsubscript{pLpA} failed to induce protective immunity in mice when challenged with either Tg1-1 or MCA-26neu tumor cells (Figure 4.12A and B), further indicating that the specificity of
Figure 4.12 Vaccination of mice with DC_neu induces stronger anti-tumor immunity. (A) FVB/N and (B) BALB/c mice (n = 10) were vaccinated twice with DC_neu and pcDNAneu, respectively. Ten days later, the immunized mice were then s.c. challenged with 3\times10^5 neu-positive Tg1-1 (A) and MCA-26neu (B) tumor cells. Mice were monitored twice weekly up to 60 days. Survival of the DC_neu-immunized group of mice (*) was significantly longer than that of the pcDNAneu-immunized group (Log rank test, \( P < 0.05 \)) in both (A) and (B) experiments. One representative experiment of two independent experiments is shown.
the protective immunity derived in DC\textsubscript{neu} immunized mice is neu specific.

4.1.11 DC\textsubscript{neu} vaccine delays breast cancer development significantly longer than DNA vaccine in transgenic mouse model:

A variety of studies have previously shown the ability for DNA vaccines to prevent spontaneous tumor formation in the aggressive breast tumor forming BALB-neuT model with varying efficacies (Rovero et al. 2001; Cappello et al. 2003; Quaglino et al. 2004a). However, there are limited studies to date examining the ability to prevent tumor formation in the FVBneuN Tg mice, containing the wildtype neu gene. These mice spontaneously develop mammary tumors in a stochastic fashion beginning around 4-5 months of age up until 8 months of age (Figure 4.13). Therefore, to determine whether the antitumor immunity derived from HER-2/neu-targeted vaccines would be able to inhibit carcinogenesis, thereby preventing tumor formation, we vaccinated FVBneuN Tg mice beginning at two and four months of age. These time points represent periods of observed atypical hyperplasia and infiltrating ductal carcinomas, respectively (Cardiff and Wellings 1999). Mice were immunized with \(1 \times 10^6\) DC\textsubscript{neu} cells s.c. and 100 µg pcDNAneu plasmid DNA i.m. every four weeks for a total of three times, respectively. Age matched controls were also used to monitor the development of spontaneous breast tumor development along, as seen in Figure 4.14A and B, in the ten mammary glands. In Figure 4.14C, the photograph of the various spontaneous tumors derived from the Tg mice over a time period ranging from four months to the end of the study or when tumors reached a maximum size of 1.5 cm in diameter is shown.

Tumor free curves were calculated based upon the first appearance of a mammary tumor detected. As shown in Figure 4.15, Tg mice immunized with DC\textsubscript{neu}
beginning at the age of 2 months (A) but not at the age of 4 months (B), significantly delayed tumor development compared to that of the DNA-immunized mice ($P<0.05$; Log-rank test). Overall, our data indicated that $\text{DC}_{\text{neu}}$-based vaccination induced stronger neu-specific preventive immunity in Tg mice than DNA vaccination and provided a significant delay in tumor formation.
Figure 4.13  Spontaneous tumor development in FVBneuN Tg mice. A total of 61 FVBneuN Tg mice were monitored closely with palpitations of mammary glands twice a week for signs of tumor developments. Detection of the first nodule greater than 3 mm was considered as a tumor. The data presented as Kaplan-Meier survival curves indicates the percentage of tumor free animals as a function of age (weeks) in forming spontaneous tumors. The appearance of the first tumor was detected as early as 20 weeks of age but as late as 48 weeks of age.
Figure 4.14 Spontaneous tumors detected in FVBneuN transgenic mice. (A) Both pictures show the same mice, but taken from different views. The mouse on the left has a single tumor (as shown with the arrow heads) located on its right hand side. The mouse on the left has two tumors present, both of which are located on the right side. These transgenic mice formed spontaneous breast tumors at approximately 7 months of age; however, the time varied, as tumors may be seen as early as 4 months or later on than 9 months in untreated mice. (B) Diagram of the ten mammary glands located on a mouse. (C). Photograph of a representative sample of spontaneous forming tumors taken from FVBneuN Tg mice at various time points ranging from 4-10 months of age and ordered in progressing size and time frame.
Figure 4.15 Vaccination of mice with DC$_{\text{neu}}$ delays tumor development in transgenic mice. FVBneuN transgenic mice ($n = 15$) were left untreated or vaccinated with DC$_{\text{neu}}$ and pcDNAneu for a total of three times (arrows) beginning at the age of two (A) and four months (B), respectively. Spontaneous-forming breast tumors were monitored weekly. The tumor free time of the DC$_{\text{neu}}$-immunized group of mice (*) was significantly longer than that of the pcDNAneu-immunized group (Log rank test, $P<0.05$).
4.1.12 Discussion

HER-2/neu is an attractive target for the development of immunotherapeutic strategies against breast cancers. HER-2 is commonly overexpressed in 30% of breast cancer cases and its cell surface expression would make it amenable to both Abs and CTL immune responses. This is just one of many reasons for developing HER-2/neu targeted therapies. Although the humanized mAb, Herceptin, has shown incredible clinical efficacy in treating metastatic breast cancers as well as in an adjuvant setting, patients do eventually become resistant to therapy and tumors begin to grow again (Nahta and Esteva 2006). Active immunotherapy strategies such DNA and DC-based vaccines are being created to rapidly induce an immune reaction against tumor cells. Such an approach may also be immunopreventative to provide lifelong protective immunity (Lollini and Forni 2003).

The form of the Ag is important in the context of Ag processing and ultimately the loading of epitope peptides onto MHC class molecules. HER-2 encodes for a 1255 aa protein and neu encodes for a 1260 aa protein; therefore, these proteins should posses several MHC class I and class II epitopes to induce an effective immune response. It was originally reported that plasmid DNA encoding a truncated rat neu possessing only the ECD and TM domain but lacking the ICD could induce antitumor immunity that was as strong as the one induced by a plasmid encoding the full-length HER-2/neu (Weiner 1999). This indicated that there is no beneficial advantage for incorporating the ICD to induce protective immunity. Several other groups have shown the ability to induce a strong, protective immunity when using only the ECD and TM domains of HER-2/neu in a DNA vaccine (Rovero et al. 2000; Piechocki et al. 2001; Pilon et al. 2001; Lee et al. 2003). The rationale for using only the ECD and TM is to address safety issues in
regards to the possibility of unwanted signaling that may occur if the ICD included an active tyrosine kinase domain or the potential cross reactivity with the other highly homologous HER family members. Evidence from clinical trials, however, have demonstrated that CD4⁺ T cells derived from peptide vaccination recognized peptide epitopes from both the ECD and the ICD of HER-2/neu, (Disis et al. 1997; Disis et al. 1999; Kobayashi et al. 2000). This suggested the ICD may represent a better immune target given (i) its limited exposure to the external environment, (ii) possession of additional helper epitopes and (iii) the lack of tolerogenic epitopes within the ECD due to ECD shedding, as observed in the sera of patients, leading to tolerance (Disis and Cheever 1998; Foy et al. 2002). In support of this assumption, it has been shown that vaccination with plasmid DNA encoding the ICD (full length) or using the ICD protein of HER-2/neu elicited antitumor immunity, but not with plasmid DNA encoding the ECD or ECD protein (Wei et al. 1999; Foy et al. 2001). More recently, it has also been reported that the antitumor immunity derived from vaccination with plasmid DNA encoding the ICD of HER-2/neu was mainly mediated by HER-2/neu-specific CTL, but not by a humoral response (Piechocki et al. 2001; Pilon et al. 2001). Pupa et al has also recently demonstrated using a xenogeneic immunization strategy that FVBneuN mice immunized with a DNA vector encoding human HER-2 ECD-TM was less efficient at preventing tumor formation, contrary to results observed using the full-length human HER-2 (Pupa et al. 2005). Conceptually, vaccination with plasmid DNA encoding the full-length of HER-2/neu or with full-length of HER-2/neu protein should have the advantage of presenting both MHC class I and class II epitopes and therefore, induce enhanced anti-HER-2/neu immune responses. In this study, we constructed an expression vector pcDNAneu and a recombinant adenoviral vector AdVneu containing
the cDNA fragment encoding the full-length of HER-2/neu for DNA-based and engineered DC-based vaccines, respectively. The gene-transfer method presented here allowed us to compare the efficacy of anti-HER-2/neu immunity elicited by a gene-modified DC-based vaccine versus a plasmid DNA-based vaccine.

It is also important to mention the rationale for choosing to use AdV-mediated transduction of DC. In this comparison study, we chose not to use the pcDNAneu plasmid DNA mediated gene transfer into DC based upon the fact that nonviral DNA transfection methods are rather inefficient, particularly in nondividing cells. This would limit the DNA trafficking into the nucleus for transcription to occur (Luo and Saltzman 2000). Furthermore, it has been reported that the transfection efficiency of DNA into well-differentiated BM-derived DC via electroporation is limited and generally toxic to the cells with a reduction in cell viability (Arthur et al. 1997; Van Tendeloo et al. 2001; Lundqvist et al. 2002). Viral vectors, especially AdV vectors, are more commonly used for high efficiency gene transfer into DC (Wu et al. 2005). AdV-mediated gene transfer has been demonstrated to be more efficient than plasmid DNA-mediated gene transfer via liposomes and electroporation (Arthur et al. 1997; Frolkis et al. 2003). Therefore, AdV$_{neu}$ was selected instead of using pcDNAneu for gene transfer into DC in this study.

We have shown that the BM-derived DC are amenable to AdV transduction and shown there is a perturbation in the maturation status. Previous reports have also shown that AdV transduction itself can mature DC (Dietz et al. 2001; Miller et al. 2002; Molinier-Frenkel et al. 2003; Okada et al. 2003a; Schumacher et al. 2004). In this study, our data also showed that transduced DC$_{neu}$ upregulated the expression of immunologically important molecules (MHC class II, CD40, CD54 and CD80) and inflammatory cytokines (IL-6, TNF-$\alpha$, IL-1$\beta$, MIP-$\beta$ and IP-10), compared to
nontransduced DC. This indicated that AdV-transduced DC are a more immunogenic form of DC with high immunostimulatory properties and capability in stimulating both NK cells and CTL (Miller et al. 2002). For example, IL-6 is required for T cell activation and can convert Tr-mediated immune suppression (Pasare and Medzhitov 2003). TNF-α is an immunoregulatory cytokine with the ability to activate both T cells (Robinet et al. 1990) and DC (Brunner et al. 2000) and to ameliorate IL-10-mediated DC inhibition (Brossart et al. 2000b). The chemokines MIP-β and IP-10, expressed by DC\textsubscript{neu}, are each chemotactic for T cells and DC (Sozzani et al. 1996). Therefore, the up-regulation of the above cytokines and chemokines play a role in DC\textsubscript{neu}-induced anti-HER-2/neu immunity.

In this study, we have also demonstrated that mice immunized with DC\textsubscript{neu} and pcDNAneu DNA predominantly secreted IFN-γ, but not IL-4, indicating induction of mainly a Th1 cellular immune response. However, the T cells from DC\textsubscript{neu} immunized mice secreted larger quantities of IFN-γ, which allowed for Ig class switching, compared to the T cells from pcDNAneu-immunized mice. On the other hand, we also found the presence of neu-specific Abs in the sera of immunized mice. The higher IFN-γ seen in DC\textsubscript{neu} immunized mice may account for higher levels of HER-2/neu-specific IgG2a than IgG1 Ab seen in DC\textsubscript{neu} immunized mice. This confirmed that DC\textsubscript{neu} induced a mixed Th1/Th2 response rather than a strict Th1 type response while DNA immunized mice seemed to favor a mild Th1 response. Moreover, we showed the immune sera containing anti-neu Ab from mice immunized with DC\textsubscript{neu} led to down-regulated HER-2/neu expression. This mechanism has been reported with the use of Herceptin (Cuello et al. 2001) and other HER-2/neu Abs (Drebin et al. 1986; Rovero et al. 2000). The Abs from the sera of DC\textsubscript{neu} immunized mice also inhibited the growth of Tg1-1 tumor cells
to a greater degree than the sera from DNA immunized mice, whereas CaD1 cell proliferation remained relatively unaffected when cultured in the presence of immune sera. In addition, DC\textsubscript{neu} vaccination stimulated stronger neu-specific CTL responses than pcDNA\textsubscript{neu} DNA vaccination. Activated T cells from mice vaccinated with DC\textsubscript{neu} showed significant neu-specific killing of 36% (at E:T of 50) to Tg1-1 cells, compared with only 12% killing activity derived from activated T cells of mice with vaccination of pcDNA\textsubscript{neu} DNA. This CTL activity was immunologically specific, as minimal cytotoxic activity was found against the neu-negative CaD1 tumor cells. Similar degree of cytotoxicity was observed in the \textit{in vivo} cytotoxicity assays. Based upon these mechanistic studies, it suggested that DC\textsubscript{neu} immunization induced a mixed cellular and humoral response that was of a greater magnitude than that observed using the DNA vaccine.

Neu-specific Abs have previously been identified in mice immunized with peptides (Jasinska et al. 2003), HER-2/neu allogeneic cell expressing IL-12 (Cefai et al. 1999; Nanni et al. 2001; De Giovanni et al. 2004), protein (Esserman et al. 1999; Renard et al. 2003), DNA (Chen et al. 1998; Amici et al. 2000; Lachman et al. 2001; Renard et al. 2003; Quaglino et al. 2004a) and DC (Chen et al. 2001a; Sakai et al. 2004). These Abs may target a series of different epitopes that can have an inhibitory or a stimulatory effect on tumor cell growth (Yip et al. 2001). This may explain some conflicting results in the literature. Some groups have stated that despite the presence of neu-specific Abs, they are not directly involved with the antitumor protection (Chen et al. 1998; Foy et al. 2001; Lindencrona et al. 2004). Contradicting this, other groups have found that Abs are an important component, along with the typical cellular response involving the generation of CD8\textsuperscript{+} CTL and CD4\textsuperscript{+} Th cells, for antitumor immune protection (Rovero
Another possible reason for this discrepancy between the actual roles of B cells, therefore Abs, is the tumor model used. There may be some other functional differences between the parental wildtype and Tg mice besides immune tolerance to neu. It has been demonstrated that both a humoral and cellular response was required to reject tumors in mice (Reilly et al. 2000; Reilly et al. 2001). Furthermore, stronger Ab responses were seen in parental FVB/N mice compared to the corresponding FVBneuN Tg counterpart (Reilly et al. 2001). More recently, B cell deficient knock out mice have allowed for further dissection of the immune response to show B cell deficient mice failing to produce anti-neu Abs and developing tumors (Nanni et al. 2004; Quaglino et al. 2004a; Quaglino et al. 2004b; Park et al. 2005). Interestingly, one study showed that even in these B cell deficient mice, approximately two-thirds of the mice were still able to form Abs of different subclasses and continued to provide a higher level of antitumor protection (Nanni et al. 2004). However, majority of these reported studies stressed the importance of B cells, thus the presence of anti-neu Abs, with IgG2a subset being the most important, and T cell-derived cytokines (i.e. IFN-γ) produced by CD4+ Th1 cells playing an important role in inhibiting tumor formation (Cappello et al. 2003; Curcio et al. 2003; De Giovanni et al. 2004; Dela Cruz et al. 2005; Lo Iacono et al. 2005; Park et al. 2005). It has also been demonstrated that the use of a mixture of IgG1 anti-HER-2/neu mAb recognizing different epitopes also provided significant protection in FVBneuN mice upon tumor challenge (Wolpoe et al. 2003) and reduced growth of human xenografts (Spiridon et al. 2002). Overall, some of the effects of the anti-neu Abs (both IgG1 and IgG2a) may range from blocking p185 receptor function and
inducing receptor down-regulation that will affect cell survival, proliferation, angiogenesis and metastasis (Katsumata et al. 1995; Yarden and Sliwkowski 2001; Spiridon et al. 2004). The Abs may also prevent the formation of heterodimers, leading to cell growth inhibition through G1 cell cycle arrest and p27 induction, prevent the cleavage of the ECD and inhibit angiogenesis. Immune mediated mechanisms may also play a role such as CDC and ADCC via the Fcγ receptor on leukocyte membranes (Baselga and Albanell 2001). In vivo, these Abs have been also been shown to mediate receptor down-modulation and diminished proliferation with decreased proliferating cell nuclear Ag (PCNA) detected, halting the progression of preneoplastic lesions (Quaglino et al. 2004a; Quaglino et al. 2004b).

More importantly, the in vivo animal studies showed the generation of antitumor immunity offered complete protection in DC_{neu} immunized mice compared to the moderate protection seen in plasmid DNA immunized mice, when challenged with neu-expressing tumor cells using two different wildtype models. FVB/N mice given the DC_{neu} vaccine were completely protected from a challenge of Tg1-1 cells whereas the DNA vaccine only protected 50% of the mice. When taken into account the general pattern observed when studying the antitumor mechanisms, the fact that DNA immunized mice were only partially protected was not surprising, since we have clearly shown that DC_{neu} immunized mice stimulated both a stronger neu-specific cellular and humoral immunity compared to their counterparts. This conclusion was further solidified using another wild-type mouse MCA-26neu tumor model to examine the induction of antitumor immune responses with prophylactic immunizations. Vaccination of DC_{neu} cells also resulted in tumor protection in 8 out of 8 mice, whereas none of the mice vaccinated with pcDNAneu rejected a tumor challenge with MCA-
26neu expressing the neu Ag. Renard et al reported similar in vivo findings in a different animal model system with 50%-80% protection seen in DNA immunized mice (Renard et al. 2003). These results indicated that immunization of both FVB/N and BALB/c mice with two rounds of DC \textsubscript{neu} vaccine provided significant amount of antitumor protection against neu-expressing tumor cell challenge.

The FVBneuN Tg mice were derived from the parental FVB/N strain and expressed the wild-type rat neu cDNA under the control of a mouse mammary tumor virus promoter (Guy et al. 1992). Female mice stochastically develop spontaneous mammary tumors in situ and small mammary tumors beginning at 2 and 4 months of age, respectively (Cifaldi et al. 2001). Because the HER-2/neu tumor Ag is endogenous to the host, this allows for development of self-tolerance to the HER-2/neu Ag, as evidenced by the poor ability of these mice to develop HER-2/neu-specific antitumor immunity following vaccination as compared with the parental FVB/N mice (Reilly et al. 2000; Renard et al. 2003). This finding was highlighted in our study whereby FVBneuN immunized with DC \textsubscript{neu} twice failed to reject the highly tumorigenic Tg1-1 tumors, even when challenged with a 100-fold lower dose (0.03x10\textsuperscript{6} cells) typically used for inducing tumor growth in FVB/N mice. Therefore, this mouse model of breast cancer that closely mimics immune tolerance that exists in some patients with cancer becomes a suitable model for evaluating HER-2/neu-targeted vaccines (Pupa et al. 2001). In this study, we demonstrated that DC \textsubscript{neu} vaccinations starting at two months of age delayed breast cancer development significantly longer in Tg mice than DNA vaccine, indicating that AdV-mediated neu-gene-engineered DC vaccine was superior to DNA vaccination in breast cancer prevention of Tg mice predestined to form mammary tumors. However, there were no cases of complete prevention of breast cancer.
development observed in DC_{neu} immunized Tg mice. The neu-specific self tolerance observed in Tg mice may account for the relatively low efficacy observed in DC_{neu} immunized FVBneuN, despite expression of IL-6 that allows for partial suppression of Tr-mediated immune tolerance. Furthermore, regardless of the vaccine used to immunize mice beginning at four months of age, negligible effects were seen on the ability to hamper spontaneous tumor development in DC_{neu} immunized compared to both DNA immunized and age-matched control mice.

The delay observed when immunizing mice with DC_{neu} at the younger age may be a result in the early generation of an effective immune response against neu but over time, the immune response declines in the presence of clinical tumors overexpressing neu (Takeuchi et al. 2004; Lo Iacono et al. 2005). This may imply that additional boosters are required later on in life to maintain the immune response or perhaps alter the vaccination schedule for life-long administration (Astolfi et al. 2005). By increasing the number of immunizations, it may activate low-avidity T cells to further stimulate more T cells after tolerization and elimination or to allow for the formation of a memory response (Lustgarten et al. 2004). Moreover, the immune response generated at an earlier age and the resulting preventative effects may also reflect the histopathologic status of the mammary glands within these mice during the time of immunization with atypical hyperplasia seen at 2 months of age whereas at 4 months of age, infiltrating ductal carcinomas are seen (Pupa et al. 2001). The decline in the immunopreventative effects of the cancer vaccine has been demonstrated by others that the age at which vaccinations were started could determine the overall protective effects (Cappello et al. 2003; Provinciali et al. 2003; Sakai et al. 2004; Pupa et al. 2005). At an older age, the immune system is less able to respond with impaired T cell proliferation and CTL
generation, cytokine secretion and Ab production (Provinciali et al. 2003). In FVBneuN mice, vaccinations started at 28 weeks of age was ineffective at preventing tumors from forming whereas vaccinations beginning at 6 weeks followed by continuous booster every 4 weeks offered the greatest degree of prevention (Boggio et al. 2000). A similar pattern was also confirmed in BALB-neuT immunized mice at a younger age offering a greater degree of protection and prevention compared to older aged mice (Boggio et al. 1998; Boggio et al. 2000).

Upon inducing a protective immune response, multifocal preneoplastic cells are inhibited (Quaglino et al. 2004a; Quaglino et al. 2004b); however, there may be some dormant tumor cells remaining. Depending upon the condition, these remaining cells may develop into neu-negative expressing tumor cells (Knutson et al. 2004), or decreased MHC class I neu-positive expressing tumor cells (Lollini et al. 1998). It may also, in the face of decreasing protective response allow for the neu expressing tumors to regrow. Also, as the tumors begin to progress, alterations in hematopoiesis may generate larger amounts of immunosuppressive immature myeloid cells (Melani et al. 2003), CD4+ T cell dysregulation and the generation of increasing number of Tr cells (Manjili et al. 2003; Ercolini et al. 2005) as seen in both mice and humans. Other factors may also include further induction of tolerance or development of immunological escape of tumor cells through the secretion of immunosuppressive cytokines such as vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β). These factors may directly impact the function of T cells or indirectly on DC (Takeuchi et al. 2004). The expression of Fas-L by tumors may also induce T cell apoptosis (Cefai et al. 2001).
Overall, the results from both of our \textit{in vivo} studies showed that FVBneuN mice are tolerant to neu but DC\textsubscript{neu} immunization was able to partially overcome this tolerance. Our results were similar in comparison to those obtained by Reilly \textit{et al} (Reilly \textit{et al.} 2000; Reilly \textit{et al.} 2001). However, the failure to completely protect and prevent tumors from forming in these mice indicated that the efficiency of HER-2/neu-targeted DC-based vaccination needs further improvement. The addition of immunostimulatory cytokines may be one avenue that deserves attention. Vaccination of DC engineered by two different AdV to simultaneously express a TAA such as HER-2/neu or gp100 along with cytokines such as TNF-\(\alpha\) (Chen \textit{et al.} 2002c), IL-12 (Chen \textit{et al.} 2001a; Okada \textit{et al.} 2005) or GM-CSF (Nakamura \textit{et al.} 2002) has shown augmented immunity compared to DC engineered to express either the TAA or cytokine alone. Therefore, DC engineered to express both tumor Ag and cytokine gene may represent a new powerful direction in DC-based vaccine to improve and strengthen antitumor immunity.

The reduced efficacy of pcDNAneu DNA vaccine in generating anti-HER-2/neu immunity compared to DC\textsubscript{neu} vaccine may be derived from the following reasons. As indicated above, immunization using naked DNA relied on the uptake of DNA by host APC (Condon \textit{et al.} 1996; Akbari \textit{et al.} 1999). Defective APC function \textit{in situ} has been documented in tumor-bearing mice and may in part explain the failure in induction of effective immunity using plasmid DNA in these studies (Chaux \textit{et al.} 1997; Troy \textit{et al.} 1998). Secondly, it has also been reported that DNA vaccination induced low level of CTL activity and often failed in breaking immune tolerance in animal models (Rovero \textit{et al.} 2000). Another important factor may stem from the reduced number of immunizations/boosters with the DNA plasmid used in our study compared to other reports that have varying immunization protocols ranging from a total of 3
immunizations to continuous boosting throughout the entire study period for the rest of their life (Amici et al. 1998; Chen et al. 1998). The number of injections may be a critical factor, as noted by Bellone et al. whereby mice immunized 3 times with an OVA-DNA vector provided a similar level of protection just as effective as a single dose of OVA I peptide pulsed DC vaccine (Bellone et al. 2000), clearly supporting the strength of DC-based vaccinations.

Taken together, we have demonstrated that AdV-mediated HER-2/neu-gene-modified DC vaccine is more potent than DNA vaccine in both protective and preventive animal tumor models in our side-by-side comparison study. Therefore, DC genetically engineered to express tumor Ag such as HER-2/neu are likely representative of a new direction in DC-based vaccine of breast cancer.
4.2 Xenogeneic approach utilizing adenovirus mediated transfer of human HER-2 into dendritic cells to overcome self tolerance to rat neu

4.2.1 HER-2 adenovirus creation and DC expression

Previous studies have utilized the human HER-2 gene for DNA-based immunization against neu-expressing tumors in both normal and Tg mouse models (Wei et al. 1999; Pilon et al. 2001). More recently, the use of human HER-2 DNA-based vaccine was shown to prevent the formation of spontaneous tumors in FVBneuN Tg mice, thus acting as a form of xenogeneic immunization (Pupa et al. 2005). Therefore, we employed similar methods in the previous section to create a recombinant AdV containing human HER-2 and subsequently used the AdV for transduction of DC. The full length HER-2 gene was cloned from cDNA obtained from a HER-2 expressing breast tumor cell line. To address the potential safety issue, site directed mutagenesis was performed to inactivate ATPase activity within the kinase domain (Ben-Levy et al. 1994; Wei et al. 1999), rendering the expressed gene kinase deficient. The recombinant adenovirus was amplified, purified by two-rounds of CsCl ultracentrifugations and dialyzed. PCR analysis was performed to verify the resulting AdV construct contained the appropriate transgene.

As shown in Figure 4.16A, the analytical gel from PCR analysis revealed that the final purified product (lane F) does indeed contain the transgene with the generation of a 2,277 bp product. Both water and plasmid backbone vector served as negative controls (lane A and B, respectively) and the shuttle vector containing the transgene as the positive control (lane C). Both the crude viral lysate (lane D) and culture media (lane E) from 293 infected cells produced similar bands, which supported the detection of HER-2
within the final purified AdV preparation. As well, expression of the HER-2mut transgene product was clearly detected in AdV\textsubscript{HER-2mut} transduced DCs (DC\textsubscript{HER-2mut}) using the anti-HER-2 specific antibody (Herceptin), generously supplied by Dr. Sami from the Saskatoon Cancer Centre, while no expression of HER-2 was detected in nontransduced DC (Figure 4.16B).

4.2.2 Examination of DC\textsubscript{HER2mut} ability to induce protective and preventive antitumor immunity in mice

To examine the utility of a xenogeneic DC vaccine expressing HER-2mut against neu expressing tumors, a proof of principle study was performed. This allowed us to determine whether prophylactic immunization of mice with a DC\textsubscript{HER2mut} vaccine induced immunoprotective effects by protecting the mice from developing tumors upon tumor challenge. Previous immunizations with DC\textsubscript{neu} in wildtype mice provided strong antitumor protective responses (Section 4.1.5; Figure 4.6) but failed to do so in the FVBneuN Tg mice (Figure 4.7), so FVBneuN Tg mice were used to determine if tolerance could be overcome against neu. We found that FVBneuN mice (n=10/group) immunized with DC\textsubscript{HER2mut} followed by a boost two weeks later showed the greatest potential with significantly enhanced survival by delaying tumor growth compared to mice injected with only PBS (P <0.0001; Log-rank). As a comparison, immunization of FVBneuN Tg mice with DC\textsubscript{neu} saw only a minimal, but still a significant delay in tumor growth compared to mice injected with PBS only (P <0.05; Log-rank). Albeit, prolonged tumor free survival was observed in all of the vaccinated mice but all of the mice eventually developed progressively growing tumors. This suggested that the tolerance to rat neu was not completely overcome.
**Figure 4.16** AdV\textsubscript{HER2mut} construction and gene expression verification. A. PCR gene analysis. The samples were loaded onto a 1% agarose gel after 30 cycling reactions. Controls include a water control (A); the backbone vector, pAdEasy-1 (B); the recombinant vector, pAdEasy\textsubscript{HER2mut} (C). Samples include the crude viral lysate from infected cells (D), the infected culture media (E) and the purified AdV\textsubscript{HER2mut} (F). The presence of the 2.1 kb band indicated the presence of the HER2 gene within the viral vectors. B. Flow cytometric analysis of HER-2 expression. The expression of HER-2 on the cell surface of DC and DC\textsubscript{HER2mut} was analyzed by staining the cells with human anti-HER-2 Ab followed by incubation with anti-human IgG-FTIC Ab (dark line). Isotype matched mAb (faint band).
Figure 4.17 Vaccination of FVBneuN mice with DC_{HER2mut} improves survival. FVBneuN mice (10 per group) were immunized twice with 1x10^6 DC_{neu} (▲) or DC_{HER2mut} (■) s.c. or with PBS (▼). Mice were challenged with 0.3x10^6 Tg1-1 cells and survival was monitored closely for tumor development and survival. A Kaplan Meier plot of survival is shown and log-rank tests performed to compare survival between the different groups. * P<0.05, ** P<0.0001; note that between DC_{HER2mut} and DC_{neu} P=0.003.
With the encouraging results seen from the previous animal study, we moved on to examine the effects of vaccinating FVBneuN Tg mice (n=15-20/group) with DC\textsubscript{\text{HER-2mut}} or PBS alone. The Tg mice were immunized with $1 \times 10^6$ DC\textsubscript{\text{HER-2mut}} cells beginning at two months of age and every 4 weeks thereafter, for a total of 3 times. The development of spontaneous tumors was closely monitored with all mammary glands palpitated weekly. We found the age-matched control mice, injected with only PBS, all developed tumors within 5 to 7 months of age (Figure 4.18A), which was within the range normally observed in our previous Tg animal study (Figure 4.13 and 4.15). Mice vaccinated with DC\textsubscript{\text{HER-2mut}} showed a significant delay in the development of its first detectable spontaneous breast tumor ($P<0.0001$; Log-rank). In addition, DC\textsubscript{\text{HER-2mut}} immunized mice developed less mammary tumors on average (2.1 mammary tumors at 38 weeks) compared to the PBS age-matched controls with 3.6 mammary tumors during the same period (Figure 4.18B). However, this alone was not sufficient to successfully prevent tumors from growing as all of the mice eventually developed spontaneous tumors.
Figure 4.18  The effect of vaccination on tumor development in transgenic mice. (A). FVBneuN mice (8 weeks old; n=15-20/group) were given three injections of 1x10^6 DC\textsubscript{HER2mut} (▲) s.c. at 4-week intervals (indicated by arrows) or with PBS (■) then continually monitored for development of spontaneous tumors. Mice with at least one mammary tumor were classified as tumor bearing. Tumor incidence was significantly delayed in DC\textsubscript{HER2mut} immunized mice (*, P<0.0001, log-rank test). (B), The mean tumor multiplicity (mean number of tumors per mouse) in both PBS and DC\textsubscript{HER2mut} immunized mice was recorded throughout the same time. *, P<0.0001 (Student’s t test).
4.2.3 Discussion

The use of xenogeneic approach in cancer vaccines attempts to utilize the “foreignness” of an Ag to overcome tolerance and mount a protective immune response against a “self” Ag (Wei 2002). Xenogeneic immunization strategies have mainly been associated with the use of DNA or protein based vaccines. Some of the Ags previously used included human gp100 (Gold et al. 2003; Yamanaka and Xanthopoulos 2005), tyrosinase related protein-1 TRP-1/gp75 (Naftzger et al. 1996; Weber et al. 1998) and EGFR (Lu et al. 2003) to overcome self-tolerance and reject syngeneic tumors in mouse animal models.

Recently, xenogeneic immunization strategies have been examined for use to target HER-2/neu. Protein immunization of rats utilizing human ICD generated significantly higher titer levels of HER-2 and neu-specific Abs as well as T cell responses compared to respective controls (Disis et al. 1998). Further studies by Pupa have shown the ability of DNA xenoimmunization in BALB-neuT Tg mice with either the full-length or the ECD-TM portion of human HER-2 to induce antitumor immune responses that delayed tumor growth (Pupa et al. 2001; Pupa et al. 2005). When the full length of HER-2 was used in the vector, VR1012/HER-2-FL, a significant delay in the formation of spontaneous tumors with over 60% remaining tumor free at 50 weeks of age was seen (Pupa et al. 2001). All of the control mice developed tumors by the 36th week of age. When only the ECD-TM portion was used in the vector, VR1012-ECD-TM, only 25% of the Tg mice remained tumor free (Pupa et al. 2005). This suggested that the inclusion of the ICD may contain additional helper epitopes to generate protective immune responses and provided a basis for us to create an AdV containing the full length HER-2 gene, AdV_{HER2mut}, for use in a DC-based vaccine. In a recent
report, an AdV containing the full-length HER-2 gene created by another group was used to directly immunize BALB-neuT Tg mice. This immunization strategy led to the induction of a humoral and cellular response that slightly delayed the onset of spontaneous tumors (Gallo et al. 2005).

The initial in vivo studies utilizing AdV mediated transfer of HER-2 into BM derived DC (DC\textsubscript{HER-2mut}) provided proof that prophylactic immunization of neu-tolerized FVBneuN mice at high risk of developing breast tumors provided a benefit to some degree. When mice were challenged with a lethal dose of $3 \times 10^5$ Tg1-1 tumor cells, DC\textsubscript{HER-2mut} immunization significantly delayed the formation of tumors and increased their overall survival whereas PBS immunized mice all succumbed to large tumors by day 27. Furthermore, we found that immunization of mice with DC\textsubscript{HER-2mut} was more effective at delaying tumor growth when compared to mice immunized with DC\textsubscript{neu}. This suggested DC\textsubscript{HER-2mut} was able to break the tolerance in FVBneuN Tg mice to rat neu. From this animal study, it showed that the use of the human HER-2 gene provided stronger protective immunity than using the syngeneic neu gene in a DC vaccine. For the prevention of spontaneous tumors from forming, immunization of mice with DC\textsubscript{HER-2mut} delayed the formation of mammary tumors and significantly reduced the number of mammary tumors formed to an average of 2.1 tumors compared to an average of 3.6 tumors seen in control group mice. Although there was a significant delay observed in DC\textsubscript{HER-2mut} immunized mice, all of the mice eventually developed tumors. Our results contrasts the results obtained by Pupa et al whereby they found 70% of their vaccinated mice remained tumor free (Pupa et al. 2005). However, it is rather difficult to compare their model system with our model system, as we did not perform DNA vaccination in our study. As well, the number of vaccinations/boosts may be an
indicator since we only used three monthly immunizations, while Pupas’ study in Tg mice received a total of 10 monthly vaccinations. It is also extremely difficult to assess the differences without determining the actual immune mechanisms involved as well as determining the role that the endogenous murine neu Ag has with autoimmunity and cross-reactivity (Concetti et al. 1996; Lin et al. 2004; Pupa et al. 2005). These immune mechanisms will need to be further analyzed to fully understand and compare the data.

Xenogeneic HER-2 immunization provides a source of Ag that not only encodes for peptides that are identical but may also be completely different from that of rat neu throughout the protein. One mechanism of action may arise from slight alterations within known peptide epitopes, acting in a heteroclitic peptide fashion. This heteroclitic peptide may have several AA residues altered that would increase the binding efficacy on MHC class molecules therefore acting as a better agonist than the native peptide in inducing immune responses (Scardino et al. 2002; Gold et al. 2003). The recently identified immunodominant MHC class I neu peptide in FVB mice is PDSLRLDSVF (RNEU_{420-429}). This peptide was altered to become PA_{SLRDL}SVF (RNEU_{420-429}A2), as determined by alanine substitution, resulting in increased T cell stimulatory activity. The modified peptide used in a DC-peptide vaccine revealed enhanced protection in FVB/N and FVBneuN mice (Ercolini et al. 2003). In comparing the HER-2 aa sequence with neu for the same region (Figure 1.1), only a single aa change (PDSL_PDLSVF) is found, which may potentially increase the binding affinity to MHC class I molecules to account for the overall improved survival seen in our experiments. The use of these heteroclitic peptide may stimulate T cells specifically recognizing not only the heteroclitic peptide sequence but also activate T cells against the native peptide (Trojan et al. 2001). It has also been recently documented that FVBneuN mice are already
tolerized to the immunodominant T cell epitope, thus failing to induce a response, (Ercolini et al. 2003; Murata et al. 2006) and this may account for the lack of overall effective protection. However, it cannot be ruled out that epitope spreading or the generation of cryptic and subdominant epitopes may also be involved with the enhanced protection observed, as previously observed when using Listeriolysin O with rat neu (Singh et al. 2005). It is also interesting to note that the aa sequences between the rat and mouse protein is 94.8% homologous (Nagata et al. 1997) and within the defined dominant epitope, there is a difference of 3 aa (PESFQDLSVF). These xenogeneic immunization strategies may potentially form cross-reactive Abs against the mouse form of neu, which remains endogenously expressed at low levels on adult mouse epithelial cells (Quaglino et al. 2004a; Pupa et al. 2005).

Overall, this strategy attempts to develop cross-reactive immune responses against a self-Ag and detailed immune protective mechanisms involved will need to be examined. Xenogeneic immunization strategies have previously been utilized successfully in DC-based vaccines. In a recent report utilizing murine BM DC transfected with DNA plasmid vectors encoding the human survivin gene, a substantial protective immunity in a C57BL/6 model of glioma was observed (Ciesielski et al. 2006). Furthermore, patients with metastatic prostate cancer were immunized with DC expressing murine prostatic acid phosphatase (PAP) in a clinical study and 11 out of 21 patients developed T cell proliferative responses (Fong et al. 2001a). More importantly, six of these patients developed clinically stabilized disease and the immune responses generated cross-reacted with the human PAP. Therefore, by combining the powerfulness of DC-based vaccines loaded with xenogeneic Ags or “altered self”, another arsenal weapon is available as a candidate for a cancer vaccine.
4.3 PART B – Enhancement of T cell immunity induced by DC phagocytosis of HSP70-transfected tumor cells on antitumor immunity

4.3.1 Tumor cell apoptosis induced by *in vitro* irradiation

To study the potential effect of HSP70 in DC-mediated antitumor immunity, we selected two tumor cell lines EG7 and MCA-26 (MCA) based upon their expression of HSP70 (Figure 4.19A) in this study. EG7 cells expressed HSP70 while MCA cells lacked the expression of HSP70. In addition, we constructed an expression vector pcDNA-HSP70 and further transfected MCA-26 tumor cells with this vector. As shown in Figure 4.19A, the transfected tumor cell line MCA-26/HSP70 (MCA/HSP) expressed HSP70 after vector transfection. Next, MCA, MCA/HSP and EG7 tumor cells were irradiated with 5,000 and 1,000 rad, respectively, to induce tumor cell apoptosis. MCA tumor cell apoptosis occurred in two phases, an early and late phase, in relation to time. The early phase was characterized by expression of PS on the cell membrane as detected by Annexin V-FITC and the late phase characterized by expression of HSP70 detected by anti-HSP70 antibody (Chen et al. 2001c), at 12 and 48 hr after irradiation, respectively (Figure 4.19B and C, respectively). These cells were termed MCA-E for cells during the early apoptotic phase and MCA-L for cells in the late apoptotic phase. Both MCA/HSP and EG7 tumor cells expressed HSP70 before irradiation, but not PS. After irradiating and in the early phase of apoptosis, these cells expressed PS and HSP70, whereas, in the late phase of apoptosis, enhanced expression of both PS and HSP70 was detected (Figure 4-19 B and C). Based upon the different stages of apoptosis, these tumor cells were termed MCA/HSP-E, MCA/HSP-L, EG7-E and EG7-L, respectively.
Figure 4.19 Induction of tumor cell apoptosis by irradiation. A. HSP70 expression in tumor cells. Non-treated tumor cells were stained with anti-HSP70 antibody and expression detected by flow cytometry. Next, the MCA, MCA/HSP and EG7 tumor cells were irradiated in vitro with 5,000 and 1,000 rad, respectively, cultured in DMEM plus 10% FCS for 12 and 48 hr, and then harvested. The original (dotted lines) and apoptotic (solid lines) tumor cells were then analyzed using Annexin V-FITC (B) and anti-HSP70 antibody (C) by flow cytometry. One representative experiment of two is shown.
4.3.2 Enhanced maturation of DC with phagocytosis of apoptotic tumor cells expressing HSP70

DC used in this study were derived from mouse bone marrow cells cultivated in culture medium in the presence of IL-4 and GM-CSF. These DC showed (i) typical morphologic characteristic of DC with numerous dendrites and (ii) the expression of MHC II, CD54 (ICAM-1), CD80 and CD86 (Figure 4.20), indicating that they were relatively mature DC (Chen et al. 2002b). After physical contact with apoptotic MCA tumor cells in culture, DC displayed phagocytosed apoptotic tumor cell in the cytoplasm, as shown by electron microscopy (Figure 4.21). To quantify the amount of apoptotic cells phagocytosed by DC, CFSE-labeled apoptotic MCA-E and MCA/HSP-E tumor cells (in early phase of apoptosis) or apoptotic MCA-L tumor cells (in late phase of apoptosis) were used for physical contact with DC in culture followed by flow cytometric analysis. Approximately 44-48% of DC displayed the phagocytosed CFSE-apoptotic tumor cells in their cytoplasm by flow cytometric analysis (Figure 4.22), indicating that BM-derived DC phagocytosed an equal amount of apoptotic cells in both the early and late phases of apoptosis, irregardless of HSP70 expression.

To verify whether further DC maturation was accompanied by phagocytosis of apoptotic tumor cells expressing HSP70, DC were further subjected to flow cytometric analysis. DC_{MCA-E} with phagocytosis of MCA-E tumor cells without expression of HSP70 expressed similar amount of MHC II, ICAM-1, CD80 and CD86 as DC without phagocytosis, when comparing the levels in Figure 4.23 with Figure 4.20. Both DC_{MCA-L} and DC_{MCA/HSP-E}, which had phagocytosed apoptotic MCA-L or MCA/HSP-E tumor cells expressing HSP70, respectively, displayed an upregulated expression of the above molecules, compared to DC_{MCA-E} with phagocytosis of apoptotic MCA-E tumor cells.
Figure 4.20 Phenotypic analysis of cultured dendritic cell. Bone-marrow derived DC were cultured in the presence of GM-CSF and IL-4. Flow cytometric analysis was performed to examine the cell surface expression of MHC class II (Ia^b), CD54 (ICAM), CD80 and CD86. Irrelevant isotype-matched antibodies were used as controls (light shaded dotted line). One representative experiment of two is shown.
Figure 4.21 Ultrastructural observation of apoptotic tumor cells and dendritic cells (DC). (A) Apoptotic MCA tumor cells were induced by irradiation (5,000 rad) treatment. Note the marked cytoplasmic condensation and blebs that form apoptotic bodies (arrow). (B) The apoptotic tumor cell (arrow) within the cytoplasm of DC that was co-cultured with apoptotic tumor cells overnight.
Figure 4.22 Flow cytometric analysis of apoptotic tumor cells phagocytosed by dendritic cells. DC (solid lines) were incubated with CFSE-labeled apoptotic tumor cells for 24 hr at 37°C, purified with Ficoll-Paque gradient then analyzed by flow cytometry. DC alone were used as the control (dotted lines).
without HSP70 expression (Figure 4.23). In fact, DC\textsubscript{MCA/HSP-E} had a more significant increase in the expression of these molecules compared to DC\textsubscript{MCA-L} and DC\textsubscript{MCA-E}. DC\textsubscript{MCA/HSP-L}, DC\textsubscript{EG7-E} and DC\textsubscript{EG7-L} with phagocytosis of apoptotic MCA/HSP-L, EG7-E and EG7-L tumor cells expressing HSP70 all displayed an up-regulation of the above molecules (similar to the data seen in Figure 4.23), indicating that the expression of HSP70 on apoptotic tumor cells further stimulated DC maturation after DC phagocytosis. Interestingly, DC\textsubscript{EG7-E} displayed more OVA peptide-MHC class I complex (pMHC I) expression than DC\textsubscript{EG7-L}, as detected by flow cytometry using anti-pMHC Ab obtained from Dr. Germain from National Institutes of Health (NIH; Bethesda MD), even though they expressed similar amounts of MHC I Ag (Figure 4.24). This may be indicative of the ability for DC\textsubscript{EG7-E} to cross-present OVA more efficiently to T cells than DC\textsubscript{EG7-L} as there would be more peptide-bound to the grooves of MHC molecules to activate T cells.

### 4.3.3 Enhanced CD8\textsuperscript{+} T cell proliferation induced by DC with phagocytosis of HSP70-expressing tumor cells in early phase of apoptosis

DC are known to be potent stimulators of primary mixed lymphocyte reaction (MLR) and induce the proliferation of allogeneic CD8\textsuperscript{+} T cells \textit{in vitro} (Inaba et al. 1987). To assess this function, we compared DC which had phagocytosed apoptotic tumor cells with or without HSP70 expression in regards to their effect on primary allogeneic MLR. As shown in Figure 4.25A, both DC\textsubscript{MCA-L} which had phagocytosed apoptotic MCA-L and DC\textsubscript{MCA/HSP-E} which had phagocytosed MCA/HSP-E tumor cells expressing HSP70 more strongly stimulated allogeneic C57BL/6 T cell proliferation in comparison to DC\textsubscript{MCA-E} with phagocytosis of MCA-E lacking HSP70.
Figure 4.23: Phenotypic analysis of DC which had phagocytosed apoptotic tumor cells by flow cytometry. DC with phagocytosis of apoptotic MCA-E (early phase of apoptosis), MCA-L (late phase of apoptosis), MCA/HSP-E (early phase of apoptosis) were termed DC\textsubscript{MCA-E}, DC\textsubscript{MCA-L}, and DC\textsubscript{MCA/HSP-E} and analyzed to measure surface expression (solid lines) of MHC class II antigen, ICAM-1, CD80 and CD86 molecules by flow cytometry. Irrelevant isotype-matched antibodies were used as controls (dotted lines). One representative experiment of two is shown.
Figure 4.24  pMHC I complex expression. DC with phagocytosis of apoptotic EG7 tumor cells in early or late phase of apoptosis (solid lines) were analyzed using anti-MHC I and pMHC I antibodies, respectively, by flow cytometry. Irrelevant isotype-matched antibodies were used as controls (dotted lines). One representative experiment of two is shown.
Figure 4.25 Mixed lymphocyte reaction. (A) Irradiated DC\textsubscript{MCA-E}, DC\textsubscript{MCA-L}, DC\textsubscript{MCA/HSP-E} and DC\textsubscript{MCA/HSP-L} which had phagocytosed apoptotic MCA and MCA/HSP tumor cells starting with $0.4 \times 10^5$ cells/well and its reciprocal dilutions were added to $1 \times 10^5$ allogeneic C57BL/6 T cells. Irradiated DC\textsubscript{EG7-E} and DC\textsubscript{EG7-L} which had phagocytosed apoptotic EG7 tumor cells starting with $0.4 \times 10^5$ cells/well) and its reciprocal dilutions were added to (B) allogeneic T cells or (C) CD8$^+$ T cells ($1 \times 10^5$ cells/well) derived from BALB/c or OTI mice, respectively. Cells were co-cultured for 3 days. [$^3$H]-thymidine uptake after overnight incubation is expressed as the mean of three determinations. *, $P<0.05$ versus cohorts of DC\textsubscript{MCA-E} or DC\textsubscript{EG7-L} (Student’s $t$ test). One representative experiment of three is shown.
expression ($P<0.05$; Student’s $t$ test). In addition, $\text{DC}_{\text{MCA/HSP-L}}$ was also shown to efficiently stimulate T cells just as effectively as $\text{DC}_{\text{MCA/HSP-E}}$ did. This suggested that DC with phagocytosis of apoptotic MCA tumor cells in the late phase of apoptosis are more immunogenic than DC with phagocytosis of apoptotic tumor cells in early phase of apoptosis, when MCA tumor cells lack the induced endogenous expression of HSP70. It also showed that when increasing levels of HSP70 expression was present, as seen in DC phagocytosis of the MCA-HSP-E and –L cells, allogeneic T cell stimulatory activity was comparable to each other.

Another MLR assay was performed to assess the stimulatory effect of $\text{DC}_{\text{EG7-E}}$ and $\text{DC}_{\text{EG7-L}}$ on allogeneic BALB/c T cells. As shown in Figure 4.25B, both $\text{DC}_{\text{EG7-E}}$ and $\text{DC}_{\text{EG7-L}}$ with phagocytosis of endogenous HSP70-expressing apoptotic EG7 tumor cells stimulated allogeneic T cell proliferation to a similar extent. Furthermore, to assess the stimulatory effect of $\text{DC}_{\text{EG7-E}}$ and $\text{DC}_{\text{EG7-L}}$ on OVA-specific CD$^8^+$ T cells, we further performed OVA-specific T cell proliferation assay using CD$^8^+$ T cells derived from OVA-specific TCR-Tg OTI mice. As shown in Figure 4.25C, $\text{DC}_{\text{EG7-E}}$ significantly enhanced OVA-specific T cell proliferation $\textit{in vitro}$ in comparison to $\text{DC}_{\text{EG7-L}}$ ($P<0.05$; Student’s $t$ test). To further confirm this, we conducted an $\textit{in vivo}$ T cell proliferation experiment using OVA-specific tetramer staining to enumerate the number of OVA-specific T cells. As shown in Figure 4.26, vaccination of mice with $\text{DC}_{\text{EG7-E}}$ and $\text{DC}_{\text{EG7-L}}$ stimulated 2.5 % and 1.5 % OVA-specific CD$^8^+$ T cells of the total CD$^8^+$ T cell population using OVA-specific tetramer staining. This indicated that DC with phagocytosis of apoptotic EG7 tumor cells endogenously expressing HSP70 during the early phase of apoptosis is more immunogenic than DC with phagocytosis of apoptotic EG7 tumor cells in the late phase of apoptosis.

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Figure 4.26 In vivo T cell proliferation assay. C57BL/6 mice were also i.v. immunized with irradiated $\text{DC}_{\text{FG7-E}}$ and $\text{DC}_{\text{EG7-L}}$ ($5 \times 10^5$ cells) which had phagocytosed apoptotic EG7 tumor cells. Six days subsequent to immunizations, tail blood samples were incubated with PE-H-2K^b/OVA I tetramer and FITC-anti-CD8 Ab according to the company’s protocol and samples were analyzed by flow cytometry. One representative experiment of three is shown.
4.3.4 Enhanced CD8⁺ CTL responses induced by DC with phagocytosis of HSP70-expressing tumor cells in early phase of apoptosis

To examine the cytotoxic effect of CTL responses, we performed chromium release assays. Splenocytes from mice immunized with either DC_{MCA-E}, DC_{MCA-L}, DC_{MCA/HSP-E}, DC_{MCA/HSP-L}, DC_{EG7-E} and DC_{EG7-L} were co-cultured with irradiated MCA or EG7 tumor cells, respectively. After 4 days in culture, T lymphocytes were harvested and analyzed by flow cytometry. These T lymphocytes, which consisted mainly of CD8⁺ T cells were used as effector cells in a chromium release assay. As shown in Figure 4.27A, T lymphocytes derived from mice vaccinated with DC_{MCA-L} which had phagocytosed apoptotic MCA tumor cells expressing HSP70 significantly displayed stronger cytotoxicity to MCA tumor cells (34 % specific killing at an E:T cell ratio of 50) than those of mice vaccinated with DC_{MCA-E} that phagocytosed early phase HSP70-negative expressing apoptotic MCA tumor cells (20 % specific killing at an E:T cell ratio of 50) (\( P < 0.05 \); Student’s \( t \) test). On the other hand, T lymphocytes derived from mice vaccinated with DC_{MCA/HSP-E} had a slight but still a significantly stronger cytotoxicity to MCA tumor cells than DC_{MCA/HSP-L} (\( P < 0.05 \); Student’s \( t \) test) (Figure 4.27B). As shown in Figure 4.27C, DC_{EG7-E} also induced significantly more efficient T cell cytotoxic activity towards EG7 tumor cells (47% specific killing at an E:T cell ratio of 25) compared to than DC_{EG7-L} (33% specific killing at an E:T cell ratio of 25) (\( P < 0.05 \); Student’s \( t \) test). Overall, our data indicated that vaccination of DC which had phagocytosed HSP70-expressing apoptotic tumor cells in early phase of apoptosis can induce more efficient tumor-specific CTL responses than DC with phagocytosis of HSP70-expressing apoptotic tumor cells in the late phase of apoptosis or apoptotic tumor cells in early phase of apoptosis lacking the endogenous induced HSP70
Figure 4.27  Cytotoxicity assay. Spleen lymphocytes were harvested from mice one week subsequent to immunization of (A) DC_{MCA-E} and DC_{MCA-L}, (B) DC_{MCA/HSP-E} and DC_{MCA/HSP-L}, (C) DC_{EG7-E} and DC_{EG7-L}, and cultured with irradiated MCA and EG7 tumor cells for 4 days, respectively. T cells were then harvested and used as effector cells, whereas {^{51}Cr}-chromate radiolabeled MCA and EG7 tumor cells were used as target cells. To confirm MCA and EG7 tumor specific T cell cytotoxicity, {^{51}Cr}-labeled A20 and EL4 cells were used as target controls. Each point represents the mean of triplicates. *, $P<0.05$ versus cohorts of DC_{MCA-E}, DC_{MCA/HSP-L} or DC_{EG7-L} (Student’s $t$ test). The experiment was repeated once with similar results.
expression. The above CTL responses were specifically against MCA and EG7 tumor cells with minimal cell cross reactivity with A20 and EL4 tumor cells, respectively, (Figure 4.27 A, B & C).

4.3.5 Enhanced antitumor immunity induced by vaccination of DC with phagocytosis of HSP70-expressing tumor cells in early phase of apoptosis

To examine whether DC which had phagocytosed HSP70-expressing apoptotic tumor cells were capable of inducing enhanced antitumor immunity in vivo, mice were vaccinated twice s.c. with the above DC then challenged with MCA or EG7 tumor cells. As shown in Figure 4.28A, 5 out of 10 mice vaccinated with DC_MCA-L which had phagocytosed apoptotic MCA-L tumor cells, were protected against MCA tumor cell challenge, compared to 20% (2 out of 10 mice) vaccinated with vaccination of DC_MCA-E. However, mice vaccinated with DC_MCA/HSP-E that phagocytosed HSP70-expressing apoptotic tumor cells protected 90% of mice from MCA tumor cell challenge, in comparison to the modest 60% protection seen in mice vaccinated with DC_MCA/HSP-L (Figure 4.28B). Interestingly, similar results were obtained from groups of mice vaccinated with DC_EG7-E and DC_EG7-L. As shown in Figure 4.28 C, DC_EG7-E vaccination provided complete protection [10 out of 10 mice (100%)] from EG7 tumor challenge, whereas DC_EG7-L vaccination resulted in only 70% (7 out of 10) protection. This clearly indicated that the vaccination of DCs that had phagocytosed HSP70-expressing apoptotic tumor cells in early phase of apoptosis induced the strongest protective antitumor immunity in both MCA and EG7 tumor models.
Figure 4.28 Vaccination of mice with DC with phagocytosis of apoptotic tumor cells. Mice (n = 10 per group) were vaccinated s.c. with irradiated (A) DC\(^{\text{MCA-E}}\) and DC\(^{\text{MCA-L}}\), (B) DC\(^{\text{MCA/HSP-E}}\) and DC\(^{\text{MCA/HSP-L}}\), (C) DC\(^{\text{EG7-E}}\) and DC\(^{\text{EG7-L}}\), which had phagocyted apoptotic MCA, MCA/HSP and EG7 tumor cells in early or late phase of apoptosis. Mice injected with PBS were used as controls. Ten days after final immunization, mice were challenged by s.c injection of 5x10^5 MCA or EG7 tumor cells. Animal mortality and tumor growth were monitored daily for up to 8 weeks. One representative experiment of three is shown.
4.3.6 Discussion

Radiation has been found to induce tumor cell apoptosis through both the death receptor and the mitochondrial pathways (Guan et al. 2001; Schumacher et al. 2001; Friedman 2002), which is one of the methods favored for tumor Ag release. There has been several phases characterized in programmed cell death (Medzhitov and Janeway 1997; Somersan et al. 2001). This ranges from early phase apoptosis with PS exposure on the cell surfaces to late phase apoptosis, also defined as secondary necrosis, characterized by DNA damage revealed by using PI staining (Somersan et al. 2001) and the expression of HSP (Feng et al. 2001). HSPs belong to a family of inducible intracellular molecular chaperone proteins that act as key elements in the protein-folding machinery of the cells and are involved with crucial immunological functions (Castelli et al. 2004).

Srivastava and Amato first identified HSP as the component in tumor lysates, which protected mice from a subsequent tumor challenge (DeLeo and Srivastava 1985). Initial studies focused on vaccination with endogenous HSP purified from tumor cells. Despite encouraging results with this strategy in mouse models (Tamura et al. 1997; Kovalchin et al. 2001) and in humans (Janetzki et al. 2000; Belli et al. 2002), purification of HSP from tumor cells is a labor-intensive and time-consuming procedure. Other strategies involve purification of recombinant HSP from bacterial or mammalian cells (Roman and Moreno 1996; Blachere et al. 1997). There are, however, some drawbacks to these strategies including the requirement for known tumor Ags to be associated with HSP and the contamination of preparations with immunogenic bacterial products (Bausinger et al. 2002; Gao and Tsan 2003). Vaccination of tumor cells engineered to express or secrete HSP preserves the primary advantage of purified HSP.
(i.e., the broad spectrum of chaperoned tumor peptides that includes unknown tumor Ag) without the cumbersome process of physically purifying HSP samples. However, transfection of tumor cells with plasmids coding for HSP sequences has given contradictory results. In some experimental models, the immunogenicity of transfected tumor cells is increased upon exogenous overexpression of HSP leading to enhanced antitumor immune responses (Todryk et al. 1999; Wang et al. 2002; Massa et al. 2004; Wang et al. 2004b), whereas in other models, the opposite is noted with increased cell tumorigenicity (Jaattela 1995; Gurbuxani et al. 2001). This discrepancy may be explained by the protective activity of HSP, which helps to prevent cells from triggering apoptosis in response to stressful stimuli (Jaattela 1999). To avoid the possible protumorigenic activity of transfected HSP, modifications have been made to alter the intracellular localization of HSPs. The cytosolic HSP70 and ER-resident HSP family member, gp96, have been converted into transmembrane proteins (Zheng et al. 2001; Chen et al. 2002a). Secretable HSP70 from tumor cells has been shown to improve immunogenicity (Massa et al. 2004). These modified HSP forms continued to maintain their ability to activate the immune responses against parental tumor cells. Moreover, in all of these strategies the tumor antigens are taken-up by DC and cross-presented to T cells resulting in the generation of antitumor immune responses.

An alternative to the previous strategy is to directly use a DC-based antitumor vaccine and incorporating HSP70 expression in tumor cells. In vitro-generated DC can phagocytose apoptotic tumor cells via specific cellular receptors when culturing DC in the presence of apoptotic tumor cells. DC that phagocytose the apoptotic tumor cells would thus be able to present tumor antigens to T cells and induce antitumor immunity. We have previously demonstrated that apoptotic tumor cells with secondary necrosis
induced DC maturation and subsequent vaccination of DC with phagocytosis of these apoptotic tumor cells in late phase of apoptosis stimulated efficient antitumor immunity in comparison to phagocytosis of early phase apoptotic tumors (Chen et al. 2001c). This finding has been confirmed by Ip and Lau, which demonstrated that DC that phagocytose early and late phase apoptotic cells retain their homing ability to LN. However, DC that take up late phase apoptotic cells are more functionally mature resulting in T cell responses whereas the uptake of early apoptotic cells may result in T cell tolerance (Ip and Lau 2004). Taken this into consideration, the potential critical role of HSP70 expression in our model system has not been fully investigated. Reports in the literature have shown necrotic tumor cells expressing HSP70 can promote DC maturation whereas apoptotic cells cannot (Basu et al. 2000; Sauter et al. 2000). This leaves the possibility that the effects of dying tumor cells on DC maturation and function may not only be dependent on the type of cell death (apoptosis versus necrosis), but also on the phase of tumor cell apoptosis (early apoptosis versus secondary necrosis) as well as the association of HSP70 expression.

In this study, we demonstrated that (i) apoptotic tumor cells expressing HSP70 and late phase apoptotic tumor cells stimulated DC maturation after DC phagocytosis of these apoptotic tumor cells, and (ii) DC with phagocytosis of apoptotic tumor cells expressing HSP70 and late phase apoptotic tumor cells induced stronger antitumor immunity compared to DC that phagocytose early phase apoptotic tumor cells. In both of our tumor models, MCA cells expressed endogenous HSP70 only during the late phase of apoptosis whereas EG7 tumor cells endogenously expressed HSP70 and MCA/HSP overexpressed HSP70. DC that phagocytosed MCA/HSP-E tumor cells resulted in a more mature DC phenotype in comparison to MCA-L and resulted in
similar levels of T cell proliferation observed in the MLR assays. Also, higher levels of pMHC I molecules were detected on the cell surface of DC$_{EG7-E}$ that led to higher T cell proliferation of CD8$^+$ OT I T cells both \textit{in vitro} and \textit{in vivo}. These results were consistent with recent findings from our group using a combined radiation therapy and DC-based vaccine in a J558 mouse myeloma tumor model (Chen et al. 2005). Interestingly, we also demonstrated that our DC-based vaccine using apoptotic tumor cells expressing HSP70 in the early phase of apoptosis induced tumor-specific CTL responses more efficiently that specifically recognized the appropriate target cells. Furthermore, the vaccine using DC that phagocytose early phase apoptotic tumor cells expressing HSP70 improved vaccine efficacy \textit{in vivo} compared to using DC with late phase apoptotic tumor cells in two MCA/HSP and EG7 tumor model systems by providing a higher level of protective antitumor immunity when given prophylactic immunization followed by tumor cell challenge.

The results from our study may be explained based upon the immunogenic properties of HSP70. It is known that HSP70 is able to trigger a “danger signal” by binding to DC receptors such as TLR4 (Asea et al. 2002; Vabulas et al. 2002) and CD14 (Asea et al. 2000) through inducing NF$\kappa$B activity to up-regulate costimulatory molecules of DC and induce secretion of proinflammatory cytokines (Basu et al. 2000). In addition, HSP70 inherently possess a cytokine/chemokine immunostimulatory sequence within the peptide binding domain that allows for the production of TNF-$\alpha$, IL-12 and CCL5 by DC, which also further enhances DC maturation (Wang et al. 2005b). The dual role of HSP70 as an antigenic peptide chaperone (Chandawarkar et al. 1999) and as a danger signal (Basu et al. 2000) makes HSP70 especially important in DC-based vaccination for inducing antitumor immune responses. Secondly, DC with
phagocytosis of apoptotic EG7 tumor cells expressing HSP70 in early phase of apoptosis can present more pMHC I complexes to T cells, as shown in this study.

There have been numerous controversial reports in the literature that has shown necrotic tumor cells induced stronger tumor-specific CTL responses compared to apoptotic tumor cells in some reports (Basu et al. 2000; Sauter et al. 2000), whereas, in some other tumor models, DC charged with apoptotic, but not necrotic tumor cells induced efficient vaccines in vivo (Goldszmid et al. 2003). The discordance between the previous studies does not provide a clear distinction as to the optimal source of tumor Ag for DC phagocytosis of tumor cells. It is perhaps that the discrepancies between these controversial findings may be derived from the different tumor models used in these studies, in which tumor cells may or may not express HSP70, and the levels of HSP70 expression in early and late phase of apoptosis may vary throughout the different types of tumor cell model systems. As well, the amount of HSP70 induced may be variable based upon the methods used to induce necrosis and apoptosis.

Taken together, our data demonstrated that HSP70 expression in apoptotic tumor cells further stimulated DC maturation. Furthermore, DC with phagocytosis of apoptotic tumor cells expressing HSP70 in the early phase of apoptosis more efficiently induced tumor-specific CTL responses and immunity than DC with phagocytosis of apoptotic tumor cells in late phase of apoptosis. These results may have an important impact in designing DC-based antitumor vaccines.
5.0 CONCLUSIONS AND FUTURE DIRECTIONS

DC are the most powerful APC in the initiation and activation of immune responses. To date, DC have been widely utilized in cancer vaccines to generate an effective antitumor immune response both in murine models and in human clinical studies. We have shown in this thesis the multitude of tumor Ag sources loaded onto DC including the use of replication-deficient adenovirus expressing tumor Ag for transduction of DC and the use of DC with phagocytosis of apoptotic tumor cells expressing HSP70. We demonstrated both types of tumor Ag sources in our DC-based vaccines were able to stimulate protective and preventive antitumor immune responses.

HER-2/neu has proven to be an attractive Ag for immunotherapeutic targeting. In our study, we found that the use of AdV-neu-transduced DC induced DC maturation by up-regulating the expression of MHC class II, costimulatory, adhesion molecules as well as a variety of pro-inflammatory cytokines. DC engineered to express neu induced a mixed Th1/Th2 response; whereas, immunization of naked DNA plasmid expressing neu resulted in a low to mild induction of a Th1 response. Moreover, immunization of parental FVB/N and Tg FVBneuN mice twice with DC-neu afforded the greatest protection upon tumor challenge in contrast to DNA immunization that only resulted in partial to no protection in tumor challenged mice. In Tg FVBneuN mice, immunization with DC-neu in younger mice provided a greater delay in the formation of spontaneous tumors when compared to aged match control and mice immunized with DNA. Both DC-neu- and DNA-immunized mice, when immunized at an older age, failed to delay the
spontaneous tumor development in FVBneuN Tg mice. The reduced therapeutic efficacy of DC\textsubscript{neu} seen in FVBneuN Tg mice may be due to the neu-specific self immune tolerance developed in the mice.

The issue of self-immune tolerance has become a critical avenue of exploration in determining the reasons for why the promising results obtained from animal models often fail in being repeatable when studies are performed in humans. This problem can be addressed through development of Tg mice (both knock-in and knock-outs) that allow for expression of a “foreign” Ag as an endogenous Ag, and development of Ag-specific self-immune tolerance. We demonstrated that DC\textsubscript{neu} was only able to partially overcome the neu-specific tolerance within the Tg mice. To improve the vaccine and address the role of Tr cells, a combination of immune modulating agents may be used that would act synergistically for overcoming the tolerance factors derived from Tr cells and potentially maximizing the antitumor immune responses of the combinational immunotherapies (Emens et al. 2005). One simple method may be to delete Tr cells using a mAb for CD25 in combination with the immunotherapy strategy (Li et al. 2003; Comes et al. 2006). Another method may be a combinatorial therapy using immunotherapy combining chemotherapeutic agents such as paclitaxel, doxorubicin and Cy (Machiels et al. 2001; Eralp et al. 2004). It has been demonstrated that low doses of Cy reduces the number of Tr cells and suppresses the function of Tr (Ercolini et al. 2005; Lutsiak et al. 2005). Reports by Jaffee’s group have previously shown that low doses of Cy with allogeneic 3T3/neu cells expressing GM-CSF enhanced the efficacy of the vaccine and delayed tumor growth in FVBneuN mice (Machiels et al. 2001). More recently, they have also shown Cy treatment increases the number of high avidity T cells.
recognizing the immunodominant neu epitope RNEU_{420-429} in FVBneuN mice (Ercolini et al. 2005).

In our study, we showed that plasmid DNA vaccination provided minimal protection and preventative effect in the tumor models used. However, alternative strategies could be used to increase the effectiveness of DNA vaccines. One method is the use of a prime-boost strategy that involves priming with a DNA vaccine followed by the use of a viral vector to generate high levels of CD8^{+} effector and memory T cells (Woodland 2004). This approach has been recently applied in a therapeutic model of neu expressing tumor cells resulting in high levels of cellular and humoral immunity (Wang et al. 2005a). There are also different alternatives to the classical prime boost strategy such as the use of DC with or without a viral boost (Badovinac et al. 2005) or initially priming with AdV transduced DCs followed by peptide-pulsed DCs (Tuettenberg et al. 2003). All of these methods have shown the ability to generate higher number of CD8^{+} T cells and to provide protection in the \textit{in vivo} animal studies. Moreover, the number of boost required is also an important factor as it may assist in expansion of long-lived tumor specific immunity, especially in face of short-lived immune responses (Knutson et al. 2002; Palucka et al. 2005)

Another method to enhance the overall effectiveness of DC vaccines is the inclusion of cytokines and chemokines to act as adjuvants. Typically, Th1 promoting cytokines can be used to directly transduce DCs by adenovirus-mediated gene transfer. On the other hand, it has also been demonstrated that injection of soluble cytokines also provide adjuvant effects (Disis et al. 2003). The most commonly used cytokines for co-immunization is IL-12 and GM-CSF that results in augmentation of the Ag-specific induced immune response. Other cytokines and chemokines have also been used in
conjunction with DC vaccines with success in various model systems. These cytokines
include TNF-α (Chen et al. 2002c; Liu et al. 2004), IL-18 (Xia et al. 2004), Flt-3L (Liu
et al. 2003) and SLC/CCL21 (Yang et al. 2004). Interestingly, higher transduction
efficiency can be achieved using RGD fiber modified AdV (Okada et al. 2001b; Okada
et al. 2005) and this may allow for dual AdV transduction of DC. This area warrants
further consideration to further improve the DC_{nu} vaccine to be able to overcome the
self-tolerance found in FVBneuN mice.

In the case of HSP70, it truly plays a dynamic role in DC maturation and DC-
based vaccines. Based upon the collective results from the presented studies, one may
potentially include the use of both HSP70 and rat neu for use in a cancer vaccine.
Another DC based vaccine could be made using a neu-expressing tumor cell line (e.g.
Tg1-1) modified to overexpress HSP70 for enhancement of neu Ag loading. Another
method is based upon a recent report demonstrating that a DNA based vaccine encoding
a fusion protein consisting of human HSP70 linked to the ECD of rat neu (forming
NeuEDhsp70) increased the survival and reduced the metastatic potential of tumor cells
in neu Tg mice (Kim et al. 2005a). Alternatively, this fusion protein could also be
utilized in a DC-based vaccine.

Overall, we have examined variations in designing a DC-based cancer vaccine to
provide an effective antitumor immunity in our various tumor animal models. Our
results continue to validate the concept of a DC-based vaccine and will have an
important impact on the design of future of DC-based vaccines. This will play an
important role for future development of immunotherapeutics in a clinical setting.
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