Development of immunotherapy against prostate cancer using lentivirally-transduced dendritic cells expressing murine erbB2 as a model tumor-associated antigen

by

Miriam Esmat Mossoba

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Medical Biophysics
University of Toronto

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Miriam E. Mossoba  
Doctor of Philosophy, 2008  
Graduate Department of Medical Biophysics  
University of Toronto  
Thesis Title: Development of immunotherapy against prostate cancer using lentivirally-transduced dendritic cells expressing murine erbB2 as a model tumor-associated antigen

Abstract:

Prostate cancer is a leading cause of cancer deaths in North American men. Current treatments are often not curative, particularly in cases of advanced metastatic disease. Immunotherapy is a promising approach to treating cancer as it harnesses the immune system’s ability to mount potent responses against tumor-associated antigens (TAAs). Dendritic cells (DCs) play a central role in mediating antigen-specific immunity and have been recently used with some success in clinical trials. The difficulties associated with obtaining sufficient quantities of DCs from cancer patients provided the rationale for developing low-dose DC-based immunotherapy approaches in my thesis project. DCs were genetically engineered using a lentiviral vector (LV) to express erbB2tr, a kinase-deficient version of erbB2. The human form of erbB2, HER2/neu, is overexpressed in 20% of primary prostate tumors and 80% of their metastases, making this TAA an attractive target. Using this LV system, efficient transgene delivery into DCs was achieved without compromising DC function or phenotype. Administering low prime and boost doses ($2 \times 10^5$ or $2 \times 10^3$) of LV-transduced DCs to mice yielded potent and long-term anti-tumor responses against murine prostate tumors engineered to overexpress erbB2tr. The $2 \times 10^5$ DC dose yielded complete tumor protection and was associated with humoral and cellular responses. The $2 \times 10^3$ dose also offered complete protection in some mice,
suggesting that we had reached a lower threshold DC dose. This novel finding prompted us to determine if co-transducing DCs with an additional LV carrying the cDNA for an immunomodulatory factor could augment the efficacy of our low-dose strategy. We chose to test both the DC survival-enhancing RANKL protein and DC function-enhancing IL-12 in combination with erbB2tr. Although DCs co-transduced with the LV/RANKL and LV/erbB2tr did not appear to offer enhanced anti-tumor benefits in a prophylactic setting, co-transduction with LV/IL-12 and LV/erbB2tr did. The incorporation of IL-12 into the low-dose immunization strategy led to robust long-term tumor protection and relatively high levels of Th1 immunity. This is the first demonstration of the efficacy of low-dose DC-mediated immunotherapy using lentiviral vectors as gene transfer tools. These studies establish a platform for DC-mediated therapies that can be realistically translated to the clinic.
Acknowledgments

I first wish to thank all of my previous supervisors for leading me to the path to graduate school. I also thank my 10th grade biology teacher, Mrs. Bernard, for introducing me to the concept of gene therapy. I endured many years of waiting to actually work on gene therapy. An enormous thank you goes to my graduate supervisor, Dr. Jeffrey Medin. I am very fortunate to have been accepted into Dr. Medin’s lab and I am so grateful for all he has done for me. His mentorship continues to direct my career. The lab environment he created taught me many things about the ‘real world’ of science without coddling me.

Thank you to Drs. Barber and Ohashi for their guidance during my PhD, especially towards the end. Their help was truly invaluable. I would also like to acknowledge all my friends and family, as well as all past and present members of the Medin lab for their advice and help, particularly during the insanely late nights in the lab. Special thanks go to Drs. Chris Siatskas, Makoto Yoshimitsu, and Jagdeep S Walia, who played a big part in my education.

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<td>adenovirus</td>
</tr>
<tr>
<td>Ad5</td>
<td>adenovirus serotype 5</td>
</tr>
<tr>
<td>AIRE</td>
<td>autoimmune regulator</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ADT</td>
<td>androgen-deprivation therapy</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>BAFF</td>
<td>B cell activating factor belonging to the TNF family</td>
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<tr>
<td>BIM</td>
<td>Bcl-2 Interacting Mediator of Cell Death</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMT</td>
<td>bone marrow transplant</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>FLIP</td>
<td>caspase-8 homologue FLICE-inhibitory protein</td>
</tr>
<tr>
<td>cppt</td>
<td>central polyurine tract</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund's Adjuvant</td>
</tr>
<tr>
<td>CTEC</td>
<td>cortical thymic epithelial cell</td>
</tr>
<tr>
<td>CAR</td>
<td>coxsackie-adenovirus receptor</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<tr>
<td>CTLA4</td>
<td>cytotoxic T-lymphocyte antigen 4</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
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<td>early region 1</td>
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<td>EF1α</td>
<td>Elongation Factor 1α</td>
</tr>
<tr>
<td>FcRs</td>
<td>Fc receptor</td>
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<td>helper T</td>
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<tr>
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<td>hematopoietic stem-progenitor cell</td>
</tr>
<tr>
<td>HIFU</td>
<td>high-intensity focused ultrasound</td>
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<tr>
<td>HRPC</td>
<td>hormone-refractory prostate cancer</td>
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<td>HER</td>
<td>human epidermal growth factor receptor family</td>
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<td>HIV-1</td>
<td>human immunodeficiency virus type 1</td>
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<td>IDO</td>
<td>indolamine 2,3-dioxygenase</td>
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<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
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<td>IL-12</td>
<td>interleukin-12</td>
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<tr>
<td>erbB2tr</td>
<td>kinase-deficient form of erbB2</td>
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<tr>
<td>LV</td>
<td>lentiviral vector</td>
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<tr>
<td>LFA-3</td>
<td>Leukocyte Function Antigen-3</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>LHRH</td>
<td>luteinizing hormone-releasing hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>mTEC</td>
<td>medullary thymic epithelial cell</td>
</tr>
<tr>
<td>MART-1</td>
<td>melanoma antigen recognized by T cells 1</td>
</tr>
<tr>
<td>MIICs</td>
<td>MHC class II-rich compartments</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MLR</td>
<td>mixed lymphocyte reaction</td>
</tr>
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<td>MVA</td>
<td>modified vaccinia virus Ankara</td>
</tr>
<tr>
<td>MPR</td>
<td>mouse prostate reconstitution</td>
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<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
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<tr>
<td>NT</td>
<td>non-transduced</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NIK</td>
<td>nuclear factor-κB-inducing kinase</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature</td>
</tr>
<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PALS</td>
<td>peri-arteriolar lymphatic sheath</td>
</tr>
<tr>
<td>TSA</td>
<td>peripheral tissue-specific antigen</td>
</tr>
<tr>
<td>PI-3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PIA</td>
<td>proliferative inflammatory atrophy</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate-specific antigen</td>
</tr>
<tr>
<td>PSCA</td>
<td>prostate stem cell antigen</td>
</tr>
<tr>
<td>PSMA</td>
<td>prostate-specific membrane antigen</td>
</tr>
<tr>
<td>PIN</td>
<td>prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>RIP</td>
<td>rat insulin promoter</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor κB ligand</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T</td>
</tr>
<tr>
<td>RRE</td>
<td>rev response element</td>
</tr>
<tr>
<td>SIN</td>
<td>self-inactivating</td>
</tr>
<tr>
<td>SHP1</td>
<td>SH2-domain-containing protein tyrosine phosphatase 1</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-domain-containing inositol phosphatase</td>
</tr>
<tr>
<td>SP</td>
<td>single positive</td>
</tr>
<tr>
<td>ss</td>
<td>single-stranded</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SA</td>
<td>splice acceptor</td>
</tr>
<tr>
<td>SD</td>
<td>splice donor</td>
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<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-α-converting enzyme</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRAMP</td>
<td>TRansgenic Adenocarcinoma Mouse Prostate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TAP1</td>
<td>transporter associated with antigen processing 1</td>
</tr>
<tr>
<td>TRICOM</td>
<td>TRIad of COstimulatory Molecules</td>
</tr>
<tr>
<td>TIL</td>
<td>tumor infiltrating lymphocyte</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor/Nodes/Metastases</td>
</tr>
<tr>
<td>TAAs</td>
<td>tumor-associated antigens</td>
</tr>
<tr>
<td>TRAF6</td>
<td>tumor-necrosis factor-receptor-associated factor 6</td>
</tr>
<tr>
<td>TKI</td>
<td>tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TRP2</td>
<td>tyrosine-related protein 2</td>
</tr>
<tr>
<td>VSV-G</td>
<td>vesicular stomatitis virus glycoprotein</td>
</tr>
<tr>
<td>REL-B</td>
<td>v-rel reticuloendotheliosis viral oncogene homolog B</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>WPRE</td>
<td>woodchuck hepatitis virus post-transcriptional regulatory element</td>
</tr>
</tbody>
</table>
Some of the text presented in this chapter has been published in the following review article:

1.1 Prostate cancer

1.1.1 Development

Prostate cancer remains one of the leading cancers in men, with an estimated 186,320 new cases in the US and 24,700 in Canada this year [1, 2]. The rates of prostate cancer vary worldwide, but African-American men have relatively high incidence rates and associated death rates [1] possibly due in part to elevated levels of viral infections [3]. Environmental exposures including dietary carcinogens [4] bacterial and viral infections [3, 5] that could lead to inflammation within the prostate are associated with a higher prostate cancer risk.

The progression from normal epithelial cells to invasive carcinoma begins with the development of a state called proliferative inflammatory atrophy (PIA) [6, 7]. PIA can be caused by several genetic or epigenetic changes. Examples include the inactivation of tumor suppressor genes, such as NKX3.1 [8] and CDKN1B [9] as well as the activation of oncogenes like MYC and hTERT [10]. Abnormal methylation patterns in CpG islands [11] may also contribute to overall genetic instability resulting in the appearance of a PIA state. PIA is associated with the infiltration of inflammatory cells, such as lymphocytes, macrophages, and neutrophils, which can release reactive oxygen and nitrogen species. Exposure to these reactive species can trigger DNA damage and cell injury, possibly aiding the progression from PIA to low-grade prostatic intraepithelial neoplasia (PIN) [12]. As its name suggests, PIN is characterized by the formation of neoplastic cells. The genetic instability within these cells can then lead to an accumulation of genetic changes and a lesion known as high-grade PIN [12]. Subsequent migration of these neoplastic cells into areas beyond the prostate gland defines the final step towards the development
of invasive carcinoma and ultimately metastatic disease. The extent of metastases is measured using the Tumor/Nodes/Metastases (TNM) system [13], where T1 and T2 clinically define organ-localized cancer and T3 and T4 indicate metastasis. Histological assessment of the tumor cells is reported as a Gleason score of 1-10, with a value of 10 indicating the most abnormal looking cells [14].

1.1.2 Treatments

The TNM and Gleason grading systems help determine what treatment option will be used for prostate cancer. Conventional treatments include surgery, radiotherapy, high-intensity focused ultrasound (HIFU), chemotherapy, cryosurgery, and hormone ablative therapy. Each of these options carries the risk of significant side effects [15-20], prompting the need for novel treatment development.

Surgical removal of the prostate gland (radical prostatectomy) for clinically-localized prostate tumors is associated with low rates of disease recurrence [21]. Due to the risk of incomplete tumor resection, other treatment modalities may be chosen over surgery for patients with more advanced disease including locally-advanced T3 cancer, in which the tumor has grown beyond the prostate organ but not to distant sites [22]. Furthermore, administering neo-adjuvant treatment such as hormonal therapy prior to radical prostatectomy has not been proven to offer a clear clinical benefit to patients with locally-advanced prostate cancer [23].

External beam radiotherapy is also often used for locally-advanced cases, but follow-up studies have demonstrated that up to 90% of treated patients experience tumor persistence [24]. However, the combination of hormonal therapy, such as luteinizing hormone-releasing hormone (LHRH) analog treatment, with external beam radiotherapy
has been shown to offer patients improved survival relative to radiotherapy alone [25]. Brachytherapy is an alternative radiotherapy treatment, involving the implantation of small radioactive rods (or seeds) into the prostate gland and this form of therapy has recently been shown to yield promising results with respect to 5-year survival rates [26].

When patients present with metastatic prostate cancer, androgen-deprivation therapy (ADT) can help control the spread of the disease, but eventually hormone-refractory disease often ensues [27]. The mechanisms of this transition from a hormone-sensitive to a hormone-refractory prostate cancer (HRPC) are poorly understood. Currently, it is unclear whether administration of chemotherapy to HRPC patients is beneficial [28].

1.1.3 Experimental mouse models

Experimental mouse models of prostate cancer can be categorized into orthotopic and ectopic models. To generate orthotopic transgenic models that develop prostate cancer, viral oncogenes may be expressed in prostate tissue or the pathways involved in prostate cancer may be genetically manipulated. One notable example is the TRansgenic Adenocarcinoma Mouse Prostate (TRAMP) mouse, which develops epithelial tumors in the dorsolateral prostate via the SV40 large T antigen expression driven by the prostate-specific rat probasin promoter [29]. Generating transgenic models through the genetic manipulation of prostate cancer signaling pathways is generally more common, as several target genes have been identified. For example, mice that express the protein kinase Akt specifically in the prostate under the probasin promoter develop PIN [30]. This model offers the advantage of being able to test therapeutics specifically against the Akt signaling pathway, although this model does not develop invasive or metastatic disease.
A more aggressive transgenic model was generated by the expressing a mutant androgen receptor (AR) gene (AR-E231G) in the mouse prostate [31]. These mice develop metastatic prostate cancer and can therefore be useful for studying the efficacy of metastasis-targeted therapeutics.

An ectopic transgenic mouse model of prostate cancer was created in which only the prostate is transgenic for MYC and RAS oncogenes [32]. This model is known as the mouse prostate reconstitution (MPR) model and is generated in three steps. First, the fetal urogenital sinus, from which the murine prostatic lobes develop, are isolated. Then, it is infected with retroviruses to express MYC and RAS. Finally, it is implanted under a host animal’s renal capsule. There it becomes a fully mature prostate that forms poorly-differentiated prostatic adenocarcinomas [32]. Furthermore, if the host animals are p53-deficient, then metastases form in a pattern that is similar to that in prostate cancer patients [33].

Several cell lines have been derived from the above-described transgenic models. For example, TRAMP-C1 [34] and RM-1 [35] are murine prostate tumor cell lines derived from TRAMP and MPR (on a C57BL/6 background) mouse models, respectively. Injecting these lines into the mouse prostate or the dorsolateral flank generates orthotopic or ectopic syngeneic mouse models, respectively. Injecting human prostate cell lines into mice, on the other hand, creates xenogeneic models. Examples of commonly used human cell lines are DU145 [36], PC3 [37], and LNCaP [38], which were derived from metastatic prostate tumors. The use of xenogeneic mouse models may be of limited value for immunotherapy studies if the host animals are immunodeficient.
1.2 Immunology of Cancer

1.2.1 Natural self-tolerance against tumor-associated antigens (TAAs)

Although some antigens expressed by tumor cells are mutant proteins, the majority of TAAs identified thus far are wild-type antigens that are also expressed by normal cells and are therefore self-antigens. Examples of TAAs are prostate stem cell antigen (PSCA), prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA) and carcinoembryonic antigen (CEA). The immune system is equipped with many mechanisms to ensure that cells, which express self-antigens including TAAs, are spared from immune attack. These mechanisms fall under the categories central and peripheral tolerance.

During central tolerance processes, positive selection of T cells takes place in the thymic cortex following interaction between thymocytes and cortical thymic epithelial cells (cTECs) [39]. As immature T cells move within the cortex, they have the opportunity to encounter a self-peptide-MHC complex on cTECs and to become positively selected. Studies have shown that the thymocytes at this stage are CD4⁺CD8⁺ double positive (DP) T cells and that their interaction with cTECs leads to the formation of CD4⁺ or CD8⁺ single positive (SP) T cells. This transition is also associated with their migration into the thymic medulla, and is in part mediated by upregulation of the chemokine receptor CCR7 [40]. Here, medullary thymic epithelial cells (mTECs) and DCs interact with the developing T cells during negative selection. The expression of co-stimulatory molecules and peripheral tissue-specific antigens (TSAs) by mTECs allow the developing T cells that enter the medulla to interact with antigens that are expressed in the periphery. If the affinity of the interaction is high, then these T cells may undergo
clonal deletion or instead develop into a regulatory T cell. Thus, the expression of TSAs serves as a critical step in guiding the immune system to define self-antigens and to ensure that tolerance is established against them. The transcriptional regulator AIRE (autoimmune regulator) helps drive the expression of TSAs [41]. Its importance is underscored by the fact that mutations in AIRE in mice cause the autoimmune disorder polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome, in which clonal deletion is defective and multi-organ autoimmunity occurs [42, 43].

Proper differentiation and function of mTECs is dependent on factors such as tumor-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) [44] and nuclear factor-κB (NF-κB)-inducing kinase (NIK) [45, 46], which help regulate members of the NF-κB family [47, 48]. The essential role of mTECs in mediating central tolerance is highlighted by the autoimmune disorders that develop in mice that have deficiencies relating to mTECs. For example, mice that are deficient in v-rel reticuloendotheliosis viral oncogene homolog B (REL-B) [49, 50] or TRAF6 [44], or that have a mutation in the NIK gene [45, 46] have a small medulla lacking mTECs. These mice display normal positive selection, but have impaired negative selection, leading to autoimmunity and/or severe inflammation [44-46, 51]. The DCs that reside in the medulla play a role in cross-presenting TSAs produced by mTECs to developing T cells. This process of cross-presentation is critical because it allows DCs to load self-peptides derived from exogenous self-antigens onto major histocompatibility (MHC) class I molecules without expressing TSAs themselves. Evidence for this process came from experiments using the transgenic RIP-OVA mouse model, which expresses ovalbumin (OVA) from the rat insulin promoter (RIP) [52]. Gallegos and Bevan (2004) demonstrated that BM-derived
antigen-presenting cells are capable of capturing OVA from mTECs and cross-presenting it to developing T cells in the thymus to mediate clonal deletion [52]. DCs also express co-stimulatory molecules, including CD80 and CD86, which have been shown to aid in the process of clonal deletion. For example, studies where CD80 or CD86 were eliminated or neutralized have demonstrated that self-reactive T cells were not properly purged [53, 54].

The depletion of lymphocytes that display high-affinity self-antigen recognition during central tolerance processes does not preclude the escape of some potentially self-reactive lymphocytes. Peripheral tolerance mechanisms provide an additional layer of protection against autoimmunity. Thymus-produced CD4^+CD25^+ regulatory T (Treg) cells help maintain peripheral self-tolerance, as they can recognize self-antigens including TAAs [55, 56]. Depletion of Treg cells can result in autoimmune disease [57] and when cancer patients are Treg cell-depleted, TAA-specific circulating effector T cells are more readily detected in the peripheral blood [58]. Conversely, Treg cells can be expanded for therapeutic purposes in treating autoimmune diseases [59, 60].

In addition to suppression by Treg cells, other mechanisms like anergy are in place to help maintain peripheral tolerance. One intrinsic mechanism of anergy is antigen-receptor down-regulation. For example, in B cells, defects in the transport of newly formed IgM from the ER to the cell surface leads to tolerant B cells [61, 62]. Alternatively, negative feedback processes can elevate the threshold of proteins needed to achieve lymphocyte activation, such as via the recruitment of SH2-domain-containing protein tyrosine phosphatase 1 (SHP1), a negative regulator of B cell antigen receptor signaling [63, 64]. Expression of inhibitory receptor CD5 in B and T cells can help
recruit SHP1 and inhibit further antigen-receptor signaling and activation [65-67]. SH2-domain-containing-inositol phosphatase (SHIP) has also been implicated in the inhibition of B cell activation. This mechanism involves the binding of SHIP to inhibitory receptor FcγRIIB [68], whose deficiency in mice leads to autoimmunity [69]. In T cells, cytotoxic T-lymphocyte antigen 4 (CTLA4) is another inhibitory receptor whose expression is induced when the TCR is highly self-reactive [64]. CTLA4 competes with CD28 for binding to co-stimulatory B7 molecules on APCs and inhibits T cell activation.

Potentially self-reactive lymphocytes can also be regulated by extrinsic mechanisms. For example, B cells can be eliminated from the periphery following competition with less reactive B cells. Prolonged ligation of a self-reactive BCR can trigger an increase in pro-apoptotic BIM (for Bcl-2 Interacting Mediator of Cell Death) expression, which would require elevated pro-survival BAFF (for B cell activating factor belonging to the TNF family) expression levels for the B cell to be rescued from cell death [70]. Failure to produce enough BAFF would result in the self-reactive B cell in being out-competed by less reactive B cells, whose dependence on BAFF production for survival is less [71].

1.2.2 Evasion of cancer from immunity

In addition to overcoming self-tolerance mechanisms, the immune system must also circumvent immune-evasion strategies used by tumors. Tumor immunity is a complex process in which tumors and the immune system regulate each other through several different mechanisms. As a result of the selective pressures exerted by the immune system, the tumors that successfully persist and develop into more advanced cancers are derived from cells that were able to evade the immune system. This
phenotypic shaping of tumor cell populations by the immune system is called ‘immunoediting’ [72].

Tumor cells have been found to evade elimination by the immune system through a variety of mechanisms. Many tumors avoid recognition by cytotoxic T lymphocytes (CTLs) through loss of MHC class I molecules and can develop into advanced cancer, as is often the case with lung cancer [73]. Other cancers, such as colorectal carcinoma [74], exhibit a down-modulation of proteins involved in antigen-processing, including transporter associated with antigen processing 1 (TAP1) [75]. Due to the subsequent lack of antigen presentation, a T-cell mediated immune response is evaded and tumors may become more advanced. It is intriguing that even if an effective T cell response is initiated against tumor cells, tumor lysis may be avoided. The mechanism for this process involves the secretion of serine-protease inhibitor PI9 [76, 77] by tumor cells, preventing effector T cells from releasing perforin and other cytolytic granules that permeabilize tumor cell membranes and initiate tumor cell death. In addition, tumor cells can resist apoptosis by down-regulating the death receptor Fas [78] or even by up-regulating the caspase-8 homologue FLICE-inhibitory protein (FLIP) for enhanced tumor survival [79].

Other than defensive mechanisms that tumors use to avoid elimination, several ‘immunosubversion’ (active immune-suppressive) mechanisms against the immune system have also been reported. Tumors may inhibit CD8+ T cell proliferation [80] and promote CD4+ T cell apoptosis by expressing indolamine 2,3-dioxygenase (IDO) [81]. Tumors may also secrete immunosuppressive cytokines and other factors, including IL-10 and prostaglandin E2, which have been reported to inhibit APC function [82]. Finally, solid tumor masses can also physically exclude immune cells [83]. The immune system
exerts a selective pressure on tumor cells such that the tumor cells that evade immunity are selected.

1.2.3 Immunotherapy against cancer

Improving patients’ immune responses against TAAs is a major goal of cancer immunotherapy, which can be classified into passive and active forms. Passive immunotherapy entails administering patients with pre-formed immune complexes (such as cytokines or antibodies) or with cells that possess anti-tumor reactivity. This approach has been effectively tested in animal models and clinical trials. For example, the anti-HER2/neu monoclonal antibody, Herceptin (trastuzumab), has been successfully tested as a first-line therapy in breast cancer patients that over-express the cell surface TAA HER2/neu, a member of the human epidermal growth factor receptor family [84]. It is thought that Herceptin binds to the extracellular domain of HER2/neu, which induces its downregulation via endocytosis and inhibits important signaling pathways (ras-Raf-MAPK and PI3K/Akt), thereby blocking tumor cell-cycle progression [85]. Another example of passive immunotherapy is the use of anti-CTLA-4 antibody, which functions by antagonizing the T cell inhibitory molecule CTLA-4 in order to help prolong anti-tumor T cell responses. Anti-CTLA-4 therapy has shown some efficacy against cancers such as melanoma and prostate cancer in clinical trials [86, 87].

Adoptive transfer of cells that mediate direct anti-tumor reactivity began with the demonstration that in vitro propagation of lymphoid cells using IL-2 could yield a population of cells termed lymphokine-activated killing (LAK) cells [88, 89]. In murine models and clinical trials, LAK cells were shown to have the ability to lyse tumors in a non-MHC restricted manner [90, 91]. In recognition of the antigen-specific recognition of
tumors by T cells, efforts were subsequently made to derive tumor-specific T cell lines and T cell clones from tumor-infiltrating lymphocytes (TILs) [92, 93]. The evaluation of adoptive therapy using TILs was shown to be 100-fold more effective than the adoptive transfer of LAK cells in murine models [94]. Since the first report demonstrating the regression of cancer using TILs in melanoma patients [95], this form of passive immunotherapy been used to treat a variety of other cancers with some success [96].

In active immunotherapy, the goal is to endow patients with the ability to create or strengthen an existing immune response towards antigen(s). This approach can be achieved in many ways including immunization with autologous tumor cells [97], tumor lysates [98], specific TAA proteins [99], TAA-derived peptides [100], or even DNA [101] or viruses [102] encoding TAAs. Another way to achieve active immunotherapy is by injecting patients with DCs presenting the TAA(s) of interest. DCs are considered to be the most potent antigen-presenting cells in the immune system [103]. They are critical for initiating and sustaining strong immune responses. They also allow the formation of memory T cells and B cells for later recall responses.

### 1.3 Dendritic cells (DCs)

#### 1.3.1 Origin and development

The origin of murine DCs is the bone marrow, where at least two distinct DC precursors reside, myeloid and lymphoid. These DC subsets have different phenotypes, locations, and other characteristics. Although both express surface markers such as CD11c, CD80, CD40, and MHC class II molecules, lymphoid DCs exclusively express CD8α [104]. Both DC subsets are found in the spleen and lymph nodes, but lymphoid DCs are mainly found in their peri-arteriolar lymphatic sheath (PALS) areas that are T
cell-rich, while myeloid DCs are usually located in the marginal zone. Although lymphoid DCs are less phagocytic than myeloid DCs, both have the ability to prime T cells efficiently in vivo [105, 106].

Evidence for a myeloid origin of DCs came from early studies showing that bone marrow (BM) myeloid precursors could give rise to macrophages, granulocytes, and DCs [107]. Other studies indicated that DCs residing in lymphoid tissues had a lymphoid origin, since they expressed markers associated with lymphoid cells including CD8α, CD4, and CD25 [108]. More recent in vitro and in vivo studies have demonstrated that common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) can each give rise to lymphoid (CD8α+) and myeloid (CD8α−) DCs [106]. This finding suggests that although the origin of all DCs is the hematopoietic stem cell, DCs appear to possess the unique property of being able to develop from both myeloid and lymphoid differentiation pathways and that the subtype of a DC may not necessarily reveal its lineage origin.

Whereas lymphoid DC precursors that form in the BM migrate to the thymus to continue their development, myeloid DC precursors migrate into peripheral tissues and take up antigens. Lymphoid DCs remain in the thymus to mediate central tolerance, as described in section 1.2.1. Myeloid DCs remain in the periphery until they receive a maturation signal, which leads to a down-regulation of receptors that take part in antigen uptake, such as Fc receptors (FcRs) [109], and an up-regulation of proteins needed for T-cell priming, such as CD80 and CD86 molecules of the immunoglobulin superfamily [110, 111]. CD80 and CD86 may influence the differentiation pathway, expansion, or cytokine production of helper T (Th) cells, depending on its antigenic experience; these
molecules may help naïve T cells secrete IL-4 and differentiate into Th2 cells, while Th1 cytokine production is mediated by CD80 and CD86 in the presence of IL-2 [112]. DC maturation signals are inflammatory cytokines, such as TNF-α, or microbial-derived antigens having pathogen-associated molecular pattern (PAMP) motifs. PAMPs interact with pattern recognition receptors, such as Toll-like receptors (TLRs), expressed by DCs. Lipopolysaccharide (LPS), for example, is a cell membrane component of Gram-negative bacteria and interacts with TLR4 on DCs [113]. During the subsequent maturation process, DCs present their captured antigens in the form of peptides bound to MHC molecules on their cell surface [114]. Concurrently, the maturing myeloid DCs migrate to secondary lymphoid organs [115, 116]. There they interact with lymphocytes using their MHC-peptide complexes and co-stimulatory molecules to induce a primary immune response before later undergoing apoptosis [117].

1.3.2 The role of DCs in inducing antigen-specific immunity

The ability of DCs to induce potent immune responses is due in part to the high expression of MHC and costimulatory molecules on their surface relative to other antigen-presenting cells (APCs) such as B cells [118]. To prime an antigen-specific immune response, DCs process antigens intracellularly and present them in the context of MHC molecules to T cells bearing the cognate antigen-specific T-cell receptor (TCR), providing T cells with a ‘signal 1’. In vitro assays have indicated that the level of peptide-MHC molecules presented on the DC surface can help influence the differentiation pathway of Th cells; low peptide doses support Th1 cell development, while higher doses promote Th2 cell development [119, 120]. Animal studies of infectious diseases have demonstrated that the bacterial immunization dose can determine whether Th1 or Th2
immunity develops; low antigen doses are associated with Th1 immunity, while high
doses yield a both Th1 and Th2 responses [121, 122]. Furthermore, presentation of low
densities of pigeon cytochrome C (PCC) antigen on APCs has been found to efficiently
activate Th1 memory cell responses [123].

The presentation of antigenic peptides in the context of MHC class I and II
molecules is dependent on the nature of the antigen and how it is acquired by the DC. In
the classical endogenous pathway, peptides from self-antigens and intracellular pathogens
are loaded onto MHC class I molecules for subsequent presentation to CD8+ T cells.
Endogenous antigens are ubiquitinated and then cleaved in the proteasome into peptides
[124]. With the aid of TAP transporters, TAP1/2, peptides are translocated to the
endoplasmic reticulum for further processing and binding to MHC class I molecules
[125]. In addition to MHC class I presentation of endogenous antigens, DCs have the
ability to ‘cross-present’ [126] exogenous antigens, such as phagocytosed antigens, in the
context of MHC class I molecules, in a TAP-independent or -dependent manner [127-
129].

For DCs to prime CD4+ T cells, peptides are presented in the context of MHC class
II molecules. DCs collect soluble antigens via macropinocytosis or endocytosis and
particulate antigens via phagocytosis [109, 130]. Captured antigens are broken down
within endosomes and the resulting polypeptides are transported to MHC class II-rich
compartments (MIICs) for subsequent binding with MHC class II molecules [131, 132].
With the aid of cathepsin S in MIICs, the invariant chain that is associated with MHC
class II molecules is cleaved off to finalize peptide loading before the MHC-peptide
complex is transported to the surface of maturing DCs [133].
While mature DCs deliver ‘signal 1’ to T cells to activate them, they also deliver a ‘signal 2’ in order to sustain T cell activation. ‘Signal 2’ is provided by the interactions between costimulatory molecules like CD80 and CD86 on DCs with CD28 on T cells and helps to sustain T cell activation and survival [134, 135].

More recently, a ‘signal 3’ has been described as a signal that is delivered from the APC to the T cell to help instruct its differentiation pathway. Interleukin-12 (IL-12), for example, is produced by DCs to promote the development of CD4+ Th1 cells and CD8+ CTLs [136], while Notch1 and Notch2, for example, signal Th2 cell development [137, 138]. Cytokine secretion by activated Th cells leads to the activation of many other cell types including CTLs [139-141] and B cells [142-144]. The release of Th1 cytokines [145] such as IFN-γ, IL-2, and TNF-α participates in the development of effector CTLs within secondary lymphoid organs. Subsequent migration of activated CTLs into the periphery allows them to migrate towards target cells and to lyse them. In contrast to the Th1 arm of cellular immunity, the release of Th2 cytokines [145] such as IL-5 and IL-13 contributes to B cell activation. Activated B cells migrate out of secondary lymphoid organs into the periphery to become mature plasma cells, capable of secreting antibodies that target the pathogens or cells carrying the appropriate antigens. Thus, DCs are important cells for inducing and regulating the specificity, strength, and type of immune reactions.

1.4 Non-viral DC manipulations for immunotherapy

Several types of DC modifications have been tested to initiate or enhance active immunotherapy against cancer. These schemas can be broadly classified into three groups: peptide- or lysate-pulsing; RNA or DNA transfections; and viral transductions.
DC-pulsing involves co-incubating DCs with whole tumor lysates or with synthetic or natural peptides against TAAs [146, 147]. This form of DC modification has shown some efficacy in animal models [148]. Since the early use of peptide-pulsed DCs in cancer patients [149], many clinical trials have assessed the use of DCs pulsed with a single peptide or a cocktail of peptides derived from TAAs. Two recent Phase I clinical trials used HLA-restricted carcinoembryonic antigen (CEA)-derived peptides to pulse autologous DCs from colorectal cancer patients [150, 151]. Both of those trials showed an increase in the frequency of CEA-specific T cells, but did not show reduction in tumors. In another Phase I trial, melanoma patients were given DCs loaded with peptides derived from four TAAs (MART-1, tyrosinase, MAGE-3, and gp100). These patients experienced no measurable increase in antigen-specific T-cell responses and no objective clinical benefit [152]. Tumor lysate-pulsed DCs have also shown modest immunotherapeutic results in clinical trials. For example, in a recent Phase I trial for hepatocellular carcinoma, 4 of 31 patients experienced a partial anti-tumor response due to DC vaccination and 17 patients were found to have stabilized disease after treatment [153], as defined by set guidelines [154]. Potential reasons for less-than-complete responses include the limited repertoire offered by peptide-pulsing and the general instability of the MHC/peptide complex following peptide- or lysate-loading.

To help overcome such potential limitations associated with pulsed DCs, other methods to modify DCs have been developed. Transfections of DNA or RNA [155, 156] into DCs have been directly compared to DC-pulsing methods using animal models. Comparative studies have shown unequivocally the advantages of using nucleic acid modification methods [157]. Reasons for this include greater MHC-peptide stability.
(because of their de novo formation), a greater repertoire of peptides derived from each antigen presented, and the additional possibility of co-expressing adjuvant proteins [158]. That said, it is not entirely clear whether plasmid or genomic DNA-loaded DCs are clinically more effective against tumors than DCs transfected with in vitro-generated or whole cell RNA [159]. There is a paucity of clinical trials on DNA-transfected DCs, but in vivo mouse studies and in vitro human cell experiments indicate potential efficacy in generating TAA-specific CTL responses [160, 161]. One of the first reported clinical trials using DCs transfected with tumor RNA did not show dramatic clinical responses [162]. However, trials using DCs transfected with in vitro-transcribed RNA encoding TAAs, such as PSA, have demonstrated enhanced T-cell responses against this antigen. In one study, a complete but transient clearance of circulating tumor cells was achieved in 3 of 3 analyzed patients [163, 164].

1.5 Generation of recombinant viruses used for transducing DCs

1.5.1 Overview

Viruses are efficient vehicles for introducing foreign DNA into host cells. Several types of virus have been exploited for delivering genes to DCs. Viral genomes have been modified in a number of ways to render them useful for gene transfer applications. Genes required for viral replication are removed and structural/packaging sequences are separated from the viral backbone into helper plasmids. Removal of viral sequences also allows for the introduction of a transgene expression cassette. Together, these viral modifications reduce the chances of homologous recombination re-creating a wild-type virus. Of note, helper plasmids lack a packaging domain, so that they themselves cannot be packaged into a viral particle. Transfection of expression and helper plasmids into
packaging cell lines produces recombinant virions that can be used to transduce target cells, such as DCs. Here, I focus on three commonly used recombinant viruses in this context: adenoviruses, poxviruses, and retroviruses.

1.5.2 Adenoviruses

Adenoviruses (Ad) are linear double-stranded (ds) DNA viruses. Following entry into a cell after binding to the coxsackie-adenovirus receptor (CAR), they transcribe their early region 1 (E1) genes, which are needed to initiate viral gene expression and genome replication. Genome replication is also dependent on the expression of E2 and E4 genes. The E3 region is dispensable for viral replication. Structural proteins necessary for encapsulating newly-formed viruses are made late in the life-cycle. First generation Ad vectors were E1-deleted to prevent viral replication. In second and third generation Ad vectors, E2, E3, and/or E4 genes were also deleted to help reduce Ad immunogenicity. Further deletions led to the development of ‘gutless’ Ad vectors, which depend on a helper virus to provide essential genes in trans. The cloning capacity of these vectors is up to 35 kb. Ad vectors have been widely used in the clinic; between 1989 and 2007, 342 gene therapy trials have used this strategy [165]. Transgene expression in DCs is transient due to the episomal nature of Ad infection [166]. Interestingly, it has been reported that binding of the fiber knob domain of Ad to CAR on DCs can actually induce DC maturation [167]. Because human DCs express very low levels of CAR, Ad vectors have been developed to permit efficient DC transduction [168]. Ad5f35, for example, is an Ad serotype 5 (Ad5) vector containing the knob domain derived from Ad35 and has been shown to efficiently transduce human DCs [169, 170].
1.5.3 Poxviruses

Vaccinia, Fowlpox, and Canarypox belong to the poxvirus family. Their linear dsDNA genomes are 100- to 300-kb long and have a hairpin loop at both ends. Attenuated strains of these viruses have been created. The modified vaccinia virus Ankara (MVA), for example, was isolated after over 500 passages in chicken embryonic fibroblasts. By the end of these passages, approximately 31 kb of its genome was lost, leading to impaired viral replication [171]. Attenuated strains of fowlpox virus and canarypox virus, such as TROVAC and ALVAC, respectively, were derived in a similar manner [171]. Between 1989 and 2007, 93 vaccinia and 88 other poxvirus gene therapy clinical trials were carried out [165]. One of the major advantages to choosing recombinant vaccinia is that it can carry over 25 kb of foreign DNA. There are reports, however, that vaccinia virus-transduced DCs exhibit reduced antigen-presenting function [172, 173].

1.5.4 Retroviruses

Retroviruses are 7- to 11-kb linear single-stranded (ss) RNA viruses. Following host cell entry, the RNA genome is reverse-transcribed into dsDNA and stably integrated into the host genome. This property of integrating into host genomes is advantageous for gene therapy since long-term expression can be achieved in transduced cells and their progeny. Onco-retroviruses and lentiviruses both belong to the Retroviridae family, but the latter are more complex. Their genomes consist of long terminal repeat (LTR) sequences at both ends, as well as structural proteins, polymerases, integrases, and surface glycoproteins that are encoded by the Gag, Pol, and Env genes, respectively. Onco-retroviruses and lentiviruses can carry up to 8 kb and 9 kb of exogenous DNA,
respectively [174, 175]. Whereas onco-retroviruses require actively dividing host cells for integration and efficient gene expression, lentiviruses have the ability to integrate into the genome of slowly-dividing cells since they have a more stable pre-integration complex. It is reported that 307 gene therapy trials used onco-retroviruses between 1989 and 2007 [165]. Extensive work with lentiviruses in mouse models is now beginning to be extended to gene therapy clinical trials, 11 of which have been completed and published to date [165]. Lentiviral vectors have been used in the studies described in this thesis and will be described in more detail.

The most widely used lentiviral vector (LV) for gene transfer into DCs is based on the human immunodeficiency virus type 1 (HIV-1). In addition to carrying the Gag, Pol, and Env genes of simple retroviruses, lentiviruses also contain a number of additional regulatory and accessory proteins and sequences such as Rev, Tat, central polypurine tract (cppt) and others. To reduce the risk of forming replication competent retroviruses, lentiviral vectors have been designed such that many accessory genes have been deleted or separated onto different plasmids [176] (Figure 1.1). To further increase vector biosafety, a 400-bp deletion in the 3’ long terminal repeat (LTR) U3 region has been introduced that renders the virus self-inactivating (SIN). Following transduction of target cells and provirus integration, this inactive U3 region is present in the 5’ LTR as well [177]. This modification also helps eliminate residual LTR promoter activity and prevents the formation of full-length viral transcripts [178, 179]. It also allows for the use of internal promoters that can achieve tissue-specific gene expression.

Recombinant lentiviruses can be produced by triple transfection of 293T cells using the following three plasmids (Figure 1.1): (1) The transfer vector, which encodes the
transgene of interest (reporter gene or therapeutic gene, for example) driven by an internal promoter, as well as cis-acting sequences required for packaging ($\psi$), reverse transcription, and proviral integration; (2) The packaging vector, which carries Gag and Pol sequences under the control of a constitutive promoter (eg. CMV); (3) The envelope vector, which contains a sequence encoding an envelope glycoprotein such as the vesicular stomatitis virus glycoprotein (VSV-G). Transfected 293T cells release viral particles into the cell culture medium. Pseudotyping viral particles with VSV-G allows for a broad tropism of infectivity and enables concentration by ultracentrifugation to produce high titers. Functional viral titer can be measured by transducing 293T cells with serially-diluted viral preparations followed by quantifying the percentage of transgene-positive cells by flow cytometry. Alternatively, particle titer can be determined by ELISA to quantify the levels of p24 antigen, which is present in the capsid. Other titration methods using real-time quantitative PCR of RNA isolated from the viral particles or from the transduced target cells have also been developed [180, 181].
Figure 1.1 Schematic diagram of a three-plasmid system used to generate recombinant lentiviruses. The transfer vector contains an expression cassette for the transgene(s) driven by the internal promoter Elongation Factor 1α (EF1α), the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), the packaging signal (ψ), splice donor (SD) and acceptor (SA) sites, and the rev response element (RRE). The packaging vector (pCMV ΔR8.91) encodes Gag, Pol, and other viral genes. The envelope vector (pMD.G) contains the VSV-G envelope sequence needed to pseudotype the recombinant lentivirus.
1.6 Mouse model studies using virally-transduced DCs

In this section, recent (published in the last 5 years) mouse model studies using DCs that have been transduced with adenoviruses, onco-retroviruses, or lentiviruses are discussed. A summary of these studies is shown in Table 1.1.

1.6.1 Studies using DCs transduced with recombinant adenoviruses

Results from several studies employing recombinant Ad vectors are generating varied immune responses but outcomes are encouraging overall. A paper by Steitz et al. (2006) [182] has shown that the use of DCs transduced with Ad carrying the melanoma enzyme tyrosine-related protein 2 (TRP2) was far more efficient in generating a TRP2-specific immune response compared to a single peptide derived from TRP2 (TRP2aa180-188). In that study, mice were first immunized with $2.5 \times 10^5$ transduced or peptide-pulsed DCs. One week later, they were challenged with $4 \times 10^5$ B16 melanoma cells to generate a model for lung metastases. Induction of CD4$^+$ T cells was found to be critical for the observed expansion of peptide-specific CD8$^+$ T cells. Complete protection from tumors was reported for mice immunized with the transduced DCs. This study highlights the potential of using Ad-transduced DCs for overcoming tolerance to a defined TRP2 peptide.

In another metastatic model of melanoma, Broder et al. (2003) [183] used recombinant Ad to transduce DCs with Melanoma Antigen Recognized by T cells-1 (MART-1) as the TAA. $5 \times 10^5$ DCs were injected 1 week before 500 B16 melanoma cells were implanted in the brain. MART-1-specific CTL immunity was achieved along with improved survival, although only 2 of 23 mice experienced complete protection. It is difficult to reconcile this report with the previous one and this underscores the
<table>
<thead>
<tr>
<th>Virus</th>
<th>Immunization Schedule</th>
<th>Rt. of Injection</th>
<th>TAA(s)</th>
<th>Cancer Model</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad</td>
<td>1 injection of 2.5 x 10⁶ DCs</td>
<td>i.v.</td>
<td>Murine TRP2</td>
<td>• 4 x 10⁶ B16 melanoma cells were injected 1 week post-immunization</td>
<td>• Complete tumor protection in 3 of 5 mice</td>
<td>[165]</td>
</tr>
<tr>
<td>Ad</td>
<td>1 injection of 5 x 10⁶ DCs</td>
<td>s.c.</td>
<td>Human MART-1</td>
<td>• 5 x 10⁶ melanoma cells were implanted in the brain 1 week post-immunization</td>
<td>• Complete tumor protection in 2 of 23 mice</td>
<td>[166]</td>
</tr>
<tr>
<td>Ad-RGD</td>
<td>1 injection of 1 x 10⁶ DCs</td>
<td>i.d.</td>
<td>Human gp100</td>
<td>• 2 x 10⁶ B16 melanoma cells were injected 1 week post-immunization</td>
<td>• Complete tumor protection in 4 of 6 mice at 60 days post-immunization</td>
<td>[167]</td>
</tr>
<tr>
<td>Ad</td>
<td>1 injection of 1 x 10⁶ DCs</td>
<td>s.c.</td>
<td>Truncated rat neu</td>
<td>• S.c.gene-transgenic mice, which develop spontaneous mammary tumors</td>
<td>• Complete tumor protection in 14 of 21 mice</td>
<td>[168]</td>
</tr>
<tr>
<td>Ad</td>
<td>3 daily injections of 1 x 10⁶ DCs</td>
<td>i.t.</td>
<td>Murine p53</td>
<td>• Sarcoma tumors were chemically induced using MethA or M1 cells were injected with MCA207 fibrosarcoma cells.</td>
<td>• Complete tumor regression in 5 of 4 DC-p53 treated MethA-injected mice</td>
<td>[169]</td>
</tr>
<tr>
<td>Ad</td>
<td>2 injections of 1 x 10⁶ DCs</td>
<td>i.t.</td>
<td>N/A</td>
<td>• Sarcoma tumors were chemically induced using MethA or M1 cells were injected with C57BL/6 cells.</td>
<td>• Complete tumor regression in 5 of 4 DC-p53 treated MethA-injected mice</td>
<td>[170]</td>
</tr>
<tr>
<td>Ad</td>
<td>1 injection of 1 x 10⁶ DCs</td>
<td>i.t.</td>
<td>N/A</td>
<td>• Mice were injected with GL261 glioma tumors</td>
<td>• Increased level of DC cross-presentation</td>
<td>[171]</td>
</tr>
<tr>
<td>Ova-RV</td>
<td>2 injections of 4 x 10⁶ DCs</td>
<td>i.p.</td>
<td>Human PSA or PSMA</td>
<td>• 1 or 3 x 10⁴ PSA tumor or PSMA were injected; 1 or 16 weeks post-immunization</td>
<td>• Antigen-specific humoral and cellular responses correlated with robust tumor protection</td>
<td>[172]</td>
</tr>
<tr>
<td>LV</td>
<td>1 injection of 5 x 10⁶ DCs</td>
<td>Footpad injection</td>
<td>Chicken OVA</td>
<td>• 2 x 10⁶ B16-OVA melanoma tumors were injected 3 days before DC-immunization</td>
<td>• Complete tumor protection in 4 of 7 mice involving both CD8+ and CD4+ T cells</td>
<td>[173]</td>
</tr>
<tr>
<td>LV</td>
<td>1 injection of 5 x 10⁶ DCs</td>
<td>s.c.</td>
<td>Murine TRP2</td>
<td>• 1 x 10⁶ B16-P10 melanoma cells were injected 10 days post-immunization</td>
<td>• Complete tumor protection in 4 of 7 mice involving both CD4+ and CD8+ T cells</td>
<td>[174]</td>
</tr>
<tr>
<td>LV</td>
<td>N/A</td>
<td>N/A</td>
<td>Influenza HA</td>
<td>• Mice were first challenged with A/20-HA-B-cell lymphoma or Flora tumor cells, then given a BMT with HA-transduced HSCs.</td>
<td>• Complete tumor protection in 4 of 7 mice involving both CD8+ and CD4+ T cells</td>
<td>[175]</td>
</tr>
<tr>
<td>LV</td>
<td>2 injections of 2 x 10⁶ or 2000 DCs</td>
<td>i.p.</td>
<td>Truncated murine erbB2</td>
<td>• 2 x 10⁶ B16 expressing RM 1 prostate tumors were injected 8 weeks post-immunization</td>
<td>• Complete tumor protection in 6 of 16 and 2 of 6 mice immunized with 2 x 10⁶ and 2000 DCs, respectively</td>
<td>[176]</td>
</tr>
<tr>
<td>LV</td>
<td>1 injection of 1 x 10⁶ DCs</td>
<td>Footpad injection</td>
<td>TRP2</td>
<td>• 2.5 x 10⁶ TRP2+ B16 tumors were injected a.c. into mice</td>
<td>• Complete tumor protection in 6 of 16 and 2 of 6 mice immunized with 2 x 10⁶ and 2000 DCs, respectively</td>
<td>[177]</td>
</tr>
</tbody>
</table>

**Table 1.1: Efficacy of transduced DCs in mouse studies**
importance of tumor location, since some locations are immune-privileged [184]. Broder et al. used a greater number of DCs to protect mice against a considerably lower tumor burden. Although a CTL response was measured, the lack of tumor protection in an immune-privileged organ, the brain, may illustrate the need for combining immunotherapy with a brain-targeting strategy. It is unclear whether the induced levels of immunity against MART-1 would have been sufficient to protect against tumor challenge at another site.

As mentioned, in order to improve the efficiency of Ad entry into immune cells, mutations have been made to Ad vectors. The low expression of the Ad-receptor CAR on DCs prompted the development of a fiber mutant Ad vector (AdRGD). This mutant uses αv-integrin on DCs to gain efficient cell entry. Employing AdRGD to transduce DCs to express the melanoma-associated antigen gp100, Okada et al. (2003) [185] found better results compared to conventional Ad. In their experimental model, mice were immunized with 1x10^6 DCs and then challenged with 2x10^5 C57BL/6-derived B16 melanoma cells one week later. The mutant fiber form of this Ad virus allowed for a higher gene transduction efficiency compared to the conventional Ad vector used for most gene therapy studies. Greater protection from tumor challenge was also achieved using the fiber-mutant; 4 of 6 mice remained tumor-free for 60 days post-immunization compared to 1 of 6 mice given the conventional Ad-gp100-transduced DCs. There was also a correlation between enhanced protection and enhanced CTL and NK cell activities against the gp100-expressing melanoma tumors [185].

Ad-transduced DCs have also been tested in transgenic tumor models, including a breast cancer model. Using Ad encoding the extracellular and transmembrane domains
of neu, the rat homologue of the human TAA HER2/neu, transduced DCs were found to be effective in delaying the onset of mammary tumors in neu-transgenic mice [186]. Whereas all mice that received non-transduced DCs had palpable tumors at 28 weeks, 14 of 21 mice that received 1x10^6 DCs were tumor-free. It is interesting to note that a humoral mechanism was primarily involved in mediating this anti-tumor effect [186].

Another study tested Ad-p53 transduced DCs injected in three consecutive daily injections of 1x10^6 cells directly into palpable MethA sarcoma tumors or MCA-207 fibrosarcoma tumors [187]. Although 5 of 8 mice bearing MethA tumors underwent complete tumor regression following DC-p53 treatment, 4 of 8 mice that received non-transduced DCs also showed complete regression. Only 2 of 5 mice harboring MCA-207 cells and immunized with DC-p53 cells survived for 80 days post-tumor challenge. Only 1 of 4 tumor-bearing mice that received uninfected DCs survived. It is unclear why in this study the control groups such a high level of tumor regression.

To enhance the effectiveness of DCs, the use of cytokine gene-modified DCs has been explored. For example, Tatsumi et al. (2003) [188] used two intratumoral injections of 1x10^6 DCs transduced with Ad carrying both IL-12 and IL-18 to effectively treat local and distant CMS4 or MethA sarcoma tumors. IL-12 and IL-18 are pro-inflammatory cytokines that help elicit Th1/Tc1-type immunity. Using this combination of cytokines, the viability of injected DCs was extended and splenocyte production of IFN-γ was enhanced [188]. Although the DCs were not engineered to express a TAA, the authors hypothesized that the extended DC viability allowed for a greater opportunity to cross-present tumor antigens.
Another promising approach uses intratumorally-injected DCs that are transduced with Ad vector encoding IFN-α. This cytokine can improve overall DC function and even help promote cross-priming of T cells [189]. Kuwashima et al. (2005) [190] tested the therapeutic efficacy of DCs transduced with Ad and thereby engineered to express IFN-α. Mice with intracranial GL261 glioma tumors were injected intratumorally with 1x10⁵ transduced DCs. Potent anti-tumor responses were measured through IFN-γ production and cytolytic activities of draining LN cells.

1.6.2 Studies using DCs transduced with recombinant onco-retroviruses

Although many earlier studies have investigated the use of onco-retroviruses in transducing DCs for inducing anti-tumor immunity, relatively few have been done in the last few years. This trend may be due to the increasing popularity of lentiviruses, which share many properties of onco-retroviruses and have the added benefit of dramatically more efficient transduction and the ability to transduce slowly-dividing cells. On the other hand, onco-retroviruses have been well studied and production can be standardized relatively easily. A previous study was published from our laboratory showing the use of onco-retrovirally transduced DCs in protecting mice from tumor development as well as inducing regression of palpable tumors [191]. DCs were genetically modified to express one of two prostate TAA s, PSA or PSMA. In tumor protection experiments, 4x10⁵ PSA- or PMSA-transduced DCs were injected into mice, followed by tumor challenge using specifically engineered cells (1x10⁶ or 3x10⁶ cells) at either 1 or 18 weeks later. Mice exhibited potent humoral and cellular responses against PSA or PSMA and yielded robust protection against tumors. Immunological memory was also observed. In a more therapeutic setting examining effects on pre-existing tumors, 5x10⁶ PSA-expressing
tumor cells were administered s.c. before immunizing mice at 7 and 37 days later with PSA-transduced DCs. Near complete elimination of PSA-tumors was observed, whereas control tumors continued to increase in size over time [191].

1.6.3 Studies using DCs transduced with recombinant lentiviruses

A few papers describing outcomes after LV transduction of DCs in murine models have been published in recent years. LVs are efficient in transducing DCs and unlike Ad, they are reported neither to affect the maturational state of DCs nor to elicit virus-specific immunity, unless unusually high MOI values (around 500) are used [192, 193].

A recent article showed some therapeutic efficacy of LV-transduced DCs expressing a model antigen (ovalbumin (OVA)) against B16 melanoma engineered to express the soluble form of OVA. Mice were inoculated with 2x10^5 tumor cells and then immunized with 5x10^5 vector-transduced or OVA peptide-pulsed DCs three days later. While the pulsed DCs did not confer a survival advantage over control groups, OVA-transduced DCs allowed mice to survive longer and strongly inhibited tumor growth, although these mice did not show actual tumor regression in that study [194].

In another investigation targeting therapy for melanoma, DCs were transduced with a LV encoding murine TRP-2 to assess the protective effects of 1x10^5 genetically modified DCs given to animals [195]. Ten days later, mice were injected with 1x10^5 B16-F10 melanoma cells. Strong protection against tumor challenge was achieved and 4 of 7 mice were free of tumors up to 80 days post-immunization; it was then further shown that effect was dependent on both CD8^+ and CD4^+ T cells [195].

Although the classical approach of testing transduced DCs in mouse models has been to inject BM-derived DCs before or after tumor inoculation, an alternative strategy
has been recently undertaken by Cui et al. and published in 2003 [196]. In that study, hematopoietic stem-progenitor cells (HSPCs) were LV-transduced to engineer expression of the model antigen hemagglutinin (HA) and transplanted into irradiated recipient mice bearing B-cell lymphoma A20-HA tumors. This step produced transduced DCs of donor-origin in the lymphoid organs of recipient mice. Subsequent adoptive transfer of \(2.5 \times 10^7\) HA-specific splenocytes from transgenic mice carrying HA-specific CD4\(^+\) or CD8\(^+\) T cells was used to clear A20-HA tumor cells. At 20 weeks post-bone marrow transplant (BMT), mice receiving transduced DCs had a better survival rate than those receiving non-transduced DCs (50\% vs. 10\%, \(P=0.026\)). Using a similar approach, Xhang et al. (2008) treated pulmonary metastases in a mouse model using bone marrow cells that were LV-transduced to express HA [197]. One week following inoculation of mice with \(1 \times 10^6\) HA-expressing Renca cells, \(4 \times 10^6\) LV-modified bone marrow cells and \(1 \times 10^7\) splenocytes were transplanted into lethally-irradiated recipients. Another week later, mice were treated with DC stimulators GM-CSF and/or CpG-containing oligonucleotides. This approach efficiently activated antigen-specific T cells and improved the survival of tumor-bearing mice. Compared to control mice, mice that received HA-expressing LV-transduced bone marrow cells as well as both adjuvants yielded up to 50\% survival by day 53 compared to just 10\% survival by day 33, demonstrating the utility of this approach in a therapeutic setting [197].

To expand the applications of LV-transduced DCs in immunotherapy, methods have been developed using LVs to help DCs overcome tolerance to self-TAAs. Shen et al. (2004) [198] generated an LV encoding a small interfering RNA (siRNA) that down-regulated the expression of the suppressor of cytokine signaling (SOCS) 1. SOCS1
functions as a negative regulator of DC antigen presentation. Silencing SOCS1 by transducing DCs with LV-SOCS1-siRNA and then pulsing these DCs with TRP2 peptide led to a significant enhancement of antigen-specific and potent anti-tumor immunity [198]. In their experimental model, TRP$^+$ B16 tumor-bearing mice were treated with 1 injection of $1 \times 10^6$ LV-transduced, TRP2 peptide-pulsed DCs. Tumor growth was nearly completely blocked and this result correlated with TRP2-specific CTL activity, as measured by IFN-γ ELISPOT and CTL assays. Thus, regulating the extent of tumor antigen presentation on DCs by LV transduction of SOCS1 siRNA has been shown to be a useful finding in enhancing DC-mediated immunotherapy.

1.7 Clinical trials using DCs transduced with recombinant viruses

In this section, recent clinical studies using DC that have been transduced with recombinant viruses are discussed below. Table 1.2 shows a summary of these trials.

1.7.1 Trials using DCs transduced with recombinant adenoviruses

In 2006, Antonia et al. reported the results of a clinical trial testing the effects of immunizing 29 patients having advanced small cell lung cancer with DCs that had been transduced with a recombinant Ad vector and engineered to express wild-type p53 [199]. Most patients received 3 DC injections consisting of $2.4 \times 10^5$ to $5 \times 10^6$ p53$^+$ DCs. Of the 29 patients in the study, 15 of them produced p53-specific T-cell immune responses. Antibodies against Ad were also measurable in responsive patients, indicating the presence of an anti-viral immune reaction. The only clinical benefit observed was a partial response in 1 patient. Interestingly however, chemotherapy treatment following the immunotherapy regimen resulted in 18 patients responding with an objective clinical response. The authors postulate that this is due to chemotherapy inducing: (1) the down-
### Table 1.2: Efficacy of transduced DCs in clinical trials

<table>
<thead>
<tr>
<th>Virus</th>
<th>TAA</th>
<th>Cancer</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad</td>
<td>p53</td>
<td>Advanced small cell lung cancer</td>
<td>- Heightened immunity in 17/29 patients</td>
<td>(182)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Clinical benefit in 1 patient with immunotherapy alone</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Clinical benefit in 18 patients following subsequent chemotherapy</td>
<td></td>
</tr>
<tr>
<td>MVA</td>
<td>Tyrosinase</td>
<td>Advanced stage melanoma</td>
<td>- Heightened immunity in 5/6 patients</td>
<td>(183)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Tumor reduction in 15 patients</td>
<td></td>
</tr>
<tr>
<td>Fowlpox</td>
<td>CEA</td>
<td>Colorectal cancer or NSCLC</td>
<td>- Heightened immunity after 1 immunization in 10/14 patients</td>
<td>(184)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Tumor regression in 1 patient and disease stability in 5 patients</td>
<td></td>
</tr>
</tbody>
</table>
regulation of tumor-derived immunosuppressive factors; (2) the up-regulation of p53 in tumor cells for enhanced recognition by CTLs; and/or (3) the up-regulation of perforin or granzyme production by CTLs for improved tumor killing. The combination of these two treatment modalities may reveal the potential for achieving better clinical responses.

### 1.7.2 Trials using DCs transduced with recombinant vaccinia viruses

A recent Phase I study used MVA to transduce DCs in order to engineer expression of the melanoma TAA, human tyrosinase [200]. Six Stage IV melanoma patients were immunized with \(1 \times 10^8\) gene-modified DCs by i.v. injection three times given 14 days apart. Tyrosinase (368-376) and VV H3L (184-192) antigen-specific IFN-\(\gamma\) secreting effector cells were detected by ELISPOT in 5 patients. Memory T cells specific for tyrosinase were also produced. The patient demonstrating the most response experienced reduction in one tumor nodule and remained tumor-free for over 850 days following surgical resection of this nodule. Side effects of the treatment included a mild fever in 3 patients, mild erythema at the site of injection in 5 patients, and vitiligo in 2 patients.

### 1.7.3 Trials using DCs transduced with recombinant fowlpox viruses

In 2005, Morse et al. reported the results from a Phase I trial for treating 11 colorectal cancer patients and 3 non-small cell lung cancer (NSCLC) patients using DCs transduced with fowlpox virus encoding CEA combined with a triad of costimulatory molecules (collectively referred to as TRICOM) [201]. TRICOM consists of B7.1 (CD80), intercellular adhesion molecule 1 (ICAM-1 or CD54), and Leukocyte Function Antigen-3 (LFA-3 or CD58). Of the 14 patients, 12 received at least 1 immunization cycle. Ten patients experienced an increase in the frequency of CEA-specific CD4\(^+\) and CD8\(^+\) T cells, although administration of a second round of immunization did not lead to
any further increase. One patient had minor regression of adenopathy and 5 patients had disease stabilized for at least 3 months following the first immunization. Grade 3/4 toxicities that were observed could not be directly attributed to immunizations.

Overall, clinical trials using virally-transduced DCs appear to be safe and able to confer heightened immunity specifically towards tumor-associated antigens to patients. Although there are several advantages of this form of immunotherapy, the rates of objective clinical tumor responses seem to be low and emphasize the need for further developing this approach. Clinical trials testing other forms of immunotherapy, such as peptide alone or peptide-pulsed DCs have also shown limited success, altogether having an average objective clinical response of about 2.6% [202].

1.8 HER2/neu (erbB2) as a model TAA

1.8.1 Biology of HER2/neu

HER2/neu belongs to the human epidermal growth factor receptor (HER) family, which also includes HER1 (EGFR), HER3, and HER4 [203]. The murine homolog of HER2/neu is often referred to as erbB2 and the rat homolog is often referred to as neu, since the HER2/neu gene was first cloned from a rat neuroglioblastoma [204]. All HER members are Type I transmembrane proteins consisting of extracellular, transmembrane, and intracellular domains. They homodimerize or heterodimerize allowing ligands to bind and stabilize their extracellular domains. Ligand binding results in activation of the intracellular tyrosine kinase domains and initiation of numerous possible downstream signaling events. Interestingly, HER2/neu is considered an orphan receptor, as it lacks the ability to bind ligands, while HER3 lacks a functional kinase domain. However, the heterodimer formed between these two receptors generates a highly active receptor [205]
and HER2/neu seems to be the preferred binding partner for the other HER proteins [203]. Each receptor dimer possesses a different collection of tyrosine phosphorylation sites and accordingly sequesters different signaling molecules. The best-studied HER2/neu-influenced signal transduction pathways involve mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI-3K) [206, 207].

Attributed functions of the HER family of proteins so far are broad, as they include cell differentiation, migration, proliferation, and survival [208, 209]. The embryonic lethality associated with erbB2-knockout mice emphasizes the essential role of this protein during normal development [210]. Furthermore, there are three main areas of the body whose development is highly dependent on HER2/neu: the cardiovascular system, the nervous system, and the mammary glands [203]. Evidence for a role for erbB2 in the cardiovascular system includes the finding that mice having a conditional erbB2 mutation using the established Cre-Lox system, exhibit severe dilated cardiomyopathy [211]. In the nervous system, the ablation of erbB2 using Cre-Lox technology results in the failure of peripheral motorneuron axons to form mature neuromuscular junctions as they enter the muscle [212]. Among the many stages of mammary gland development, erbB2 function is thought to be crucial during puberty; expression levels decrease thereafter and remain low even during pregnancy, lactation, and involution. The role of erbB2 at these later stages is still important, since adult animals that are transgenic for a dominant-negative intracellular-deficient rat homolog of erbB2 mutant have problems lactating, owing to failure of alveolar expansion [209].
1.8.2 Association with cancer

Overexpression of HER2/neu occurs in about 20% of primary prostate cancer cases and 80% of their metastases [213]. HER2/neu is also overexpressed in several other epithelial tumors, including breast, lung, and ovarian cancers [206, 214-216]. The oncogenic effects of HER2 arise from activation of the MAPK and PI-3K signal transduction pathways, leading to increased mitosis and the inhibition of apoptosis [206, 207]. \textit{In vitro} it has been shown that HER2/neu overexpression in cells can lead to chemo-resistance and even enhanced invasiveness [217-219]. Several studies have reported that cancer patients with elevated HER2/neu levels have relatively poor prognoses [220, 221]. Although the majority of HER2/neu-overexpressing cancers have amplified copies of the HER2/neu gene, mutational activation of HER2/neu has also been reported in some lung cancers [222].

1.8.3 HER2/neu-targeted therapeutics

Many of the therapies targeting HER2/neu have originally been designed for treating breast cancer. Two main approaches have been developed: (1) the monoclonal anti-HER2/neu antibody Herceptin (trastuzumab) and (2) small molecule general inhibitors of tyrosine kinase. When Herceptin is used as a first-line treatment, it is effective in reducing metastatic tumors in up to 26% of HER2/neu\textsuperscript{+} breast cancer patients [223, 224]. Its mechanism of action is not entirely clear, but it is thought to involve binding to the extracellular domain of HER2/neu in order to trigger internalization and prevent signaling from the cell surface [225, 226], which in turn may lead to cell cycle arrest [227]. The efficacy of Herceptin has been evaluated for advanced prostate cancer patients in clinical trials, but thus far it has not shown to offer benefit [228]. Efforts are
underway to understand the resistance of cancers to Herceptin. Current hypotheses for this resistance include the activation of alternative signaling pathways (such as via insulin-like growth factor-I receptor) that promote proliferation and metastasis, and the idea that Herceptin is unable to bind HER2/neu due to receptor masking by proteins like MUC4, a membrane-associated mucin [229].

Small molecule tyrosine kinase inhibitors (TKIs) of HER2/neu such as lapatinib has been used in the clinic for treating HER2/neu+ breast cancer, but offered limited efficacy even though it was confirmed to at least effectively suppress MAPK activity [230, 231]. It appears that most TKIs are generally unreliable single agent treatments, perhaps due to the incomplete suppression of HER2-signalling [206].

1.9 Immunomodulatory proteins

1.9.1 Interleukin-12 (IL-12)

Interleukin-12 (IL-12) is a pleiotropic cytokine that functions as a key regulator of innate and adaptive immunity. It is a 70-kDa heterodimer composed of two covalently linked subunits, p35 and p40, and is mainly expressed by DCs, macrophages, and other hematopoietic phagocytic cells. During T cell development, IL-12 induces the differentiation of CD4+ T cells into Th1 cells. Activated Th1 cells secrete IFN-γ, which in turn inhibits Th2 proliferation. Conversely, IL-10 and TGF-β produced by Th2 cells block Th1 activation by mechanisms that include inhibiting IL-12 synthesis [232, 233]. During host defense against pathogens, IL-12 production by APCs can initially be triggered by ligation of Toll-like receptors by bacterial products [234]. Subsequent amplification of IL-12 production occurs following APC contact with antigen-specific T cells via CD40L-CD40 and MHC-peptide-TCR interactions [234]. Ligation of the IL-12
receptor leads to signaling through the phosphorylation and activation of JAK2 and TYK2 [235]. Activation of these Janus kinases is followed by the phosphorylation of STAT3 and STAT4 [236, 237]. These transcription factors are then translocated to the nucleus, resulting in activation of T cells and secretion of IFN-γ, which in turn activates APCs to clear the pathogen.

IL-12 has also been shown to have an impact when used to treat primary and metastatic tumors in mouse models [238-240] and clinical trials [241-243]. Its anti-tumor effects are directly linked to its ability to induce IFN-γ secretion from lymphocytes. IFN-γ may act directly on tumor cells to increase tumor antigen processing and presentation on MHC class I molecules, and thus make tumors more susceptible to T cell recognition [244]. IFN-γ may also affect stromal and endothelial cells in the tumor microenvironment to inhibit angiogenesis and tumor invasion. This process involves the upregulation of chemokines such as IP-10 and MIG [245, 246], which can recruit effector cells that in turn remodel the extracellular matrix and inhibit matrix metalloprotease (MMP) expression [247]. IFN-γ may also inhibit angiogenesis by down-regulating the expression of adhesion molecules, such as the integrin αVβ3, by endothelial cells [248].

The multiple anti-tumor effects that IL-12 offers has provided the rationale for using IL-12 to treat cancer. The success of its use has varied, but has provided some patients objective tumor responses. For example, in a recent Phase II clinical trial, when non-Hodgkin’s lymphoma patients were given IL-12 alone, 21% of patients exhibited objective responses and had elevated levels of circulating CD8+ levels [249]. IL-12 therapy has also been used in combination with other anti-tumor agents, including Herceptin. When this treatment combination was used in a Phase I trial in patients with
HER2+ tumors, 6% of patients had objective responses that were also associated with an increase in IFN-γ secretion by NK cells [250].

Despite the modest success achieved by IL-12 therapy, there have been reports of toxicity associated with it due to the high levels of IFN-γ secreted by lymphocytes. To overcome the toxic effects of systemic IL-12 in patients, gene therapy studies have been designed to achieve local IL-12 production at the tumor site and only low levels of systemic IL-12 [251]. In one clinical trial [252], patients with metastatic gastrointestinal carcinomas received intra-tumoral injections of DCs that were engineered to express IL-12 by recombinant Ad. Among the 17 patients, 1 patient experienced a partial response. In another study [253], plasmid DNA encoding IL-12 was injected directly into lesions of 9 malignant melanoma patients. The outcome was that the treatments were well tolerated and 2 patients had stable disease and 1 patient achieved complete remission. Carefully selecting combinatorial approaches using IL-12 may help extend its efficacy in treating cancer.

1.9.2 Receptor activator of nuclear factor κB ligand (RANKL)

RANKL (also called tumor necrosis factor (TNF)-related activation-induced cytokine [TRANCE]) is a member of the TNF ligand family and its receptor is receptor activator of nuclear factor κB (RANK). The RANKL-RANK system is known to play important roles in the immune system, bone metabolism, and the vascular system. In the immune system, activated T cells express both membrane-bound and soluble forms of RANKL. Soluble RANKL is generated through cleavage of the membrane-bound form by the protease TNF-α-converting enzyme (TACE) [254]. Both forms of RANKL bind to RANK expressed by DCs, leading to signal transduction via the NF-κB and c-Jun N-
terminal kinase pathways [255]. These signaling events result in the upregulation of the anti-apoptotic factor Bcl-xL in DCs, leading to the enhancement of DC survival [255, 256] and even protection from FasL-mediated DC apoptosis [257]. RANKL also has the ability to increase DC abundance in draining lymph nodes and enhance antigen-specific effector and memory CTL responses [256, 258]. Engagement of RANK on T cells also triggers signaling through the NF-κB pathway, again leading to enhanced cell survival. Secretion of osteoprotegerin (OPG) by DCs helps down-modulate these signaling events, as OPG acts as a decoy receptor.

The effects of T-cell-derived RANKL are not restricted to regulating DCs and T cells. RANKL expressed by T cells can also activate RANK on osteoclast precursors to promote their development into mature osteoclasts to mediate bone loss [259-262]. Bone metabolism is further regulated through the ligation of RANKL by osteoclast-expressing RANK and OPG produced by stromal cells and osteoblasts [263, 264]. The upregulation of RANKL or RANK expression has been associated with the onset of several bone diseases including post-menopausal osteoporosis [265] and myeloma bone disease [266]. In the vascular system, RANKL is expressed by endothelial cells, which also express RANK. In these cells, RANK engagement by RANKL leads to signaling via the PKB/Akt pathway to promote endothelial cell survival [267]. Blocking of this process due to OPG production is associated with cardiovascular disease [268]. Thus for all three systems, the relative levels of RANKL, OPG, and RANK must be well-balanced to achieve proper regulation of immunity as well as bone and vascular development.
1.10 Hypothesis and goal of thesis project

DCs have a natural ability to elicit potent antigen-specific immunity. The work in my thesis investigates how DCs can be manipulated and used to prevent against the development of cancer. Given the difficulties associated with generating or obtaining DCs from cancer patients to perform immunotherapy [269-271], we sought to develop low-dose DC-based approaches that may translate to feasible doses on a human scale. We hypothesized that if DCs could be made to efficiently express the self-antigen erbB2 through lentiviral transduction, then we could use relatively low doses of the engineered DCs as an immunotherapy vaccine to induce erbB2-specific immunity and offer anti-tumor effects in a mouse model of prostate cancer.

As described in Chapter 2, an experimental murine model was first developed to test the ability of genetically engineered DCs to protect mice from forming ectopic prostate tumors. In Chapter 3, I describe expanded research that investigated whether immunomodulatory molecules could be incorporated into the vaccination strategy to help strengthen anti-tumor immunity.
Chapter 2:
Tumor protection following vaccination with low doses of lentivirally transduced DCs expressing the self-antigen erbB2

A version of text presented in this chapter has been published in the following original article:


I performed all experiments for the work presented in this chapter, except for N Buxhoeveden and JE Foley, who ran the multiplex immunoassays, and C Ying, who prepared the lineage-specific antibodies. JS Walia assisted with the thymidine incorporation assay and R Head assisted with handling of the mice.
2.1 Abstract

Gene therapy strategies may accelerate the development of prophylactic immunotherapy against cancer. We synthesized a lentiviral vector encoding a kinase-deficient form of erbB2 (erbB2tr) to efficiently transduce murine DCs. Murine erbB2 models a clinically-relevant tumor-associated self-antigen; its human homolog (HER-2/neu) is overexpressed in breast cancer and 80% of metastatic prostate cancers. Following one infection, ~47% of DCs overexpressed erbB2tr. To determine whether low doses of transduced DCs could protect mice from tumors, we performed prime/boost vaccinations with 2x10³ or 2x10⁵ erbB2tr-transduced DCs. Six weeks post-vaccination, mice were simultaneously challenged with the aggressive RM-1 prostate cancer cell line and an erbB2tr-expressing variant (RM-1-erbB2tr). Whereas control mice developed both tumors, all recipients of 2x10⁵ erbB2tr-transduced DCs developed only wild-type RM-1 tumors. One-third of mice vaccinated with just 2x10³ erbB2tr-transduced DCs also demonstrated erbB2tr-specific tumor protection. Protection against RM-1-erbB2tr tumors was associated with sustained levels of anti-erbB2tr antibody production and also correlated with erbB2tr-specific Th1 cytokine secretion. Depletion of CD4⁺, CD8⁺, or NK cells prior to tumor challenge underscored their role in mediating tumor protection. We conclude that administration of DCs expressing a self-antigen through efficient lentivirus-based gene transfer activates cellular and humoral immunity, protecting host animals against specific tumor challenge.
2.2 Introduction

Cancer immunotherapy aims to overcome the inability of the immune system to efficiently protect against the establishment of tumors or reject established tumors. Dendritic cells (DCs) are potent antigen-presenting cells that have been widely used to initiate or enhance tumor-associated antigen (TAA)-specific immune responses in animal models and clinical settings. Numerous reports show that modifying DCs via TAA peptide- or tumor lysate-pulsing can induce anti-tumor immunity [148, 150-153]. Transfecting DCs with nucleic acid sequences encoding TAAs carries the advantage of inducing immunity towards a larger repertoire of naturally-derived MHC class I and II compatible peptides. Comparative studies have shown that by transfecting DCs with RNA, stronger anti-tumor effects can be achieved than by pulsing DCs with peptides [155, 157, 272, 273].

Viral transduction of DCs offers similar advantages as RNA transfection, with the added potential benefits of more efficient transgene delivery and stable transgene expression, depending on the choice of virus. Retroviruses, including onco-retroviruses and lentiviruses, can also be used to transduce DCs with one or more genes. Lentiviruses are well-suited for transducing DCs because they are capable of efficiently transducing slowly-dividing cells. Their integration into the host genome provides a way to generate long-term stable transgene expression. In a few recent reports, lentiviruses have been used to transduce murine and human DCs with TAAs [194, 195, 274-276]. Overall, murine studies that have used virally-transduced DCs for immunotherapy have demonstrated encouraging anti-tumor efficacy [277]. However, the majority of murine
studies published to date use human or rat homologs of TAAs instead of species-matched TAAs.

In the current study, we chose to use a naturally occurring splice variant of the murine erbB2 antigen (erbB2tr) as our target TAA in a mouse model of prostate cancer. In this setting, murine erbB2 represents a true self-antigen, which better mirrors the clinical setting. The human homolog of erbB2 (HER-2/neu) is overexpressed in 20% of primary prostate tumors and 80% of patients with metastatic prostate cancer, making this TAA a clinically relevant target for immunotherapy [213]. HER-2/neu is also overexpressed in other malignancies including breast, ovarian, and lung tumors [278-280]. A naturally occurring kinase-truncated variant of HER-2/neu has also been described in human tumor cells [281].

Our aim was to generate immunity towards the self-antigen erbB2 in mice using DCs that were genetically engineered to express erbB2tr. We hypothesized that vaccinating mice with lentivirally-transduced DCs could impart long-term erbB2-specific immunity and protection against subsequent challenge with erbB2-expressing tumors. In our model we used an aggressive RM-1 prostate tumor cell line that we have modified to express erbB2tr. We chose to focus on low-dose vaccination strategies, as limited availability of syngeneic DCs may be a factor in human vaccination protocols. This study provides a platform for the development of a low-dose DC immunotherapy strategy using LVs as gene transfer tools engineering expression of target TAAs.
2.3 Materials and Methods

2.3.1 Lentiviral vector (LV) construction and preparation of high titer stocks

The enhanced green fluorescent protein (enGFP)-containing LV pHREF-GW-SIN (LV/enGFP) was described previously [282]. To construct an erbB2tr-containing recombinant LV (LV/erb), the enGFP cDNA sequence was excised from pHREF-GW-SIN by \textit{EcoRI} (New England Biolabs, Beverly, MA) digestion and replaced with the cDNA sequence for erbB2tr (GI:28386210). This erbB2tr sequence was amplified by PCR from the Invitrogen pYX-Asc plasmid (IMAGE 5702040) with Taq polymerase (both Invitrogen, Burlington, ON), ligated into PCR-Script Amp(+) SK(+) (Stratagene, La Jolla, CA), and excised by \textit{EcoRI} digestion.

LV particles were generated by calcium-phosphate transfection of 293T cells (kindly provided by Dr. Michele Calos, Stanford University, CA) with the plasmids pCMVDR8.91, pMD.G [283], and either LV/enGFP or LV/erb. Viral supernatants were collected at 24 and 48 hrs post-transfection, filtered using a 0.45 um filter, and concentrated at 19,000xg for 2 hrs using an Optima L-100 XP Ultracentrifuge (Beckman Coulter Canada Inc., Mississauga, ON). Concentrated virus preparations were serially diluted and titered on 293T cells by FACS analysis as previously described [284].

2.3.2 Mice and cell lines

C57BL/6 (Jackson Laboratories, Bar Harbor, ME) and BALB/c (Charles River, Wilmington, MA) mice were bred and housed under specific pathogen-free conditions at the UHN Animal Resource Centre. RM-1 cells, a murine prostate cancer cell line syngeneic to the C57BL/6 strain, were kindly provided by Dr. Timothy Thomson.
The clonal RM-1-erbB2tr cell line was generated by transducing RM-1 cells to overexpress a kinase-truncated form of erbB2 (erbB2tr) and then isolating single cell clones. For these transductions, an onco-retroviral pUMFG-erbB2tr vector was constructed (Mossoba and Medin, unpublished) and transfected into the E86 packaging cell line to generate virus-producing E86 cells, as previously described [285]. In vitro growth characteristics of RM-1-erbB2tr vs. WT RM-1 cells were nearly identical. All animal experiments were performed under a protocol approved by the Animal Care Committee at the UHN.

2.3.3 Murine DC generation and transduction

DCs were generated according to Lutz et al. (1999) [286] with slight modifications. Briefly, bone marrow was flushed from femurs and tibiae of C57BL/6 mice using a 25G needle. Red blood cells (RBCs) were lysed using RBC Lysing Buffer (Sigma, St. Louis, MO). Remaining cells were plated in 10-cm petri dishes at a concentration of 2x10^5 cells/ml in a total volume of 10 ml/dish. DC media consisted of RPMI with 10% FBS (PAA Laboratories, Etobicoke, ON), 1% penicillin/streptomycin, 5x10^{-5} M 2-mercaptoethanol (both from Sigma), 40 ng/ml rmGM-CSF and 5 ng/ml rmIL-4 (both from Peprotech, Rocky Hill, NJ). Cells were infected on day 3 of culture with either LV/erb or LV/enGFP, or left uninfected. Half-volume media changes were done every other day starting on day 4. On day 7, 50 ng/ml of rmTNF-α (Peprotech) was added for 24-48 hrs of DC maturation.

2.3.4 Flow cytometric analysis of DCs, tumor cells, and splenocytes
DCs and tumor cells were stained with an anti-erbB2 primary antibody (Ab4, Oncogene Science, Tarzana, CA) and a PE-conjugated poly-adsorption goat anti-mouse Ig secondary antibody (BD Biosciences Canada, Mississauga, ON) and cell surface expression of erbB2tr was measured using a FACS Calibur (BD). For phenotypic analysis of DCs, the following BD antibodies were used with appropriate isotype controls: PE- or FITC- conjugated anti-CD11c (clone HL3), purified anti-CD80 (clone 1G10), and FITC-conjugated anti-CD86 (clone GL1), FITC-conjugated anti-I-A^b (clone AF6-120.1). For analysis of splenocytes, the following antibodies were used with appropriate isotype controls: anti-CD8-FITC, anti-TNF-α-PE, anti-CD4-PerCP, anti-IFN-γ-APC, anti-IL-4-PE, anti-IL-2-APC. Levels of regulatory T cells were measured by cell surface staining with anti-CD4-FITC, anti-CD25-APC, and intracellularly with anti-FoxP3-PE according to manufacturer’s instructions (BD Biosciences).

2.3.5 Allogeneic Mixed Lymphocyte Reaction

Transduced and control DCs were harvested on day 9 of culture and dosed with 30 cGy in a Gammacell 3000 Elan ^137^Co irradiator (Nordion International Inc., Ottawa, Canada). Freshly isolated splenocytes from C57BL/6 and BALB/c mice were B cell-depleted using goat anti-mouse Ig magnetic beads (Dynal, Brown Deer, WI). The remaining T cell-enriched population was plated in triplicate in a 96-well U-bottom plate (BD) at 2x10^5 cells per well in T cell media. Next, serially-diluted irradiated DCs (range of 0 to 0.6x10^5 cells/well) were added. Following 4 days of co-incubation, 1 uCi of [^3^H]methyl-thymidine was added to each well for 20 hrs. Thymidine incorporation was measured using a Beckman LS 1801 Liquid Scintillation Counter (Beckman).
2.3.6 Immunizations and tumor inoculations

C57BL/6 mice were injected i.p with $2 \times 10^5$ or $2 \times 10^3$ DCs transduced with LV/erbB2tr, LV/enGFP, or non-transduced controls in 200 ml of PBS. As a positive control, one group of 5 mice was injected with $2 \times 10^5$ erbB2tr-transduced DCs along with CFA (Sigma). These immunizations were repeated 2 weeks later. Six weeks after the second immunization, 6 of 10 mice in each cohort were challenged with bilateral tumors and the remaining mice were sacrificed for splenocyte cytokine secretion analyses (see below). For the tumor challenge, each mouse was injected s.c. with $2 \times 10^5$ RM-1-NT and RM-1-erbB2tr cells (in 200 ml of PBS) in the dorsal left and right flanks, respectively. Starting six days later, the length (l), width (w), and height (h) of each tumor was measured by caliper on a daily basis. Tumor volume was calculated by multiplying $l \times w \times h$.

2.3.7 Measurement of anti-erbB2tr antibody from mouse plasma

Approximately 200 ul of blood was collected weekly from the tail vein of each mouse into EDTA-coated tubes (Sarstedt, Montreal, Canada). Plasma was isolated by centrifugation at 18,000xg at 4°C for 20 min. Plasma anti-erbB2 measurements were performed using a flow cytometry-based ELISA method we developed that was based on that described by Piechocki et al. (2002) [287]. Briefly, RM-1-erbB2tr and wild-type (WT) RM-1 cells were first stained with diluted plasma samples or primary Ab4 antibody (above) for 1 hr on ice followed by 2 washes with PBS. Secondary staining with PE-conjugated poly-adsorption goat anti-mouse antibody was done for 1 hr on ice, again followed by 2 PBS washes. 7-AAD was added to each sample to exclude dead cells from
flow cytometric analysis. The mean fluorescence intensity (MFI) value in the FL2 channel was measured on a FACS Calibur for each sample. A standard curve was generated by plotting Ab4 antibody concentration versus the MFI values of the Ab4-stained RM-1-erbB2tr cells. This curve was used to convert MFI values of plasma anti-erbB2 levels from each mouse cohort into antibody concentration values. Each experiment was performed three times and the SD of the means was calculated.

2.3.8 Cytokine secretion assays

Spleens from immunized and naïve control C57BL/6 mice were dissociated into single-cell suspensions and treated with RBC lysis buffer. RBC-depleted splenocytes were cryopreserved in freezing medium (90% FCS, 10% DMSO), then thawed when needed using a method described by Maecker et al. (2005) [288]. Briefly, cryovials were warmed to 37°C in a waterbath and the contents diluted dropwise with an equal volume of warm media. Diluted cells were transferred to a 50 ml tube containing 8 ml of warm media per cryovial of added cells and centrifuged at 290xg for 7 min. Cell pellets were resuspended and brought to a final concentration of 5x10^6 cells/ml in RPMI medium containing 10% FCS, 1% penicillin/streptomycin, 1% minimal essential amino acids (Invitrogen), and 5x10^{-5} M 2-mercaptoethanol. Next, 200 ml of cell suspensions were transferred to each well of 96-well round-bottom plates (BD) and incubated at 37°C for 18 hrs. Splenocytes were then collected from each well, counted, and plated in 24-well plates at 3x10^6 cells per well in 1 ml. Approximately 2x10^5 freshly prepared DCs that were left non-transduced or that were transduced with LV/erb, LV/enGFP were added to each well. Co-cultures were incubated at 37°C for 24 hrs and supernatants were collected and stored at -20°C. IFN-γ, IL-2, TNF-α, IL-4, and IL-10 levels were measured from
thawed supernatant samples by Bio-Plex multiplex sandwich immunoassays according to
the manufacturer’s protocol (Bio-Rad Laboratories, Hercules, CA).

2.3.9 CD4⁺, CD8⁺, and NK cell depletion

Five mice per group were immunized two times (two weeks apart) with either
erbB2tr- or enGFP-transduced DCs at the dose of 2x10⁵ or 2x10³ cells per immunization.
Approximately 6 weeks post-immunization, mice were depleted of CD4⁺, CD8⁺, or NK
cells by antibody administrations and bilaterally challenged with ~0.1x10⁵ RM-1-NT and
RM-1-erbB2tr tumors (s.c.). For CD8⁺ T cell depletion, mice were injected i.p. with
200ug of anti-CD8 (clone 2.43) 3 days and 1 day before bilateral tumor inoculations.
After tumor injections, mice were given 200ug of antibody once per week. For CD4
depletion, mice were injected with 400ug of anti-CD4 (clone GKI.5) 3 days and 1 day
before bilateral tumor challenge. After tumor challenge, the dose of anti-CD4 was
reduced to 200ug per mouse and anti-CD4 was administered every 3-4 days. For NK cell
depletion, mice were given 200ug of anti-NK1.1 (clone PK136) 2 days and 1 day before
bilateral tumor inoculations. After tumor injections, mice were injected with 200ug of
anti-NK1.1 per mouse every 3-4 days. All i.p. injections were prepared in PBS in a final
volume of 400ul per mouse. Depletion of all three cell lineages from peripheral blood of
recipients was verified by flow cytometry in 7 animals per depletion group.

2.3.10 Statistical analysis

Student’s t tests were used to perform pairwise comparisons. Differences in means
were considered statistical significance at P< 0.05.
2.4 Results

2.4.1 DCs are efficiently transduced with lentivirus

A lentiviral transfer vector encoding erbB2tr, a truncated (kinase-deficient) form of the murine self-antigen erbB2 (LV/erb) was constructed (Fig. 2.1); an enGFP LV was previously described [284]. Titers of produced LVs usually approximated between $5 \times 10^6$ and $3.6 \times 10^8$ functional infectious viral particles per ml. To determine the transduction efficiency of LV/erb, we infected BM-derived murine DCs on day 3 of in vitro culture. In an initial pilot experiment, we determined that between 20% and 70% of a DC population was productively infected after one overnight incubation with LV/erb. Using these erbB2tr-transduced DCs, we performed an in vivo pilot study designed to test the efficacy of prime/boost vaccinations with LV-transduced DCs in mediating erbB2tr-specific anti-tumor immunity. After observing the potent antigen-specific effects of erbB2tr-expressing DCs in vivo, we initiated a second set of larger-scale experiments to corroborate our pilot study. Freshly-derived DCs were transduced and their expression levels of erbB2tr and enGFP were monitored over time. On culture day 7 for DCs used in the first immunization, we observed that 32.6% of transduced DCs were erbB2tr$^+$ and 47.9% were enGFP$^+$, respectively (Fig. 2.2a). By day 9, when DCs were injected, erbB2tr$^+$ and enGFP$^+$ cells had decreased to 16.7% and 22.3%, respectively. For the second immunization, we also checked expression levels at day 5 and found that 47.4% of DCs were erbB2tr$^+$ and 70.2% were enGFP$^+$ (Fig. 2.2b) at that time. The percentage of
Figure 2.1. Schematic diagram of lentiviral vector (LV) constructs used in these studies. LTR, long-terminal repeat; SD, splice donor site; SA, splice acceptor sites; $\psi$, RNA packaging signal; EF1$\alpha$, elongation factor 1 $\alpha$ promoter; SIN LTR, Self-inactivating LTR; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; Gag, viral structural proteins; RRE, Rev response element.

Figure 2.2. Transduction efficiency of LV/erb and LV/enGFP on DCs. BM cells were cultured in the presence of GM-CSF and IL-4. DC maturation was induced with TNF-$\alpha$ on culture day 8. Transductions were performed on culture day 3 using either erbB2tr- or enGFP-encoding lentiviruses. Transgene expression on DCs used for the first (A) and the second (B) scheduled immunizations was monitored by flow cytometry. Numbers indicate the percentage of transgene-positive cells for each filled histogram compared against appropriate isotype or non-stained controls (outlined histograms).
erbB2tr+ DCs decreased steadily to 33.7% on day 7 and 2.7% on day 9. The percentage of enGFP+ DCs was 79.7% and 73.7% on days 7 and 9, respectively.

2.4.2 Lentiviral transduction does not alter DC phenotype or allostimulatory capacity

To determine whether transducing DCs with our recombinant LVs at reasonable MOIs led to changes in phenotype, we first performed flow cytometry to compare the expression of typical surface molecules on mature transduced and control DCs. We assessed the percentage of cells expressing the myeloid marker CD11c, MHC II molecule I-A\textsuperscript{b}, along with co-stimulatory molecules CD80 and CD86. DCs used for the first scheduled vaccinations expressed similar levels of CD11c; 68.1% of the non-transduced DC cultures were CD11c+ compared to 75.7% and 70.2% for erbB2tr- and enGFP-transduced DC cultures, respectively (Fig. 2.3a). Further comparisons revealed that the percentage of CD11c+ I-A\textsuperscript{b+} DCs was nearly identical between non-transduced and LV/erb-transduced DCs (39.3% vs. 38.1%, respectively). A minor difference in the percentage of CD11c+CD80+ DCs was measured from non-transduced compared to erbB2tr-transduced cultures (39.5% vs. 45.5%, respectively). Similarly, 37.5% of non-transduced DCs and 43.8% of erbB2tr-transduced DCs were CD11c+CD86+ (Fig. 2.3a).

The DCs generated for the second vaccination exhibited similar trends (Fig. 2.3b). The percentage of CD11c+ cells in the control cultures was 91.1%, compared to 90.3% for erbB2tr-transduced DCs, and 79.8% for enGFP-transduced DCs. The percentages of
Figure 2.3. Transducing DCs with LV/erb and LV/enGFP does not affect DC phenotype or allostimulatory capacity. BM-derived DCs were transduced on culture day 3 and matured on culture day 8. Transduced and non-transduced (NT) immature (day 5 and/or 7) and mature (day 9) DCs prepared for the first (A) and the second (B) immunizations were stained with antibodies recognizing CD11c, I-A^b, CD80, and CD86 and analyzed by flow cytometry. Numbers indicate the percentage of positive cells in the indicated density plot quadrants. Mature day 9 DCs from C57BL/6 mice were also cocultured with [³H]thymidine-pulsed syngeneic (C57BL/6) or allogeneic (BALB/c) splenocytes to compare the allostimulatory capacity of non-transduced DCs to either (C) erbB2tr- or (D) enGFP-transduced DCs. Co-cultures were plated in triplicate and the mean ± SD values are shown.
CD11c⁺ I-Aᵇ⁺ were similar for control and erbB2tr-transduced DCs (60.0% vs. 67.0%, respectively). Comparing the percentages of DCs expressing costimulatory molecules, we found that 52.3% of non-transduced DCs and 59.0% of erbB2tr-transduced DCs were CD11c⁺ CD80⁺. A slight difference in the CD11c⁺ CD86⁺ percentage was also detected between the control and transduced DCs (62.3% vs. 65.0%, respectively).

To determine whether transduction with LV/enGFP or LV/erb affected DC function, we compared the ability of non-transduced and transduced DCs to induce a response in an allogeneic mixed lymphocyte reaction (MLR). We cultured H-2ᵇ⁻-expressing DCs (transduced and control) with either H-2ᵈ splenocytes from BALB/c mice or H-2ᵇ splenocytes from C57BL/6 mice and measured splenocyte proliferation by thymidine incorporation (Figs. 2.3c,d). No significant differences were found between the allostimulatory capacities of non-transduced DCs and either LV/enGFP- or LV/erb-transduced DCs.

2.4.3 Vaccination with low doses of LV-modified DCs generates antigen-specific tumor protection

Although many studies employing DC-vaccination strategies [277] evaluate tumor protection around 1-2 weeks post-vaccination, we chose to investigate the long-term benefits of a prime/boost vaccination strategy by challenging mice ectopically with RM-1 prostate tumors 6 weeks after the second vaccination. RM-1 cells grow aggressively in vivo, providing a stringent model for assessing tumor growth after vaccination; subcutaneously implanting just 10⁴ RM-1 cells will yield palpable tumors within 1 week and can compromise mouse survival by 10 days post-implantation [289]. The RM-1
tumor cell line lacks endogenous erbB2 expression according to our FACS analysis (Fig. 2.4a). Therefore, we generated a clonal cell population of erbB2-tr-expressing RM-1 cells (RM-1-erbB2tr) by onco-retroviral transduction followed by clonal isolation (Fig. 2.4a).

In our aforementioned pilot study, we first tested the efficacy of three immunizations using doses of 2x10⁵ and 2x10³ of erbB2-tr-transduced DCs to protect against subsequent challenge with erbB2-expressing tumors. We vaccinated mice three times with either erbB2-tr-transduced or non-transduced DCs. Two weeks after the third vaccination, we injected mice with non-transduced RM-1 cells (RM-1-NT) on one dorsal flank and RM-1-erbB2tr cells on the opposite dorsal flank in order to generate a bilateral tumor model in the same individual. Whereas many tumor protection studies utilizing virally-transduced DCs typically inject between 0.5x10⁶ and 1x10⁶ DCs per immunization [277], the focus of this pilot study was to investigate the possible benefits of using markedly lower doses of transduced DCs. In that initial pilot study, we observed that both erbB2 immunization regimens offered considerable protection against erbB2-tr-expressing RM-1 tumors specifically, compared to that obtained using non-transduced DCs.

To examine these low-dose outcomes in more detail, we next performed a larger study. We again used the dose of 2x10⁵ DCs for immunizing one cohort of mice, and the 100-fold lower dose of 2x10³ DCs for another. Importantly, however, we also reduced the number of vaccinations per animal to just two. We injected mice twice with either control, erbB2-tr-transduced, or enGFP-transduced DCs, two weeks apart. We next inoculated animals with the same tumor challenge described in our pilot study above. As a positive control, one group of mice was immunized twice with 2x10⁵ erbB2-tr-
Figure 2.4. Immunization with low doses of DC-erbB2tr generates potent anti-tumor responses. Cell lines RM-1-erbB2tr and non-transduced RM-1 (RM-1-NT) were stained with anti-erbB2 antibody and analyzed by flow cytometry (A). Mice were immunized twice with either $2 \times 10^5$ (B) or $2 \times 10^3$ (C) DCs and then inoculated with RM-1-erbB2tr and RM-1-NT cells on opposite flanks. Plotted values are the mean ± SEM of 5 or 6 mice per group, except for the positive control group (n=3). *P<0.05, vs DC-erbB2tr vaccination; **P<0.005, vs DC-erbB2tr vaccination.
transduced DCs mixed with the Complete Freund's Adjuvant (CFA) emulsion. Using this prime/boost strategy, none of the 6 mice that were immunized with $2 \times 10^5$ erbB2tr-transduced DCs showed RM-1-erbB2tr tumor growth, whereas RM-1-NT tumors grew rapidly in each animal (Fig. 2.4b). In contrast, the control naïve mice and mice immunized with $2 \times 10^5$ non-transduced or enGFP-transduced DCs developed both RM-1-NT and RM-1-erbB2tr tumors with an aggressive growth profile that necessitated euthanasia within 2 weeks. Strikingly, significant tumor protection from RM-1-erbB2tr tumors was also observed in mice that were immunized with the 100-fold lower dose of $2 \times 10^3$ erbB2-transduced DCs (Fig. 2.4c). In this group, 2 of 6 mice displayed complete tumor protection until the point of sacrifice at 2 weeks post-tumor challenge, and 2 of 6 mice showed reduced RM-1-erbB2tr growth compared to control cohorts.

### 2.4.4 Mice vaccinated with DC-erbB2tr show strong antigen-specific humoral immunity

To begin investigating potential mechanisms by which DC-erbB2tr immunizations could break tolerance against erbB2tr, we collected blood from each mouse on a weekly basis and measured the plasma levels of anti-erbB2 antibodies. As shown in Figure 2.5a, mice immunized with CFA + erbB2tr-transduced DCs (positive control) began producing modest levels of anti-erbB2tr antibodies. Following the second vaccination, these mice showed steadily increasing titers that peaked at about 45 days after the first DC injection. Relatively high antibody levels were detected up to 70 days post-prime vaccination when the mice were sacrificed. In our experimental groups, mice injected twice with $2 \times 10^5$ erbB2tr-transduced DCs showed a rapid increase in antibody titer after the boost.
vaccination. Indeed, within 10 days, the average anti-erbB2tr titer rose to over 5 times the average level in control mice. After this peak, a steady decline was measured, but specific anti-erbB2tr antibodies were still detectable at day 50, when mice were inoculated with tumors. In the non-vaccinated group of mice, challenge with RM-1-erbB2tr tumors caused a slight increase in antibody titers, revealing weak background immunogenicity of these tumors. Injecting the lower dose of $2 \times 10^3$ transduced DCs did not lead to detectable anti-erbB2tr antibodies at least within the sensitivity limits of this assay, despite the anti-tumor effects observed above (Fig. 2.5b).

2.4.5 Analysis of cytokine secretion from splenocytes

To further evaluate mechanisms, we harvested the spleens from naïve and immunized mice 6 weeks after the second vaccination. We re-stimulated splenocytes in vitro for 24 hrs with freshly prepared transduced or control DCs and analyzed culture supernatants for production of Th1 (IL-2, IFN-γ, and TNF-α) and Th2 (IL-4 and IL-10) cytokines. We found that recipients of $2 \times 10^5$ erbB2tr-transduced DCs produced greater levels of IL-2, IFN-γ, and TNF-α following in vitro re-stimulation with erbB2tr-transduced DCs relative to controls (Fig. 2.6). In contrast, this erbB2tr-specific cellular response was absent from the supernatants of all other mouse cohorts. To quantify the levels of antigen-specificity of the Th1 response, we calculated a ‘specificity index’ by normalizing cytokine concentration results from each group to the values obtained from re-stimulation with non-transduced DCs (Fig. 2.6). We found that splenocytes from mice vaccinated with $2 \times 10^5$ erbB2tr-transduced DCs produced approximately 370-fold more IL-2 after re-stimulation with erbB2tr-transduced DCs than with non-transduced DCs. An
Figure 2.5. DC-erbB2tr immunization dose of $2 \times 10^5$ cells causes a sustained erbB2-specific humoral response. Plasma samples from naïve and immunized mice were pooled according to cohort and quantitatively tested for the presence of anti-erbB2 antibodies by a flow cytometry-based ELISA. Graphs show a comparison between anti-erbB2tr levels from control groups and either (A) $2 \times 10^5$ or (B) $2 \times 10^3$ DC dose groups. Plotted values are the mean ± SEM of three independent assays. *$P<0.01$, vs vaccination with DC-NT, DC-enGFP, or no vaccination.
Figure 2.6. Immunization using DCs transduced with LV-erb offers antigen-specific Th1 immunity. Splenocytes from naïve and immunized mice were co-cultured in triplicate with LV-transduced or non-transduced DCs for 24 hours and supernatants were analyzed for IL-2, IFN-γ, and TNF-α by Bio-Plex multiplex sandwich immunoassays. Specificity index values were calculated by normalizing cytokine concentration values from each 24-hr re-stimulation condition to the values obtained from re-stimulation with non-transduced DCs.
even greater specificity index value was calculated for the relative increase in IFN-γ; over 1100-fold more IFN-γ was produced after erbB2tr-specific re-stimulation. We also found a large (635-fold) increase in TNF-α production following *in vitro* re-stimulation with erbB2tr-transduced DCs relative to controls. Levels of IL-4 and IL-10 in the co-culture supernatants were generally very low and specificity towards erbB2tr was not observed.

We confirmed the specificity of our cytokine responses towards erbB2tr antigen using fractionated splenocytes. Six weeks after immunizing mice with erbB2tr- or control-DCs, percentages of CD4+ or CD8+ T cells producing IL-2, IFN-γ, TNF-α, or IL-4 were measured by intracellular flow cytometry (*Fig. 2.7*). Splenocytes from mice that received 2x10⁵ erbB2tr-transduced DCs produced high levels of IL-2 and IFN-γ. In this immunization cohort, approximately 57% of CD8+ cells and 48% of CD4+ cells were IL-2+, compared to < 9% of cells from all negative control groups. In addition, 13.3% and 5.3% of splenocytes from this cohort were CD8+IFN-γ+ and CD4+IFN-γ+, respectively, compared to ≤ 2% of splenocytes from all negative control groups. With the exception of the positive control group, production of TNF-α and IL-4 were quite low in CD8+ and CD4+ splenocytes among all cohorts, with levels ranging from about 1% to 4% double positive cells. Despite not observing a clear pattern of TNF-α production specifically towards erbB2tr, our overall findings of Th1 cytokine upregulation from fractionated splenocytes supports our erbB2-specificity index data in Figure 2.6.

### 2.4.6 Role of CD4+, CD8+, and NK cells in mediating tumor protection

To evaluate the importance of CD4+, CD8+, and NK cells in determining tumor protection, we repeated our study using mice depleted of these cell lineages. First, mice
were immunized two times (two weeks apart) with either erbB2tr- or enGFP-transduced DCs at the dose of 2x10^5 or 2x10^3 cells per immunization. Approximately 6 weeks post-immunization, mice were depleted of CD4^+, CD8^+, or NK cells by antibody administrations and bilaterally challenged with RM-1-NT and RM-1-erbB2tr tumors. As expected, RM-1-NT tumor growth was not significantly affected by cell depletion (Fig. 2.8a). Whereas RM-1-erbB2tr tumor growth was inhibited in non-depleted recipients of 2x10^5 erbB2tr-transduced DCs, there was some enhanced growth following all three depletion conditions (Fig. 2.8b). Interestingly, mice that were immunized with 2x10^3 erbB2tr-transduced DCs relied on a protection mechanism that appeared to be highly dependent on CD4^+ T cells. In this cell depletion study, the use of enGFP-transduced DCs as control vaccines did not yield clear differences in tumor growth between depleted and non-depleted conditions, but overall rates of tumor growth in this iteration of the experiment were slower than expected.

To further understand the mechanism of tumor protection, we used ELISAs to analyze the production of IFN-γ and IL-4 in the supernatants of cultured splenocytes harvested from depleted and non-depleted immunized mice (Figs. 2.8c,d). Although cytokine levels were generally difficult to detect in all mice that were immunized with 2x10^3 erbB2tr-transduced DCs, we were able to measure cytokines produced by splenocytes from mice immunized with the higher dose of DCs. Production of IFN-γ was reduced by 44.5-fold in NK cell-depleted mice and 3.3-fold in CD4^+ T cell-depleted mice compared to non-depleted mice (Fig. 2.8c). CD8^+ T cell depletion also yielded a relatively small reduction in IFN-γ splenocyte secretion on average (1.2-fold), but was associated with a high level of variability (Fig. 2.8c). The depletion of each of the three
lineages also correlated with an increase in IL-4 production in mice that received $2 \times 10^5$ erbB2tr-transduced DCs (Fig. 2.8d). This finding reflects how Th1/Th2 cross-regulation can upregulate Th2 immunity when Th1 cytokine production is low.

To evaluate the role of a fourth cell type in mediating anti-tumor immunity, we studied the impact of gene-modified DC immunization on the behavior of regulatory T cells (Treg cells), defined as CD4$^+$$CD25^+$$FoxP3^+$ cells. We used flow cytometry to investigate whether there were changes in Treg levels in spleens of all immunized and naïve mice. However, significant differences in Treg levels between experimental groups were not detected.
**Figure 2.7.** Cytokine production analysis of fractionated splenocytes shows erbB2tr-specific Th1 immunity. Mice were immunized twice with non-transduced, enGFP-transduced, or erbB2tr-transduced DCs (two weeks apart) and then 6 weeks later, 4 of 10 mice were sacrificed from each group to check the cytokine levels from their splenocytes. Percentages of (A) CD8+ or (B) CD4+ T cells producing IFN-γ, IL-2, TNF-α, and IL-4 were measured by intracellular flow cytometry.
Figure 2.8. Involvement of CD4+, CD8+, and NK cells in mediating RM-1-erbB2tr tumor protection. Five mice per group were first vaccinated with 2x10^5 or 2x10^5 erbB2tr- or enGFP-transduced DCs two times, two weeks apart. Approximately 6 weeks after the last immunization, mice were depleted of CD4+, CD8+, or NK cells, or left non-depleted, and injected bilaterally with RM-1-NT and RM-1-erbB2tr tumors. (A) RM-1-NT or (B) RM-1-erbB2tr tumor volumes were calculated based on caliper measurements of length x width x height. Graphed values are the mean ± SEM of 4 or 5 mice per group. Splenocytes were prepared from each mouse at two weeks post-tumor challenge and cultured at a concentration of 2x10^6 cells/ml for 48 hrs. Analysis of (C) IFN-γ and (D) IL-4 production was done in triplicate by ELISA.
2.5 Discussion

This study is the first to demonstrate the use of lentivirally transduced DCs in an immuno-gene therapy cancer model targeting the self-antigen erbB2 in mice. Despite the growing number of DC-mediated immuno-gene therapy studies, complete protection against cancer has been mostly elusive [277]. One limitation likely relates to the level of antigen presentation by DCs. Thus, the method of engineering DCs to present TAAs may play an important role in the potency of immunotherapy schemas. Our use of recombinant retroviral vectors encoding full-length or large portions of TAAs may permit transduced DCs to present a broad repertoire of natural immunogenic tumor antigen peptides in stable MHC complexes. Indeed, we have previously demonstrated the utility of DCs transduced with onco-retroviruses to express xenogeneic human prostate TAAs in mediating anti-tumor immunity in mice [191]. In our current study, we employed an LV-based system and found that DCs could be even more efficiently transduced to express antigens, without compromising their phenotype or function. This finding is especially important given that the functional effects of lentiviral transduction of murine DCs with erbB2 have not been previously investigated and our LV/GFP transduction efficiency is higher than those reported by other groups [194, 290].

To address the possibility of oncogenic effects due to the use of integrating vectors, many groups have already extensively assessed the safety of lentiviral transduction [291-294]. In addition, although genetically modifying DCs to express murine erbB2 is original, data from other DC studies employing human or rat homologs have shown that this type of manipulation does not lead to oncogenic effects in DCs [186, 295-297]. To further decrease the possibility of affecting the target myeloid DC population by
overexpressing a heterologous signaling molecule, we employed a kinase-deficient version of erbB2 for our lentiviral construct. Although recent evidence suggests that some cases of acute myeloid leukemia may have a myeloid DC origin [298, 299], myeloid DCs may be less amenable to oncogenesis than plasma cytoid DCs, whose malignant counterparts are well documented [300-303]. In the future, gene therapy protocols may also incorporate additional safety mechanisms, such as the tmpk/AZT suicide gene therapy strategy that our laboratory developed [304].

While monitoring enGFP and erbB2tr levels in transduced DC cultures, we observed a decrease in transgene-positive DCs between culture day 5 and 9. The decrease was more pronounced in DC populations transduced with erbB2tr compared with enGFP. Some researchers have attributed this phenomenon to the elimination of genetically-modified DCs by T cells that contaminate DC cultures [305]. The possibility that this elimination occurred in our cultures cannot be ruled out, since our DC generation protocol allows for the persistence of low levels of lymphocyte contaminants [286]. However, the finding that enGFP levels can remain stable over time does not fully support this notion. Instead, it may simply point to the possibility that peptide formation from protein degradation within DCs occurs more readily for some antigens than for others. Our flow cytometry data, which shows decreased whole antigen expression, may be capturing the transition from whole protein expression to peptide formation as DCs develop into mature APCs.

It is important to consider the number of DCs being employed for immunization strategies, as an effort to minimize the DC dose could improve the feasibility of employing such strategies in clinical settings. Availability of effective patient DCs may
be a limiting factor [269-271]. We used only 2 immunizations, in order to decrease the number of DCs required, and were still able to induce specific immune-mediated anti-tumor responses. As stated previously, typical in vivo immunotherapy approaches involving virally transduced DCs administer between 0.5x10^6 to 1x10^6 DCs per immunization [277]. We found that a dose as low as 2x10^3 DCs offered partial protection against erbB2tr-expressing tumors, suggesting that the minimum effective DC dose falls in the range between our tested doses. In addition, these results were obtained in a relatively long-term setting, as mice were tumor challenged 6 weeks after the last immunization. As a direct result of testing these low-dose DC vaccinations, we also gain the ability to establish a workable threshold that can be used as a sensitive read-out in future studies to determine if other co-manipulations further enhance the immunotherapy effect.

In agreement with the recent results of Sakai et al. (2004) [186], we also observed a humoral response in vaccinated mice that correlated with tumor protection in the 2x10^5 DC-dose group. Mice vaccinated with 2x10^3 erbB2tr-transduced DCs had comparatively low antibody titers. In addition, long-term Th1 immunity was observed in the 2x10^5 DC-dose mice. It was not detected in the 2x10^3 DC dose cohort despite the finding that 2 of 6 of these mice were protected from developing RM-1-erbB2tr tumors and an additional 2 mice had markedly reduced tumor volumes compared to controls. This may reflect the detection limit of the assays, and may also point to the very sensitive nature of this system. It may be that specific anti-tumor immune responses can be induced with very subtle changes to the immune marker profiles. In our Th1 assays, the erbB2tr specificity of cytokine production in the 2x10^5 DC-erbB2tr cohort is clear. Note that we did detect
low levels of Th1 reactivity towards enGFP, likely because enGFP is a xeno-antigen and therefore inherently foreign to the mouse species. That we did not observe significant levels of Th2 cytokines at 6 weeks post-vaccination is consistent with the waning of anti-erbB2tr antibody levels over time.

Our DC vaccination strategy using erbB2-transduced cells was well tolerated in animals. Although the self-antigen erbB2 is naturally expressed at varying levels throughout the mouse body including the lungs, intestines, and brain [306], manifestations of auto-immune toxicity were not observed in the 10 weeks after initiation of the vaccination schedule. Other groups have also reported that anti-tumor immunity in mouse models can occur without damaging normal tissues, even when self-antigens are targeted [307-309]. Although the exact reasons for the lack of observed auto-immune disease in these studies are not clear, it is thought that it may relate to the higher than normal level of antigen-presentation by over-expressing tumor cells [308, 310, 311] or to differences in antigen recognition between tumor cells and normal cells [312].

In conclusion, our results show that vaccination using relatively low doses of DCs transduced with a recombinant LV to express a truncated form of HER-2/neu can safely and effectively protect mice against tumor development in an antigen-specific manner. This is the first implementation of this stable gene transfer method for this broadly important antigen in tumor biology. Tumor protection was associated with antigen-specific cellular and humoral immunity. The recombinant LV system we utilized served as an efficient gene transfer vehicle, which did not adversely affect DCs. Efforts to develop low dose DC-immunotherapy strategies will be of value in clinical situations where patient DCs may be scarce. Such cancer immunotherapy vaccines may be
particularly applicable before tumors are established and in early stage disease, and could reduce the need for more intensive treatments with systemic toxicity such as chemotherapy or radiation therapy.
Chapter 3:
Co-transduction with an IL-12, but not RANKL, lentivector enhances low-dose DC-erbB2tr immunotherapy against erbB2-expressing prostate tumors

The text presented in this chapter has been submitted for publication to Cancer Immunology, Immunotherapy.


I performed all experiments for the work presented in this chapter, except for J Symes and VI Rasaiah, who helped run some of the Western blots, JE Foley and J Mariotti, who ran the multiplex immunoassays, and JS Walia, who constructed one of the vectors. S Loisel-Meyer assisted with measuring tumors.
3.1 Abstract

Dendritic cell (DC)-based immunotherapy has the potential to protect against tumor formation and even reduce existing tumors at primary and metastatic sites. To aid in the translation of DC-based immunotherapies to the clinic, we are developing strategies in mouse models that may be achievable on the human scale. We have previously shown that vaccination with doses as low as $2 \times 10^3$ lentiviral vector (LV)-transduced DCs could offer some animals complete antigen-specific protection against aggressive prostate tumors. In this prior model, DCs were engineered to express a kinase-truncated version of erbB2 (erbB2tr), a self-antigen that is the murine orthologue of the human tumor-associated antigen (TAA) HER2/neu. Here, we sought to test whether combining erbB2tr expression with that of immunomodulatory proteins IL-12 or RANKL, could further augment anti-tumor immunity to achieve stronger tumor protection. We hypothesized that IL-12 expression on DCs would promote erbB2tr-specific Th1 immunity and that RANKL would also enhance T cell stimulation through its known effect on DC life-span. First, we demonstrated the feasibility of co-transducing functional DCs with two LVs simultaneously, one LV encoding either enGFP or erbB2tr cDNA sequences and the other encoding IL-12 or RANKL cDNA sequences. In our mouse model of prostate cancer, we show that immunizing mice twice (two weeks apart) with low doses of DCs expressing erbB2tr and IL-12 offered long-term tumor protection. This anti-tumor effect correlated with an upregulation in Th1 immunity, as defined by IL-2, IFN-$\gamma$, and TNF-$\alpha$ secretion from stimulated splenocytes. Vaccination with DCs expressing higher levels of IL-12 alone also yielded significant tumor protection, but was not associated with erbB2tr-specific immunity. By comparison, co-expressing RANKL with erbB2tr in DCs
offered neither tumor protection nor anti-erbB2 immunity. This study reveals that IL-12 is a better modulator than RANKL in the context of low-dose DC-based immunotherapy strategies that combine elements of antigen specificity with factors generating enhancement of DC-mediated immunity.
3.2 Introduction

In recent years, dendritic cell (DC)-based immunotherapy has been shown to possess potential for the treatment of cancer [277]. DCs naturally play a critical role in controlling the balance between immunity and tolerance [103]. They can also be modified to enhance their ability to stimulate selective immune responses against specific tumor-associated antigens (TAAs). This often requires that they be manipulated in some fashion to effectively present TAA-derived peptides. While many methods exist to accomplish this task, lentiviral vector (LV) transduction allows for efficient, stable, and strong transgene expression [313].

No matter which method is employed to manipulate the antigen-presenting cells, the use of TAA-expressing DCs for prevention or regression of tumors in mouse models have generally followed a protocol of administration of high DC doses - on the order of $10^6$ cells per immunization [277]. Considering the average weight of a human and mouse to be 75 kg and 30 g, respectively, a DC dose of $10^6$ manipulated cells in a mouse on a per weight basis would be equivalent to $\sim 2.5 \times 10^9$ human DCs. Given that it is more difficult to collect DCs or generate them from DC precursors (monocytes and CD34$^+$ cells) from cancer patients than from healthy donors [314, 315] and that most clinical protocols use $\sim 10^7$ DCs per immunization [316], then mouse studies using high DC doses simply do not model treatments that can be readily applied to a clinical setting. In fact, this marked dose discrepancy may possibly explain why differential outcomes are seen between mouse studies and human clinical protocols. Indeed, the appropriate DC dose for inducing TAA-specific immunity remains unknown - especially as regards the clinical context. The optimal dose is likely variable and dependent on many factors including the
health of the donor, the inherent immunogenicity of the target TAA(s), DC purity and stage of maturity, and the ability of *ex vivo*-generated DCs modified by various methods to home to the appropriate locations within the lymphatic system.

Our laboratory has been working towards the development of a DC-based immunotherapy strategy for the prevention and treatment of prostate cancer [191, 317]. Recently we have chosen to focus on self-antigens and have targeted the TAA, HER2/neu (erbB2), as it is overexpressed in both primary and metastatic prostate tumors along with breast, lung, and ovarian cancers [206, 213, 318]. We have previously shown that a potent and specific vaccine can be made following LV-transduction of bone marrow (BM)-derived DCs engineered to express a truncated (kinase-deficient) version of erbB2 (erbB2tr) [317]. In that study, we used a bilateral murine tumor model wherein mice were injected in one flank with erbB2tr-expressing murine prostate tumor cells (RM-1-erbB2tr) and the non-erbB2tr expressing parental line (RM-1-NT) in the other. We showed that immunizing mice two times, two weeks apart, with $2\times10^5$ DCs that were LV-transduced to express erbB2tr completely protected mice from tumor challenge at six weeks post-immunization. This protection was associated with both humoral and cellular immunity. Furthermore, reducing the vaccination dose to only $2\times10^3$ cells per vaccination yielded erbB2tr-specific tumor protection in some animals. Based on these findings and this relative threshold that we uncovered, we sought here to further develop a low-dose DC-based immunotherapy strategy against prostate cancer.

To enhance the role of DCs in immunotherapy, strategies employing function-enhancing cytokines [319, 320] or other proteins that help prevent apoptosis [321], exposure to heat-shock proteins [322], or knock-down of SOCS1 expression [198] have
all been attempted. In particular, the use of Interleukin-12 (IL-12) has been well documented [251, 323]. As a pleiotropic cytokine that is expressed by many cells including DCs, IL-12 has been shown to augment several immune response pathways, including CTL- and NK-based immunity [324]. IL-12 signals through the JAK-STAT pathway to stimulate the release of Interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) from T lymphocytes and NK cells, thus enhancing their functional responses [325]. In addition, IL-12 has offered some benefit to cancer patients when used alone [249] and as an adjuvant [326]. Receptor Activator of NF-kB Ligand (RANKL) is another protein that has been implicated in DC stimulation, but its effects on BM-derived DCs towards the goal of augmenting anti-tumor immunity have not been extensively studied. It is known, however, that RANKL can upregulate expression of the anti-apoptotic molecule Bcl-xL in DCs to extend their life-span in vitro and their persistence within draining lymph nodes in vivo [327]. In addition, treatment of DCs with RANKL has been shown to improve DC function by increasing expression of inflammatory cytokines such as IL-1, IL-6, IL-12, and IL-15 [327-329]. RANKL is normally expressed on T lymphocytes and binds its cognate receptor, RANK, on DCs [330]. However, adding RANKL to DCs may allow DCs within a population to activate each other and also allows shed RANKL to activate transduced DCs in an autocrine fashion.

For the current study, we chose to co-express erbB2tr with either IL-12 or RANKL in DCs in an effort to develop a more effective low-dose DC vaccine. We hypothesized that we could combine our previously-demonstrated specificity towards erbB2tr with the immune-enhancing effects of IL-12 by co-transducing DCs with LVs carrying these two cDNAs. We successfully designed a co-transduction protocol to achieve functional
expression of erbB2 tr along with either IL-12 or RANKL in DCs, and then assayed the independent effects of LV co-transduction on DC purity, the state of DC maturation, and APC function. We next employed the co-transduced DCs in a murine prostate cancer immunotherapy model using low doses of DCs for immunizations. It was found that the DCs co-transduced with LV/erb and LV/IL-12 but not with LV/RANKL provides additional tumor protection effects compared to LV/erb-transduced DCs alone. Further, our results show this effect is associated primarily with Th1 immunity towards erbB2.

This study is an important step in refining the methods used to modulate DCs towards achieving tumor protection, and furthers our aim to facilitate translation of dose-effective DC immunotherapy into the clinic.
3.3 Materials and Methods

3.3.1 Lentiviral vector (LV) construction and preparation of high titer lentivirus

Construction of the following transfer vectors was described previously: pHR'EF-erbB2tr-W-SIN (LV/erb) [317], pHR'EF-IL-12-W-SIN (LV/IL-12) [331], and pHR'EF-GW-SIN (LV/enGFP) [282]. To construct pHR'EF-RANKL-W-SIN (LV/RL), we excised the enGFP cDNA sequence from LV/enGFP using restriction enzyme EcoRI (New England Biolabs, Beverly, MA) and inserted the cDNA encoding RANKL (kindly provided by Dr. A.K. Stewart, Mayo Clinic Arizona).

We generated LV particles by polyethyleneimine (PEI) transfection of 293T cells (kindly provided by Dr. Michele Calos, Stanford University, CA) as described previously [304] using the plasmids pCMVDR8.91, pMD.G [282], and our transfer vectors. Viral supernatants were harvested using a 0.45 µm filter at 48 hrs and 72 hrs post-transfection. Vector was then concentrated at 19,000xg for 2 hrs using an Optima L-100 XP Ultracentrifuge (Beckman Coulter Canada Inc., Mississauga, ON) and titered on 293T cells as previously described [282].

3.3.2 Mice and tumor cell lines

Male C57BL/6 mice (age 7-10 weeks) (Jackson Laboratories, Bar Harbor, ME) were housed under specific pathogen-free conditions at the UHN Animal Resource Centre. The RM-1 cell line is a murine prostate cancer cell line that is derived from the C57BL/6 strain. We generated the clonal RM-1-erbB2tr cell line, which expresses a kinase-truncated form of erbB2 (erbB2tr) previously described [317]. All mouse
experiments were performed following a protocol approved by the UHN Animal Care Committee.

### 3.3.3 Murine DC generation and transduction

DCs were generated according to Lutz et al. (1999) [286] with some modifications as already described [317]. Cells were either left uninfected or were infected on culture day 3 with one LV or with a combination of 2 LVs. Media was changed after 4 hours and then half-volume media changes were done every other day. To induce DC maturation on culture day 7, 50 ng/ml of rmTNF-α (Peprotech) was added for 24-48 hrs.

### 3.3.4 Western blot analyses

Transduced and non-transduced DCs were lysed 24 hours after addition of TNF-α in NP40 buffer (50mM Tris pH 7.4, 1mM EDTA, 150mM NaCl, 1% NP-40) containing protease inhibitors (Sigma, Oakville). Protein quantification was done using the DC Protein Assay (Bio-Rad, Hercules, CA). For Stat4 (89 kDa) and Stat4-P (89 kDa) analyses, 20 ug of protein were run on a 7% polyacrylamide gel alongside the All Blue (broad range) ladder (Bio-Rad). Following semi-dry transfer, membranes were blocked in 5% milk and stained with either purified rabbit anti-mouse Stat4 (C-20; 1:1000) (Santa-Cruz Biotechnology, Inc, Santa Cruz, CA), purified rabbit anti-mouse Stat4-P (1:500; Zymed Laboratories, South San Francisco, CA), or anti-β-actin (1:2000; Chemicon International, Temecula, CA). For RANKL (35 kDa) and Bcl-xL (27 kDa) detection, 15 ug of protein were run on a 10% gel. Following semi-dry transfer, membranes were blocked in 5% milk and probed with either biotin-conjugated rat anti-mouse TRANCE (RANKL) (1:1000; eBioscience), purified mouse anti-mouse Bcl-xL (2H12) (1:200;
eBioscience), or anti-β-actin. Membranes were probed with the appropriate HRP-linked secondary antibody and bands visualized using Western Lightning Chemiluminescence Kit (Perkin Elmer).

### 3.3.5 Analysis of transgene and cell surface marker expression on DCs

To assess erbB2tr levels on DCs using a FACS Calibur (BD Biosciences Canada, Mississauga, ON), cells were stained on culture day 5 as already described [317]. RANKL expression was assessed on day 5 using an anti-TRANCE primary antibody (IKK2/5, eBioscience, San Diego, CA) and an APC-conjugated anti-rat secondary antibody (BD). To measure IL-12 production from DCs, day 5 cell culture supernatants were harvested and analyzed using a murine IL-12 ELISA kit (BD). To analyze the cell surface profile of DCs, we used the following antibodies along with proper isotype controls: PE- or FITC- conjugated anti-CD11c (clone HL3), purified anti-CD80 (clone 1G10), and FITC-conjugated anti-CD86 (clone GL1), FITC-conjugated anti-I-Ab (clone AF6-120.1) (all from BD). For all FACS analyses, dead cells were gated out using 7-aminoactinomycin D (Sigma).

### 3.3.6 Immunizations and tumor inoculations

For tumor protection experiments, C57BL/6 mice were injected i.p with 2x10³ transduced or control DCs (in 200 ul of PBS). One group of mice was injected with 2x10³ erbB2tr-transduced DCs along with CFA (Sigma). Two weeks later, mice were immunized a second time. Five of ten mice per group were sacrificed for subsequent splenocyte cytokine secretion analyses (see below). The remaining mice were inoculated s.c. in one flank with 2x10⁵ RM-1-NT and RM-1-erbB2tr cells (in 200 ml of PBS) in
opposite flanks. Tumors were measured using a caliper and volumes were calculated by multiplying tumor length, width, and height. For the tumor regression study, mice (5 per group) were first bilaterally inoculated with $2 \times 10^5$ RM-1-NT and RM-1-erbB2tr cells. Three days later, mice were immunized i.p with $4 \times 10^5$ non-transduced or transduced DCs. Another three days later, a second round of immunizations was performed. Mice were sacrificed approximately two weeks post-tumor inoculation.

### 3.3.7 Measurement of anti-erbB2tr antibody from mouse plasma

Anti-erbB2tr antibody levels were determined using the plasma of each mouse as previously described [317]. Briefly, about 200 ul of blood were collected from each mouse using EDTA-coated tubes (Sarstedt, Montreal, Canada) and then tubes were centrifuged (18,000xg at 4°C for 20 min) to isolate the plasma. We used a modified flow cytometry-based assay [287] to determine the plasma anti-erbB2 levels relative to naïve control mice.

### 3.3.8 Cytokine secretion assays

RBC-depleted splenocytes were prepared from the spleens of mice at two-weeks post immunization or approximately two weeks post-tumor challenge. Cells were counted, diluted, and plated in 24-well plates at a final concentration of $1 \times 10^6$ splenocytes per ml for IL-12 experiments or $2 \times 10^6$ splenocytes per ml for RANKL experiments. Cells were cultured in a total volume of 1 ml of RPMI medium containing 10% FBS, 1% penicillin/streptomycin, 1% minimal essential amino acids (Invitrogen), $5 \times 10^{-5}$ M 2-mercaptoethanol, and 60U/ml of rhIL-2. Splenocytes were co-cultured with 70 Gy-irradiated RM-1-NT or RM-1-erbB2tr cells at a 10:1 splenocyte:tumor cell ratio.
As controls, splenocytes were also cultured alone or with 100ng/ml of PMA/ionomycin. Supernatants were harvested 36 hours later and kept at -20°C until thawed and analyzed for IFN-γ, IL-2, TNF-α, IL-4, and IL-10 levels by Bio-Plex multiplex sandwich immunoassays according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA).

3.3.9 Analysis of tumor-infiltrating lymphocytes

RM-1-NT and RM-1-erbB2tr tumors were harvested from each mouse in our tumor protection studies. Tumors were snap frozen in liquid nitrogen using Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, CA) and stored at -20°C. Frozen sections approximately 5 um in thickness were mounted on slides and stored at -80°C until use. Two sections per tumor were fixed in a 1:1 solution of methanol and acetone (both from Sigma). Slides were put at -80°C for 30 minutes and washed in PBS. Slides were blocked, stained first with either goat anti-mouse CD4 (L3T4) (clone GK1.5), goat anti-mouse CD8a (Ly-2) (clone 53-6.7) primary antibodies (both from eBioscience), or appropriate isotype controls, and then stained with AlexaFluor488 goat anti-rat IgG (H+L) secondary antibody (Invitrogen). Sections were also co-stained with DAPI using a final concentration of 1 ug/ml. Up to 10 images of each stained tumor section were captured on a Zeiss Axioskop 2 microscope using Axiovision 3.1 and images were analyzed using ImageJ version 1.37v.

3.3.10 Statistical analysis

Student’s t tests were used to perform pairwise comparisons. Differences in means were considered statistical significant at P< 0.05 or better as otherwise indicated.
3.4 Results

3.4.1 DC populations can be simultaneously transduced with two independent LVs to efficiently co-express a TAA and an immunomodulatory gene

To obtain DC populations that efficiently co-express a TAA and either IL-12 or RANKL as an immunomodulatory gene, we sought to develop a lentiviral transduction method that utilized two separate LVs. Instead of creating a single bicistronic LV as we have done before [284, 304, 332, 333], we rationalized that separate transductions would facilitate the testing of DCs that are genetically-engineered to express different combinations of TAAs and immunomodulatory genes. The optimized transduction protocol involved simultaneously exposing cultured DCs to pre-mixed concentrated LVs at functional MOI values typically less than 2 for a relatively short duration of 5 hours at 37°C.

We used combinations of four monocistronic LV constructs to demonstrate the feasibility and outcomes of our co-transduction protocol. Previously constructed pHR’EF-erbB2tr-W-SIN [317], which encodes a kinase-deficient version of erbB2, and the enhanced green fluorescent protein (enGFP)-encoding pHR’EF-enGFP-W-SIN [282] were used to make LV/erb and LV/enGFP viruses, respectively. In addition, we employed pHR’EF-IL-12-W-SIN (LV/IL-12) [331] and newly constructed pHR’EF-RL-W-SIN (LV/RL), which carry the cDNA sequences for murine IL-12 and murine RANKL, respectively. After producing concentrated virus from all four constructs, we performed a single tandem transduction of day 3 BM-derived DCs with either LV/erb or LV/enGFP alone or in combination with LV/IL-12 or LV/RL. As shown in Figure 3.1a,
Figure 3.1. Efficiency of transducing DCs with LVs to express enGFP or erbB2tr alone, or in combination with IL-12 or RANKL. BM cells were cultured in the presence of GM-CSF and IL-4. DC maturation was induced with TNF-α on culture day 8. Shown are transgene expression levels in DCs on culture day 5 following transductions with either LV/enGFP or LV/erb alone, or in combination with either LV/IL-12 (a,b) or LV/RL (c). Flow cytometry plots are representative of two independent experiments. Plotted IL-12 concentrations are the mean ± SEM of n=3. *P<0.005, vs DC-NT vaccination.
after transducing DC populations with LV/enGFP, 53.2% of the population was enGFP-positive in this representative analysis from one of two independent experiments. Performing co-transductions with LV/enGFP and LV/IL-12 yielded a population of cells that were 50.3% enGFP-positive. In the case of DC transductions with LV/erb, both single transductions and dual co-transductions of DCs generated a population of cells that expressed similar levels of erbB2tr - around 29.4% and 29.7%, respectively. Although the addition of LV/IL-12 did not appear to affect the levels of enGFP or erbB2tr expressed by DC populations compared to single transductions (Fig. 3.1a), there were differences in the levels of IL-12 produced among these different conditions (Fig. 3.1b). Less than 100 pg/ml of IL-12 was measured in control culture supernatants of non-transduced DCs or DCs that were transduced only with LV/erb or LV/enGFP alone. The addition of LV/IL-12 to the co-transduction protocol increased the levels of IL-12 to ~800 pg/ml. By contrast, DC populations transduced only with LV/IL-12 secreted far higher amounts of IL-12, reaching a concentration of >2000 pg/ml in culture supernatants.

When DC populations were transduced to express either enGFP or erbB2tr alone or in combination with RANKL, transgene levels were measured by flow cytometry in two independent experiments. As shown in Figure 3.1c, transducing DCs with LV/enGFP alone generated a population of cells that were measured to be 81.3% enGFP-positive. Co-transducing DCs with LV/enGFP and LV/RL yielded a population of cells that was 23.9% enGFP-positive and 47.8% enGFP- and RANKL-positive, respectively. When LV/erb was used to transduce DCs, 52.9% of the population expressed erbB2tr. Adding LV/RL to the transduction procedure kept the levels of erbB2tr-expressing cells to 46.3%. DCs that were genetically engineered to express RANKL alone were 43.5%
transgene-positive, compared to 37.3% for those that were engineered to co-express erbB2tr and RANKL.

Having established the feasibility of co-transducing DC populations with LVs encoding a TAA and an immunomodulatory protein, we aimed to verify that the IL-12 and RANKL proteins being expressed from DCs were functional. One of the signaling effects that IL-12 expression elicits in DCs is the phosphorylation of transcription factor Stat4 [334, 335]. We performed Western blot analyses to confirm that lysates of LV/IL-12-transduced DCs contained higher levels of Stat4-P than lysates of DCs transduced with LV/enGFP or LV/erb alone or left non-transduced (Fig. 3.2a). We also performed Western blot analyses to determine the relative levels of the anti-apoptotic protein Bcl-xL, which has been reported to become upregulated in cultured DCs in the presence of exogenous RANKL [328]. As expected, Bcl-xL levels were elevated in the lysates of all DC groups that were transduced with LV/RL compared to those transduced with LV/enGFP or LV/erb alone or left non-transduced (Fig. 3.2b).

3.4.2 DCs expressing erbB2tr and IL-12 or RANKL exhibit varying levels of DC markers

Here we aimed to monitor the potential differences in DC marker expression due to the influence of IL-12 and RANKL expression. For this, we measured levels of CD11c, co-stimulatory molecules CD80 and CD86, and the MHC class II molecule I-A^b_. Although we expected IL-12 to mediate enhancement of DC marker expression [336, 337], we actually observed the opposite effect according to flow cytometric analyses. As shown in Figure 3.3a, decreases in the percentages of cells expressing these molecules ranged from about 10% to 30%. By contrast, transducing DCs with LV/RL
Figure 3.2. Effect of IL-12 and RANKL on DC signaling. Lysates were prepared from non-transduced and transduced DCs on culture day 8 (at 24 hours post-TNF-α addition). (a) Lysates from DCs transduced with LV/IL-12 were evaluated for Stat4 phosphorylation. (b) Lysates from DCs transduced with LV/RL were evaluated for Bcl-xL upregulation. Optical density (OD) values shown were normalized to β-actin OD values.
Figure 3.3. Effect of transducing DCs with (a) LV/IL-12 or (b) LV/RL on the cell surface expression of DC markers. BM-derived DCs were transduced on culture day 3 and matured on culture day 8. Transduced and non-transduced (NT) mature DCs were stained with antibodies against CD11c, I-A$^b$, CD80, and CD86 and analyzed by flow cytometry.
either led to an enhancement in DC purity and maturation state or offered no clear modulation (Fig. 3.3b).

**3.4.3 Vaccination using DC-erbB2tr/IL-12, but not DC-erbB2tr/RANKL, elicits erbB2tr-specific anti-tumor protection**

We have previously demonstrated that immunizing mice with $2 \times 10^5$ DCs transduced with LV/erb could completely protect against the development of the aggressive RM-1-erbB2tr prostate tumors in an antigen-specific manner [317]. In that study, we also tested the 100-fold lower dose of $2 \times 10^3$ DC-erbB2tr cells and discovered that complete tumor protection could still be achieved in some animals (in 2 of 6 mice with 2 other animals demonstrating reduced tumor volumes). To evaluate whether this low dose could be made more effective, we tested the immunomodulatory effects of IL-12 and RANKL when co-expressed with erbB2tr on DCs.

Mice were immunized twice, two weeks apart with $2 \times 10^3$ DCs, and then six weeks later inoculated bilaterally with RM-1-erbB2tr cells in one hind flank and with wild-type RM-1-NT cells in the opposite flank. We first compared the vaccination efficacies of DCs transduced with enGFP or erbB2tr with and without IL-12 (Fig. 3.4a). The best inhibition of RM-1-erbB2tr tumor development was achieved with DC-erbB2tr/IL-12 vaccinations. In this cohort, all 5 mice displayed robust protection specifically against RM-1-erbB2tr, but not RM-1-NT tumors. Mice that were administered DC-IL-12 also exhibited a moderate degree of RM-1-erbB2tr tumor protection over the 13 days of observation (Fig. 3.4a), but vaccinations with DC-enGFP/IL-12 offered no significant erbB2tr-specific tumor protection. As expected, all
Figure 3.4. Immunizing mice with low doses of DC-erbB2tr/IL-12 leads to robust tumor protection. Following two DC immunizations of $2 \times 10^3$ cells given two weeks apart, mice were inoculated with RM-1-erbB2tr and RM-1-NT. Tumor volumes were calculated (LxWxH) from caliper measurements for each RM-1-erbB2tr (a,c) and RM-1-NT (b,d) tumor to assess the effects of IL-12 and RANKL on tumor protection, respectively. Plotted values are the mean ± SEM of 4 or 5 mice per group. *P<0.05, vs ‘No DCs’ group; **P<0.01, vs ‘No DCs’ group.
vaccinated and naïve mice developed RM-1-NT tumors in an uninhibited manner (Fig. 3.4b), showing that the immune response generated is specific towards RM-1-erbB2tr tumors.

In separate experiments, we next assessed the immunotherapeutic impact of co-expressing erbB2tr and RANKL in DCs. Although mice that were immunized with DC-RL or DC-erbB2tr/RL seemed to exhibit the most resistance to RM-1-erbB2tr tumor growth at 9 days, this trend was not sustained to the time when mice had to be sacrificed (Fig. 3.4c). By that point, none of the experimental test groups displayed any significant tumor protection. As a control, we had included a cohort of mice that were immunized with a combination of Complete Freund’s Adjuvant (CFA) and 2x10^3 DC-erbB2tr cells. We observed that this vaccination group yielded the most robust protection from RM-1-erbB2tr growth and was sustainable over the 11 days of observation, thus it is unlikely that there were problems with the DC cultures themselves that led to the lack of protection in the mice vaccinated with DC-RL or DC-erbB2tr/RL. As before, mice in all vaccination cohorts allowed RM-1-NT tumors to grow without restraint (Fig. 3.4d).

3.4.4 Tumor protection was not associated with increased anti-erbB2tr antibody response

To determine whether anti-tumor immunity correlated with an erbB2tr-specific humoral response, we evaluated the levels of anti-erbB2tr antibody in the plasma of all vaccinated mice relative to the baseline levels in mice that were not injected with DCs. As shown in Figure 3.5a, vaccinations using DC-NT and DC-enGFP did not produce significant levels of anti-erbB2tr antibodies, as one might expect, and neither did immunizing with the low dose of DC-erbB2tr cells with or without the aid of CFA. In accordance with the well-
Figure 3.5. Assessment of humoral immune response against erbB2 in vaccinated and non-vaccinated mice. The presence of anti-erbB2 antibodies in mouse plasma were determined using a flow cytometry-based ELISA. Levels of antibodies in IL-12 (a) or RANKL (b) experiments were normalized to ‘No DC’ treatment cohort. Plotted values are the mean ± SEM of 4 or 5 mice per group.
established role of IL-12 in skewing immune responses towards a Th1 type [338], we did not measure an appreciable relative level of anti-erbB2tr antibodies in any of the IL-12-immunized cohorts. Although the vaccinations themselves did not seem to lead to humoral immunity, challenging the vaccinated mice with RM-1-erbB2tr tumors lead to a slight rise in titers in most cohorts, particularly in the DC-erbB2tr cohort (Fig. 3.5a).

Similarly, in our assessment of anti-erbB2tr antibody titers that could result from RANKL immunomodulation, we did not find a significant change in antibody titers throughout the time course of the study (Fig. 3.5b). Following the second vaccination, there seemed to be small titer increases in the majority of cohorts, but these were not found to be statistically significant.

**3.4.5 DC-erbB2/IL-12 vaccinated mice show strong erbB2tr-specific Th1 immunity following tumor challenge**

Next we were interested in evaluating which cytokines were upregulated as a result of introducing IL-12 into our low-dose prime/boost immunization strategy. We examined the Th1 (IL-2, IFN-γ and TNF-α) and Th2 (IL-4 and IL-10) cytokine concentrations secreted from splenocytes of sacrificed mice, which were cultured with either RM-1-erbB2tr cells or RM-1-NT cells for 36 hours. First, 5 of 10 mice from each cohort were sacrificed two-weeks post DC vaccination and we calculated the average relative increases in cytokine concentrations upon stimulation to assess the levels of erbB2tr-specific immunity. Here we found that neither Th1 nor Th2 cytokines were appreciably upregulated (less than 2-fold increases) (Fig. 3.6a,b). However, after measuring cytokine levels in all remaining mice two weeks post-tumor challenge, we found that all three Th1
Figure 3.6. Vaccinations with DCs transduced with LV/erb and LV/IL-12 offers erbB2tr-specific Th1 immunity. Splenocytes obtained from naïve and immunized mice (n=4 or 5) were co-cultured with either RM-1-erbB2tr or RM-1-NT cells for 36 hours. Using Bio-Plex multiplex sandwich immunoassays, levels of IL-2, IFN-γ, and TNF-α were assessed two weeks post-immunizations (a) or post-tumor inoculations (b). Similarly, levels of IL-4 and IL-10 were determined two weeks post-immunizations (c) or post-tumor inoculations (d). Cytokine concentrations from RM-1-erbB2tr co-cultures were normalized to those from RM-1-NT co-cultures. Plotted values are the mean ± SEM of 4 or 5 mice per group. *P<0.10, vs. DC-NT treatment group.
cytokines were strongly upregulated (approximately 10- to 30-fold) in the supernatant of stimulated splenocytes in mice that were immunized with DC-erbB2tr/IL-12, but not in any other vaccination group (Fig. 3.6c). Interestingly, even DC-IL-12 mice that displayed tumor protection did not show this Th1 response. In contrast, post-tumor challenge Th2 cytokine levels as measured from stimulated splenocytes in mice from all vaccination cohorts were generally very low (~2-fold) similar to the cytokine levels measured in the stimulated splenocytes from the naïve control cohort (Fig. 3.6d).

We also tested whether immunizing mice with RANKL had any detectable effects on cytokine secretion profiles from stimulated splenocytes. Two weeks post-vaccination, only one cohort, DC-erbB2tr, showed considerable IFN-γ upregulation, having an average fold-increase of 7.8 over background (Fig. 3.7a). The levels of Th2 cytokines from stimulated splenocyte cultures were also very low, averaging less than 2.5-fold increases over background (Fig. 3.7b). Post-tumor challenge, both Th1 and Th2 cytokine levels were also low (all less than 3.3-fold over background), similar to levels from cells of naïve mice (Figs. 3.7c,d).

To further investigate possible reasons that IL-12 offered tumor protection while RANKL did not, we compared the levels of CD4\(^+\) and CD8\(^+\) tumor infiltrating lymphocytes (TILs) in the RM-1-NT and RM-1-erbB2tr tumors of mice in select cohorts. In mice immunized with DC-enGFP/IL-12 or DC-erbB2tr/IL-12, no significant differences in CD4\(^+\) TIL levels were measured between tumors within either immunization group. In addition, the CD8\(^+\) T cell levels in RM-1-erbB2tr tumors were similar in both cohorts, but RM-1-NT tumors of mice vaccinated with DC-erbB2tr/IL-12 displayed higher percentage of CD8\(^+\) TILs (Fig. 3.8a). We also assessed levels of TILs in
Figure 3.7. Neither Th1 nor Th2 immune responses against erbB2tr was upregulated in mice following immunizations with DC-erbB2tr/RANKL. Secretion of IL-2, IFN-γ, and TNF-α from splenocytes stimulated with tumor cells for 36 hrs was assessed two weeks post-immunizations (a) or post-tumor challenge (b). Concentrations of IL-4 and IL-10 were also measured from stimulated splenocyte cultures at two weeks post-immunizations (c) or post-tumor challenge (d). Cytokine concentrations from RM-1-erbB2tr co-cultures were normalized to those from RM-1-NT co-cultures. Plotted values are the mean ± SEM of 4 or 5 mice per group.
Figure 3.8. Mice vaccinated with DC-erbB2tr exhibited systemically high tumor-infiltrating CD8+ lymphocytes. Shown are the percentages of CD4+ and CD8+ TILs in mice immunized with DCs co-expressing (A) IL-12 or (B) RANKL with enGFP or erbB2tr.
mice immunized with either DC-enGFP/RL or DC-erbB2tr/RL. Mice immunized with DC-erbB2tr/RL neither had elevated levels of CD4$^+$ nor CD8$^+$ TILs compared to DC-enGFP/RL mice (Fig. 3.8b).

3.4.6 DC-erbB2tr/IL-12 immunizations fail to reduce established aggressive tumor growth

Having shown the potential for vaccination with DC-erbB2tr/IL-12 in protecting mice from developing erbB2tr-expressing tumors, we further tested the ability of a dose of 4x10$^5$ transduced DCs to cause rejection of aggressive tumors already present in vivo at the time of immunization. Given the aggressive nature of RM-1 cells, we rationalized that this moderately high treatment dose might hedge against the possibility of an overwhelming tumor burden from developing before the administered immunotherapy could elicit an anti-erbB2 adaptive immune response. We began by inoculating mice bilaterally with RM-1-NT and RM-1-erbB2tr tumors and then 3 days later, we immunized mice with 4x10$^5$ transduced DCs, followed by a second immunization another 3 days later. As expected, RM-1-NT tumors grew well in all immunization cohorts, however even this higher dose of DC-erbB2tr/IL-12 cells did not reduce RM-1-erbB2tr tumors and neither did any other immunization types tested (Fig. 3.9a,b). Thus, it was not surprising when we measured splenocyte secretion of IFN-γ and IL-4 by ELISA that we saw no appreciable changes in production of these cytokines in any of the tested immunization groups relative to control, mock-immunized mice (Fig. 3.9c,d).
Figure 3.9. Immunization of tumor-bearing mice with DCs transduced with LV/erb and LV/IL-12 does not delay tumor growth. Mice were inoculated bilaterally and immunized with 4x10^5 DCs. Tumor volumes were measured for RM-1-NT (a) and RM-1-erbB2tr (b). Levels of IFN-γ (c) and IL-4 (d) were determined by ELISA from 36-hour cultured splenocytes. Plotted values are the mean ± SEM of 5 mice per group.
3.5 Discussion

The prevention and treatment of aggressive and metastatic cancers remains an elusive task. DC-based immunotherapy is one modality that has shown considerable promise in recent years in pre-clinical models and many clinical trials [277]. Although the efficacy of DCs in eliciting anti-tumor immunity has been well established, one hindrance to their use in clinical trials is the difficulty in generating modified DCs in the required large numbers [164, 339]. We were the first to demonstrate that when DCs were transduced with LV/erb, a DC dose as low as $2 \times 10^3$ cells could be effective in vaccinating mice against RM-1-erbB2tr growth [317]. Using the same calculation as discussed, $2 \times 10^3$ murine DCs would be equivalent to $5 \times 10^6$ human DCs, which is less than the average immunization dose of $\sim 10^7$ DCs, and thus our work models a clinically feasible protocol. Continuing our goal to develop an effective low-DC-dose vaccination, we hypothesized that co-transducing DCs with LV/erb and an immunomodulatory gene such as IL-12 or RANKL could enhance the efficacy of our strategy towards mounting an antigen-specific anti-tumor response. To test our hypothesis, we first established the feasibility of simultaneously transducing murine BM-derived DCs with two different LVs, in order to engineer expression of both a TAA and an immunomodulatory protein. To our knowledge, we are the first to show the feasibility of co-transducing DCs with two separate LVs; we developed a protocol that not only achieves efficient transduction, but also avoids the cell death that can be associated with transducing DCs with high viral loads [340].

One advantage to employing separate vectors to encode the antigen and immunomodulatory cDNAs of choice is that in principle, it would allow us to control the
ratio of each vector and hence factor expression levels in transduced cells. Importantly, IRES elements often used in bicistronic vectors may have reduced downstream transgene expression, thus generating lower levels of the desired co-factor.

In performing our DC transductions, we noticed that transducing DCs with LV/IL-12 alone yielded much higher IL-12 production than with LV/IL-12 combined with LV/erb or LV/enGFP. This phenomenon occurred despite the fact that we consistently used the same relative number of LV/IL-12 viral particles for each transduction. It may reflect the possibility that we have simply reached a ceiling in the number of viral particles that can productively enter the target cells. Despite this discrepancy in IL-12 production, we demonstrated the ability of DC populations that express both erbB2tr and IL-12 to protect mice from developing aggressive ectopic tumors.

Tumor protection in our DC-erbB2tr/IL-12 treated mice was specifically against erbB2tr, as demonstrated by the unrestricted growth of RM-1-NT tumors in comparison to reduced growth of RM-1-erbB2tr tumors. We further showed that expressing erbB2tr and IL-12 together in DCs offered strong antigen-specific Th1 immunity. In addition, DCs expressing the higher level of IL-12 alone also offered significant tumor protection, without evidence of a Th1 response. Thus, it appears that the combination of erbB2tr and IL-12 provided a synergistic effect. The efficacy of these strategies is underscored by the lack of both tumor protection and significant immune responses following vaccination with DC-erbB2tr alone or with DC-enGFP/IL-12, which expressed similar IL-12 levels as DC-erbB2tr/IL-12. It was not surprising that the relatively high IL-12 production from DC-IL-12 therapy alone was sufficient to protect against RM-1-erbB2tr tumor
development; others have shown that IL-12 alone can have anti-tumor effects [249, 251]. To achieve RM-1-erbB2tr tumor protection with a DC vaccine that produced less IL-12 than the DC-IL-12 vaccine itself required co-expression of erbB2tr. Thus, the lack of specific protection offered by DC-enGFP/IL-12 may be due to the fact that it lacked sufficient IL-12 expression or the directed combination of erbB2tr and IL-12.

The finding that Th1 immunity was the predominant immune response towards erbB2tr following immunizations with DCs co-transduced to express erbB2tr and IL-12 is in agreement with the role of IL-12 in promoting Th1 responses. Based on the mechanism of Th1 differentiation, the ligation of IL-12 receptor on T cells by IL-12 triggers the release of IFN-γ, which in turn leads to the activation of transcription factor T-bet [341]. Activated T-bet not only modulates the opening of chromatin around the IFN-γ locus to increase IFN-γ production for further Th1 development, but it also inhibits the activation of transcription factor GATA-3, which is a main regulator of Th2 development [342]. Our finding that the presence of IL-12 primarily lead to Th1 immunity without evidence of significant Th2 immunity supports this model of Th1/Th2 differentiation.

Although the same DC generation and transduction protocols were applied to all DC cultures used in our study, we found non-transduced control DCs did not consistently exhibit the same upregulation of maturation markers in our studies. In addition, we found that transducing DCs with RANKL alone increased expression of DC markers relative to non-transduced DCs; yet marker expression was not altered when RANKL was combined with another LV. This held true for co-transduction of LV/RL with either LV/enGFP or LV/erb. Due to these differences in DC maturation status, it is not clear whether this
could have partly accounted for the lack of immunotherapy enhancement by RANKL. However, similar to a recent report that demonstrated the inability of RANKL to improve anti-tumor immunity [343], we did not find that it aided anti-erbB2 immunity in our prophylactic tumor model as neither tumor protection nor enhanced Th1/Th2 immunity towards erbB2tr were detected.

It is important to note that while we have previously reported the ability of our low-dose DC-erbB2tr immunization strategy alone to offer partial protection against RM-1-erbB2tr in a prophylactic tumor model [317], we did not find that it faired as well in our current study. This finding may point to the possibility that in this current system with this antigen and tumor model, the dose of $2 \times 10^3$ transduced DCs may represent a threshold value around which immune effects are measurable and adequate enough to offer benefit. It follows that slight variations in different preparations of DC cultures at this threshold dose may determine whether the critical level of anti-erbB2tr immunity can be achieved. Indeed our present data emphasizes the need for addition of immunity-enhancing IL-12, which is sufficient to tip the balance towards tumor protection. On the other hand, similar to a recent report that indicated the lack of benefit that RANKL had on improving anti-tumor immunity [343], we did not find that it aided anti-erbB2 immunity in our prophylactic tumor model as neither tumor protection nor enhanced Th1/Th2 immunity towards erbB2tr were detected.

Although DC-erbB2tr/IL-12 vaccinations effectively prevented tumor growth in an antigen-specific manner, this immunization strategy failed when used to regress established tumors, despite using the dose of $4 \times 10^5$ DCs per immunization. Reasons for this finding may relate to the highly aggressive nature of RM-1 cells [289]; suggesting
that analyses using a slower-growing tumor model may show greater efficacy. Alternatively, specific inhibitory mechanisms due to the presence of these tumor cells themselves may have dampened the immune response [344], especially since significant differences in cytokine levels were not found between groups.

When developing anti-tumor immunotherapy strategies in a mouse model, it is essential that the therapies themselves are clinically practical. Since obtaining large quantities of DCs from cancer patients for manipulations carries inherent limitations, we aimed to develop a DC vaccine that would be effective at a dose low enough to be actually achievable on a human scale. Testing the low dose of 2x10^3 DCs sets an important precedent in the field of cancer immunotherapy, as current studies of this kind typically use 500-fold higher doses. We are also the first to demonstrate the feasibility of co-transducing DCs with two separate LVs, an approach that facilitates fine-tuning of the dosing of key components. In our current study, we measured the effects of combining erbB2tr and either IL-12 or RANKL expression on LV-transduced DCs. The synergistic effects of co-expressing erbB2tr and IL-12 on DCs led to strong erbB2tr-specific Th1 immunity and tumor protection. By contrast, we did not find that RANKL was a valuable immunomodulator. Overall, our study provides a basis for the development of clinically feasible and effective immunotherapy strategies using low doses of LV-transduced DCs.
Chapter 4:

Discussion
4.1 Summary of findings

The role of DCs in eliciting potent antigen-specific immunity is well recognized [103, 345]. Their use in generating anti-tumor effects in mouse models remains an important area of research, as the knowledge gained from these studies can be useful in developing therapies for cancer patients. Unfortunately, given the difficulties associated with obtaining and generating DCs from cancer patients, the use of typical murine DC doses on the order of $10^6$ cells is not realistically translatable to the human scale. The goal of the research described in this thesis was to develop an effective immunotherapy strategy using low doses of DCs to target erbB2-expressing prostate tumors in a mouse model. It was hypothesized that if DCs could be engineered to efficiently express erbB2 via lentiviral transduction, then the combined intrinsic potency of DCs could allow lower numbers of DCs to be sufficient to produce appreciable anti-tumor effects. The TAA erbB2 was chosen as the target antigen due to its upregulation in many cancers, including prostate cancer.

While many techniques exist for generating DCs that present TAA-derived peptides [316, 346], viral transduction remains one of the most efficient methods of engineering expression of TAAs in DCs for subsequent peptide presentation [313]. Transduction of cells using LVs carries many advantages over other methods including the ability to transduce slowly-dividing cells, stable transgene expression, and low toxicity for primary cells [313, 347]. Taken together, LV transduction of DCs is an ideal approach to efficiently generate DCs that express TAA-derived peptides and has been the method of choice for my thesis projects described herein.
The utility of DCs in directing TAA-specific immune responses has been established for many years. However, the efficacy of low doses of DCs that have been LV-transduced to express a TAA in the prevention of tumor formation is unknown. In Chapter 2, I have shown that LV-transduction of DCs is efficient, since one overnight transduction could yield a population of DCs that was approximately 47% positive for kinase-truncated murine erbB2, erbB2tr. Use of the truncated version of this TAA reduced the likelihood of obtaining oncogenic signaling events in transduced DCs. In addition, LV transduction of DCs did not extensively alter either the cell surface marker profile on DCs, or their ability to induce an allogeneic mixed lymphocyte reaction. The efficacy of low doses of LV/erb-transduced DCs in protecting mice from developing ectopic tumors was also demonstrated in Chapter 2. The dose of $2 \times 10^5$ cells in a prime/boost vaccination strategy completely protected mice from developing erbB2tr$^+$ RM-1 tumors, but not control tumors. This antigen-specific anti-tumor effect correlated with an anti-erbB2tr antibody response as well as an upregulation in Th1 immunity. Using the 100-fold lower dose of $2 \times 10^3$ LV/erb-transduced DCs also offered some mice antigen-specific tumor protection, but evidence of either humoral or cellular immunity was not found.

The finding that partial and complete tumor protection could be achieved with $2 \times 10^3$ and $2 \times 10^5$ LV/erb-transduced DCs suggested that there might be a lower threshold dose of complete protection between the tested doses. In Chapter 3, I have investigated this possibility by testing whether co-transducing DCs to express erbB2tr and either IL-12 or RANKL could create an enhanced DC vaccination such that the low dose of $2 \times 10^3$ DCs could actually lead to robust tumor protection and detectable immune responses. IL-
12 is well known for its ability to skew immunity towards Th1 [324, 325] and was chosen with the hypothesis that it could enhance immunity against erbB2tr. RANKL is better known for its ability to enhance DC function by enhancing its life-span and persistence in draining lymph nodes [330]. The hypothesis was that combining the antigen-specific factor of expressing erbB2tr on DCs with the immunomodulatory factor of expressing either IL-12 or RANKL on DCs would augment the ability of the $2 \times 10^3$ DC dose in the context of tumor protection and possibly even regression. In Chapter 3, the feasibility of co-transducing DCs was shown and that combining LV/erb and LV/IL-12, but not LV/RL, offered Th1 upregulation and strong tumor protection, but not regression. As with the findings of Chapter 2, the $2 \times 10^3$ low-dose DC immunotherapy did not lead to any significant humoral responses.

Although the DC doses used in our experiments are markedly lower than those used by others investigating DC-mediated cancer immunotherapy, the concept of low immunization doses in the context of infectious diseases is well studied. Animal models of mycobacterial or Leishmania infection have been used to demonstrate that low antigen doses can lead to Th1 polarized immune responses [121, 122]. Thus, the finding that low numbers of DCs expressing TAA can also lead to a Th1 phenotype may reflect a similar mechanism of T cell activation. Parallels between the two systems may be useful in understanding the mechanism of inducing TAA-specific cellular immunity at low antigen doses.

Overall, investigating the efficacy of low DC vaccine doses sets a good example for developing clinically-relevant immunotherapy approaches. In the case of erbB2, the use of IL-12 as an immunomodulatory adjuvant appears to be advantageous and
underscores the feasibility and importance of finding potent adjuvants to combine with TAA.s. To my knowledge, this is the first reported use of such low doses of DCs. Its successful application in a prophylactic setting provides a platform for further low-dose immunotherapy advancements.

4.2 Future directions

4.2.1 Modifying the immunization strategy

To carry forward the research described in this thesis, one could modify the immunization strategy to potentially improve its anti-tumor potency. Whereas I tested the effects of 1 prime and boost immunization set, one could evaluate whether performing multiple boost immunizations could strengthen a recall response against erbB2tr. Designing this type of study would provide useful information, since injecting DCs several times is an approach that is used in the clinic [316, 346].

An alternative way to modifying the immunization strategy is to engineer expression of multiple TAA.s on DCs via LV transduction. Our lab has already demonstrated the prophylactic and therapeutic benefits of administering DCs expressing TAA.s such as PSA and PSMA [191]. Testing a combinatorial approach with multiple antigens may prove more effective against prostate tumors than one TAA alone. In my thesis, the rationale for using just one TAA, erbB2, was to establish the feasibility of LV transduction methods and to demonstrate anti-tumor effects as a proof-of-principle. Choosing additional TAA.s to target should take into account its expression profile on normal tissues and tumors, as well as its inherent immunogenicity. Ideally, the TAA(s) of choice would be tumor-specific.
Instead of or in addition to expressing two or more TAAs on DCs, one could also investigate the effects of incorporating one or more immunomodulatory components into the immunization strategy. The validity of such an approach has been affirmed in clinical trials against prostate and colon cancer using immunizations with a TRIad of COstimulatory Molecules (TRICOM; B7-1, ICAM-1 and LFA-3) [348] and supports the rationale for developing this area of research. The positive results from incorporating IL-12, but not RANKL, into the immunization strategy developed in my thesis emphasizes the importance of carefully choosing which immunomodulatory components should be used. While in these experiments the enhancement effects associated with IL-12 were investigated, it would be interesting to test the effects of several other cytokines including IFN-α and other type I interferons, which have proven to be helpful in enhancing DC function [190, 349]. Since the induction of potent immune responses is dependent on using mature DCs, factors to enhance their maturation status could also be considered. The effects of fusing HSP70 with human papilloma antigen E7, for example, was shown to protect mice from developing tumors [350]. The clinical benefit of using heat-shock protein gp96 was also demonstrated in melanoma clinical trials [351, 352]. Finally, one could knockdown specific proteins to achieve a potentially stronger vaccine. One example is knocking down SOCS1 in DCs, since its expression in DCs has been attributed to maintaining immune tolerance [334, 353].

Taking into consideration that the peripheral tolerance mechanisms need to be overcome for DCs to work more effectively, studies have shown that injecting anti-CD25 antibodies into mice to deplete Treg cells can enhance the effects of DC-mediated immunotherapy [354]. Thus, an interesting experiment would be to test the efficacy of an
approach using low doses of TAA-presenting DCs to determine whether such a strategy can produce stronger protection or even tumor regression without the aid of a cytokine adjuvant.

4.2.2 Modifying the prostate tumor model

To better evaluate the efficacy of the vaccines developed in this thesis, one could use a more stringent and more clinically-relevant tumor model. For example, one approach would be to factor in the complication of age into the tumor model by using mice that are near the end of their life expectancy; prostate cancer often occurs in elderly men [355] and their weakened immune system [356] may best be modeled with an old mouse [357]. Another way of altering the tumor model to make it more stringent and clinically-relevant would be to use a slower-growing tumor cell line that causes immune suppression, such as via TGF-β secretion, since prostate cancer is generally slow-growing and immunosuppressive [358]. RM-1 tumor cells do secrete this cytokine [359], but grow very rapidly, thus making it difficult to assess whether tumor protection is sustained in a bilateral model. Nevertheless, as already mentioned, the aggressive nature of this cell line provided a stringent model for evaluating the potency of the immunotherapy strategies developed in this thesis. Although injecting tumor cell lines to generate ectopic tumors is a popular approach for modeling cancer having mice strains, such as the TRAMP mouse [29] that develop orthotopic prostate tumors, may serve as a more accurate model, depending on the expression levels of erbB2 in TRAMP mice. It is not clear whether the prostate tumors that develop in these transgenic mice are erbB2⁺, but it has been recently established that at least their premalignant dorsolateral prostates
are erbB2⁺ [360]. Thus, it would be worth testing the prostate tumors of TRAMP mice for erbB2 expression by Western blot analysis, for example. If the naturally growing orthotopic tumors are indeed erbB2⁺, then the next logical step would be to use TRAMP mice to evaluate erbB2-targeted immunotherapy strategies.

### 4.2.3 Testing new applications

Although I performed experiments using a prostate cancer model, other cancer models could be used instead, since human HER2/neu is overexpressed in ovarian, lung, and breast cancer, for example. Tumors originating from any of these other organs may express unexpected levels of immune-evading factors or display other properties that would make them inherently different from prostate-derived tumors. Thus, using other tumor models may give different results than those described in this thesis.

In the case of breast cancer, HER2/neu is already a successful therapeutic target; the monoclonal antibody Herceptin is currently in clinical use [361]. However, in cases where Herceptin is ineffective against HER2/neu-positive breast tumors, perhaps the use of a DC-based immunotherapy strategy could be worth investigating, since it is not clear why some patients respond to Herceptin better than others. If, for example, Herceptin is unable to bind to HER2/neu due to changes in HER2/neu conformational states following binding with other EGFR family members, then a DC-mediated approach could perhaps overcome this issue. DCs prime T cells using peptides derived from whole antigen, and could therefore be effective even in situations of altered HER2/neu conformations.
Towards the goal of applying LV-transduced DCs in immunization strategies against other tumors, our laboratory is already working with a colon cancer mouse model and targeting the TAA carcinoembryonic antigen (CEA).

An LV/erb-transduced DC immunization strategy could also be valuable in combination with other treatment modalities. For example, to combine immunotherapy with tumor resection, one could first allow tumors to form in mice, then resect the tumors and inject LV/erb-transduced DCs to help eliminate any remaining tumor cells. This approach would mirror the clinical setting of surgical tumor resection and subsequent attempts to prevent relapse. Alternatively, DC immunizations could be administered with chemotherapy or radiation therapy, as these may lead to enhancement of anti-tumor effects [362, 363]. Thus, developing a hybridized approach using novel DC immunotherapy and standard therapies for the prevention and treatment of cancer may prove to be a valuable direction of research to pursue.

**4.3 Conclusions**

DC-mediated immunotherapy is a rapidly-expanding field of research. Many studies using animal models have shown that tumors can be effectively targeted and eliminated using TAA-presenting DCs. Achieving the same success has been more difficult in clinical settings. Reasons for this discrepancy are unclear, but may point to the shortcomings of experimental vaccines in their translatability from mouse to human, or the animal models themselves in their ability to truly mimic cancer patients. Alternatively, it may be that clinical trials are being performed on patients with a disease
state that is simply too advanced for DC-mediated immunotherapy to consistently provide objective clinical responses.

The thesis work presented herein contributes to the growing field of immunotherapy. Unlike many of the currently published mouse model studies, however, my main goal was to develop a clinically-feasible immunotherapy approach. To this end, lentiviruses were employed to efficiently transduce primary DCs without causing cellular toxicity. In addition, I tested relatively low doses of murine DCs that would translate to reasonable human DC doses. Choosing erbB2 as the TAA of interest was an important component of the immunotherapy developed here; since it represents a true self-antigen and would thus require peripheral tolerance mechanisms to be overcome in order to achieve positive anti-tumor results. We are the first to report the successful use of such low doses of DCs. Moreover, using IL-12 as an immunomodulatory protein, we were able to boost the efficacy of the $2 \times 10^3$ DC dose to a level where strong erbB2-specific Th1 immunity tumor protection was achieved. It is hoped that the work presented in this thesis can provide the basis for developing even more potent immunization strategies worthy of being translated to the clinic.

A great proportion of cancer immunotherapy research is focused on the optimization of the DC therapy. A retrospective look at how DC vaccines have evolved since they were first used in the clinic over a decade ago shows that many significant changes have been made. Whereas early vaccines often consisted of DCs that were pulsed with a single type of tumor antigen-derived peptide [364], many of the more recent vaccines utilize transfected or transduced DCs [200, 365] in an effort to achieve more efficient antigen presentation of a wider variety of peptides. Furthermore, many
recent DC immunotherapy clinical protocols use DCs that not only present peptides from multiple TAAs, but also express co-stimulatory molecules or adjuvant cytokines [348, 366].

Although the DC vaccines themselves have become more sophisticated, the immune status of cancer patients also need to be appropriately addressed. It seems that the next step for the field of cancer immunotherapy is to expand in scope to address the role of the host. The patient’s immune system is likely as important in determining the success of an immunotherapy strategy as the therapy itself. If one considers the DC vaccine to be a key and the patient to be a lock, then it becomes clear that both must be in a good working condition; if either component is rusty, then the key may not fit into the lock. Thus, if the host is not able to receive or respond to the DC therapy, then the patient cannot be adequately treated. Recently, attention has been given to specifically tackling the host’s immune tolerance status. Methods to overcome peripheral tolerance may create a better setting for administered DCs to prime antigen-specific anti-tumor responses. For example, the Treg-depletion protocol using recombinant IL-2 diphtheria toxin conjugate DAB(389)IL-2 has resulted in encouraging clinical outcomes when combined with a DC vaccine [367]. Thus, designing feasible immunotherapy approaches in animal models and patients that can be combined with methods that simultaneously address the tolerant state of the patient’s immune systems would be useful contributions to the field of cancer research.
References


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