CHARACTERIZATION OF DENDRITIC CELLS TRANSDUCED WITH VENEZUELAN EQUINE ENCEPHALITIS VIRUS REPLICON PARTICLES AS THERAPEUTIC CANCER VACCINES

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ABSTRACT

TIMOTHY P. MORAN: Characterization of Dendritic Cells Transduced with Venezuelan Equine Encephalitis Virus Replicon Particles as Therapeutic Cancer Vaccines
(Under the direction of Jonathan S. Serody and Robert E. Johnston)

Cancer vaccines seek to harness the specificity of T and B lymphocytes for reduction of tumor burden, as well as prevention of recurrent disease by establishing immunological memory. Because of their ability to initiate adaptive immune responses, dendritic cells (DCs) presenting tumor antigens have frequently been used as cancer vaccines. Unfortunately, induction of therapeutic responses in cancer patients has been sporadic, suggesting that current DC vaccines are unable to surmount tolerance against tumor antigens. The transduction of DCs with recombinant viral vectors may be a viable strategy for augmenting the ability of DC vaccines to break tolerance, as this approach can be used to efficiently deliver tumor antigens to DCs in the context of an immunostimulatory viral infection. Therefore, we have investigated the potential of DCs transduced with Venezuelan equine encephalitis virus replicon particles (VRPs) as cancer vaccines. VRPs could efficiently transduce human and murine immature DCs *ex vivo*, leading to high-level transgene expression, DC maturation, secretion of proinflammatory cytokines and efficient presentation of VRP-encoded antigens to T cells. VRP-transduced DCs (VRP-DCs) expressing a truncated neu oncoprotein stimulated neu-specific T cell and antibody responses and induced regression of established tumors in nontolerant mice. In contrast, VRP-DCs failed to induce robust antitumor responses in mice tolerant to neu, and were likewise unable

to inhibit tumor growth. Depletion of $CD4^+CD25^+$ regulatory T cells (T_{reg}) improved the effectiveness of VRP-DC vaccines in tolerant mice, demonstrating that VRP-DCs alone were unable to overcome T_{reg} activity. Furthermore, provision of tolerant mice with neu-specific T cells from nontolerant mice did not augment vaccine efficacy, indicating that tolerogenic mechanisms are dominant over effector T cell activity. These results demonstrate that while highly immunogenic, virally-activated DCs cannot break tolerance against self/tumor antigens. Moreover, these findings imply that potent DC vaccines alone are unlikely to induce therapeutic antitumor immunity unless additional measures are undertaken to inhibit immunoregulatory mechanisms.

To Arlene,

who caringly listened even when she wasn't interested

And to Ian,

who provided the impetus for finishing this work

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LIST OF ABBREVIATIONS

Ad	Adenovirus
APC	Antigen presenting cell
ACT	Adoptive cell therapy
BCG	Bacille Calmette-Guérin
BHK	Baby hamster kidney cell
CTL	Cytotoxic T lymphocyte
CY	Cyclophosphamide
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FMP	Influenza matrix protein
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage-colony stimulating factor
HA	Hemagglutinin
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iMC	Immature myeloid cell
LC	Langerhans cell
LPS	Lipopolysaccharide

mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
MMTV	Murine mammary tumor virus
mRNA	Messenger ribonucleic acid
neuET	neu extracellular-transmembrane domains
neuET-DC	DC transduced with VRPs encoding neuET
NFκB	Nuclear factor kappa B
NK	Natural killer cell
nsP	Nonstructural protein
null-DC	DC transduced with VRPs lacking a transgene
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
pi	Post-infection
RNA	Ribonucleic acid
RRV	Ross River virus
SFV	Semliki Forest virus
SIN	Sindbis virus
TAA	Tumor-associated antigen
TDLN	Tumor-draining lymph node
TGF	Transforming growth factor
T_H	T helper cell
TIR	Toll/interleukin-1 receptor

TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_R1	T regulatory type 1 cell
T_{reg}	$CD4^+CD25^+$ regulatory T cell
VEE	Venezuelan equine encephalitis virus
VEGF	Vascular endothelial growth factor
VRP	VEE replicon particle
VRP-DC	VRP-transduced dendritic cell
VV	Vaccinia virus

CHAPTER ONE

INTRODUCTION

DENDRITIC CELL BIOLOGY

Discovery of dendritic cells (DCs)

DCs were originally described by Steinman and Cohn as a "novel cell type" found in peripheral lymphoid organs of mice (279). The cells comprised a minor population (1.0-1.6%) of the murine spleen and were morphologically distinct from granulocytes, macrophages and lymphocytes. While these cells were heterogeneous in morphology, they were predominantly characterized by branching cellular processes extending from the cell body, and were thus referred to as "dendritic" cells (279). DCs were phenotypically and functionally characterized as: *i*) being a glass adherent and low-density cell population, *ii*) having little endocytic activity in comparison to macrophages; *iii*) having a low *ex vivo* proliferative capacity but a high *in vivo* turnover rate as determined by uptake of ^{3}H-thymidine; *iv*) lacking lymphocyte or monocyte surface markers; and *v*) originating from bone marrow-derived precursors (279-281).

While initially described in the spleen, it soon became clear that DCs were also present in several other lymphoid and nonlymphoid tissues. In rodents, DCs were identified in multiple tissues, including the epidermis (where they are referred to as Langerhans cells) (259), heart (122), lungs (262), lamina propria (230), peripheral lymph nodes (279), and thymus (13). In humans, DCs were first identified in the peripheral blood, where they comprise <0.1% of mononuclear cells (300). DCs were subsequently found in several other human tissues, including tonsils (123), lung (262) and skin (245). Thus, DCs are dispersed throughout the body in both lymphoid and nonlymphoid compartments, an attribute that is essential for their sentinel function.

DC subsets

DCs were initially described as a population of cells with a heterogeneous morphology, although it was unknown if this equated to different functional subpopulations as seen for T lymphocytes. With the advent of fluorescence-activated cell sorting (FACS), it became clear that DCs could be subdivided into different populations based upon the expression of specific cell surface markers (61). Furthermore, the various DC subpopulations appeared to have varied functions. However, it was not clear whether the different DC subpopulations represented distinct activation stages of a single lineage of cells or were derived from different precursor populations. Current evidence suggests that a mixture of both models is involved in the development of the different DC subpopulations (269).

Mature murine DCs are classically characterized by expression of CD11c (the αX integrin subunit of complement receptor 4) and moderate to high expression of MHC class II molecules (200). DCs also express various levels of the T cell costimulatory molecules CD40, CD80 (B7.1) and CD86 (B7.2) (3, 128). Other markers that have been useful for differentiating DC subpopulations include CD11b (the αM integrin subunit of complement receptor 3), CD4, CD8 (αα homodimers) and CD205 (DEC^-205) (3, 128, 152).

In the murine spleen, two major populations of DCs have been routinely identified: DCs that express CD11b but are negative for CD8αα ($CD8^-$ DCs), and DCs that express CD8αα but lack CD11b expression ($CD8^+$ DCs). In addition, $CD8^-$ DCs can be further divided into $CD4^+$ and $CD4^-$ subtypes (3, 128). $CD8^-$ and $CD8^+$ DCs have been historically referred to as myeloid and lymphoid DCs, respectively, which was based upon their proposed hematopoietic lineage of origin (5, 142). However, several studies have shown the ability of

common myeloid and lymphoid precursors to give rise to all types of DCs, indicating that DC development is more complex than originally proposed (195, 269, 313).

In addition to the three DC populations found in the spleen, murine skin-draining lymph nodes also contain two other DC subtypes: $CD11b^{+}CD4^{-}CD8^{lo}CD205^{hi}$ and $CD11b^{+}CD4^{-}CD8^{-}CD205^{+}$ cells (128, 269). The former represent Langerhans cells (LCs) from the epidermis, while the latter are likely derived from interstitial DCs that have migrated to the lymph node. Recently, a sixth population of DCs has been described in mouse spleen and lymph nodes (8). These cells, referred to as plasmacytoid DCs, are $CD11c^{lo}$ and express the unconventional markers CD45 (B220) and Ly6C. While plasmacytoid DC precursors do not morphologically resemble typical DCs, they can differentiate into functional antigen presenting cells (APCs) and secrete copious amounts of type I interferon (IFN) following viral infection.

Besides expressing different surface markers, the DC subtypes of the spleen also appear to have distinct functions. While both $CD8^{+}$ and $CD8^{-}$ DCs can stimulate T cell proliferation, the type of T cell response induced appears to depend upon the subclass of DC presenting the antigen (192). Injection of antigen-bearing $CD8^{+}$ DCs resulted in the secretion of T_H1 cytokines [i.e. interleukin (IL)-2 and IFN-γ] by $CD4^{+}$ T cells in the draining lymph node. In contrast, stimulation of $CD4^{+}$ T cells by $CD8^{-}$ DCs resulted in the secretion of T_H2 cytokines (i.e. IL-4 and IL-5). The ability of $CD8^{+}$ DCs to stimulate T_H1 responses was dependent upon high level IL-12 secretion by these DCs, whereas $CD8^{-}$ DCs only produced minimal levels of IL-12 following activation (192, 236). The DC subpopulations also differ in their ability to capture and present antigen to T cells. $CD8^{+}$ DCs, but not $CD8^{-}$ DCs, are capable of cross-presenting cell-associated antigen to $CD8^{+}$ T cells *in vivo* (68). Consistent with their

cross-priming ability, $CD8^+$ DCs are also responsible for inducing tolerance against exogenous antigens in the peripheral lymphoid system (20). Furthermore, $CD8^+$ DCs appear to be responsible for initiating CTL responses against several different types of viruses, regardless of the inoculation route (21).

In humans, the identification of different DC subsets is hindered by a lack of fresh tissue specimens other than peripheral blood. Finding human equivalents to the different murine DC populations has also been difficult since human DCs do not express CD8αα. However, DCs in the human thymus do appear to resemble their murine counterparts in that they primarily express lymphoid rather than myeloid markers (301). It is well known that human peripheral blood contains at least three different DC precursors: $CD34^+$ stem cells, $CD14^+$ monocytes and $CD123^+CD45RA^+$ plasmacytoid cells (269). $CD34^+$ stem cells are usually found at low numbers in the peripheral blood, but are enriched in umbilical cord blood or bone marrow. In the presence of granulocyte/macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β), $CD34^+$ cells can be differentiated into $CD1a^+$ DCs with a LC-like phenotype characterized by expression of Birbeck granules, CD207 (Langerin) and E-cadherin (45, 286). $CD34^+$ stem cells can also give rise to DCs that lack Birbeck granules and resemble interstitial or dermal DCs (45). $CD14^+$ monocytes can differentiate into $CD11c^+CD11b^+$ immature DCs after 6-7 days of culture in the presence of GM-CSF and IL-4 (255). These cells can be further differentiated into functionally mature DCs by treatment with monocyte-conditioned media, TNF-α, CD40L or lipopolysaccharide (LPS) (22, 255). The final precursor—$CD123^+CD45RA^+$ plasmacytoid cells—was one of the few cell types identified in humans before mice (116). These cells are most notable for their ability to secrete large amounts of

type I IFN following stimulation with viruses (270). However, plasmacytoid cells can differentiate into DCs when cultured in the presence of IL-3 and CD40L (116). To date, most studies of human DC populations involve *in vitro* culture of precursors in the presence of exogenous growth factors, and therefore more research is needed to verify the *in vivo* relevancy of these experiments.

DC function

The immunological role of DCs was first hinted at when DCs were identified as the primary stimulators of the mixed leukocyte reaction (MLR)—being at least 100-fold more potent than lymphocytes or macrophages (282). The observation that DCs were potent stimulators of the MLR suggested that DCs may be specialized for initiating lymphocyte-dependent immune responses. Indeed, DCs were subsequently shown to be capable of priming naïve antigen-specific $CD4^+$ T helper (146) and $CD8^+$ cytotoxic T lymphocytes (CTL) (148). The ability of DCs to activate T cells is dependent upon several functional attributes of DCs, including antigen capture and processing, migration to T cell areas of lymphoid tissues, high-level expression of proteins involved with antigen presentation and T cell stimulation, and secretion of immune-modulating cytokines.

DCs are the prototypic APC, meaning that their main function is to capture antigen from the environment and then process the antigen into peptides for presentation to T lymphocytes. To aid in this process, DCs are strategically deployed throughout cutaneous and mucosal sites in an immature state. Upon migration into peripheral tissues, DCs begin sampling the extracellular environment for antigens via macropinocytosis, phagocytosis and receptor-mediated endocytosis (12, 254). DCs possess several molecules that assist in each

of these mechanisms of antigen capture. Immunoglobulin Fc and complement receptors allow the capture of antigen-antibody complexes or the phagocytosis of opsonized microbes. Lectins, such as CD206 (mannose receptor), CD207 and CD209 (DC-SIGN), recognize specific carbohydrate domains present on microbes or glycosylated antigens, leading to engulfment of the antigen (107).

Upon antigen capture, DCs must determine whether the antigen is from an insidious foreign entity, such as a pathogenic microbe, or the antigen is an innocuous self-protein from a normally dying cell. This fateful choice will ultimately determine how the antigen is presented to T cells. To aid in this decision, DCs can express an array of germ line-encoded receptors that recognize pathogen-associated molecular patterns (PAMPs) found on many microbes. These receptors—known as Toll-like receptors (TLR) due to their homology with the *Drosophila* Toll protein—can discriminate multiple PAMPs on microbial molecules, including zymosan and peptidoglycan (TLR2); viral double-stranded RNA (TLR3); LPS (TLR4); flagellin (TLR5); viral single-stranded RNA (TLR7-8); and bacterial or viral DNA containing unmethylated cytosine-guanosine motifs (TLR9) (2). TLRs are transmembrane proteins with an extracellular domain composed of leucine-rich repeats involved with ligand binding, and an intracellular region containing a Toll/IL-1 receptor (TIR) domain (1). Upon ligand binding, most TLRs dimerize and recruit the adapter protein MyD88 through TIR-TIR domain interactions, which in turn recruits the kinase IRAK-4. IRAK-4 initiates a cascade of downstream signaling events that culminate in the translocation of nuclear factor kappa B (NFκB) to the nucleus, resulting in the transcription of genes involved with inflammation and cell survival. Some TLRs also utilize a MyD88-independent pathway involving either TIRAP (TLR4) or TRIF (TLR3), which results in IRF3 or late-stage NFκB activation (1).

Regardless of the signaling pathway used, activation of TLRs ultimately informs the DC that the endocytosed antigen is likely from a pathogen, and should therefore be presented in a stimulatory manner to T cells. The process of DC maturation is thus initiated, which involves a coordinated series of events. Although cells may transiently increase macropinocytosis immediately following TLR activation (308), the endocytic capacity of DCs is eventually down-regulated (254, 255). Endocytosed antigen is then processed and loaded onto intracellular MHC class II molecules, which are rapidly relocated to the cell surface (254). Remarkably, DCs can discriminate what antigen to present at the level of the phagosome, which is dependent upon the concurrent presence of TLR ligands with the endocytosed antigen (28). The presentation of antigen is accompanied by increased expression of cell-adhesion molecules (i.e. ICAM-1 and LFA-3) and costimulatory molecules (i.e. CD40, CD80 and CD86), both necessary for optimal stimulation of T cells (48, 178, 255). Finally, activated DC can secrete proinflammatory and immune-modulating cytokines such as TNF-α, IL-6, IL-1β and IL-12p70. These cytokines are not only involved with molding the ensuing T cell response (e.g. IL-12p70 induction of T_H1 responses) (135), but can also inhibit the activity of suppressor T cells, thus facilitating effector T cell activity (229).

Once the DC has captured antigen, it must then transport the antigen to the regional lymph node where naïve or memory T cells await. Migration of antigen-bearing DCs to the lymph nodes is dependent upon maturation-induced expression of chemokine receptor CCR7, which allows DCs to enter afferent lymphatic vessels and traverse to the draining lymph nodes (97). Upon entering the lymph node, DCs migrate to the paracortical areas of the lymph node and position themselves around high-endothelial venules (9). Here, DCs transiently and

randomly interact with 500-5000 T cells per hour—an amazing yet necessary process if the DCs are to encounter the rare antigen-specific T cell (32). Once a T cell recognizes its cognate antigen presented by a DC, stable contacts are formed for up to 12 hours, during which time the T cell becomes activated and secretes IL-2 and IFN-γ (199). The T cell eventually disengages from the DC and begins the process of cellular expansion, during which it will embark down a pathway of differentiation into either an effector or memory cell.

The above description of how DCs initiate T cell responses was predicated upon the encounter of the DC with a microbe-associated antigen. However, what happens when a DC encounters a self-antigen? Studies in the 1990s demonstrated that bone marrow-derived APCs could capture and present antigen from dying pancreatic islet cells under steady-state conditions (173, 174). Interestingly, cross-presentation of antigen to reactive $CD8^+$ T cells resulted in T cell deletion rather than activation, suggesting that APCs may be responsible for maintaining peripheral tolerance (174). In agreement with this finding, studies by Steinman and Nussenweig demonstrated that targeting antigen to immature DCs led to antigen-specific T cell anergy or deletion (30, 125). Other studies have demonstrated that presentation of antigen by immature DCs can induce the development of IL-10 secreting T cells with regulatory properties (69). Taken together, it appears that presentation of antigen by DCs in the absence of maturation cues results in T cell tolerance. However, the erroneous presentation of self-antigen by mature DCs does not necessarily lead to irrevocable autoimmunity, as these DCs can induce expansion of regulatory T cells capable of suppressing the immune response (318).

DENDRITIC CELLS AND CANCER IMMUNOTHERAPY

A brief history of cancer immunotherapy

The hypothesis that the immune system could control carcinogenesis was postulated as early as 1909 by the esteemed German scientist Paul Ehrlich (79). In the 1950s and 1960s, several studies demonstrated that carcinogen-induced tumor cells could be recognized by the immune system of syngeneic mice (219). In 1953, Foley observed that removal of methylcholanthrene-induced sarcomas from mice led to a state of immunological memory against the tumor, as these mice were now resistant to subsequent challenge with the same sarcoma (94). Other studies demonstrated that the transfer of sera or lymphoid cells from tumor-bearing mice to naïve syngeneic animals would result in protection from subsequent tumor challenge (219). These observations suggested that immunogenic molecules of unknown identity were present on transformed cells, which could lead to tumor rejection. These molecules were thus referred to as tumor-specific transplantation antigens (158). Based upon these early studies of tumor immunity, Burnett developed the tumor "immunosurveillance" hypothesis, which postulates that the immune system of higher vertebrates is essential for recognizing and eliminating transformed cells throughout life (39).

Over the next several years, the immunosurveillance hypothesis was a contentious topic. While it was generally accepted that the immune system was important in controlling tumors spawned by oncogenic viruses, its ability to restrain the development of spontaneous tumors of epithelial origin was debatable. Studies in the 1970s using thymectomized mice, mice treated with anti-lymphocyte sera, or athymic nude mice did not yield convincing data that supported tumor immunosurveillance (79). Therefore, the notion of tumor

immunosurveillance went out of vogue for nearly 20 years. In the mid 1990s, new studies using gene-targeted mice deficient for IFN-γ, perforin, or recombination activating gene-2 added credence to the immunosurveillance hypothesis, as these mice develop more spontaneous- and chemically-induced tumors than their wild-type counterparts (263). These studies also supported the rationale that the immune system could be harnessed to eradicate neoplastic cells, an idea known as cancer immunotherapy.

One of the earliest examples of cancer immunotherapy was described by William Coley in the 1890s (227). Coley treated several sarcoma patients with a formulation of bacterial derivatives, known as "Coley's toxin," resulting in tumor regression in about 10% of cases. Coley's work eventually led to the use of Bacille Calmette-Guérin (BCG) for tumor immunotherapy, which is still a standard treatment for superficial bladder cancer. In retrospect, it is likely that Coley's toxin contained TLR ligands that primarily activated constituents of the innate immune system—an important component of tumor immunity (34).

The ability to coerce the adaptive immune system to specifically target tumor cells became realistic in the 1980s and 1990s with the discovery of several antigens that were either solely or preferentially expressed by spontaneous tumors (299). These tumor-associated antigens (TAAs) are often derived from mutated or overexpressed oncogenes or tumor-suppressor genes, which include *ras*, *p53* and *c-erbB-2* (*neu*). Other TAAs are derived from alternatively glycosylated proteins, such as the mucin MUC-1, or aberrantly expressed fetal genes such as carcinoembryonic antigen (CEA). In particular, malignant melanomas express a plethora of TAAs, ranging from proteins involved with melanocyte differentiation (i.e. MART-1, tyrosinase, and gp100) to proteins that are normally expressed in testicular tissue (i.e. MAGE-1 and LAGE) (246).

Armed with this new knowledge of TAAs, researchers performed several clinical trials aimed at inducing therapeutic tumor immunity against these proteins. Multiple strategies for immunizing patients against TAAs have been employed, including MHC class I-restricted peptides, irradiated tumor cells, DNA plasmids, and recombinant viral vectors (89). Unfortunately, cancer vaccines have been generally ineffective in the clinical setting. A recent review by Rosenberg *et al.* claimed that the objective response rate for 440 patients enrolled in cancer vaccine studies was only 2.6% (248). While the exact number has been debated by other scientists, the overall success rate of cancer vaccines has been undeniably low.

DCs as cancer vaccines

It was recognized in the 1980s that DCs possessed a unique ability to prime naïve $CD8^+$ and $CD4^+$ T cells. The enhanced immunostimulatory capacity of DCs is due to several factors, including highly efficient capturing and processing of antigen; high level expression of intracellular MHC class II molecules, which are quickly relocated to the cell surface following maturation; expression of an arsenal of costimulatory molecules, including CD40, CD80, and CD86; and secretion of immunomodulatory cytokines such as IL-6, IL-12p70 and type I IFN (12). It was not long after their discovery that researchers proposed the use of DCs for tumor immunotherapy. It seemed reasonable that these potent APCs might be capable of activating and expanding tumor-specific T cells in patients. At that time, though, the clinical use of DCs as cancer vaccines seemed impractical, since DCs were present at very low frequencies in peripheral blood (<0.1% of leukocytes) and appeared to be terminally differentiated cells that were incapable of expansion (300).

Sources of DCs for Immunotherapy. The use of DCs as immunotherapeutic reagents moved closer to reality when methods for isolating and expanding DCs from precursors were developed in the early 1990s—first in mice and then in humans. In 1992, Inaba *et al.* described a method for expanding murine DCs from precursors in the blood and bone marrow in the presence of GM-CSF (144, 147). The functionality of these DCs was confirmed in a subsequent study where the DC precursors were pulsed with killed BCG mycobacterium *in vitro* (143). The DCs were capable of endocytosing the bacterium, and then processing and presenting BCG-derived epitopes to primed T cells *in vitro*. Furthermore, injection of BCG-loaded DCs into naïve mice primed BCG-specific T cells in the draining lymph node, although it was not demonstrated that the adoptively transferred DCs had directly presented the antigen. Several other methods for generating large numbers of DCs from murine bone marrow precursors have been published (33, 189, 196, 275), although in general, most of these methods are based upon the protocol originally described by Inaba *et a.l* in 1992. One notable difference is the addition of IL-4 during the culturing process, which minimizes the outgrowth of granulocytes and macrophages and increases the yield of fully differentiated DCs (196, 275).

The generation of large numbers of DCs from human peripheral blood precursors was originally described by Banchereau and colleagues in 1992. The authors demonstrated that $CD34^+$ hematopoietic stem cells isolated from peripheral blood could be expanded and differentiated into $CD1a^+$ cells with a LC-like morphology after 12 days of culture in the presence of GM-CSF and TNF-α (44). Parallel studies by Schuler and colleagues demonstrated that the addition of IL-4 further enriched the DC yield by suppressing the

growth of monocytes (244). Unfortunately, the normal frequency of $CD34^+$ cells in peripheral blood is very low (<0.1%), and therefore patients must be treated with drugs that mobilize bone marrow precursors and subsequently undergo leukopheresis to generate sufficient numbers of $CD34^+$ stem cells for *ex vivo* culture and differentiation into DCs (95). Appreciable numbers of human DCs can also be generated from peripheral blood $CD14^+$ monocytes (22, 255, 326). Human monocytes cultured for 6-7 days in the presence of GM-CSF and IL-4 differentiate into immature DCs; the cells can be further matured with TNF-α or monocyte-conditioned media.

DCs generated from either $CD14^+$ monocytes (mono-DCs) or $CD34^+$ stem cells (CD34-DCs) have been used in several clinical trials (64 and see below). While there is no data to suggest that one population of DCs is therapeutically superior to the other in patients, some researchers have described differences in the ability of mono- or CD34-DCs to stimulate antigen-specific T cells (87, 204). However, a recent comparison of DCs derived from either monocytes or $CD34^+$ cells from the same individual found that while mono-DCs expressed higher levels of CD86 and HLA-DR, both mono- and CD34-DCs were functionally equivalent in an allogeneic MLR (290). However, the yield of DCs was greater when using $CD34^+$ cells as a precursor population, suggesting that CD34-DCs may be preferable when large numbers of DCs are required.

Preclinical and clinical evaluation of DC vaccines. Several *in vitro* studies in the 1980s had demonstrated that DCs were the principle APC responsible for priming antigen-specific $CD4^+$ and $CD8^+$ T cells (278). Based on this finding, Inaba *et al.* demonstrated that murine splenic DCs pulsed with antigen could induce primary T cell responses following injection

into naïve syngeneic mice (145). This was one of the first studies to support the notion of using antigen-pulsed DCs for immunotherapy. Earlier studies had suggested that antigen-pulsed APCs could induce tumor-specific immunity in animals, but the role of DCs in the APC population was not directly evaluated (112, 265).

The first report of antigen-pulsed DCs used specifically for the induction of tumor immunity *in vivo* was published in 1994 by Flamand *et al.* (93). The authors demonstrated that immunization with splenic DCs pulsed with idiotypic antibody as a tumor antigen could protect mice from challenge with lymphoma cells. Shortly thereafter, Mayordomo *et al.* demonstrated that vaccination with peptide-pulsed DCs generated from bone marrow precursors could induce prophylactic and therapeutic tumor immunity in three different animal tumor models, although two of the tumors expressed model antigens (196). Several other researchers have published similar findings regarding the ability of DCs to induce tumor immunity *in vivo* (49, 223, 235, 277, 328). While these results were encouraging, the relevancy of both the tumor antigen used and the animal model in which the vaccinations were performed was conveniently disregarded. Furthermore, most of the studies evaluated the ability of DC vaccines to protect mice from subsequent tumor challenge rather than their ability to inhibit growth of established tumors.

Preclinical studies describing DCs as potential immunotherapeutic agents for cancer generated significant excitement in the medical community, and quickly led to several clinical trials. The results from an initial patient study were published by Hsu *et al.* in 1996 (136). This study involved four patients with follicular lymphoma who had been previously treated with chemotherapy. Tumor biopsies from the patients were used to generate tumor-specific (and therefore patient-specific) idiotype antibody. The idiotype antibody was used

to pulse DCs that had been isolated from peripheral blood mononuclear cells (PBMCs) by density centrifugation. The antigen-pulsed DCs were then injected intravenously into the patients. The patients received 3-4 monthly DC vaccinations, and were also boosted with soluble protein every two weeks following each DC vaccination. All vaccinations were well tolerated, and all of the patients developed proliferative PBMC responses to their respective idiotype antibodies. Evaluation of the patients after completing the vaccine regimen indicated that one patient was in clinical remission, one patient had a minor response with some regression of diseased lymph nodes, and the other two patients exhibited no significant change in disease. While this study only included four patients, it supported the clinical use of DC vaccines for the treatment of cancer.

Since the original DC vaccine trial reported by Hsu *et al.*, several more studies have been undertaken to evaluate the efficacy of DC vaccines in other types of cancer, including breast, colon, prostate, renal and malignant melanoma (11, 64, 67, 168, 209, 284). It has often been difficult to compare the results of different DC clinical trials, since the studies differ in the type of cancer that is being treated, the origin and maturation of the DCs being used, the type and source of tumor antigen, the immunological assays used to measuring tumor-specific T cell activity, and even the criteria used for evaluating clinical responses. Overall, the clinical response rate for DC vaccines has been low, yet is potentially higher than that seen with other vaccine regimens. Focusing on melanoma alone, a recent review by Rosenberg *et al.* reported that DC vaccines have resulted in a 9.5% remission rate, compared to a mean of 3.1% for peptides, viral vectors and tumor cell vaccines combined (248). One must also remember that the majority of DC vaccine trials have been performed in patients with extensive and refractory disease, and therefore complete tumor regression following any type

of treatment would be highly extraordinary. Instead of seeing the low success rate of DC vaccines as a deterrent for their use, some researchers have viewed it as an argument for the development of improved DC vaccines (10).

Virally-transduced DCs as cancer vaccines

One of the most frequently used methods for loading DCs with tumor antigens is to pulse the DCs with MHC class I-restricted peptides—typically those restricted to HLA-A2 molecules (95). This method is often preferred because it is relatively simple to generate clinical-grade peptides of 8-10 amino acids in length. Most tumor antigen-derived peptides were originally identified via acid elution of peptides present on the surface of tumor cells, or through the use of mathematic algorithms that predicted sequences with high-binding affinities for MHC class I molecules (64). While dozens of MHC class I-restricted peptides have been mapped for several different tumor antigens, this approach for antigen loading has several drawbacks. First, MHC class-I restricted peptides can only be used for patients of a specific MHC haplotype. Second, focusing the immune response against only one or two MHC class I-restricted peptides can select for tumors with mutations in those specific peptides, thus leading to immune escape. Third, the use of MHC class I-restricted peptides precludes the involvement of MHC class II-restricted $CD4^+$ T_H cells in the nascent anti-tumor response. Antigen-specific $CD4^+$ T cells are necessary for the licensing of DCs through CD40-CD40L signaling, thus resulting in more efficient activation of $CD8^+$ CTL (23). Additionally, $CD4^+$ T_H cells are required for the activation of tumoricidal macrophages and the generation of T-dependent antibodies against tumor antigens (60, 140). While inclusion of $CD4^+$ T cells in the anti-tumor response could be achieved by pulsing DCs with MHC

class II-restricted peptides, relatively few $CD4^+$ T_H cell epitopes have been characterized for tumor antigens.

Because of the limitations associated with MHC class I-restricted peptides, several alternative strategies for loading DCs with tumor antigens have been employed, including pulsing DCs with recombinant protein or killed tumor cells (223, 235, 296), transfecting DCs with tumor messenger RNA (mRNA) (212), or fusion of DCs with tumor cells (111). These approaches have some benefits, but are also plagued with certain drawbacks. DCs pulsed with recombinant protein do not induce significant $CD8^+$ T cell responses, since uptake and cross-presentation of soluble protein on MHC class I molecules is typically inefficient (68). Cross-presentation of exogenous antigen can be enhanced, however, by feeding DCs antigen-antibody complexes (239) or antigen-heat shock protein complexes (272). Using whole killed tumor cells or tumor cell lysates as an antigen source is potentially advantageous because both known and unknown tumor antigens could be delivered to DCs. Unfortunately, tumor cell lysates can also contain immunosuppressive proteins that inhibit DC activation (90), and apoptotic cells have been shown to suppress DC maturation (257). Furthermore, loading DCs with killed tumor cells relies on the availability of either primary tumor isolates or closely related tumor cell lines. Similar to pulsing DCs with killed tumor cells, transfection of DCs with tumor mRNA, or fusion of DCs with tumor cells, can lead to presentation of unidentified but potentially immunogenic epitopes. However, these methods carry the risk of presenting self antigens that are not specifically associated with the tumor, which could lead to autoimmunity (132). Finally, most of these methods for antigen delivery do not result in DC maturation, and therefore codelivery of maturation stimuli is required.

The use of viral vectors for the delivery of antigens to DCs has several theoretical advantages. First, viral transduction can be significantly more efficient that other physical methods of gene delivery, such as electroporation or chemical transfection (7). Second, viral vectors can be used to deliver intact tumor antigens containing both MHC class I- and II-restricted epitopes (319, 322, 330). The presentation of multiple epitopes would induce a broader T cell response, thus decreasing the likelihood of tumor immune escape through mutations in immunodominant epitopes. Third, viral vectors can be used in patients of all MHC haplotypes. Fourth, viral vectors can induce DC maturation through stimulation of TLRs (i.e. TLR-3, 7, 8 and 9) or cytoplasmic viral recognition proteins (i.e. RIG-I or Mda5) (156). DC maturation can occur via direct activation of the NFκB pathway by viruses, or indirectly through autocrine or paracrine secretion of type I IFN (133). Fifth, viral vectors can induce secretion of type I IFN, which can enhance the activity of innate immune effectors such as macrophages and NK cells (129, 194). Furthermore, type I IFN can act directly on $CD4^+$ and $CD8^+$ T cells to augment clonal expansion (124, 167) and can enhance the cross-presentation of antigen to CD8+ T cells (179). Finally, viral vectors have been shown to inhibit the activity of regulatory T cells and thus lower the threshold for breaking tolerance (320).

Several different viral vectors have been evaluated for *ex vivo*-transduction of human DCs. Some of the more promising and clinically relevant virally-derived vectors are described here.

Adenovirus. Adenoviral (Ad) vectors are non-integrating DNA vectors that can accommodate large fragments of heterologous DNA (~37 kb) (314). Ad vectors can

transduce murine DCs and human mono-DCs *ex vivo*, although a very high MOI (>1000) is required for efficient transduction (~95%) due to low expression of the coxsackievirus and Ad receptor by DCs (7, 240). Strategies to enhance DC transduction efficiency by altering the tropism of Ad vectors have met with some success (295). Transduction of human DCs with Ad vectors does not significantly disrupt DC maturation or antigen presentation (240, 325). Human DCs transduced with recombinant Ad vectors encoding tumor antigens activate CTLs specific for several tumor antigens *in vitro*, including MART-1 (40), p53 (215), and CEA (56). Multiple preclinical studies have evaluated the efficacy of Ad-transduced DCs as tumor vaccines in mice (314). These studies have generally shown that Ad-transduced DCs induce appreciable $CD4^+$ and $CD8^+$ T cell responses against tumor antigens, and can partially inhibit the growth of established poorly-immunogenic tumors (155). To date, no clinical trials have been performed with Ad-transduced DCs.

Vaccinia virus. Vaccinia virus (VV) is a member of the *Poxviridae* family of viruses, which are large double-stranded DNA viruses of ~200 kb in size. Replication-defective VV vectors have been shown to transduce human immature and mature DCs with moderate efficiency (mean 30.9% at an MOI of 2.5) (151). Furthermore, human DCs transduced with VV vectors were capable of stimulating melanoma-specific CTL *in vitro* (76). However, VV appears to inhibit DC maturation and induces apoptosis, likely due to the multiple immune-modulating proteins expressed by poxviruses (82). Despite the derogatory effects of VV on DCs, VV vector-transduced human CD34-DCs have been evaluated in a phase I clinical trial (70). Six stage IV melanoma patients received four injections of 1×10^8 VV-transduced DCs over a two month period. Treatments were well tolerated and melanoma-specific $CD8^+$ T cells were

detected in four or five patients. One patient receiving adjunct surgery also demonstrated a partial clinical response.

Retroviruses and lentiviruses. Retroviral vectors are most often associated with gene therapy due to their stable integration in the host genome, but they have also been utilized for transduction of human DCs. The major limitation of retroviral vectors is their inability to transduce non-dividing cells, thus precluding their use for transducing mono-DCs. However, retroviruses can be used to transduce proliferating $CD34^+$ precursors, which can then be differentiated into DCs (291). Transduction efficiency is relatively limited (10-20%), but transduced DCs are functional and can stimulate both $CD4^+$ and $CD8^+$ T cells specific for the vector-encoded antigen (177, 330). Unlike classical retroviral vectors, lentiviral vectors have the capacity to infect both dividing and quiescent cells, including human mono- and CD34- DCs (118). Transduction efficiency of human DCs ranges from 20-99%, depending upon the source of the DCs and the maturation status (78). Human DCs transduced with lentiviral vectors do not exhibit significant functional deficits, and can stimulate tumor-specific $CD8^+$ T cells *in vitro* (91, 288). *In vivo* studies in mice have indicated that lentiviral-transduced DCs are superior to peptide-pulsed DCs at inducing immunity against a model tumor antigen (127). Furthermore, transfer of CD34-DCs transduced with a lentiviral vector encoding green fluorescent protein (GFP) induced GFP-specific T cell responses in non-human primates (172). Based on these studies, vaccination with lentiviral vector-transduced DCs may be a promising strategy for tumor immunotherapy. However, issues with large-scale production and questionable safety of lentiviral vectors are significant barriers to clinical application (78).

Alphaviruses. Alphaviruses are single-stranded, positive-sense RNA viruses of ~11 kb in length and belong to the *Togaviridae* family of viruses (see below for a detailed description). While studies by MacDonald and Johnston had shown that alphaviral vectors derived from VEE could target murine DCs *in vivo* (190), the ability of an alphaviral vector to transduce human DCs *ex vivo* was first reported by Gardner *et al.* in 2000 (104). Using replicon vectors derived from Sindbis virus (SIN), the authors identified a single mutation in the E2 glycoprotein that conferred tropism for human mono-DCs (18% transduction efficiency at an MOI of 50). Transduction of human immature DCs with SIN replicons resulted in DC maturation, but the ability of transduced DCs to stimulate human T cells was not assessed. Vectors derived from Semliki Forest virus (SFV) have been used to infect murine DCs *ex vivo*, although the virus had to be treated with a synthetic polymer for efficient transduction (53). SFV-infected DCs have also been evaluated for tumor immunotherapy. Vaccination of mice with murine DCs pulsed with SFV replicons encoding IL-12 or IL-18 resulted in prolonged survival in a metastatic brain tumor model (316, 317), although the DCs were used primarily for adjuvant activity rather than for antigen presentation. The major limitation of current alphaviral vectors is their inefficiency at transducing DCs—an attribute that is likely due to type I IFN sensitivity rather than an inability to target DCs (250).

Other viruses. Vectors derived from human herpes simplex virus (HSV) can be engineered to encode large fragments of heterologous DNA and can efficiently transduce a variety of cells, including DCs (90% transduction at MOIs of 1-5). However, HSV infection inhibits maturation of DCs and is highly cytopathic (202, 253), thus limiting its utility as a viral

vector. Vectors derived from adeno-associated viruses (AAV) have also been used to transduce human mono-DCs, although the transduction efficiency varied widely (2-55%) at an MOI of 100. AAV-transduced DCs did not exhibit any functional deficits, and were capable of stimulating $CD4^+$ and $CD8^+$ T cells specific for vector-encoded tumor antigens *in vitro* (55, 289).

VENEZUELAN EQUINE ENCEPHALITIS VIRUS

Overview of Venezuelan equine encephalitis virus (VEE)

VEE is a member of *Togaviridae* family of viruses, and specifically falls within the alphavirus genus. VEE can be further classified as a New World alphavirus, which also includes Eastern and Western equine encephalitis viruses; these viruses have the potential to cause febrile disease and encephalitis in equines (i.e. horses, donkeys and mules) and humans. Old World alphaviruses include SIN, SFV, Ross River virus (RRV), Chikungunya virus, and O'nyong-nyong virus. Human infection with Old World alphaviruses is often characterized by fever, rashes and/or arthralgias. All alphaviruses are transmitted by arthropod vectors, and are thus capable of replicating in both vertebrate and invertebrate hosts (88).

VEE is normally an enzootic infection, cycling between mosquitoes and small rodents in subtropical regions of the North and South America. However, certain serotypes of VEE, namely I-AB and I-C, can cause epizootics/epidemics (305). Epizootic VEE infection of equines was initially described in the 1930s in northern South America, with the virus being first isolated in 1938 (17, 171). Although the ability of VEE to infect humans had been first reported in 1943 in two laboratory personnel working with the virus (43), the virus was not identified as a cause of human epidemics until the 1950s (256). From 1938 to 1972, epizootic/epidemic outbreaks of VEE occurred every 5-10 years throughout northern South America, Central America, Mexico and even the southern United States. During this time, the development of vaccines against VEE—first from formalin-inactivated virus, and then from a live-attenuated virus (TC-83)—significantly reduced epidemics amongst livestock

(305). No major outbreaks were reported after 1972 until 1992, when epizootic disease reappeared in western Venezuela (242). Not long after, one of the largest epizootic/epidemic outbreaks involving up to 100,000 people in Venezuela and Columbia occurred in 1995 (306). The ability to cause epizootic/epidemic disease, along with the potential to be used for biological warfare, has made VEE a focus of intensive research.

VEE structure and replication

VEE is an enveloped, single-stranded, positive-sense RNA virus. The genome of VEE is 11,477 nucleotides in length and contains a 5' methylguanosine cap and a 3' polyadenylated tail. The genome is divided into two regions: the 5' two-thirds encodes the nonstructural proteins (nsP1-4) involved with viral replication, while the 3' one-third encodes the structural proteins (capsid, E3, E2, 6K and E1) required for virion assembly (285). The RNA genome has significant secondary structure, and is encased in a T=4 icosahedral nucleocapsid consisting of 240 copies of the capsid protein (57). The nucleocapsid is surrounded by a lipid bilayer derived from the host cell plasma membrane, which also contains the viral glycoprotein spikes projecting outward from the viral core in a T=4 icosahedral symmetry (225). Recent cryoelectron micrographs of other alphaviruses indicate that the E1 glycoprotein lies tangential to the plasma membrane surface and forms an icosahedral scaffold, while the E2 glycoprotein predominantly forms the protruding spikes (181, 234).

No single receptor has been definitively identified for VEE. Because of its wide tropism for both vertebrate and invertebrate cells, it is likely that VEE either uses a ubiquitously expressed and evolutionarily conserved receptor, or uses multiple receptors for binding and entry. Some candidate receptors for VEE include the laminin-binding protein (187), heparin

sulfate (25) and c-type lectins (159). *In vitro* passage of VEE can lead to mutations in the E2 glycoproteins resulting in the use of heparin sulfate as a receptor (25, 66). While heparin sulfate-binding VEE exhibits enhanced viral binding and penetration *in vitro*, these viruses have reduced virulence *in vivo*, arguing against heparin sulfate as a natural receptor. Recent studies by Klimstra *et al.* have identified the c-type lectins DC-SIGN and L-SIGN as putative receptors for Sindbis virus (159)—an observation that is likely relevant for other alphaviruses. Carbohydrate modifications of the virus appeared to be important for binding to DC-SIGN, since virus produced in insect cells or mammalian cells defective in enzymatic glycosylation, and therefore lacking complex carbohydrates, exhibited higher infectivity of DC-SIGN-expressing cells. However, it was not clear if the enhanced infectivity of DC-SIGN-binding viruses was due to more rapid binding and internalization, or rather to enhanced replication through down-regulation of the antiviral response. The latter is supported by observations that binding of pathogens to DC-SIGN can inhibit DC activation (108).

After binding to its receptor on the cell surface, VEE is internalized into coated vesicles through an endocytic pathway. As the pH of the endosomes decreases, the E1 glycoprotein undergoes conformational changes resulting in fusion of the viral envelope with the endosome (181, 234, 285). While most of the knowledge concerning alphavirus entry was gleaned from studies of SIN and SVF, recent studies have suggested that VEE may enter cells somewhat differently. Using retrovirus pseudotyped with VEE envelope glycoproteins, Kolokoltsov *et al.* demonstrated that the viruses exited the endocytic pathway at the late-endosome stage and were resistant to cholesterol depletion (166). In contrast, SFV has been shown to exit early-endosomes in a process that is highly dependent upon cholesterol in the

host membrane (35). It is also possible that VEE may enter cells through a mechanism that is independent of membrane fusion and endocytosis, as recently demonstrated for SIN by Dennis Brown and colleagues (226).

Upon entry into the cytoplasm, the viral genome is uncoated from the nucleocapsid and is directly translated by host ribosomes into a polyprotein consisting of nsP1-3 or nsP1-4, depending on whether the ribosome reads through an opal stop codon located between nsP3 and nsP4 (285). The nonstructural proteins mediate the processes of viral replication and transcription, and possibly interact with cellular proteins to modulate host cell translation. nsP1 possesses guanine-7-methyltransferase and guanyltransferase activities, and is also involved with attachment of the viral replicase complex to cell membranes. nsP2 is a multifunctional protein, with helicase and NTPase activity in the N-terminus region and thiol protease activity in the C-terminus region; the latter is responsible for proteolytic processing of the nsP precursor protein. Interestingly, a significant proportion of nsP2 is found in the nucleus, where it can interact with host nuclear proteins and possibly modulate host cell transcription and/or translation (203). nsP3 is a phosphoprotein that has an essential but unknown role during viral replication. nsP4 is the core viral RNA-dependent RNA polymerase responsible for viral replication and transcription (285).

Following the initial translation of the viral genome, the nsP polyprotein is first cleaved in *cis* at the nsP3/4 site to yield a complex consisting of P123 and nsP4, which is thought to initiate minus-strand synthesis (268, 285). Minus-strand synthesis dominates during the first 3-4 hours following infection, but then becomes undetectable as positive-strand synthesis arises. The cessation of minus-strand synthesis is due to continued processing of the P123 into nsP1 and P23, and finally into all four individual nsPs (180). The individual nsPs, along

with several putative host factors, form the replication complex, which is thought to be confined to the cytoplasmic surface of endosome-derived vesicles known as cytopathic vacuoles type I (98). The replication complex produces new positive-sense genomic RNA by binding to a promoter in the minus-strand complementary to the 5' untranslated region of the genome. The replicase complex also drives transcription of a 26S subgenomic mRNA encoding the structural polyprotein downstream of a strong promoter located after nsP4. Subgenomic mRNA is made in significant excess to full-length genomic RNA, resulting in production of large amounts of capsid and viral glycoproteins (285). The capsid protein is self-cleaved from the structural polyprotein, while the PE2 (a precursor protein composed of E2 and E3) and E1 glycoproteins are proteolytically processed in the endoplasmic reticulum by host furin-like proteases. The processed E1 and E2 viral glycoproteins are transported through the Golgi complex to the plasma membrane, undergoing several posttranslational modifications during the voyage including glycosylation (161). As mentioned above, the type of carbohydrates added to the glycoproteins differs between vertebrate and invertebrate host cells, and may ultimately dictate tropism for the virus.

During virion assembly, the capsid protein specifically interacts with a packaging signal sequence in the nonstructural gene region, which results in selective encapsidation of full-length genomic RNA rather than subgenomic RNA (285). The nucleocapsid then interacts with the intracellular domain of E2 at the plasma membrane, resulting in envelopment of the nucleocapsid and budding from the cell. Budding involves binding of preformed nucleocapsid to the glycoproteins, resulting in the release of an infectious virion from the cell. (105). Infected mammalian cells eventually die by apoptosis, which is likely caused by

a combination of inhibition of host-cell translation and direct cytotoxic effects of viral structural proteins (88).

Pathogenesis of VEE

Infection of humans or equines with VEE results in a variety of clinical manifestations, ranging from fever to fatal encephalitis. Enzootic serotypes of VEE are typically avirulent in equines, causing little or no disease. In contrast, epizootic serotypes can cause significant mortality in equines (305). In humans, the severity of disease is most dependent upon the age of the patient and the serotype of the virus (81). After a 2-5 day incubation period, humans infected with VEE can present with fever, malaise, vomiting and severe retro-orbital headache. Symptoms typically subside within one week; although some patients may go on to develop neurological sequelae including convulsions, somnolence, confusion and coma. Death occurs in less than 1% of cases, and is often accompanied by meningoencephalitis, cerebral hemorrhage and/or necrotizing vasculitis (81, 88, 305). Interestingly, VEE causes a profound peripheral lymphopenia in humans and other mammals—a clinical sign often associated with other acute viral infections (81). This finding may result from the action of IFN-α/β, which can directly deplete lymphocytes (197) or induce lymphocyte retention in peripheral lymphoid tissues through a mechanism involving CD69 and sphingosine 1-phosphate receptor-1 (267).

While VEE can infect a range of animals, studies involving mice have proven most valuable for deciphering the pathogenesis of VEE. Pathological studies in the 1960s demonstrated that mice exhibit a two-phase disease following peripheral challenge with VEE: an initial phase of replication in peripheral lymphoid and myeloid tissue followed by a

neurotropic phase that eventually resulted in death by 6-7 days post-infection (pi) (110). More recent studies by Grieder *et al.* using a virus (V3000) derived from a molecular clone of an isolate from the original Trinidad Donkey strain have more thoroughly described VEE pathogenesis in mice (114). Following foot pad inoculation, V3000 replication was detected in the draining popliteal lymph node within 4 hours. By 12 hours pi, virus was isolated from serum and nearly every visceral organ, including, spleen, heart, lung, liver, kidney and adrenal glands; pancreas and thymus had detectable virus by 18 hours pi. Viral RNA was only detected in a subset of tissues (i.e. lymphoid tissue, heart and pancreas), suggesting that viral replication was primarily limited to specific cell types. Viral titers in the peripheral tissues peaked between 24-48 hours pi, and virus was eventually cleared from the periphery by 3-4 days pi. By this time, however, V3000 had already invaded the central nervous system (CNS), with virus first detected in the brain at 2-3 days pi. Penetration of the CNS likely occurs through infection of olfactory neuroepithelium or the trigeminal nerve (52). By 6 days pi, viral RNA was detectable throughout most of the brain and was accompanied by severe neuropathology. Infected animals uniformly died by 6-7 days pi. Grieder *et al.* and others also characterized the pathogenesis of several viruses with mutations in the E1 and/or E2 glycoproteins (6, 66, 114). These mutants all had reduced virulence upon footpad inoculation, and exhibited marked differences in viral dissemination, thus providing tools for further dissecting the mechanisms of VEE pathogenesis.

As mentioned above, VEE replicated in lymphoid tissues shortly after footpad inoculation. However, the identity of the originally infected cells was unknown. In 2000, MacDonald and Johnston used a double-promoter V3000 encoding GFP (dpV3000-GFP) and VEE replicon particles (VRP, see below) to demonstrate that the virus initially infected

DCs—specifically LC from the epidermis (190). At 12 hours following foot pad inoculation of dpV3000-GFP, GFP was detected in cells within the paracortex of the lymph nodes. These cells morphologically resembled DCs, and also expressed CD205 but were negative for the lymphocyte markers B220 and CD5. Inoculation of mice with VRP encoding GFP, which undergo only one round of replication, demonstrated that the initially infected cells had a LC-like morphology. While the cells expressed CD205 and MHC class II, they did not stain positive for the DC marker CD11c. Moreover, the authors did not evaluate the cells for expression of the LC marker CD207 (Langerin) or the presence of Birbeck granules. Finally, increasing the inoculum of VRP resulted in infection of $CD11c^+$ and $CD11b^+$ cells in the paracortex, suggesting that other DC populations may be targeted at higher viral loads. Based upon this study, a model for early VEE pathogenesis was proposed. Following the transfer of VEE to a mammalian host from a mosquito bite, the virus initially infects LC at the site of inoculation. Infected LC then migrate to the peripheral draining lymph node, where viral replication continues and eventually spreads to other lymphoid and non-lymphoid cells.

With regards to immune control of VEE infection, both the innate and adaptive immune systems are essential. Early studies in hamsters demonstrated that induction of type I IFN prior to challenge with virus precluded the establishment of infection, although sensitivity to IFN was dependent upon the viral strain (150). Mice genetically deficient in the receptor for IFN-α/β die within 24-48 hours after VEE infection, further underscoring the importance of the innate immune system in controlling early viral replication (115, 309). The adaptive immune response appears to be important for clearance of virus from the periphery through the production of VEE-specific IgM that is independent of T cell help (51). While antibody

responses are not sufficient to prevent neuroinvasion and death during primary infection with wild-type VEE, they are likely essential for protection following vaccination (88). The role of $CD8^+$ CTL in VEE immunity has not been extensively investigated, but a recent study failed to identify VEE-specific CTL in infected mice, suggesting that CTL activity may not be involved with viral clearance (154). Besides conferring protection against infection, the immune system can also potentiate disease by causing immunopathology in the CNS, as witnessed for SIN and SFV (86). While VEE induces some immunopathology, the virus can also cause lymphocyte-independent destruction of the CNS in SCID mice, although the pathology of disease resembles a spongiform encephalopathy rather than fulminant encephalitis (51).

VEE replicon particles (VRP)

The advent of reverse genetics allowed the development of stable cDNA clones of RNA viruses and facilitated their genetic manipulation. The generation of full-length cDNA clones of several alphaviruses has allowed the development of expression vectors for heterologous genes. This can be accomplished by one of two ways (298). The first method involves the generation of attenuated yet propagating "double-promoter" viruses, which contain a second 26S subgenomic promoter downstream of the native E1 gene that drives expression of a heterologous gene. Since these vectors can form new progeny, they result in sustained antigen expression. However, they also carry the risk of developing mutations or undergoing genetic recombination, which could result in the formation of wild-type virulent virus. Furthermore, these vectors will produce large amounts of structural proteins, which can increase the likelihood of anti-vector immunity and interfere with immunological

responses against the vector-encoded immunogen. The second method entails the development of non-propagating "replicon" vectors. Replicon vectors are created by replacing the structural genes with a heterologous gene, resulting in a replicon mRNA. If the structural genes are then supplied in *trans*, and modified so that they do not contain the *cis*-acting packaging sequences, then the replicon mRNA can be packaged into new virions termed replicon particles. These replicon particles can only undergo a single round of replication, since they lack the structural genes necessary for the synthesis of new virions. The benefit of this approach is that vector is incapable of producing new virus, which greatly enhances safety. However, antigen synthesis is transient since it will only be produced in the initially infected cell.

The development of alphaviral replicons was first described by Xiong *et al.* using SIN (315). Replacement of the structural genes with chloramphenicol acetyltransferase (CAT) resulted in high level production of heterologous protein following transfection of cells with *in vitro*-derived transcripts. Infection of the cells with Sindbis virus, which provided the structural genes in *trans*, resulted in the packaging of the CAT-encoding replicon RNA into infectious particles. Not long after, Liljestrom and Garoff described a procedure for generating SFV replicons using packaging-deficient helper RNAs encoding the structural genes (182). Additional studies demonstrated that vaccination of mice with recombinant SFV replicons encoding influenza nucleoprotein resulted in both cell-mediated and humoral immunity (327).

In 1997, Pushko *et al.* described a method for generating VEE replicon particles (VRP) from the attenuated viral mutant V3014 (238). Using a bipartite helper system in which the capsid and glycoproteins were expressed on different RNA constructs, Pushko *et al.*

demonstrated the production of high-titer VRPs with no detectable contamination of recombinant propagating virus. The authors also showed that vaccination of mice with VRP encoding influenza hemagglutinin (HA) induced a strong antibody response and conferred protection against mucosal challenge with influenza virus. Moreover, previous vaccination with a VRP encoding Lassa virus nucleocapsid did not interfere with anti-HA antibody responses generated by subsequent immunization with HA-VRP, suggesting that anti-vector immunity was not an issue with VRP vaccination in mice. It was argued that VEE replicons may be ideal vaccine vectors due to their ability to replicate in lymphoid tissues, thus providing antigen at the site of immune initiation. Furthermore, multiple attenuating mutations in the glycoproteins had been described, which could be incorporated into VRP for additional safety.

Subsequent studies demonstrated that like replication-competent VEE vectors, vaccination with recombinant VRP could induce mucosal IgA responses (121, 294) and CTL activity (302, 311) in mice. In addition, VRP have been shown to be superior to SIN replicons at inducing antigen-specific $CD8^+$ T cells in a head-to-head comparison (231). Furthermore, vaccination of non-human primates with recombinant VRP conferred partial or complete protection against SIV (65, 153) or Marburg virus (131), respectively. VRP have also been evaluated as vectors for tumor immunotherapy. Velders ***et al.*** demonstrated that vaccination with VRP encoding the human papillomavirus 16 (HPV16) *E7* gene (E7-VRP) induced antigen-specific CTLs (302). Prophylactic vaccination with E7-VRP protected mice from challenge with an E7-expressing tumor cell line. More importantly, therapeutic vaccination with E7-VRP completely inhibited the growth of established tumors. Interestingly, vaccination with irrelevant GFP-VRP also inhibited tumor growth, which demonstrated

potential adjuvant activity of VRP but also exposed the low threshold for induction of tumor immunity in this system. Tumor immunotherapy studies involving VRP encoding the *neu* oncogene have also been performed, and will be described in detail below.

IMMUNOTHERAPY AGAINST HER-2/NEU

Overview of HER-2/neu

The neu oncoprotein was originally discovered as a 185 kilodalton phosphoprotein expressed at high levels in several neuro/glioblastoma cell lines derived from ethylnitrosourea-treated rats (222, 264). The protein was shown to share homology with the retrovirus-associated oncoprotein v-erbB (derived from avian erythroblastosis virus locus B) and the epidermal growth factor (EGF) receptor (c-erbB-1) (59, 258). Molecular cloning of the *neu* cDNA demonstrated that the oncogene encoded a transmembrane protein that was closely related to the EGF receptor (14). The oncoprotein was thus named c-erbB-2, with the human form of the protein often referred to as HER-2 (derived from *H*uman *E*GF *R*eceptor).

HER-2/neu is one of four members of the erbB receptor family, which also includes the EGF receptor (HER-1/c-erbB-1), HER-3/c-erbB-3, and HER-4/c-erbB-4 (218). The erbB receptors are expressed primarily by cells of mesodermal or ectodermal origin. The four receptors share a common structure, consisting of an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic domain with tyrosine kinase activity. There are multiple ligands for the erbB receptors, which can be divided into four groups: ligands that only bind c-erbB-1 (i.e. EGF, transforming growth factor-α, and amphiregulin); ligands that bind either c-erbB-1 or c-erbB-4 (betacellulin, heparin-binding EGF, and epiregulin); ligands that bind only c-erbB-3 [neuregulin-1 and -2]; and ligands that bind only c-erbB-4 (neuregulin-3 and -4) (321). To date, no ligands specific for c-erbB-2 have been identified. However, c-erbB-2 has potent tyrosine kinase activity, and likely potentiates the

signaling of other ligand-bound erbB receptors by heterodimerization. Indeed, c-erbB-2 is the preferential heterodimerization partner for ligand-bound c-erbB-3 (113).

Signaling via the erbB receptors is highly complex and depends upon several factors, including the type of ligand bound, the specific erbB receptors expressed, the type of homo- or heterodimers formed, the specific adapter proteins associated with the receptors, and the rate of receptor endocytosis (321). In general, binding of ligand to the extracellular domain of an erbB receptor induces receptor homo- or heterodimerization, resulting in activation of the tyrosine kinase catalytic site and subsequent autophosphorylation of tyrosine residues in the cytoplasmic domain. The phosphorylated tyrosine residues act as docking sites for several adapter proteins that initiate intracellular signaling pathways, including the mitogen-activated protein kinase and the phosphatidylinositol-3-OH/Akt pathways. These pathways ultimately modulate expression of genes involved with cell survival, proliferation and migration (321). erbB signaling is also salient during development, as illustrated by the embryonic lethality of null mutations in all of the individual erbB receptors (218).

Shortly after its discovery, it was noted that the *neu* oncogene was amplified and overexpressed in various human tumor cell lines, suggesting that HER-2/neu may be involved with the development of cancer (99, 260). It was presumed that amplification of *neu* leads to increased HER-2/neu concentration on the cell surface, thus increasing the spontaneous formation of receptor homodimers. Transforming mutations in the transmembrane region that increase the spontaneous homodimerization of HER-2/neu have been identified in rodents (307); however, no similar mutations have been routinely recognized in cancer patients. Although recent studies have identified mutations in the tyrosine kinase domain of c-erbB-2 in a minority of patients with non-small cell lung cancer,

these mutations were not associated with amplification of the gene (283). Overexpression of HER-2/neu also increases heterodimerization with coexpressed erbB family members, which significantly augments the signaling capacity of these receptors and increases their tumorigenic potential (218).

The relationship between *neu* amplification and human breast cancer was first published in 1987 by Slamon *et al.* (273). The authors identified *neu* amplification as a negative prognostic factor for human breast cancer, which was subsequently confirmed in several large studies (249). Current data indicates that HER-2/neu is overexpressed in about 15-30% of all cases of breast adenocarcinoma (41). HER-2/neu is also overexpressed in several other types of malignancies including ovary, pancreas, gastric, kidney and lung cancer and multiforme gliomoblastoma (141).

Several transgenic mouse models have been developed for studying the role of neu in tumorigenesis. These transgenic mice have also proven invaluable for tumor vaccine studies, since they provide a model for evaluating vaccine efficacy under conditions of immunological tolerance. The first neu transgenic mouse was described by Muller *et al.* in 1988 (208). The authors developed transgenic mice (FVB/neu-T) on the FVB/N genetic background that expressed a mutated and constitutively activated form of rat neu (neu-transforming or neuT) under the murine mammary tumor virus (MMTV) promoter. Overexpression of the neu-T protein had a profound affect on tumorigenesis, as female FVB/neu-T mice uniformly and synchronously formed tumors in all mammary glands by 14 weeks of age. A year later, Bouchard *et al.* generated similar transgenic mice on a BALB/c background, with neu-T expression driven by the MMTV long terminal repeat (31). In contrast to the results of Muller *et al.*, these mice developed mammary tumors stochastically

between 5-10 months of age. In 1992, Guy *et al.* generated transgenic FVB/N mice that expressed the wild-type rat neu protein (neu-nontransforming or neu-N) under the MMTV promoter (120). Unlike FVB/N mice expressing neu-T, FVB/neu-N mice developed focal tumors after a long latency period. At one year of age, 30% of FVB/neu-N mice remained tumor-free, whereas all of the FVB/neu-T mice had developed tumors by 14 weeks of age. Subsequent studies demonstrated that tumor formation in FVB/neu-N mice was frequently associated with somatic mutations in the *neu* gene, resulting in increased tyrosine kinase activity and transforming potential (271). A neu transgenic mouse on the BALB/c background was characterized by Boggio *et al.* in 1998 (29). These mice were derived from a transgenic CD-1 male breeder that expressed neu-T under control of the MMTV long terminal repeat, thus resulting in accelerated tumor growth (185). Unlike FVB/neu-T mice, which only formed tumors in mammary tissue, BALB/neu-T mice form multifocal adenocarcinomas in breast tissue, salivary and Harderian glands and the epididymis by 33 weeks of age. Most recently, Piechocki *et al.* developed transgenic mice on the C57BL/6 background that expressed human c-erbB-2 under the whey acidic protein promoter (B6/HER-2) (232). B6/HER-2 transgenic mice did not develop spontaneous mammary tumors, but were immunologically tolerant to HER-2/neu, making them an acceptable model for evaluating HER-2/neu vaccines.

Strategies for HER-2/neu immunotherapy

Monoclonal antibody (mAb) therapy. Because HER-2/neu is overexpressed on the cell surface of many cancers, it appeared to be an ideal target for mAb therapy. Early studies using mAb specific for rat neu demonstrated that antibody treatment inhibited growth of neu-

overexpressing tumor cell lines both *in vitro* and *in vivo* (74, 75). Subsequent studies using anti-HER-2/neu mAb generated in mice showed similar results for human tumor cell lines, although the exact mechanism of action was unclear (139). Because murine antibodies are of little clinical use due to their immunogenicity in humans, a "humanized" version of a murine anti-neu antibody was created by cloning the antigen-binding domains of a murine IgG2a mAb into the human IgG1 backbone (42). Phase II clinical trials utilizing this recombinant antibody, which is referred to as trastuzumab (Herceptin), demonstrated that treatment of metastatic breast cancer patients with trastuzumab alone resulted in a 15% objective response rate (58). A phase III randomized clinical trial showed that when given with first-line chemotherapy, trastuzumab significantly increased the objective response rate and survival in patients with HER-2/neu-overexpressing metastatic breast cancer, although cardiac toxicity was an uncommon but serious side effect (274). Based on these studies, the United States Food and Drug Administration approved the use of trastuzumab either alone or in conjunction with chemotherapy for the treatment of metastatic breast cancer with HER-2/neu overexpression. While trastuzumab has shown promising results in the clinic, the overall objective response rate is relatively low and resistance to the drug frequently occurs after one year of treatment (210).

Peptide/protein immunization. While several studies demonstrated the anti-tumor effects of manufactured antibodies against HER-2/neu, it was not clear if HER-2/neu was normally immunogenic in cancer patients. An early study of breast cancer samples described a positive correlation between HER-2/neu amplification and lymphocyte infiltration, suggesting that cellular immunity against HER-2/neu may exist (292). The presence of anti-

neu immunity in cancer patients was first characterized by Disis *et al.* in 1994, who described the presence of neu-specific antibodies and $CD4^+$ T cells in patients with HER-2/neu^+ breast cancer (71). An important implication of this report was that immune responses against an overexpressed self antigen could exist in the absence of apparent autoimmunity, which supported the notion that self proteins could be successfully targeted for vaccination.

Induction of neu-specific T cell responses by vaccination could conceivably be accomplished with either whole protein or MHC-restricted peptides. An early study by Disis *et al.* reported that immunization with neu-derived peptides, but not whole protein, could overcome tolerance and induce T cell responses in rats (72). Thus, early efforts to vaccinate against HER-2/neu primarily employed immunogenic peptides. The first MHC class I-restricted peptide was identified in 1993 by Ioannides *et al.*, who described CTLs from ovarian cancer patients that recognized an HLA-A2-restricted epitope corresponding to amino acids 971-980 of HER-2/neu (149). Shortly thereafter, an immunodominant HLA-A2-restricted nonapeptide—E75 (amino acids 369-377)—was discovered (92). Since then, several more HER-2/neu-derived epitopes restricted to both MHC class I and II molecules have been identified (16).

Several clinical trials evaluating the immunogenicity of neu-derived peptides have been undertaken. While vaccination with HER-2/neu peptides was uniformly safe, induction of meaningful neu-specific T cell responses was typically absent. Zaks and Rosenberg showed that immunization of patients with E75 and incomplete Freund's adjuvant could induce E75-specific $CD8^+$ T cells, but these T cells failed to recognize neu-expressing tumor cells (324). Similarly, injection of E75 peptide with GM-CSF could induce E75-specific T cells, but the responses were short-lived and were undetectable by 5 months after vaccination (163).

Studies involving the addition of MHC class II-restricted T helper cell epitopes to the vaccine formulation have fared slightly better. Knutson *et al.* demonstrated that immunization with peptides containing both MHC class I and II epitopes resulted in the induction of neu-specific CTLs that could lyse neu-expressing tumors (164). Furthermore, neu-specific CTLs could be detected up to a year after vaccination. These clinical trials clearly demonstrate that vaccines containing HER-2/neu-derived peptides can elicit neu-specific T cell responses in cancer patients. However, no objective clinical responses in vaccinated patients have been reported to date (15).

As noted above, vaccination with whole protein failed to break tolerance against HER-2/neu. However, targeting neu protein to the MHC class I presentation pathway of professional APCs may help address the ineffectiveness of whole protein vaccination. Several strategies for targeting neu to APCs have been employed, including the incorporation of neu protein into hydrophobized polysaccharides complexes (119), the combination of neu with heat shock proteins (193), and the generation of chimeric CD152-neu molecules (243). These strategies have yet to be tested in the clinical setting.

Whole tumor cell vaccines. The use of neu-overexpressing tumor cells for inducing neu-specific immunity has been primarily restricted to preclinical animal models. In 1999, Cefai *et al.* characterized an allogeneic vaccine consisting of BALB/c fibroblasts transduced with the neu protein (47). This vaccine could prevent the formation of tumors in FVB/neu-T mice. Nanni *et al.* described the efficacy of an allogeneic neu-expressing tumor cell vaccine in BALB/neu-T mice (213). When combined with systemic injections of IL-12, the vaccine significantly inhibited the development of spontaneous tumors through an IFN-γ-dependent

mechanism. In 2000, Jaffee and colleagues described an irradiated neu-expressing 3T3 cell vaccine that had been transduced with GM-CSF (3T3neuGM). The vaccine was highly immunogenic in nontolerant syngeneic FVB/N mice, but was only slightly efficacious at inhibiting growth of adoptively transferred neu^+ tumor cells in tolerant FVB/neu-N transgenic mice (241). Subsequent studies have demonstrated that the efficacy of 3T3neuGM vaccines can be enhanced when combined with anti-neu mAb or chemotherapy (191, 312).

Although whole cell tumor vaccines have shown promising results in preclinical models, their actual use in the clinical setting is hampered by a frequent unavailability of autologous tumor cells (276). While allogeneic tumor cells can be used, they often lead to allospecific rather than tumor antigen-specific T cell responses. In addition, allogeneic tumor vaccines may lack neoantigens present in the patient's own tumor. Nonetheless, clinical trials using allogeneic tumor cells for vaccination against neu have been recently described (73). Allogeneic vaccines were well tolerated and even appeared to induce tumor-specific T cell responses in some instances, although it was not clear whether the T cell responses were directed against alloantigens or against neu. Furthermore, no clinical responses were observed.

DC vaccines. Both preclinical and clinical studies have evaluated the ability of DC vaccines to induce neu-specific immunity. Methods for loading DCs with antigen include pulsing with HER-2/neu-derived peptides (37, 67, 168, 261), viral transduction (54, 80, 201, 252, 330), and transfection with tumor-derived RNA (207).

Several preclinical evaluations of DCs loaded with HER-2/neu-derived peptides have been performed. In 2000, Serody *et al.* evaluated the immunogenicity of DCs pulsed with either wild-type or modified GP2 peptides (amino acids 654-662) in transgenic $A2K^b$ mice, which express a chimeric MHC class I molecule containing the α1 and α2 domains of human HLA-A2 and the α3 domain of murine $H2\text{-}K^b$ (261). The authors found that DCs presenting the modified GP2 induced better CTL responses in comparison to DCs presenting the wild-type peptide. The route of delivery of the DC vaccine did not seem to affect the magnitude of the immune response, but DC vaccines given intradermally resulted in more rapid induction of CTL activity. Interestingly, weekly DC vaccination appeared to diminish the overall CTL response, which could be avoided if the vaccine was given every three weeks. While this study yielded important data concerning the logistics of DC vaccination, it did not evaluate the activity of DC vaccines in tolerant mice. Recent studies by Lustgarten *et al.* examined the ability of peptide- or whole tumor-pulsed DCs to inhibit the growth of preexisting tumors in $A2K^b$ × FVB/neu-N mice, which are tolerant to neu (62, 188). Multiple DC vaccinations resulted in modest inhibition of tumor growth; this could be slightly enhanced with adjunct anti-OX40 antibody and IL-2 therapy.

Few virally-transduced DC vaccines have been studied in relevant animal models for neu immunotherapy. In 2001, Chen *et al.* described the vaccination of FVB/N mice with DCs transduced with adenovirus encoding neu (54). DC vaccination resulted in protection from challenge with neu^+ tumors and even suppressed growth of established tumors when given therapeutically. However, the authors did not test the efficacy of the vaccine in tolerant neu transgenic mice, although a recent study published in 2006 showed that prophylactic vaccination with adenovirus-transduced DCs slightly delayed the formation of spontaneous

tumors in FVB/neu-N transgenic mice (50). The most compelling data concerning the efficacy of virally-transduced DCs for neu immunotherapy was published by Sakai *et al.* (252). In this study, DCs from BALB/c mice were transduced with Ad encoding the extracellular-transmembrane domains of rat neu (neuET). Vaccination with Ad-transduced DCs delayed spontaneous tumor formation in BALB/neu-T mice and reduced the number of mammary tumors. Furthermore, vaccination induced appreciable anti-neu IgG responses, but only low-level $CD8^+$ T cell responses. Unfortunately, the authors did not assess vaccine efficacy when given therapeutically to mice with established tumors.

Human DCs have also been transduced with viral vectors encoding the HER-2/neu protein or derivative peptides. Transduction of CD34-DCs with retrovirus encoding HER-2/neu was only moderately efficient (~15%), but resulted in processing and presentation of HER-2/neu-derived epitopes to both $CD8^+$ CTL and $CD4^+$ T helper cells (330). Transduction of mono-DCs with recombinant influenza virus encoding the E75 epitope was also inefficient, yet the transduced DCs were capable of stimulating E75-specific CTL clones that exhibited some lytic activity against a HER-2/neu^+ tumor cell line (80).

The immunogenicity of DCs presenting epitopes from HER-2/neu has been evaluated in the clinical arena. In 2000, Brossart *et al.* vaccinated metastatic breast cancer patients with mono-DCs pulsed with either MUC-1- or HER-2/neu-derived peptides (37). Two of six patients receiving HER-2/neu peptide-pulsed DCs developed neu-specific $CD8^+$ T cells. However, no clinical responses were noted. In 2002, Kono *et al.* vaccinated gastric carcinoma patients with mono-DCs pulsed with E75 peptide alone (168). Six of nine patients developed neu-specific T cells, although cytolytic activity was only present in two of the responders. One of the patients who developed neu-specific T cells with cytolytic activity

had a partial clinical response. A more recent trial was published by Dees *et al.* in 2004 (67). In this study, patients were treated with CD34-DCs pulsed with either wild-type GP2 or an altered GP2 peptide with enhanced binding to HLA-A2. Two of ten patients developed modest GP2-specific $CD8^+$ T cell responses, but no cytolytic activity was measured. Interestingly, two separate patients exhibited partial clinical responses.

Recombinant viral vectors. The use of a recombinant virus expressing neu was first reported in 1987 by Bernards *et al.* (26). The authors used a VV vector encoding rat neu to induce protective anti-neu antibody responses in rats, resulting in protection from challenge with xenogeneic 3T3 cells expressing neu. Since then, other viral vectors encoding neu proteins or peptides have been evaluated primarily in nontolerant animal models.

In addition to their use for transduction of DCs (see above), recombinant Ad vectors have also been used to directly immunize animals. Palmer *et al.* described the efficacy of intratumor inoculation of Ad vectors encoding a kinase-dead mutant of rat neu in wild-type mice (224). The vector appeared to inhibit tumor growth through induction of neu-specific immunity and through direct cytolytic effects on tumor cells. Another study in 2005 demonstrated that xenogeneic vaccination with an Ad vector encoding neu slightly delayed tumor formation and decreased the mean number of tumors formed in BALB/neu-T mice (103). In that same year, Park *et al.* observed that multiple vaccinations with Ad encoding neuET significantly delayed autochthonous carcinomas in BALB/neu-T mice (228). However, vaccination had to be initiated early (7 weeks of age) for any observed efficacy. Interestingly, inhibition of tumor growth did not require $CD8^+$ T cells, but instead required T helper-dependent antibody responses. Another DNA viral vector evaluated for neu

immunotherapy was derived from murine polyomaviruses (293). A single vaccination with a recombinant polyomavirus vector encoding neuET significantly delayed spontaneous tumor formation in BALB/neu-T mice; however, the clinical relevance of murine polyomavirus is unclear.

VRP encoding rat *neu* (neu-VRP) have been evaluated in three different studies. The first use of VRP for neu immunotherapy was reported by Nelson *et al.* in 2003 (214). Using a rat animal model, the authors demonstrated that prophylactic vaccination with neu-VRP protected 50% of rats from challenge with a neu-expressing tumor cell line. Vaccinated rats also rejected a second tumor challenge, suggesting that neu-specific immunity was present. However, neu-specific T cell responses were not directly measured, and it was not clear if the tumor cell line used in the study was MHC-matched. A subsequent study by Lachman and colleagues demonstrated that therapeutic vaccination with neu-VRP alone was ineffective at inhibiting tumor growth in wild-type BALB/c mice (83). However, adjunct treatment with either doxorubicin or paclitaxel modesty inhibited tumor growth. A follow-up study in 2005 demonstrated that repeated vaccination of FVB/neu-T mice with neu-VRP prevented spontaneous tumor formation up to 240 days of age, whereas all control mice had developed tumors by 140 days of age (304). However, therapeutic vaccination of tumor-bearing mice was not evaluated.

Adoptive cell therapy (ACT). The adoptive transfer of tumor-specific T cells has been of great interest, since it is one of the few immunotherapeutic strategies that has induced tumor regression in patients with advanced disease (77). The isolation and *ex vivo* expansion of high-avidity T cells that maintain the capacity to home to tumors and exert effector function

have been generally difficult, although new methods based on the use of alternative lymphoproliferative cytokines such as IL-15 have been promising (106). To increase the repertoire of neu-specific lymphocytes, human T cells have been genetically modified to express anti-neu antibody receptors, which can function as artificial T cell receptors (233). In xenogeneic animal models, human T cells expressing membrane-bound anti-neu chimeric receptors migrated to tumor sites and exerted anti-tumor activity. However, genetic manipulation of T cells can potentially lead to cellular transformation, which may limit their clinical application.

Barriers to successful HER-2/neu immunotherapy

Despite the use of multiple vaccination strategies, the overall success rate for inducing clinically relevant immunity against HER-2/neu in cancer patients has been dismal. This may be surprising to some, since several preclinical studies (see above) have shown induction of robust immune responses against neu and even regression of neu-expressing tumors. However, most of these studies have been performed in nontolerant animal models, where the threshold for inducing tumor immunity is significantly low. In studies that did use tolerant mice, most vaccinations were performed in young mice prior to tumor development—a scenario that is far from the reality of the clinic, where most cancer patients are elderly, present with established disease, and have been extensively preconditioned with chemotherapy and radiation. For tumor immunotherapy to be successful in this patient population, a better understanding of the barriers to successful vaccination is required. A few of the known impediments are described below.

Tumor-specific inhibitory factors. There are many mechanisms by which malignant cells can interfere with tumor immunity, including down-regulation of MHC class I molecules or immunogenic antigens, secretion of anti-inflammatory mediators, and expression of cell surface molecules that can directly inhibit NK or T cells (157). Most of these strategies for immune evasion have been observed for HER-2/neu-overexpressing tumors. Loss of HER-2/neu expression is the most straightforward way for HER-2/neu$^+$ tumor cells to avoid immune recognition. However, because of the essential role of HER-2/neu-signaling in tumor growth and metastasis, it was thought that loss of HER-2/neu would result in "unfit" variants that contribute little to disease. Nonetheless, studies in mice treated with anti-neu antibodies identified HER-2/neu antigen-loss variants with malignant potential, suggesting that somatic mutations conferring HER-2/neu independency may arise (162). Down-regulation or loss of MHC class I expression has also been identified in patients and mice with HER-2/neu$^+$ tumors (184, 216). Interestingly, HER-2/neu itself may be directly involved, as induced expression of HER-2/neu in tumor cells resulted in the down-regulation of proteins involved with the MHC class I presentation pathway (130). HER-2/neu signaling can induce secretion of vascular endothelial growth factor (VEGF) (160), and HER-2/neu expression has been associated with production of indoleamine 2,3-dioxygenase (206); both can have potent inhibitory effects on immune cells. Finally, spontaneous tumors in neu transgenic mice were shown to express CD95L (FasL), which correlated with apoptosis of tumor-infiltrating T cells (46). In summary, multiple tumor-derived factors could conspire against successful neu-specific immunotherapy.

Paucity of HER-2/neu-specific effector T cells. Most self-reactive T cells are centrally deleted in the thymus through the process of negative selection. Yet it is clear that some self-reactive T cells can avoid thymic selection and escape into the periphery (217, 220). Fortunately (or unfortunately for tumor vaccinologists), the functional avidity of self-reactive T cells in the periphery is usually low, and these cells remain phenotypically naïve due to antigen ignorance (169). However, self-reactive T cells can potentially be converted into potent effector cells capable of causing autoimmune disease or tumor immunity—a process in which DCs likely play a pivotal role (186). It has thus been the goal of tumor vaccines to recruit the endogenous pool of self-reactive T cells for the elimination of cancer cells.

The ability to induce effective anti-neu T cell responses in mice and humans tolerant to HER-2/neu has been difficult. Tolerant neu transgenic mice generally lack high-avidity $CD8^+$ T cells specific for neu (85, 188). Vaccination alone does little to expand the repertoire of neu-specific T cells in these mice, although combination therapy with certain chemotherapeutic drugs can enhance the expansion of high-affinity T cells (84). $CD8^+$ T cells specific for the immunodominant epitope E75 have been identified in patients with HER-2/neu-overexpressing tumors, but these cells are of low frequency (27). While neu-specific $CD8^+$ T cells can be expanded *ex vivo* using peptide-pulsed APCs, they often lack the ability to recognize and kill HER-2/neu^+ tumors (163, 324). Some investigators have attempted to circumvent tolerance to HER-2/neu through the use of modified heteroclitic peptides (261, 303). This strategy may recruit cross-reactive T cells that escaped central deletion and would not be subject to the same regulation as normal neu-specific T cells. However, studies in melanoma patients have indicated that T cells specific for heteroclitic peptides are often deficient at recognizing native peptide presented by tumor cells (287).

In the event that neu-specific $CD8^+$ T cells with high avidity can be efficiently expanded by vaccination, this may still be insufficient for effective immunotherapy. While tumor-infiltrating lymphocytes are typically considered a positive prognostic factor, malignant lesions can continue to growth even in the presence of infiltrating tumor-specific $CD8^+$ T cells (205). Furthermore, a study by Rosernberg *et al.* showed that tumors can progress unabated in melanoma patients with large numbers of melanoma-specific $CD8^+$ T cells (247). These studies strongly suggest that immune inhibitory mechanisms will trump CTL responses in cancer patients, and therefore strategies that "inhibit the inhibitors" are needed.

Regulatory T cells. The identification of T cells that could suppress immune responses was first made by Gershon *et al.* in 1972 (109). Subsequent work by North and colleagues demonstrated that these cells could inhibit antitumor immunity in animals (24). However, the confusion regarding the existence of I-J specific T cells led to a decade with little work performed evaluating the function of these cells. In 1995, Sakaguchi and colleagues described the presence of CD4+ T cells that constitutively expressed CD25, the high-affinity IL-2 receptor, and could inhibit immune responses (251).

Regulatory $CD4^+$ T cells are thought to come in two basic varieties: "natural" cells that are purposely selected in the thymus, and "induced" cells that acquire suppressor function in the periphery. Natural regulatory T cells (T_{reg}) are characterized by expression of CD25 (the α chain of the IL-2 receptor) and the transcription factor FoxP3 (134). Other markers that are preferentially expressed on T_{reg} (but also on activated effector T cells) include CTLA-4, GITR, and Lag-3 (138). While CD25 may be expendable for the development of T_{reg} (96), FoxP3 is not, as humans and mice deficient in the *FoxP3* gene lack T_{reg} and quickly succumb

to a fatal lymphoproliferative disorder (38, 310). *In vitro*, T_{reg} appear to suppress effector cells indiscriminately through a cell-contact dependent mechanism. *In vivo*, though, T_{reg} may employ different mechanisms for suppression, such as the expression of IL-10 and/or TGF-β (18, 19). Induced regulatory T cells were originally described by Groux *et al.* in 1997 (117). These regulatory cells were referred to as T regulatory type 1 (T_R1) cells, and they appeared to arise from naïve $CD4^+$ T cells following stimulation in the presence of IL-10. T_R1 cells could inhibit effector T cells both *in vitro* and *in vivo* through the secretion of IL-10 and/or TGF-β (cells secreting only the latter are also referred to as T_H3 cells). Several types of induced IL-10-producing regulatory T cells distinct from the originally defined T_R1 cells have been described, and it may be more appropriate to refer to these cells collectively as IL-10-secreting regulatory T cells (126). However, the conventional term "T_R1" cell will be used hereafter.

Because regulatory T cells were involved with maintaining tolerance to self antigens in peripheral tissues, it was not long before they were implicated as negative regulators of tumor immunity. T_{reg} and T_R1 cells can be found in tumor-draining lymph nodes and at the tumor site in both mice and humans (63, 165, 183). Adoptive transfer studies in mice have shown that T_{reg} can inhibit tumor specific $CD8^+$ T cells, resulting in progressive tumor growth (4). Moreover, several studies have demonstrated that pretreatment of mice with anti-CD25 mAb improved immune responses against transplanted tumors (221, 266, 329). Other methods for inhibiting and/or depleting T_{reg} during tumor immunotherapy include treatment with cyclophosphamide (CY) (84) or anti-GITR antibodies (165).

T_{reg} can potentially inhibit immune responses against neu in both humans and mice. In 2004, Curiel *et al.* found that T_{reg} accumulated in the tumors and ascites of ovarian cancer

patients, many of whom were HER-2/neu-positive (63). T_{reg} from these cancer patients could inhibit HER-2/neu-specific $CD8^+$ T cells *in vitro*, although it was not shown that the T_{reg} were actually specific for HER-2/neu. In 2005, Ercolini *et al.* demonstrated that treatment of FVB/neu-N mice with CY prior to vaccination protected 10-20% of mice from subsequent challenge with neu^+ tumor cells (84). The enhanced protection was thought to be due to inhibition of T_{reg}, since *i*) CY treatment reduced the number of cycling $CD4^+CD25^+$ cells; and *ii*) adoptive transfer of $CD4^+CD25^+$ T cells into vaccinated mice abrogated protection. Furthermore, the depletion of T_{reg} was accompanied by an increase in neu-specific $CD8^+$ T cells with high-avidity. This suggests that high-avidity $CD8^+$ T cells specific for tumor antigens may escape negative selection in tolerant mice, but are actively suppressed in the periphery by T_{reg}. Most recently, Nair *et al.* observed increased infiltration of spontaneous tumors with $CD4^+FoxP3^+IL\text{-}10^+$ T cells following multiple vaccinations against neu, which was associated with decreased CTL activity by intratumor $CD8^+$ T cells (211). However, no depletion studies were performed to more definitively assess the role of T_{reg}. Overall, these findings suggest that T_{reg} are likely involved with inhibiting immunity against neu, although the extent of their role remains to be determined.

Dysfunctional or suppressive APCs. In addition to inhibiting the effector arms of the immune system, tumors can also dampen the initiation of immunity by suppressing the activity of APCs, namely macrophages and DCs (100). DCs found within the tumor microenvironment frequently exhibit functional deficits in maturation and antigen presentation (102, 297). Additionally, tumors can inhibit the development of DCs from myeloid precursors through the secretion of soluble factors such as VEGF (101). Tumors

can also cause the accumulation of myeloid precursors with suppressor function—referred to as immature myeloid cells (iMCs)—in bone marrow, lymphoid tissues or at the tumor itself (36, 323). In mice, iMCs are characterized by expression of CD11b and Gr-1 (Ly6G), whereas human iMCs express CD33; both lack expression of MHC class II (100). iMC can inhibit $CD8^+$ T cell activity through a cell-contact dependent mechanism that likely involves the release of reactive oxygen species (176). iMC at the tumor site have also been incriminated in the generation of T_{reg}/T_R1 cells (137), although other studies have failed to make this connection (175).

APC defects in humans and mice with neu-overexpressing tumors have been documented. Early pathological studies found a positive correlation between high-grade HER-2/neu^+ tumors and macrophage infiltration, suggesting that macrophages may facilitate tumor growth (237). More recently, tumor-associated macrophages expressing the inhibitory molecule B7-H4 from HER-2/neu^+ ovarian cancer patients have been shown to inhibit HER-2/neu-specific $CD8^+$ T cells *in vitro* (170). A study of BALB/neu-T mice with spontaneous tumors found a positive correlation between tumor multiplicity and the number of iMCs in the blood and spleen (198). Furthermore, iMC could suppress T cell proliferation in response to anti-CD3 antibodies and IL-2. The expansion of iMC appeared to be due to VEGF secretion by neu-expressing tumor cells, as transfer of iMC to tumor-free mice resulted in contraction of the population. Further studies are required to more definitely assess the role of iMC in humans and mice bearing neu-overexpressing tumors.

DISSERTATION OBJECTIVES

Because of their potent immunostimulatory capacity, several preclinical and clinical studies have evaluated the ability of DCs to induce immunity against HER-2/neu. Although DC vaccines have been uniformly safe and well tolerated, their overall efficacy at inducing therapeutic immunity in cancer patients has been disappointing. While the ineffectiveness of DC vaccines may be viewed as a deterrent for future use, it can be conversely argued that current peptide-pulsed DC vaccines are suboptimal, likely due to their limited secretion of immunostimulatory cytokines. Thus, novel strategies for improving antigen delivery to DCs as well as maximizing DC function are warranted. Transducing DCs with recombinant viral vectors may be an ideal approach for generating more potent DC vaccines. As mentioned previously, viral vectors can be used for highly efficient delivery of intact antigen to DCs, resulting in processing and presentation of both MHC class I- and II-restricted epitopes. Moreover, viral transduction can induce robust activation of DCs through TLR-dependent and -independent mechanisms. Finally, viral vectors may provide the persistent TLR stimulation necessary for overcoming T_{reg}-mediated suppression and thus breaking tolerance against tumor antigens.

While several viral vectors have been investigated for transduction of DCs, many are hampered by inefficient transduction efficiencies, potential interference with DC function, and questionable safety. Viral vectors derived from VEE may successfully address several of these limitations. VRPs have many appealing characteristics, including: *i*) a natural tropism for DCs (190); *ii*) high-level transgene production in infected cells (238); *iii*) the inability to produce progeny virions, thus preventing potentially dangerous spread of virus in vaccinated

hosts; *iv*) an efficient *in vitro* production system that allows packaging of replicons in distinct glycoprotein coats, which can confer specific tropism and additional safety (238); *v*) a lack of preexisting anti-vector immunity in the vast majority of patients outside of certain subtropical regions; *vi*) an impeccable safety record as demonstrated in scores of studies with rodents and non-human primates (65, 131); and *vii*) an established protocol for producing the vector by Good Manufacturing Practices, thus expediting its use in a Phase I clinical trial.

Vaccination with VRPs has proven highly effective at inducing both cellular and humoral immune responses (121, 238), and has even been shown to stimulate tumor immunity in some rodent models (214, 302). The *ex vivo* transduction of DCs with VRPs may further enhance anti-tumor immune responses, as this strategy can focus antigen production in the cells responsible for initiating immune responses and can circumvent potential issues concerning anti-vector immunity. Moreover, *ex vivo* transduction of DCs allows better control of DC quality, an important issue since endogenous DCs are frequently impaired in cancer patients. The ability of VRPs to transduce DCs *ex vivo*, as well as their potential as cancer vaccines is unknown. Therefore, the objectives of this dissertation are as follows:

1) To determine if VRPs can efficiently transduce human and murine DCs *ex vivo*, and characterize the resulting effects on DC phenotype and function.

2) To determine if VRP-transduced DC vaccines expressing a truncated neu oncoprotein can stimulate tumor-specific immunity *in vivo*.

3) To determine if VRP-transduced DC vaccines expressing a truncated neu oncoprotein can overcome tolerance in FVB/neu-N transgenic mice and consequently induce therapeutic tumor immunity.

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CHAPTER TWO

A NOVEL VIRAL SYSTEM FOR GENERATING ANTIGEN-SPECIFIC T CELLS[1]

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Abstract

Dendritic cell (DC)-based vaccines are increasingly used for the treatment of patients with malignancies. While these vaccines are typically safe, consistent and lasting generation of tumor-specific immunity has been rarely demonstrated. Improved methods for delivering tumor antigens to DCs and approaches for overcoming tolerance or immune suppression to self-antigens are critical for improving immunotherapy. Viral vectors may address both of these issues, as they can be used to deliver intact tumor antigens to DCs, and have been shown to inhibit the suppression mediated by regulatory T cells (T_{reg}). We have evaluated the potential use of VEE replicon particles (VRPs) for *in vitro* antigen delivery to human monocyte-derived DCs. VRPs efficiently transduced immature human DCs *in vitro*, with approximately 50% of immature DCs expressing a vector-driven antigen at 12 hours post-infection (hpi). VRP infection of immature DCs was superior to TNF-alpha treatment at inducing phenotypic maturation of DCs, and was comparable to LPS stimulation. Additionally, VRP-infected DC cultures secreted substantial amounts of the proinflammatory cytokines IL-6, TNF-alpha and IFN-alpha. Finally, DCs transduced with a VRP encoding the influenza matrix protein (FMP) stimulated 50% greater expansion of FMP-specific $CD8^+$ CTL when compared to TNF-alpha-matured DCs pulsed with an HLA-A*0201-restricted FMP peptide. Thus, VRPs can be used to deliver antigens to DCs resulting in potent stimulation of antigen-specific CTL. These findings provide the rationale for future studies evaluating the efficacy of VRP-transduced DCs for tumor immunotherapy.

Introduction

There is significant interest in the use of DC vaccines as treatments for patients with malignancies and chronic infectious diseases (20, 43). Following activation by inflammatory cytokines or microbial products, DCs possess several characteristics that are necessary for efficient stimulation of tumor-specific T lymphocytes, including enhanced homing to lymphoid tissues, high level expression of MHC class I- and II molecules in conjunction with costimulatory molecules, and secretion of immunostimulatory cytokines (3). The ability of DCs to prime tumor-specific T cell responses has been demonstrated in various animal models (7, 18, 34). These studies have led to several clinical trials evaluating the efficacy of DCs loaded *ex vivo* with tumor-associated antigens (TAAs) to initiate protective immune responses in cancer patients (2, 8, 10, 19, 28, 39, 49). Multiple techniques have been employed for loading DCs with TAAs including pulsing with MHC class I- and/or II-restricted peptides (41, 47, 51), incubation with tumor cell lysates (41), and electroporation with tumor cell RNA (23). Unfortunately, induction of measurable and durable anti-tumor T cell responses have been infrequent in most clinical trials, suggesting that the stimulatory capacity of current DC vaccines is inadequate (42). Therefore, alternative strategies for inducing optimal DC maturation and antigen presentation are warranted.

Viral vectors that encode TAAs may provide an alternative method for delivering antigens to DCs. Delivery of an entire TAA rather than TAA-derived peptides allows processing and presentation of multiple epitopes on both MHC class I and II molecules, resulting in a broader $CD8^+$ T cell response and incorporation of $CD4^+$ T cell help (56, 58). In contrast to MHC-restricted peptide vaccines, viral vectors can be used to transduce DCs of all MHC haplotypes. Viral vectors can induce DC maturation through both TLR-dependent and -

independent pathways, resulting in up-regulation of costimulatory molecules and secretion of Th1-inducing cytokines (1, 13). Additionally, viral vectors may provide stimuli that are required for overcoming tolerance against TAAs, specifically through the down-regulation of T_{reg} activity (57).

Several viral vectors have been utilized for transducing human DCs with TAAs (5, 14-16). While some of these vectors have entered clinical trials (12), their widespread use is hampered by inefficient transduction efficiencies, interference with DC function, and induction of anti-vector responses due to pre-existing immunity (25). Because of these limitations, we have evaluated the potential use of vectors derived from Venezuelan equine encephalitis virus (VEE) for transduction of human DCs. Nonpropagating VRPs possess intriguing characteristics including *1*) significant expression of the inserted gene in infected cells (45), *2*) induction of both cell-mediated and humoral immunity (6), *3*) potential for repeated immunizations without significant induction of anti-vector immune responses (45), and *4*) potential tropism for DCs. While our group has shown that VRPs can infect murine DCs *in vivo* (33), their capacity to transduce human DCs is unknown. In this report, we demonstrate that VRPs can infect human immature monocyte-derived DCs. VRP-transduced DCs can efficiently process and present VRP-encoded antigens, leading to robust proliferation of antigen-specific T cells and acquisition of effector function. Thus, vaccines consisting of VRP-transduced DCs may prove highly effective for the induction of tumor-specific $CD8^+$ T cells.

Materials and Methods

Antibodies and Reagents

PE-conjugated monoclonal antibodies specific for human CD8 (SK2), CD11c (B-LY6), CD14 (M5E2), CD40 (5C3), CD80 (L307.4), CD83 (HB15e) and HLA-DR (G46-6) were purchased from BD Pharmingen (San Diego, CA). Anti-human CD86-PE (HA5.2B7) was purchased from Beckman Coulter (San Diego, CA). Mouse anti-influenza A matrix protein (FMP) monoclonal antibody was purchased from Serotec (Raleigh, NC). All isotype control antibodies were purchased from BD Pharmingen. Recombinant human GM-CSF, IL-4, IL-2, IL-7 and TNF-α were purchased from Peprotech (Rocky Hill, NJ). Human AB serum (HABS) was purchased from Gemini Bioproducts (Woodland, CA).

Generation of Human Monocyte-Derived DCs

Peripheral blood was obtained from volunteer donors by venipuncture and diluted 1:2 with phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over lymphocyte separation medium (ICN Biomedicals, Aurora, OH), washed twice with PBS, and resuspended in serum-free AIM-V media (Invitrogen, Carlsbad, CA). Monocytes were enriched by culturing 10^7 PBMCs/well in 6-well tissue culture plates for 2 hours. Nonadherent PBMCs were removed and cryopreserved in 90% fetal bovine serum/10% DMSO. In experiments evaluating cytokine secretion, highly purified monocytes (>90% $CD14^+$) were obtained by immunodepletion of non-monocytic cells using the Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Monocytes isolated by either method were cultured at 37°C/5% CO_2 in complete AIM-V/10% HABS supplemented with GM-CSF (800U/ml) and IL-4

(500U/ml). Fresh cytokine was added on days 3 and 6 of culture. The cells were harvested on day 6 as immature DCs, or further matured for 24-48 hours with lipopolysaccharide (LPS) (0.1 - 1μg/ml) or for 48 hours with recombinant human TNF-α (20ng/ml) added daily. All of the clinical reagents were generated under protocols approved by the Committee for the Protection of the Rights of Human Subjects at the University of North Carolina School of Medicine.

Generation of Recombinant VRPs

The production of VRPs that encode the green fluorescent protein (GFP-VRP) has been previously described (33). The absence of propagating recombinant virus was confirmed by passage in BHK cells. VRPs were concentrated from supernatants by centrifugation through a 20% sucrose cushion and resuspended in PBS. Titration of GFP-VRPs was determined by infecting BHK monolayers with 10-fold dilutions of VRPs for 16-18 hours at 37°C/5% CO_2. The infected cells were fixed with 4% paraformaldehyde and GFP-expressing cells were directly visualized by fluorescent microscopy. VRPs that encode FMP (FMP-VRP) were generated by directionally cloning the FMP cDNA (kindly provided by P. Palese, Mt. Sinai School of Medicine, New York, NY) immediately downstream of the 26S mRNA promoter of the pVR21 replicon plasmid; proper orientation was confirmed by DNA sequencing. The FMP replicon plasmid was used to generate FMP-VRPs. For titration, BHK monolayers were infected with 10-fold dilutions of FMP-VRPs for 16-18 hours at 37°C/5% CO_2. Infected cells were fixed with ice-cold methanol and sequentially stained with mouse anti-FMP monoclonal antibody, biotinylated anti-mouse IgG, and FITC-conjugated streptavidin. FITC-positive cells were directly enumerated by fluorescent microscopy.

Infection of Human DCs with VRPs

Immature or mature DCs were resuspended in serum-free AIM-V at 0.5-1.0 × 10^6 cells/ml and seeded at 1-2 × 10^5 DCs per well in 24-well ultra low attachment plates (Corning Inc., Corning, NY). For infectivity experiments, 1-2 × 10^5 DCs were infected with VRPs at different MOIs over specific time intervals as indicated in the figure legends. Infections were performed in serum-free conditions at 37°C/5% CO_2. After 1-2 hours, DCs were washed with AIM-V/10% HABS, resuspended in media supplemented with GM-CSF (800U/ml) and IL-4 (500U/ml), and cultured in 24-well ultra low attachment plates at 37°C/5% CO_2.

Flow Cytometry Analysis

For quantification of VRP transduction efficiency, GFP-VRP- or mock-infected DCs were harvested at 6, 12 or 24 hpi and washed once with cold FACS buffer (PBS/0.5% human serum albumin). DCs were fixed with PBS/1% formaldehyde before FACS analysis. In some experiments, DC viability was determined using the Fixation and Dead Cell Discrimination Kit (Miltenyi Biotec) according to the manufacturer's instructions. For phenotypic analysis, 5 × 10^4 DCs were incubated with 200μg/ml mouse IgG (Sigma) at 4°C for 20 minutes. Following blocking, the DCs were stained with 2μl of PE-conjugated specific or isotype control antibodies for 30 minutes at 4°C, washed once with FACS buffer, and fixed with PBS/1% formaldehyde. FACS data was acquired using a FACScan flow cytometer (BD Biosciences, San Jose, CA), and analyzed using FlowJo software (Tree Star, Ashland, OR).

Cytokine Assays

For evaluation of cytokine secretion by DCs, immature DCs were either mock-infected or infected with GFP-VRPs (MOI = 20) for 2 hours at 37°C/5% CO_2. Fully mature DCs were generated by treatment for either 24 hours with LPS (100 ng/ml) or 48 hours with TNF-α (20 ng/ml). Mock-infected immature DCs, VRP-infected immature DCs, or fully mature DCs were washed and seeded into 96-well flat bottom tissue culture plates at 10^5 DCs/well. Supernatants were harvested at 12, 24, 36 or 48 hours post-treatment and stored at -80°C. Quantification of IL-6, IL-8, IL-10, IL-12p70, and TNF-α in the supernatants was performed using the cytometric bead array (CBA) according to the manufacturer's instructions (BD Pharmingen, Franklin Lakes NJ). Measurement of IFN-α was determined by ELISA (Biosource International, Camarillo CA) according to the manufacturer's instructions.

Allogeneic Mixed Leukocyte Reactions (MLR)

Mock- or GFP-VRP-infected (MOI = 10) DC cultures were harvested after 1 hour of infection, washed with media, and resuspended in AIM-V/10% HABS. Decreasing numbers of DCs were added in triplicate to 1×10^5 nonadherent allogeneic PBMCs per well in 96-well round bottom plates and T cell proliferation assays were performed as previously described (55).

In Vitro Expansion of FMP-Specific T Cells

Immature DCs from HLA-A*0201-positive donors were infected for 2 hours with either GFP-VRPs or FMP-VRPs (MOI = 10) and washed with AIM-V/10% HABS media. DCs

($0.2\text{-}2 \times 10^5$) were added to 2×10^6 autologous nonadherent PBMCs per well in 24-well tissue culture plates. For comparative stimulation of T cells with peptide-pulsed DCs, TNF-α-matured or LPS-matured DCs from the same donors were incubated with 10 μg/ml of FMP peptide in AIM-V/10% HABS for 2 hours. FMP peptide-pulsed DCs were washed with media and added to autologous nonadherent PBMCs as described above. PBMCs were incubated for 7 days in AIMV/10% HABS supplemented with IL-2 (20U/ml) and IL-7 (10ng/ml). Fresh cytokine was added on days 3 and 6 of culture, and cell density was maintained at $< 2 \times 10^6$ cells/ml during the entire assay. On day 7, the responders were harvested and evaluated for either antigen-specific expansion by tetramer staining, or for specific lysis of peptide-pulsed T2 cells by a conventional ^{51}Cr release assay (48). Percent specific lysis was determined using the following formula:

Percent specific lysis = $100 \times$ [(sample cpm - spontaneous cpm) / (total cpm - spontaneous cpm)]

For tetramer staining, 1×10^6 responders were stained for 30 minutes with 20 μl of anti-human CD8-FITC (Pharmingen) and 10 μl of either PE-conjugated HLA-A*0201/Influenza M1 peptide tetramer or HLA-A2*0201/Negative tetramer (Beckman Coulter). Cells were washed with PBS, fixed with PBS/0.5% formaldehyde, and analyzed by FACS within 6 hours.

Statistical Analysis

Statistical differences were calculated using a Student's t-test when sample data distribution was parametric. Sample data that exhibited nonparametric distribution were evaluated using a Mann-Whitney rank sum test. Differences in costimulatory molecule expression between mock- and VRP-infected DCs from several donors were analyzed using a Wilcoxon signed rank test. P values ≤ 0.05 were considered significant. All statistical analyses were performed with SigmaStat 3.0 software (Port Richmond, CA).

Results

VRPs Can Efficiently Transduce Human Immature DCs

Our group has previously demonstrated that VRPs can infect mouse DCs *in vivo* following foot pad injection (33). To determine if human DCs could be infected with VRPs *in vitro*, immature monocyte-derived DCs were generated from normal donors. By day 6 of culture, DCs exhibited a typical immature phenotype ($CD11c^+$, $HLA\text{-}DR^+$, $CD86^+$, $CD14^-$, $CD40^-$, $CD80^-$) when evaluated by flow cytometry (data not shown). Immature day 6 DCs were infected with GFP-VRPs at an MOI of 10. GFP expression in DCs was first detectable at around 4 hpi, and reached a maximum between 6-12 hpi (Figure 1A).

To quantify VRP transduction efficiency, mock- or GFP-VRP-infected (MOI = 10) immature DCs were harvested at 6, 12 or 24 hpi and analyzed for GFP expression by flow cytometry. As shown in Figure 1B, VRPs could infect human immature DCs at an MOI of 10, with a mean of 10.8% expressing GFP by 6 hpi. The mean percentage of GFP-positive immature DCs peaked at 18.4% at 12 hpi and then decreased to 15.5% by 24 hpi. To determine if the maturation status of the DCs affected the transduction efficiency, immature DCs were stimulated with LPS for two days, resulting in marked up-regulation of CD40, CD80, CD83 and CD86 (data not shown). LPS-matured DCs were minimally transduced by GFP-VRPs (Figure 1B). DCs matured with TNF-α for two days were also less susceptible to VRP infection (mean 6.2% GFP-positive at 12 hpi), although not to the same degree as found using LPS-matured DCs. Thus, VRP transduction efficiency was inversely related to the degree of DC maturation.

In order to verify that the GFP-positive cells exhibited a DC phenotype, we performed two-color FACS analysis on infected DC cultures. GFP-VRP-infected DCs were harvested

at 24 hpi and stained with PE-conjugated antibodies specific for CD11c, HLA-DR and CD14. GFP-positive cells expressed high levels of CD11c and HLA-DR, and did not express the monocyte-marker CD14 (Figure 1C). To demonstrate that VRPs specifically infected immature DCs, the ability of VRPs to transduce peripheral blood T cells, B cells and monocytes was determined. We did not observe infection of $CD3^+$ T cells and $CD19^+$ B cells with GFP-VRPs (MOI = 10), and only minimal (~2%) transduction of $CD14^+$ monocytes (data not shown). Thus, VRPs specifically infected immature DCs.

We next evaluated approaches that could enhance the efficiency of VRP transduction of immature DCs. Increasing the MOI improved the transduction of immature DCs by GFP-VRPs. At an MOI of 100, approximately 50% of DCs expressed GFP (Figure 2A). The percent of GFP-positive DCs began to plateau between an MOI of 50 and 100, suggesting that transduction efficiency was near maximal. In an effort to maximize transduction efficiency at a lower MOI, we increased the duration of infection and the DC concentration during infection. By doubling both the time of infection and the DC concentration during infection at an MOI of 20, the transduction efficiency increased from a mean of 22.5% to 37.0% ($n = 3$, $p = 0.002$) (Figure 2B). Thus, immature DCs can be efficiently transduced with relatively small quantities of VRPs.

The percentage of GFP-expressing DCs began to decline between 12 and 24 hpi (Figure 1B), suggesting that VRP infection may be cytopathic to human DCs. Alphaviruses and alphaviral vectors induce apoptosis in cultured cells (31), although their ability to similarly induce cell death in human DCs is unknown. We therefore compared the viability of VRP-infected (GFP-positive) DCs to uninfected (GFP-negative) DCs in the culture by exclusion of a vital dye. VRP-infected DCs exhibited >90% viability between 6-12 hpi, and remained

~75% viable at 24 hpi (Figure 3). However, by 48 hpi only 26% of the DCs remained viable compared to 66% of the uninfected DCs. This loss in viability was associated with increased expression of annexin-V by VRP-infected DCs (data not shown), suggesting that VRP-induced apoptosis was likely responsible for the death of human DCs. In summary, the viability of VRP-transduced DCs remained high for 24 hours following infection, but steadily decreased between 24-72 hpi.

VRP Infection Induces DC Maturation and Secretion of Proinflammatory Cytokines

The studies described above indicated that immature DCs could be easily transduced with VRPs. However, immature DCs are poor stimulators of antigen-specific T cells and have been shown to induce tolerance (11, 22). Thus, we wanted to determine if VRP-infection induced maturation of immature DCs by evaluating expression of costimulatory and maturation surface markers (Figure 4). At 12 hpi, the expression of various costimulatory/maturation markers in VRP-infected DC cultures was similar to DCs that were mock-infected or treated with TNF-α. In contrast, DCs treated with a strong maturation stimulus (100 ng/ml of LPS) had upregulated CD80 and CD86 expression at this time. By 24 hpi, however, the expression of CD40, CD80 and CD86 was significantly elevated in VRP-infected DC cultures when compared to mock-infected or TNF-α-treated DCs. CD86 expression in VRP-infected DC cultures at 24 hpi was comparable to that seen with LPS treatment, while LPS induced higher levels of CD80 and CD83. Interestingly, VRP-infection induced higher levels of CD40 expression when compared to LPS treatment, a trend that was consistent in four different experiments. We next determined if VRP infection induced maturation of both infected and uninfected bystander DCs by analyzing

costimulatory/maturation marker expression on GFP-positive and -negative DCs in the culture (Table 1). The expression of costimulatory/maturation molecules was increased on both GFP-positive and GFP-negative DCs, although the latter exhibited the highest expression levels at 24 hpi. These observations indicate that VRP infection resulted in phenotypic maturation of both infected and uninfected immature DCs within the same culture.

We next evaluated secretion of proinflammatory cytokines by GFP-VRP-infected DC cultures (Figure 5). Because DC cultures generated from adherent PBMCs contained a small but significant population of contaminating lymphocytes (15-45%), we generated highly purified DCs from monocytes that had been isolated by negative selection using immunomagnetic beads. DC cultures generated by this method were >95% $CD11c^+$, and were similar to adherent monocyte-derived DCs in both surface marker phenotype and susceptibility to VRP infection (data not shown). These DCs were mock- or VRP-infected and supernatants were collected and assayed for proinflammatory cytokines at various time points post-infection. In contrast to mock-infected DCs, VRP-infected immature DCs secreted significant amounts of TNF-α, IL-6 and IFN-α at 24 to 48 hours following infection. Low but statistically significant levels of IL-12p70 were detected at later time points (36-48 hpi). IL-10 was also barely detectable in the supernatants from VRP-infected DCs, but the levels were not significantly higher than in mock-infected DC supernatants. By comparison, DCs that had been matured by either 24 hours with LPS or 48 hours with TNF-α did not secrete significant amounts of TNF-α, IL-6 and IFN-α. LPS- and TNF-α-matured DCs secreted IL-8 (Figure 5) and displayed increased costimulatory molecule expression (Figure 4), demonstrating that these cells had been activated. Additionally, incubating mature DCs

with an MHC class I-restricted peptide (FMP peptide 58-66) did not affect cytokine secretion (data not shown). In summary, VRP infection of immature human DCs induced maturation and proinflammatory cytokine secretion.

VRP-Infected Human DCs Can Stimulate Allogeneic and Antigen-Specific T Cells

To initially evaluate the functionality of VRP-infected DC cultures, we performed a standard allospecific T cell stimulation assay. DCs infected with GFP-VRP stimulated substantial proliferation of allogeneic T cells, indicating that VRP infection did not have a detrimental effect on DC function (data not shown). More importantly, we determined if VRP-transduced DCs could stimulate expansion of autologous T cells specific for a VRP-encoded antigen. For this set of experiments, we utilized recombinant VRPs expressing FMP. When autologous PBMCs were stimulated with an irrelevant VRP expressing GFP, there was no significant increase in the percentage of FMP-specific $CD8^+$ T cells (Figures 6A-B). However, stimulation of PBMCs with FMP-VRP-transduced DCs led to a significant increase in the percentage of FMP-specific $CD8^+$ T cells (Figures 6A-B). VRP-transduced DCs were highly efficient at expanding FMP-specific $CD8^+$ T cells at even low DC numbers (Figure 6C). Furthermore, the expanded FMP-specific $CD8^+$ T cells were functional as they could lyse T2 cells pulsed with the FMP peptide (Figure 6D). When we compared FMP-VRP-transduced DCs to TNF-α-matured DCs pulsed with FMP peptide, we found that FMP-VRPs were significantly more effective at inducing expansion of FMP-specific $CD8^+$ T cells compared to FMP-pulsed DCs (Figures 6A-B). However, peptide-pulsed DCs matured with a more potent stimulus (100 ng/ml of LPS) induced comparable expansion of FMP-specific $CD8^+$ T cells when compared to FMP-VRP-infected DCs (42% and 38% tetramer-positive

cells, respectively, responder:stimulator ratio = 10:1). VRP-infected DC can thus process and present vector-encoded antigens to reactive T cells, resulting in significant T cell expansion and acquisition of effector function.

Discussion

Consistent generation of tumor-specific T cell responses in patients treated with DC-based vaccines has remained elusive. The shortcomings of current vaccines are due in part to inefficient antigen loading of DC, as well as unsatisfactory induction of DC maturation and proinflammatory cytokine secretion (35). To address these issues, we evaluated the utility of vectors derived from VEE. We have demonstrated that VRPs can efficiently transduce human immature DCs *in vitro*, leading to DC maturation and secretion of proinflammatory cytokines. Furthermore, transduced DCs processed and presented a VRP-encoded antigen and stimulated significantly greater expansion of antigen-specific T cells in comparison to peptide-pulsed DCs matured with TNF-α.

Several vectors have been used for transducing human DCs with TAAs (5, 14-16). However, the potential clinical use of these vectors is hindered by poor transduction efficiencies, inhibition of DC maturation, questionable safety, and induction of detrimental anti-vector immune responses. The use of VRPs for DC transduction successfully addresses many of these concerns. We have shown that VRP transduction efficiency is appreciable at an MOI of 20; efficiency can be further enhanced at higher MOIs. VRPs have an outstanding safety record in thousands of animal experiments including both rodents and primates (9, 45). Because VEE is only endemic to specific subtropical regions, pre-existing immunity to VRPs is unlikely to be present in the majority of patients. Finally, our group and others have shown that VRPs can induce cell-mediated and humoral immune responses (6, 53). Recent work suggests that induction of both a humoral and cellular anti-tumor response may increase the effectiveness of tumor vaccines (40).

One concern from our data was the cytopathic effect of VRPs on human DCs. After terminal maturation, DCs remain viable *in vitro* for approximately 96 hours (54). However, we found that VRP-infected DCs began to lose viability within 48 hours following infection. The shortened life-span of VRP-transduced DCs could limit their effectiveness in tumor immunotherapy. However, studies have shown that DCs injected intradermally migrate rapidly (within 12-24 hours) to draining lymph nodes (37). Additionally, several groups have found that DC-T cell interactions *in vivo* occur during the first 24 hours following immunization (24, 36). As shown, the majority of VRP-infected DCs are viable during the first 24 hours following infection, which should allow sufficient time for infected DCs to migrate to regional lymph nodes and interact with T cells. Furthermore, the induction of apoptosis in transduced DCs may actually be advantageous since cross-presentation of antigen from apoptotic DCs can effectively induce antigen-specific $CD8^+$ T cells (30, 38, 46). Indeed, apoptosis was necessary for the enhanced efficacy of alphaviral replicase-based DNA vaccines in an *in vivo* tumor challenge model (30). Our group has preliminary evidence that VRP-infected DCs can generate protective immunity in a tolerant breast cancer animal model (Moran et al, unpublished observations). Thus, we do not believe that the shortened life-span of VRP-infected DCs should provide a significant impediment to their use *in vivo*.

VRP infection not only resulted in production of TAAs within the cytoplasm of human DCs, but also induced maturation of DCs and proinflammatory cytokine secretion. VRP infection resulted in up-regulation of the costimulatory molecules CD40, CD80 and CD86, and the maturation marker CD83. This is consistent with previous observations that replicons derived from Sindbis virus, a related alphavirus, induced maturation of human DCs (21). Interestingly, costimulatory/maturation marker expression was induced on both

infected and uninfected DCs within the same cultures. VRP-induced maturation of bystander DCs is potentially advantageous, as this could enhance cross-presentation of antigen from infected DCs undergoing apoptosis. In addition to phenotypic maturation, VRP infection resulted in secretion of proinflammatory cytokines including IFN-α, TNF-α, IL-6 and IL-12p70. IFN-α, TNF-α and IL-6 are important for activation of APCs, and are likely responsible for maturation of uninfected bystander DCs (26, 32). IFN-α also enhances the efficiency of cross-presentation of antigen by DCs (29), a mechanism that is important for *in vivo* priming of tumor-specific $CD8^+$ CTL (50). Furthermore, recent studies suggest that IL-6 secretion by DCs is important for inhibiting T_{reg} activity (17, 44). In contrast to VRP-infected DCs, DCs that had been terminally matured with TNF-α or LPS did not secrete significant levels of IFN-α, TNF-α, IL-6 or IL-12p70. This observation is in line with other publications describing the inability of fully matured DCs to secrete several proinflammatory cytokines (27, 52). In summary, VRPs represent a novel strategy to deliver TAAs and a strong maturation signal simultaneously to human DCs.

In the current study, we have shown that VRP-infected DCs could efficiently stimulate antigen-specific T cell responses against a VRP-encoded antigen. Furthermore, VRP-infected DCs were more efficient at expanding antigen specific $CD8^+$ T cells when compared to TNF-α-matured DCs pulsed with an MHC class I-restricted peptide. Increased costimulatory molecule expression and secretion of proinflammatory cytokines are likely responsible for the enhanced immunostimulatory capacity of VRP-infected DCs. It is also possible that VRP-transduced DCs are presenting MHC class II-restricted epitopes to $CD4^+$ T helper cells in the PBMC cultures, which would augment activation and expansion of CTL

(4). The ability of VRP-transduced DCs to stimulate $CD4^+$ T cells is under current investigation.

In conclusion, we have found that immature DCs can be readily transduced with VRPs and these DCs can induce potent antigen-specific T cell expansion. Our group is currently pursuing this strategy for vaccination of patients with cancer.

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Table 2-1

	Mock-Infected DC Cultures	VRP-Infected DC Cultures	
		GFP-positive DCs	GFP-negative DCs
CD40	4.0 (2.6 – 12.8)	6.0 (2.6 – 14.4)[a]	12.4 (4.2 – 26.1)[b]
CD80	4.1 (2.9 – 8.8)	6.4 (3.0 – 14.9)[a]	10.9 (4.2 – 18.2)[b]
CD83	3.6 (1.7 – 9.3)	5.8 (3.5 – 7.7)[a]	7.6 (3.6 – 12.5)[b]
CD86	268.5 (64.0 – 605.0)	355.5 (63.7 – 554.0)	556.5 (83.5 – 823.0)[b]

Median fluorescence intensity (MFI) of costimulatory/maturation markers following VRP infection of DCs. DC cultures were mock-infected or infected with GFP-VRP (MOI = 10-20) and evaluated 24 hours later for the expression of costimulatory/maturation markers with PE-conjugated antibodies. Numbers represent the median PE fluorescence from eight donors (minimum – maximum). In VRP-infected cultures, the MFI for infected and uninfected DCs was determined by gating on GFP-positive and GFP-negative DCs, respectively. [a]$p < 0.05$ compared to mock-infected DCs (Wilcoxon sign rank test); [b]$p < 0.01$ compared to mockin-infected DCs.

Figure 2-1

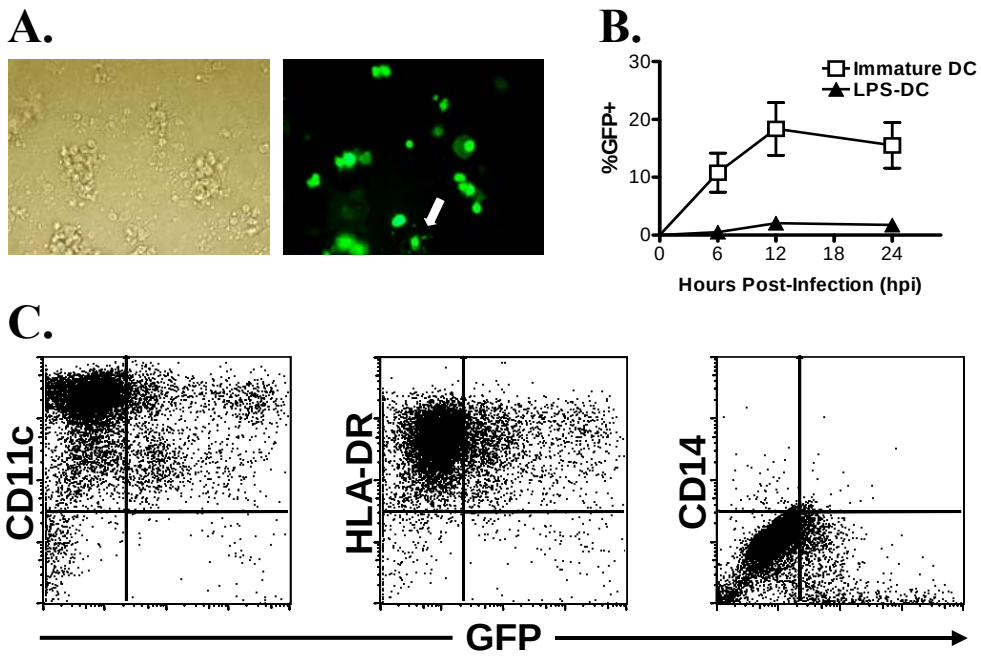
A.
B.
Immature DC
LPS-DC
%GFP+
30
20
10
0
0
6
12
18
24
Hours Post-Infection (hpi)
C.
CD11c
HLA-DR
CD14
GFP

Figure 2-1: VRPs can efficiently transduce immature human DCs. *A*) Human immature monocyte-derived DCs were infected with GFP-VRPs for 1 hour (MOI = 10). Cells were analyzed for GFP expression at 12 hpi by fluorescent microscopy. The white arrow indicates possible apoptosis of infected DCs. *B*) Infectivity of immature or LPS-matured DCs (n = 3 donors) was quantified by FACS. DCs were infected for 1 hour (MOI = 10) and GFP expression was evaluated at 6-24 hpi as indicated. Symbols represent mean percentage of GFP-positive DCs +/- standard error of the mean (SEM). *C*) DCs were infected with GFP-VRP as described above, harvested at 24 hpi, stained with PE-conjugated antibodies specific for CD11c, HLA-DR or CD14, and analyzed by two-color FACS.

Figure 2-2

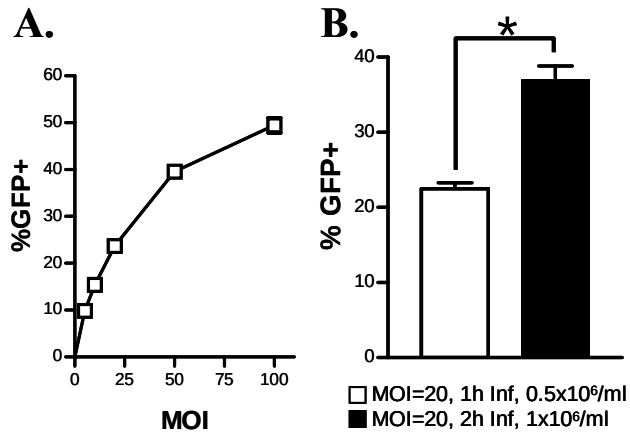
A.
%GFP+
60
50
40
30
20
10
0
0
25
50
75
100
MOI
B.
% GFP+
40
30
20
10
0
*
MOI=20, 1h Inf, 0.5x10⁶/ml
MOI=20, 2h Inf, 1x10⁶/ml

Figure 2-2: VRP infection of immature DCs is dependent upon the MOI, length of infection and cell density during infection. *A*) Immature DCs (n = 3 donors) were infected for 1 hour with GFP-VRPs at increasing MOIs. The percentage of GFP-positive cells was determined at 12 hpi by FACS. *B*) Immature DCs (n = 3 donors) were infected with GFP-VRPs (MOI = 20) for 1 hour at 0.5 x 10^6 DC/ml, or for 2 hours at 1 x 10^6 DC/ml. The percentage of GFP-positive cells was determined at 12 hpi by FACS. Graphs represent the mean percentage of GFP-positive cells +/- SEM. *p = 0.002, Student's t test

Figure 2-3

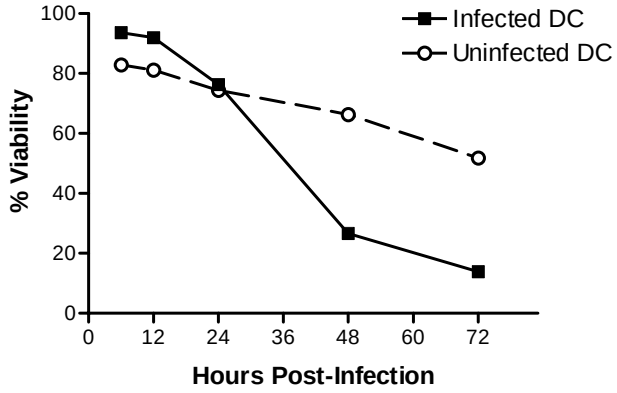
Infected DC
Uninfected DC
% Viability
100
80
60
40
20
0
0
12
24
36
48
60
72
Hours Post-Infection

Figure 2-3: VRP-infected DCs remain predominantly viable during the first 24 hours following infection. Immature DCs were infected for 2 hours with GFP-VRP (MOI = 20). At various times post-infection, viability of infected (GFP-positive) DCs and uninfected (GFP-negative) DCs was determined as described in *Materials and Methods*. Graphs represent the mean percentage of viable cells from two experiments.

Figure 2-4

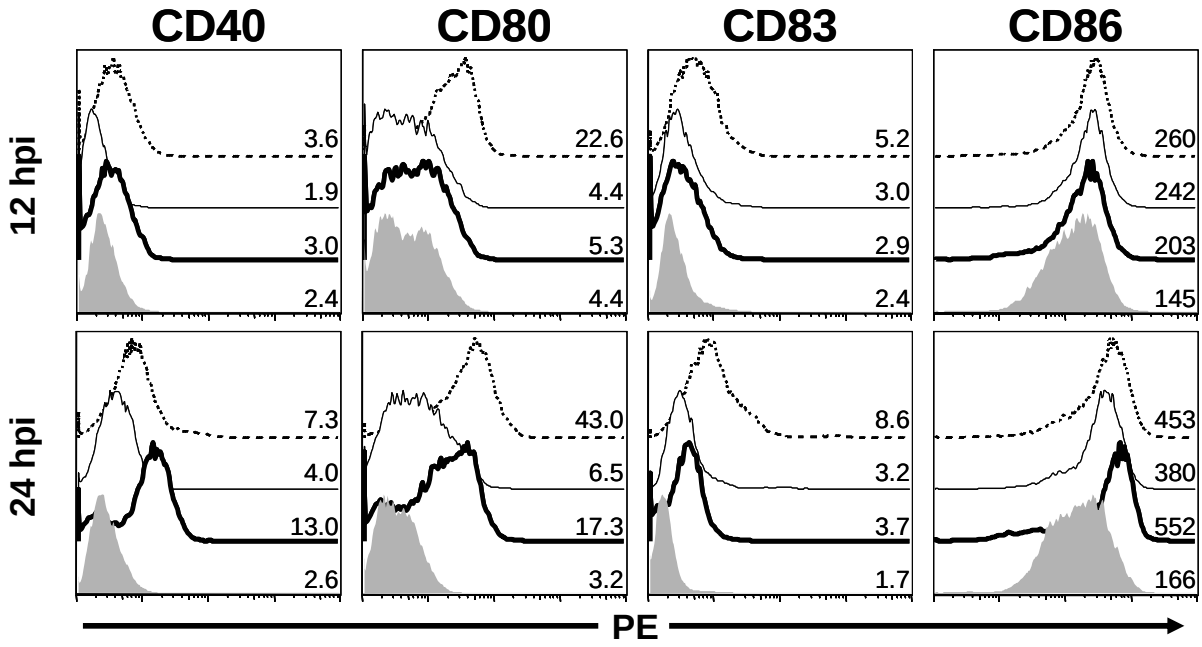
CD40
CD80
CD83
CD86
12 hpi
24 hpi
3.6
1.9
3.0
2.4
22.6
4.4
5.3
4.4
5.2
3.0
2.9
2.4
260
242
203
145
7.3
4.0
13.0
2.6
43.0
6.5
17.3
3.2
8.6
3.2
3.7
1.7
453
380
552
166
PE

Figure 2-4: VRP infection induces maturation of immature DCs. Immature DCs were either mock-infected (gray histogram), infected for 2 hours with GFP-VRPs at an MOI of 20 (heavy line), treated with TNF-α at 20 ng/ml (thin line), or treated with LPS at 100 ng/ml (dashed line). DCs were harvested at 12 or 24 hpi and stained with the indicated PE-conjugated specific antibodies. Staining with isotype control antibodies was negative. The numbers indicate the median PE fluorescence intensity. The median costimulatory/maturation marker expression in VRP-infected DC cultures includes both GFP-positive and -negative cells. Data is representative of four experiments.

Figure 2-5

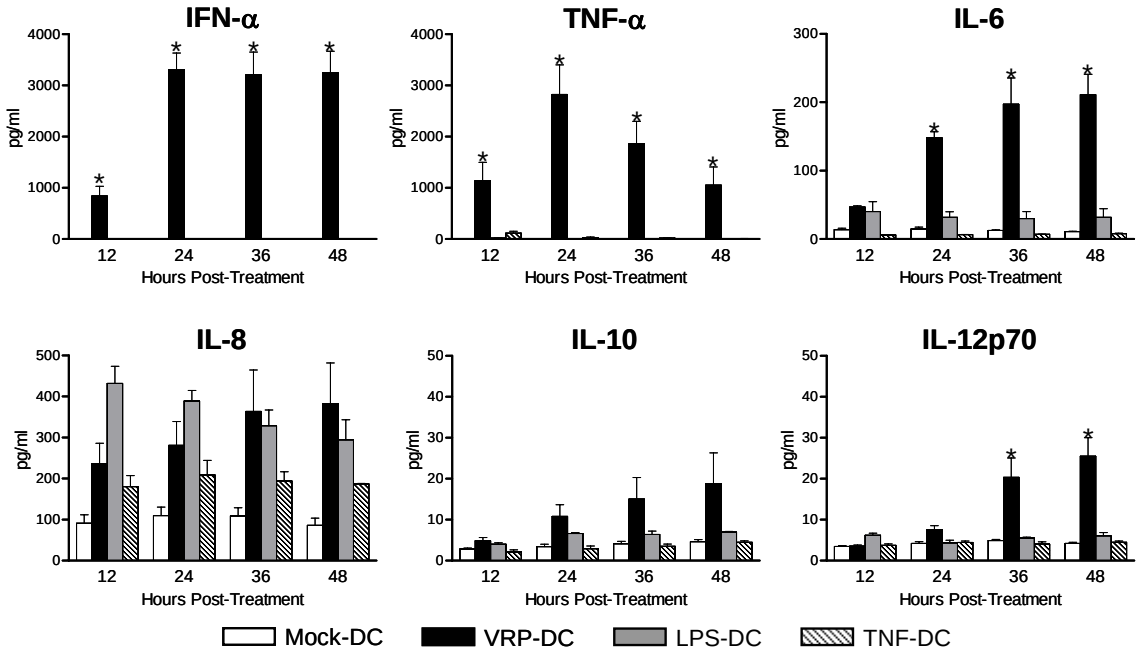
IFN-α
TNF-α
IL-6
IL-8
IL-10
IL-12p70
pg/ml
Hours Post-Treatment
12
24
36
48
Mock-DC
VRP-DC
LPS-DC
TNF-DC

Figure 2-5: VRP-infected DCs, but not fully matured DCs, secrete high levels of proinflammatory cytokines. Supernatants from immature DCs that were either mock-infected (Mock-DC) or GFP-VRP-infected at an MOI of 20 (VRP-DC) were harvested and analyzed for specific cytokines by CBA (TNF-α, IL-6, IL-12p70 or IL-8) or ELISA (IFN-α). Supernatants from DCs that had been previously matured by 24 hours of LPS treatment (LPS-DC) or 48 hours of TNF-α treatment (TNF-DC) were also analyzed. The mean cytokine concentration +/- SEM from three donors is shown. Data is representative of two experiments. *$p < 0.05$ (Student's t test) when compared to Mock-DC, TNF-DC or LPS-DC.

Figure 2-6

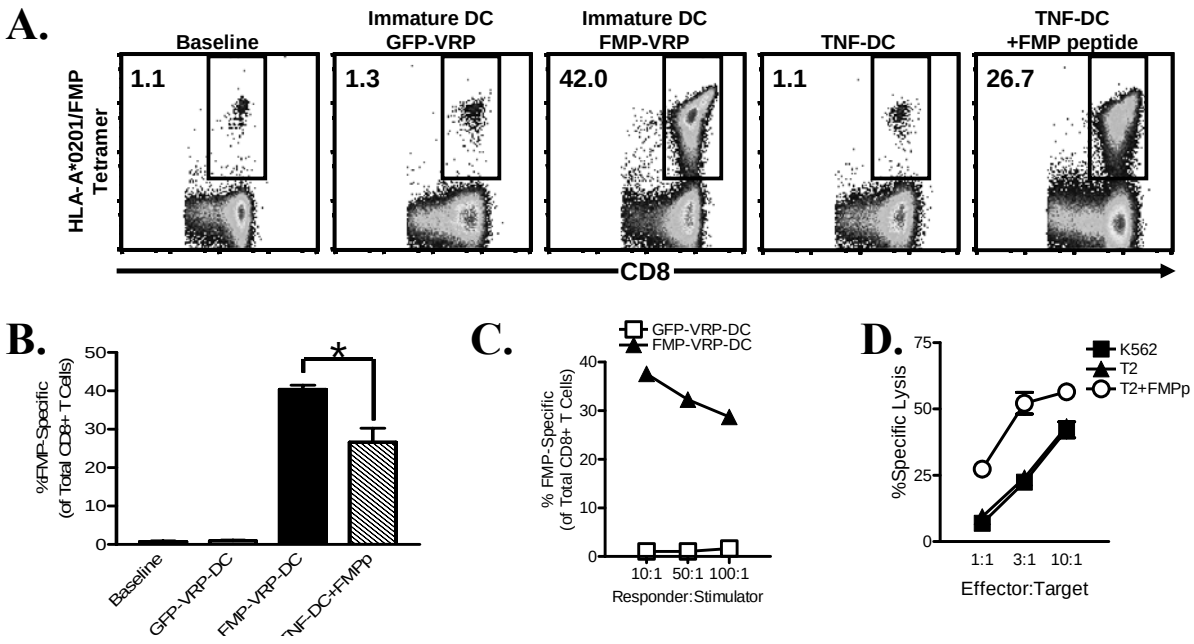
A.
Baseline
Immature DC GFP-VRP
Immature DC FMP-VRP
TNF-DC
TNF-DC +FMP peptide
1.1
1.3
42.0
1.1
26.7
HLA-A*0201/FMP Tetramer
CD8
B.
%FMP-Specific (of Total CD8+ T Cells)
Baseline
GFP-VRP-DC
FMP-VRP-DC
C.
GFP-VRP-DC
FMP-VRP-DC
% FMP-Specific (of Total CD8+ T Cells)
10:1 50:1 100:1
Responder:Stimulator
D.
K562
T2
T2+FMPp
%Specific Lysis
1:1 3:1 10:1
Effector:Target

Figure 2-6: VRP-transduced DCs stimulate greater expansion of antigen-specific $CD8^+$ CTL compared to TNF-α-matured DCs pulsed with peptide. Immature DCs were transduced with either FMP-VRPs or irrelevant GFP-VRPs for 2 hours (MOI = 20). DCs were washed and cocultured with autologous nonadherent PBMCs at various responder:stimulator ratios in the presence of IL-2 and IL-7 for 7 days. *A*) Expansion of FMP-specific $CD8^+$ T cells was determined by tetramer analysis. Baseline indicates the percentage of FMP-specific T cells before stimulation. The stimulatory capacity of VRP-infected DCs was compared to TNF-α-matured DCs (TNF-DC) that had been pulsed with FMP peptide (10 μg/ml) for 2 hours or left untreated (responder:stimulator ratio = 20:1). Numbers represent the percentage of FMP-specific cells of total $CD8^+$ T cells. *B*) Mean percent of FMP-specific $CD8^+$ T cells on day 7 of stimulation from three experiments. *$p < 0.05$ (Student's t test). *C*) Percent of FMP-specific $CD8^+$ T cells on day 7 of stimulation at various responder:stimulator ratios. One of two similar experiments is shown. *D*) PBMCs that had been stimulated with FMP-VRP-infected DCs for 7 days were assayed for effector function in a standard ^{51}Cr-release assay. Labeled FMP peptide-pulsed (open circles) or unpulsed T2 cells (solid triangles) were incubated with effector cells for 4 hours and specific lysis was calculated as described in *Materials and Methods*. Lysis of the NK-sensitive cell line K562 was similar to that found using unpulsed T2 cells indicating that the enhanced lytic activity using FMP-pulsed T2 cells was not due to NK-mediated lysis. The graphs represent the mean of triplicate wells +/- SEM. One of two similar experiments is shown.

CHAPTER THREE

VIRAL ACTIVATION OF DENDRITIC CELLS IS INSUFFICIENT FOR BREAKING TOLERANCE

Abstract

The inability of current dendritic cell (DC) vaccines to generate cytokines critical for T cell polarization and T_{reg} suppression may be responsible for their limited clinical efficacy. Previously, we have shown that human DCs transduced with Venezuelan equine encephalitis virus replicon particles (VRPs) produced significant levels of proinflammatory cytokines and were potent stimulators of T cells. In the current study, we have evaluated whether VRP-transduced DCs (VRP-DCs) could overcome tolerance against the neu oncoprotein and induce therapeutic tumor immunity *in vivo*. VRP-DCs expressing a truncated neu oncoprotein induced robust T cell and antibody responses in wild-type mice. Moreover, a single vaccination with VRP-DCs induced regression of established neu-expressing tumors. However, VRP-DCs were unable to inhibit growth of the same tumor cell line in mice tolerant to neu, despite an accumulation of IFN-γ-producing T cells at the tumor site. Vaccine efficacy in tolerant mice was enhanced by depletion of $CD4^+CD25^+$ regulatory T cells (T_{reg}), demonstrating that virally-activated DCs alone were incapable of overcoming T_{reg}-mediated suppression. Finally, provision of neu-specific T cells from wild-type mice did not improve the efficacy of VRP-DC vaccines in tolerant animals, indicating that tolerance was not entirely due to the absence of a high-avidity T cell repertoire. These results demonstrate that while highly immunogenic, virally-activated DCs cannot break tolerance against self/tumor antigens. Furthermore, our findings suggest that the ability to reject tumors is more dependent upon dominant immunoregulatory mechanisms within the host than tumor-specific inhibitory factors.

Introduction

Metastatic breast cancer remains a leading cause of cancer morbidity and mortality among women (41). Vaccination is an attractive approach for treating metastatic breast cancer, as this strategy can be used not only to eliminate malignant cells but also to prevent recurrent disease through establishment of immunological memory (15). A frequent target for breast cancer vaccines is HER-2/neu (c-erbB-2), a member of the epidermal growth factor receptor family that is overexpressed in 15-30% of breast cancers (4). Amplification of the *neu* oncogene in breast cancer patients is associated with metastasis and a poor clinical prognosis (40). Humoral and cell-mediated immunity against HER-2/neu have been detected in breast cancer patients, suggesting that tolerance against the protein may be incomplete (13). Therefore, several clinical studies aimed at inducing neu-specific immunity through vaccination with either peptides or dendritic cells (DCs) have been performed (6, 12, 22, 24, 31, 49). Although HER-2/neu vaccines have been safe and well tolerated, they have rarely yielded objective clinical responses.

The inability of cancer vaccines to induce therapeutic responses in patients with macroscopic tumors is likely due to several factors. First, tumors can secrete immunosuppressive cytokines or express inhibitory molecules, thus making the tumor environment hostile for tumor-specific T cells (1). Second, because tumor-specific T cells recognize self proteins, most that evade negative selection in the thymus are of low avidity for their cognate antigen and are thus compromised in their ability to attack tumor cells (11, 26, 27). Third, tumors have been shown to recruit leukocytes with immunoregulatory activity, which can directly inhibit tumor-specific T cells (8, 25). Clearly, the lack of

antitumor immune responses following vaccination could be due to a combination of these factors and others.

Because of their role in initiating adaptive immune responses, many researchers have focused on utilizing DCs as therapeutic cancer vaccines (3). DCs loaded with HER-2/neu derived peptides have been evaluated in clinical trials (6, 12, 24), and while they have been shown to stimulate HER-2/neu-specific $CD8^+$ T cell responses, therapeutic responses have been infrequent. The limited efficacy of peptide-pulsed DC vaccines may be due to the lack of the third signal for T cell activation—Toll-like receptor (TLR) activation and proinflammatory cytokine production (9, 10, 43). Viral vectors may provide a superior strategy for antigen-loading of DCs, as they can efficiently deliver intact protein to the DC cytoplasm, thus providing multiple MHC class I and/or II epitopes (47, 51). Furthermore, viral vectors can induce DC activation and provide the persistent TLR stimulation deemed necessary for overcoming the activity of regulatory T cells and breaking tolerance (21, 48).

We previously found that Venezuelan equine encephalitis virus replicon particles (VRP) could efficiently transduce human immature DCs, resulting in DC maturation and secretion of proinflammatory cytokines (30). Furthermore, VRP-transduced DCs (VRP-DCs) were superior to peptide-pulsed DCs at expanding antigen-specific CTLs *in vitro*, arguing that VRP-DCs may be an ideal vaccine for breaking tolerance. To expand upon our *in vitro* findings, we have examined the efficacy of VRP-DCs as therapeutic cancer vaccines in mice bearing neu-expressing tumors. We demonstrate that VRP-DCs are highly immunogenic in wild-type FVB/N mice, and can induce regression of established tumors. In contrast, therapeutic vaccination with VRP-DCs alone was unable to inhibit tumor growth in neu transgenic (neu-N) mice, which exhibit a profound tolerance against neu (35). Depletion of

T_{reg} with cyclophosphamide (CY) enhanced the effectiveness of VRP-DC vaccines, demonstrating that VRP-DCs could not independently circumvent T_{reg}-mediated suppression. Adoptive transfer of T cells from FVB/N mice into neu-N mice did not improve vaccine efficacy, indicating that the inadequacy of VRP-DC vaccination was not purely due to a lack of high-avidity T cells. Taken together, these results suggest that viral activation of DCs is insufficient to overcome immune regulation and break tolerance to a self protein, even in the presence of high-avidity T cells. Additionally, our data support a model in which tumor immunity is not directly inhibited by the tumor itself, but rather by dominant suppressive mechanisms present in the host.

Materials and Methods

Mice

FVB/N mice (age 6-7 weeks) were purchased from Jackson Laboratories. Homozygous neu-N transgenic on the FVB/N background (19) were purchased from Jackson Laboratories and maintained in our animal breeding facility. All mice used were 6-12 weeks of age.

Cell lines and peptides

NIH-3T3 (American Type Culture Collection), 3T3*neu* and NT2 cells have been previously described (35). $RNEU_{420-429}$ (PDSLRDLSVF) and $NP_{118-126}$ (RPQASGVYM) peptides were purchased from New England Peptide (Gardner, MA) at >95% purity. $RNEU_{420-429}$ is the immunodominant H2-D^q-restricted epitope from rat neu (17), while $NP_{118-126}$ peptide is an H2-D^q-restricted epitope from the LCMV nucleoprotein and served as an irrelevant peptide control.

VEE replicon particles (VRP)

VRP encoding GFP (GFP-VRP) or VRP lacking a functional transgene (null-VRP) have been previously described (42). VRP encoding the extracellular-transmembrane domains (amino acids 1-697) of rat neu (neuET-VRP) were generated by cloning the neuET cDNA into the pVR21 replicon plasmid as previously described (30). VRP titer was determined by infection of baby hamster kidney (BHK) cells as previously described (30). All VRPs were packaged in the wild type (V3000) viral envelope.

Generation of VRP-DC vaccines

DCs were derived from bone marrow as previously described (33). Briefly, bone marrow from female FVB/N or neu-N mice was harvested by flushing femurs and tibias with complete R-10 media. Following treatment with ACK lysis buffer, bone marrow progenitor cells were plated at 1 x 10^6 cells per well in 6-well ultra low attachment plates (Costar, Corning, NJ) in 1.5ml of R-10 supplemented with 20 ng/ml murine GM-CSF (Peprotech, Rocky Hill, NJ). On day 3, 1.5 ml of R-10 was added along with GM-CSF and murine IL-4 (Peprotech) to a final concentration of 10 ng/ml for each cytokine. On day 5, 3 ml of R-10 was added along with GM-CSF and IL-4 to a final concentration of 5 ng/ml of each cytokine. On day 7, immature DCs were harvested and cryopreserved in 90% FBS/10% DMSO at a concentration of 5 x 10^6 cells per ml. DCs were stored in liquid nitrogen and used within 3 months of cryopreservation.

To generate VRP-transduced DC (VRP-DC) vaccines, cryopreserved DCs were quickly thawed at 37ºC and washed twice with R-10. DCs were plated in 6-well ultra low attachment plates at 10^6 cells/ml in R-10 supplemented with 5 ng/ml GM-CSF and 5 ng/ml IL-4, and cultured overnight at 37ºC/5% CO_2. The next morning, DCs were harvested, washed and diluted in RPMI-1H infection media (RPMI-1640, 1% FBS, 10mM HEPES) to 10^6 cells/ml. DCs were plated at 10^6 cells/well in 6-well ultra low attachment plates and infected with VRP at a multiplicity of infection (MOI) of 10 for 2 hours at 37ºC/5%CO_2 as previously described (30). Infected DCs were washed three times and suspended in 0.9% sterile saline. Prior to vaccination, female FVB/N or neu-N mice were anesthetized by i.p. injection of 1.3 mg ketamine HCl / 0.38 mg xylazine. VRP-DC (10^6) were injected s.c. in the mammary fat pad.

Antibodies and flow cytometric analysis

Anti-CD8-FITC (53.6.7), anti-CD25-FITC (PC61), anti-CD40-PE (1C10), anti-CD62L-APC (MEL-14), anti-MHC class I-PE (28-14-8), anti-MHC class II-PE (M5/114.15.2), anti-IFN-γ-PE (XMG1.2), and anti-FoxP3-PE (FJK-16s) monoclonal antibodies were purchased from eBioscience (San Diego, CA). Anti-CD4-PerCP (RM4-5), anti-CD80-PE (16-10A1), anti-CD86-PE (GL1), and anti-IL-10-PE (JES5-16E3) monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA). Anti-c-ErbB2/neu (Ab4) monoclonal antibody was purchased from Calbiochem (San Diego, CA). PE-conjugated H-2D^{q}/RNEU$_{420\text{-}429}$ tetramers were synthesized by the NIH Tetramer Facility (Emory University, Atlanta, GA). For phenotypic analysis of DCs, cells were washed with FACS buffer (PBS/2% FBS), stained with the indicated antibodies for 30 min at 4ºC, and fixed with 1% formaldehyde prior to analysis. For tetramer staining of lymphocytes, cells were incubated with PE-conjugated H-2D^{q}/RNEU$_{420\text{-}429}$ tetramers (1:200) for 1 hour at room temperature; anti-CD8, anti-CD3 and anti-CD62L antibodies were added during the last 15 min of incubation. Cells were then washed and suspended in 0.5% formaldehyde prior to analysis. Analysis of intracellular FoxP3 expression was performed according to the manufacturer's instructions. FACS data were acquired using a FACSCalibur flow cytometer (BD Biosciences), and analyzed using FlowJo software (TreeStar, Ashland, OR).

Cytokine secretion assays

Murine DCs were infected with GFP-VRP as described. Two hours post infection, the DCs were washed and plated into 96-well tissue-culture plates at 10^5 cells/well in a total

volume of 200 µl of R-10 supplemented with GM-CSF (5 ng/ml) and IL-4 (5 ng/ml). Supernatants were harvested at 6, 12, 24 and 48 hours post-infection. Analysis of IL-6, TNF-α and IL-10 was performed using the Murine Inflammation Cytometric Bead Array (CBA) kit (BD Pharmingen) according to the manufacturer's instructions. Analysis of IL-12p70 was performed using the BD OptEIA™ Mouse IL-12p70 ELISA Set (BD Pharmingen) according to the manufacturer's instructions. Analysis of IFNα/β was determined by a type I interferon (IFN) bioassay as previously described (45).

Detection of neu-specific $CD8^+$ T cells

Female FVB/N or neu-N mice were vaccinated with 10^6 VRP-DC as described. Mice were boosted with VRP-DC two weeks later. At one week post-boost, spleens were harvested and dissociated into single-cell suspensions. After treatment with ACK lysis buffer, splenocytes were suspended in R-10 (2×10^6 cells/ml) and stimulated with 5 µg/ml of either $RNEU_{420-429}$ or irrelevant $NP_{118-126}$ peptide for 12 h at 37ºC/5% CO_2 in the presence of 3 µg/ml brefeldin A (eBioscience). The cells were then stained for surface expression of CD8, CD3 and CD62L, fixed and permeabilized, and stained for intracellular IFN-γ. Enumeration of neu-specific $CD3^+CD8^+$ T cells was determined by subtracting the percentage of IFN-γ^+ cells in samples treated with irrelevant $NP_{118-126}$ peptide from the percentage of IFN-γ^+ cells in samples treated with $RNEU_{420-429}$ peptide.

Detection of serum anti-neu IgG

3T3 or 3T3*neu* cells were harvested with trypsin and blocked with 20 µg/ml goat IgG (Sigma) for 15 min at 4ºC. The cells were stained with two-fold dilutions of serum from

vaccinated FVB/N or neu-N mice for 1 hour at 4ºC. Cells were washed twice with FACS buffer and stained with goat anti-mouse IgG-FITC (Sigma) at a 1:200 dilution for 30 min at 4ºC. Cells were subsequently washed twice, suspended in 1% formaldehyde and transferred to 96-well V-bottom plates. The median FITC fluorescence intensity (MFI) was measured using a Guava EasyCyte cell analysis system (Guava Technologies, Hayward, CA). Specific staining of neu was determined by subtracting the MFI of 3T3 cells from the MFI of 3T3*neu* cells. The concentration of neu-specific IgG in sera was calculated using a standard curve generated with Ab4 monoclonal antibody.

Therapeutic vaccination with VRP-DC

For each tumor challenge experiment, a fresh vial from the same lot of cryopreserved NT2 tumor cells was thawed and passaged *in vitro* for 5-10 days. NT2 cells were harvested with trypsin, washed twice with HBSS, and suspended to an appropriate concentration in HBSS. FVB/N or neu-N mice were challenged with 2×10^6 or 5×10^4 NT2 cells, respectively, in the mammary fat pad. FVB/N or neu-N mice were vaccinated 7 or 3 days post-tumor challenge, respectively, with VRP-DC as described above. In some experiments, mice were boosted every 14 days for a total of three vaccinations. Tumor growth was monitored 2-3 times weekly by measuring the major and minor axes with metric calipers.

In T_{reg}-depletion experiments, neu-N mice received i.p. injections of CY (100 mg/kg) two days after tumor challenge. Mice were vaccinated two days later with VRP-DCs as described. For adoptive transfer experiments, CD25-depleted splenic T cells (>95% purity) were isolated from FVB/N mice as previously described (46). Two days following CY

treatment, neu-N mice received i.v. injections of 1-2 x 10^7 CD25-depleted T cells. Mice were then vaccinated the next day with VRP-DCs.

In vivo depletion of lymphocytes

$CD4^+$ or $CD8^+$ T cells were depleted by i.p. injection of 0.5 mg of GK1.5 or 53.6.72 (Bio Express, West Lebanon, NH), respectively. Control mice received i.p. injections of 0.5 mg rat IgG (Sigma). Injections were given on days -6, -4 and -2 prior to tumor challenge, and depletions were maintained by antibody injections every 4-5 days thereafter. Depletion of specific T cell populations was verified by FACS analysis of splenocytes from select mice (data not shown).

Isolation of tumor-infiltrating lymphocytes (TILs)

Tumors from vaccinated mice were excised, disrupted with a razor blade, and incubated under constant agitation for 1 h with collagenase A (2.5 mg/ml), DNase I (17 μg/ml) and glass beads at 37ºC. Undigested material was removed using a 100 μm nylon cell strainer. The single cell suspension was washed, suspended in 44% Percoll (Sigma), layered on a Lympholyte-M density gradient (Cedarlane Laboratories, Hornby, ON, Canada), and centrifuged for 30 min at 2500 rpm, 25ºC. TILs banding at the Percoll-Lympholyte interface were removed and washed. For intracellular cytokine staining, TILs were stimulated for 4 h with PMA (5 ng/ml) and ionomycin (500 ng/ml). TILs were stained for surface expression of CD4 and CD8, fixed and permeabilized, and stained for intracellular expression of IFN-γ or IL-10. For evaluation of CTL activity, $CD8^+$ TILs were isolated using anti-CD8 magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The

$CD8^+$ TILs were then used as effectors in a standard ^{51}Cr-release assay as previously described (39).

Statistical analysis

Statistical differences for costimulatory molecule expression, cytokine expression, T cell and antibody responses and cellular tumor infiltrates were calculated by a two-tailed Student's t test. Differences in tumor delay and survival were determined by Kaplan-Meier survival analysis. All statistical analyses were performed with GraphPad Prism® 3.0 software. For all analyses, a P value ≤ 0.05 was considered significant.

Results

Characterization of VRP-DC vaccines

We initially investigated the ability of VRPs to transduce murine bone marrow-derived DCs *in vitro*. Preliminary studies using GFP-VRP demonstrated that transgene expression in VRP-transduced murine DCs exhibited similar kinetics as in VRP-transduced human monocyte-derived DCs, with expression peaking at 6-12 hours post-infection (data not shown). Interestingly, the transduction efficiency for murine DCs was enhanced in comparison to human DCs. 40-60% of murine DCs were transduced at an MOI of 10, whereas only 35-40% of human DCs could be transduced at an MOI of 20 (30). We also determined if murine DCs could be efficiently transduced with a VRP encoding the tumor antigen neuET. At 12 hours post-infection, 59% of murine DC expressed high levels neuET as determined by staining for surface and intracellular expression of the antigen (Figure 1A).

We next characterized the phenotype of murine DCs following VRP transduction. VRP-DCs exhibited increased expression of the costimulatory molecules CD40, CD80 and CD86 in comparison to mock-infected DCs (Figure 1B). VRP-induced expression of CD40 and CD86 was comparable to treatment with 100 ng/ml LPS, whereas induction of CD86 by VRPs was slightly less than that seen with LPS. We also evaluated cytokine secretion by DCs following VRP infection (Figure 1C). At 24 hours post infection, VRP-transduced and LPS-treated DCs secreted comparable levels of TNF-α, whereas LPS-treated DCs secreted significantly more IL-6 over a 48 hour period. Neither LPS-treated nor VRP-infected DCs secreted appreciable levels of IL-10. Surprisingly, VRP infection of murine DCs did not induce significant IL-12p70 secretion, which is in contrast to our previous studies with human DCs (30). However, VRP infection was a potent inducer of type I IFN, as VRP-

transduced DCs secreted nearly 1000-fold more IFN-α/β than LPS-treated DCs. DCs treated with VRP that had been inactivated with ultraviolet light were similar to mock-infected DCs in terms of phenotype and cytokine secretion (data not shown), indicating that the effects of VRP infection were not due to a contaminant in the VRP preparation. Overall, VRP infection of murine DCs resulted in high-level transgene expression, phenotypic maturation, and secretion of several proinflammatory cytokines, which are all important characteristics of a potent DC vaccine.

VRP-DC vaccines induce neu-specific immunity in FVB/N mice

To validate the immunogenicity of VRP-DC vaccines *in vivo*, we immunized FVB/N mice with DCs transduced with neuET-VRP (neuET-DCs). At seven days post-boost, spleens were harvested and evaluated for neu-specific $CD8^+$ T cells by intracellular IFN-γ staining. Mice vaccinated with neuET-DCs had a significant population of $CD8^+CD62L^-$ cells specific for the immunodominant peptide $RNEU_{420-429}$ (Figure 2A-B). The lack of reactivity against the irrelevant peptide $NP_{118-126}$ confirmed the specificity of the $CD8^+$ T cells for neu. $CD8^+$ T cells specific for $RNEU_{420-429}$ were absent in untreated mice (data not shown) and mice vaccinated with DCs transduced with null-VRP (null-DCs) (Figure 2A-B).

Because humoral immunity is important for controlling the growth of neu-expressing tumors (36), we evaluated the ability of neuET-DC vaccines to induce neu-specific antibody responses. Mice vaccinated with neuET-DCs had significant levels of serum anti-neu IgG, whereas anti-neu IgG was undetectable in mice vaccinated with null-DCs (Figure 2C). Thus, VRP-DC vaccines have the capacity to induce both humoral and cell-mediated immunity against neu *in vivo*.

VRP-DC vaccines induce therapeutic immunity against established tumors in FVB/N mice

While DC vaccines can frequently protect mice from subsequent tumor challenge or spontaneous tumor development (7, 38), few studies have evaluated their efficacy after therapeutic vaccination of mice with preexisting tumors. The ability of DC vaccines to inhibit the growth of established tumors is arguably more relevant for determining potential clinical efficacy, since the majority of cancer patients present with existent disease. Therefore, we sought to evaluate the ability of VRP-DC vaccines to inhibit the growth of established tumors in FVB/N mice. Mice were challenged with 2×10^6 NT2 tumor cells and then vaccinated with neuET-DCs seven days later when tumor size was approximately 50 mm^2. A single vaccination with 1×10^6 neuET-DCs resulted in inhibition of tumor growth, and induced regression in the majority of treated mice (Figure 3A). Vaccination with neuET-DCs significantly prolonged survival of tumor-bearing FVB/N mice when compared to vaccination with null-DCs ($p = 0.0045$).

Because effective tumor immunity has been associated with increased lymphocytic infiltration of tumors (14), we characterized the TILs isolated from the tumor site (Figure 3B). Mice vaccinated with neuET-DCs had an increased percentage of $RNEU_{420-429}$-specific $CD8^+$ T cells as determined by tetramer staining in comparison to mice receiving null-DCs. Mice vaccinated with neuET-DCs also had an increase in the percentage of IFN-γ^+ $CD8^+$ and $CD4^+$ T cells at the tumor site, indicating a possible mechanism for tumor clearance.

To more specifically evaluate the role of T cells in mediating inhibition of tumor growth, we depleted mice of either $CD4^+$ or $CD8^+$ T cells prior to vaccination (Figure 3C). We found that $CD4^+$ T cells were crucial for effective tumor immunity, as all CD4-depleted mice

experienced rapid tumor growth and had to be euthanized within four weeks of tumor-challenge (Figure 3C). Interestingly, tumor immunity was only partially dependent upon $CD8^+$ T cells, as 62.5% of CD8-depleted mice survived to day 60 post-tumor challenge ($p = 0.0002$ v. rat IgG-treated mice). Because $CD4^+$ T cells are important for the development of antibodies against neu, we analyzed serum anti-neu IgG responses in mice depleted of either $CD4^+$ or $CD8^+$ T cells (Figure 3D). CD4-depleted mice had no detectable serum anti-neu IgG, indicating that CD4 help was critical for the development of neu-specific humoral immunity. Interestingly, CD8-depleted mice had a modest but significant decrease in serum anti-neu IgG levels ($p = 0.042$). Taken together, the presence of tumor-specific $CD8^+$ T cells does not appear to be an absolute requirement in our vaccination model, whereas $CD4^+$ T cells are essential for inhibition of tumor growth.

VRP-DC vaccines fail to induce therapeutic immunity against neu in tolerant neu-N mice

The previously described experiments demonstrated that a single vaccination with VRP-DCs induced therapeutic tumor immunity in nontolerant mice. We next explored the ability of VRP-DC vaccines to induce neu-specific immunity under conditions of immunological tolerance in neu-N transgenic mice. In contrast to FVB/N mice, neu-N mice did not develop $CD8^+$ T cells specific for $RNEU_{420-429}$ following vaccination with neuET-DCs (Figure 4A). Vaccination with neuET-DCs also did not induce neu-specific CTL as determined by a standard ^{51}Cr-release assay using 3T3*neu* cells as targets, suggesting that $CD8^+$ T cells specific for subdominant neu epitopes were not present (data not shown). Vaccination of neu-N mice with neuET-DCs did induce very modest anti-neu IgG responses (Figure 4B), which was over 20-fold less than that seen in nontolerant FVB/N mice (Figure 2C).

As previous investigators had suggested that the generation of proinflammatory cytokines was critical in the immune rejection of tumors, we evaluated the ability of VRP-DC vaccines to inhibit the growth of tumors in neu-N mice. Female neu-N mice were vaccinated with neuET-DCs three days following tumor challenge, and were boosted every two weeks for a total of three vaccinations. Tumor growth in neu-N mice vaccinated with neuET-DCs was nearly identical to that seen in mice vaccinated with null-DCs or left untreated, indicating that VRP-DC vaccines could not induce therapeutic immune responses against neu (Figure 4C). Given the poor therapeutic responses in neu-N mice, we investigated if VRP-DCs could induce an immune response at the tumor site. Surprisingly, we found that neu-N mice vaccinated with neuET-DCs had a significantly increased percentage of $CD8^+$ TIL producing IFN-γ following stimulation with PMA/ionomycin in comparison to untreated mice ($p = 0.0162$) or mice vaccinated with null-DCs ($p = 0.0197$) (Figure 4D). However, no $RNEU_{420-429}$-specific $CD8^+$ T cells were detectable by tetramer staining, indicating that $CD8^+$ TIL specific for the immunodominant neu epitope were absent (data not shown). Furthermore, the $CD8^+$ TIL were incapable of specifically lysing 3T3*neu* cells in a standard ^{51}Cr-release assay, demonstrating that the CTLs were functionally impaired (Figure 4E). Interestingly, IFN-γ^+CD4^+ TIL were elevated in both null-DC- and neuET-DC-vaccinated mice in comparison to untreated mice, although the increase was not statistically significant (4D). Overall, these results demonstrate that VRP-DC vaccines alone are incapable of overcoming tolerance to neu in neu-N transgenic mice, despite the induction of IFN-γ-producing $CD8^+$ T cells at the tumor site.

Depletion of $CD4^+FoxP3^+$ T_{reg} with CY enhances the efficacy of VRP-DC vaccines

The ineffectiveness of VRP-DC vaccines in neu-N mice suggested that DC therapy alone was unable to overcome tolerogenic mechanisms. Previous studies have demonstrated that regulatory T cells, including $CD4^+FoxP3^+$ T cells (T_{reg}), play an important role in mediating tolerance against neu in neu-N mice (16). Because T_{reg} have been shown to preferentially migrate to the tumor site in HER-2/neu^+ ovarian cancer patients (8), we investigated whether T_{reg} similarly accumulate in NT2 tumors following vaccination. We found increased numbers of $CD4^+FoxP3^+$ T cells but not $CD4^+IL\text{-}10^+$ T cells (Figure 5A) at the tumor site in mice vaccinated with neuET-DC, suggesting that T_{reg} may be recruited to suppress immune responses within the tumor.

We next sought to determine if inhibiting T_{reg} function would enhance the efficacy of VRP-DC vaccines. Interestingly, treatment of tumor-bearing neu-N mice with anti-CD25 monoclonal antibody prior to vaccination did not result in decreased tumor growth (Supplemental Figure 1). However, the use of anti-CD25 antibodies for T_{reg} depletion is likely suboptimal, since this approach would also deplete recently activated $CD4^+$ effector T cells that are clearly necessary for efficient induction of tumor immunity in our model (Figure 3C). Therefore, we attempted to inhibit T_{reg} activity by treating mice with low-dose CY, which preferentially decreases the number and function of T_{reg} (16, 28). We found that tumor-bearing mice treated with CY (100 mg/kg) exhibited a significant decrease in the percentage of $CD4^+FoxP3^+$ T cells in the tumor-draining lymph node (TDLN) (Figure 6B), whereas total numbers of $CD4^+$, $CD8^+$ and $CD19^+$ cells were unaffected (data not shown). Tumor-bearing mice were treated with CY and vaccinated with VRP-DCs two days later. Mice pretreated with CY and vaccinated with neuET-DCs demonstrated a significant delay in formation of palpable tumors in comparison to CY-treated mice vaccinated with null-DCs

(Figure 5C, p = 0.036). However, CY-treatment did not significantly increase overall survival of vaccinated mice due to late rapid growth of tumors (p = 0.1375, Figure 5D). The rapid tumor growth was accompanied by a significant rebound of $CD4^+FoxP3^+$ T cells in the tumor-draining lymph node and at the tumor site of CY-treated mice, indicating that T_{reg}-depletion with CY was transient (Figure 5E). A second injection of CY two-weeks after vaccination did not improve survival. We were unable to identify $RNEU_{420-429}$-specific $CD8^+$ T cells in the tumors of CY-treated animals vaccinated with neuET-DCs (data not shown). In summary, depletion of T_{reg} with CY treatment prior to therapeutic VRP-DC vaccination significantly inhibits early tumor growth. However, long-term control of tumor growth is not improved, likely due a resurgence of T_{reg} at the tumor site.

Provision of tolerant mice with a neu-specific T cell repertoire does not improve VRP-DC vaccination

Peripheral neu-specific T cells in neu-N mice are generally of low avidity, which could significantly inhibit the efficacy of VRP-DC vaccination (27). In contrast, FVB/N mice possess high-avidity neu-specific T cells that can be effectively expanded following vaccination (17 and Figure 2A-B). Therefore, we sought to determine if supplying neu-N mice with a naïve T cell repertoire containing high-avidity neu-specific T cells would improve the efficacy of VRP-DC vaccines. Two days following CY treatment, tumor-bearing neu-N mice were infused with either saline or 1 x 10^7 CD25-depleted T cells and vaccinated with neuET-DCs on the following day. Tolerant mice receiving adoptively transferred T cells exhibited neither a significant delay in tumor formation (p = 0.234) nor significantly increased survival (p = 0.358) (Figures 6A-B). Increasing the T cell dose to 2 x

10^7 did not alter survival, indicating that the lack of vaccine efficacy was not due to insufficient numbers of adoptively transferred T cells (data not shown). These results demonstrate that vaccine efficacy cannot be improved by providing tolerant mice with high-avidity T cells specific for neu, and suggest that in our model, regulatory mechanisms dominantly suppress the activity of effector T cells.

Discussion

The optimal DC vaccine would generate significant quantities of antigen allowing expansion of functional antigen-specific T and B cells in concert with the production of polarizing proinflammatory cytokines. Our previous studies with human DCs demonstrated that VRP-transduction resulted in DC activation and secretion of immunostimulatory cytokines, leading to efficient stimulation of antigen-specific T cells. Thus, we were interested in determining if this platform would break tolerance in neu-N transgenic mice and inhibit the growth of existing tumors.

Here, we demonstrate that VRPs efficiently transduce murine bone marrow-derived DCs, resulting in maturation and cytokine production by infected cells. VRP-DC vaccines were highly immunogenic in wild-type FVB/N mice, and were capable of inducing both cellular and humoral immunity against neu. A single vaccination with VRP-DCs expressing neuET could induce therapeutic immune responses against established tumors, resulting in tumor regression. However, VRP-DC vaccines were incapable of generating robust $CD8^+$ T cell and antibody responses against neu in tolerant neu-N transgenic mice, which was associated with an inability to inhibit tumor growth in these mice. Thus, the same tumor and vaccine induced significantly different immune responses in tolerant and nontolerant animals, which differ only in the transgenic expression of neu.

Yang *et al.* had previously shown that viral infection alone or lentiviral transduced DCs given with anti-CD25 mAb could break tolerance to influenza HA in C3H-BALB/c F1 mice and that breaking tolerance using DCs without T_{reg} depletion required persistent LPS exposure and TLR stimulation (48). Given these findings and our previous data demonstrating significant proinflammatory cytokine induction by DCs following viral

transduction, we sought to determine if vaccination with VRP-DCs could break tolerance against neu. Despite the generation of proinflammatory cytokines by our virally-transduced DCs, we were unable to overcome tolerance in neu-N transgenic mice. VRP-DCs differed from LPS-treated DCs in the production of IL-12 and in the quantity of IL-6 generated. Thus, it is conceivable that the VRP-DCs could not overcome tolerance due to insufficient production IL-6, which is necessary for inhibiting T_{reg}-mediated suppression (34). Alternatively, the generation of IL-12 may be critical to overcoming immune regulation, although studies have shown that IL-12 production by the DC vaccine itself was not necessary for activation of CTLs *in vivo* (44). Regardless, our data suggests that type I IFN production by DCs vaccines is insufficient to break tolerance, since VRP-transduced DCs secreted copious amounts of IFN-α/β.

Since neu-specific T cells in neu-N mice are of low frequency and avidity (17, 27), we reasoned that VRP-DC vaccination was ineffective due to the absence of a high-avidity effector T cell repertoire. If this were true, we would expect to find that the administration of naïve splenocytes from nontolerant mice followed by immunization would induce tumor regression. However, the administration of a significant number of CD25-depleted naïve T cells from nontolerant mice did not break tolerance in vaccinated mice. These data suggest that tolerance in neu-N mice is an active process that blocks the expansion of naïve T cells. Moreover, our data is consistent with tolerance being mediated by dominant immunoregulatory mechanisms rather than the absence of a T cell repertoire specific for a self/tumor antigen. While our findings suggest that tolerogenic mechanisms can inhibit the priming of naïve high-avidity T cells, the ability to inhibit the effector function of activated neu-specific $CD8^+$ T cells is currently unknown. T_{reg} can effectively suppress the activity of

adoptively transferred melanoma-specific CTL that had been previously activated, indicating that tolerance may dominate over effector function (2). Nonetheless, future studies need to evaluate the ability of T_{reg} to inhibit neu-specific effector T cells in neu-N mice.

We investigated potential mechanisms for the active suppression of an immune response to neu in neu-N mice. There are several populations of cells that may mediate immune suppression *in vivo* including thymus-derived $CD4^+CD25^+FoxP3^+$ T_{reg} (37), peripherally-induced Tr1 cells (18) and myeloid suppressor cells (5). Although VRP-DC vaccines alone were incapable of inhibiting tumor growth in tolerant mice, their efficacy was enhanced when combined with CY treatment. Low-dose CY can decrease the number and function of T_{reg} (16, 28), and can unmask high-avidity T cells specific for neu (16). As seen in other studies, T_{reg} depletion by CY was transient in tumor-bearing mice (28). Furthermore, the immune-modulating effects of CY were most evident when the drug was given prior to vaccination, which is consistent with previous reports (29). Because T_{reg} numbers can rebound quickly in tumor-bearing mice, better methods for long-term T_{reg} depletion are needed. The use of anti-CD25 antibodies for durable inhibition of T_{reg} is unlikely to be effective, since these antibodies can also abrogate the activity of $CD25^+$ effector T cells (23). The use of agonistic antibodies specific for glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) may be a better strategy for overcoming T_{reg} activity during the course of tumor immunotherapy (23).

It is interesting that although tumor growth was unaffected, vaccination with neuET-DCs resulted in increased numbers of IFN-γ-producing $CD8^+$ and $CD4^+$ T cells at the tumor site, suggesting that vaccination was inducing some degree of antitumor immunity. However, the $CD8^+$ T cells were dysfunctional as they were not able to lyse neu-expressing cells in a

standard CTL assay, indicating that IFN-γ production by TILs is not an accurate predictor of tumor immunity. In addition, the elevated numbers of IFN-γ^+ $CD8^+$ T cells at the tumor site was accompanied by a concomitant increase in $CD4^+FoxP3^+$ T cells. These observations are consistent with a recent report by Nair *et al.*, who witnessed an increased number of IFN-γ-secreting $CD8^+$ T cells with impaired cytolytic activity within spontaneous tumors of neu-N mice that had been repeatedly vaccinated against neu (32). Moreover, the authors reported a parallel increase in the number of $CD4^+CD25^+FoxP3^+$ T cells at the tumor site. The study by Nair *et al.* and the results of this current report suggest that vaccination against neu in tolerant mice results in concurrent activation of both effector and regulatory T cells within the tumor environment. These observations are in line with studies showing that tumor-specific effector cells and regulatory cells are simultaneously activated in tumor-bearing mice (20, 50). Altogether, these findings have serious implications for cancer vaccines, as they suggest that vaccination can unintentionally induce expansion of regulatory T cells that will impede tumor immunity and may actually expedite tumor growth.

In conclusion, we have shown that VRP-DC vaccines are highly immunogenic in wild-type mice, eliciting effective therapeutic immunity against established tumors. Unfortunately, VRP-DC vaccines alone were incapable of inhibiting tumor growth in tolerant mice, even when mice were provided with a repertoire of high-avidity T cells specific for neu. Depletion of T_{reg} with CY enhanced the efficacy of VRP-DC vaccines in tolerant mice, although the effect was transient due to resurgence in T_{reg} numbers following vaccination. These results are consistent with the pervasive failure of DC vaccines at consistently inhibiting tumor growth in the setting of established disease, and indicate that viral activation of DCs cannot independently overcome T_{reg} activity. Therefore, future studies should

combine DC vaccination with therapies that either reduce T_{reg} activity or decrease the sensitivity of effector T cells to T_{reg}-mediated suppression.

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Figure 3-1

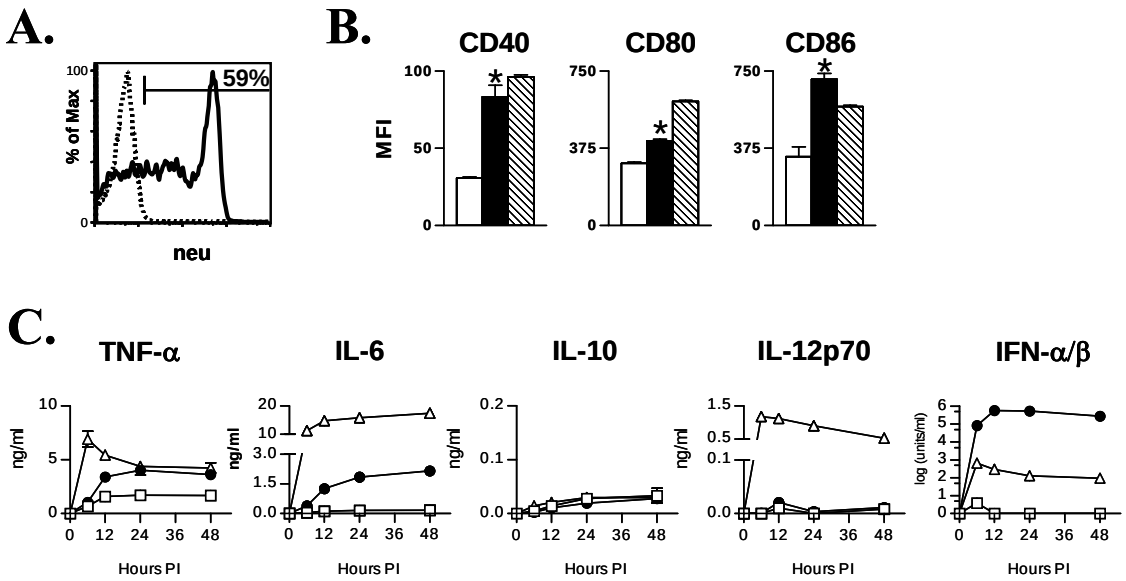

A.
B.
C.
59%
% of Max
neu
CD40
CD80
CD86
MFI
TNF-α
IL-6
IL-10
IL-12p70
IFN-α/β
ng/ml
log (units/ml)
Hours PI

Figure 3-1: VRP transduction results in high-level transgene expression and DC activation. *A.* DCs were transduced with neuET-VRP at an MOI of 10. At 12 h post-infection, DCs were stained for surface and intracellular expression of neuET. *B.* DCs were infected with either GFP-VRP (solid bars) or mock-infected with viral diluent (open bars). DCs treated with 100 ng/ml LPS (hatched bars) were included as a positive control for maturation. At 24 h post-infection, DCs were stained for CD40, CD80, and CD86. Expression of costimulatory molecules on VRP-infected DCs was determined by gating on GFP^+ DCs. Bars represent the mean +/- SEM (n = 4 per group). One of four similar experiments is depicted. *C.* DCs were either mock-infected (open squares), infected with GFP-VRP at an MOI of 10 (closed circles), or treated with 100 ng/ml LPS (open triangles). Cytokine levels were evaluated at the indicated times post-infection as described in *Materials and Methods*. *$p<0.0005$ v. mock-DCs, Student's t test.

Figure 3-2

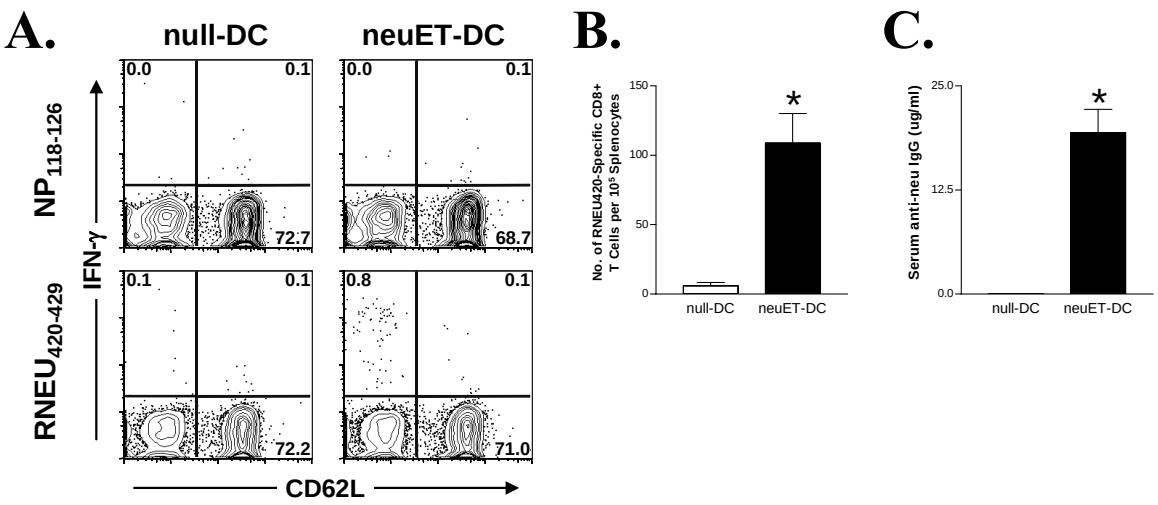

A.
null-DC
neuET-DC
0.0
0.1
72.7
0.0
0.1
68.7
0.1
0.1
72.2
0.8
0.1
71.0
NP118-126
RNEU420-429
IFN-γ
CD62L
B.
No. of RNEU420-Specific CD8+ T Cells per 10^5 Splenocytes
150
100
50
0
*
null-DC
neuET-DC
C.
Serum anti-neu IgG (ug/ml)
25.0
12.5
0.0
*
null-DC
neuET-DC

Figure 3-2: Vaccination of FVB/N mice with VRP-DCs induces neu-specific T cell and antibody responses. *A-B*. Female FVB/N mice (n = 6 mice per group) were vaccinated s.c. in the mammary fat pad with 1 x 10^6 DCs that had been transduced with either null-VRP (null-DCs) or neuET-VRP (neuET-DCs). Mice were similarly boosted two weeks later. At seven days post-boost, splenocytes were isolated and stimulated with either $RNEU_{420-429}$ peptide or irrelevant $NP_{118-126}$ peptide for 12 h, and then assayed for intracellular IFN-γ expression. *A*. Representative staining of gated $CD3^+CD8^+$ T cells from mice vaccinated with either null-DC or neuET-DCs. *B*. The frequency of $RNEU_{420-429}$-specific $CD8^+$ T cells was determined by subtracting the frequency of $NP_{118-126}$ specific T cells. Bars represent the mean +/- SEM. Data is representative of two experiments. *C*. Serum levels of anti-neu IgG were determined as described in *Materials and Methods*. Bars represent the mean +/- SEM. *$p<0.001$, Student's t test.

Figure 3-3

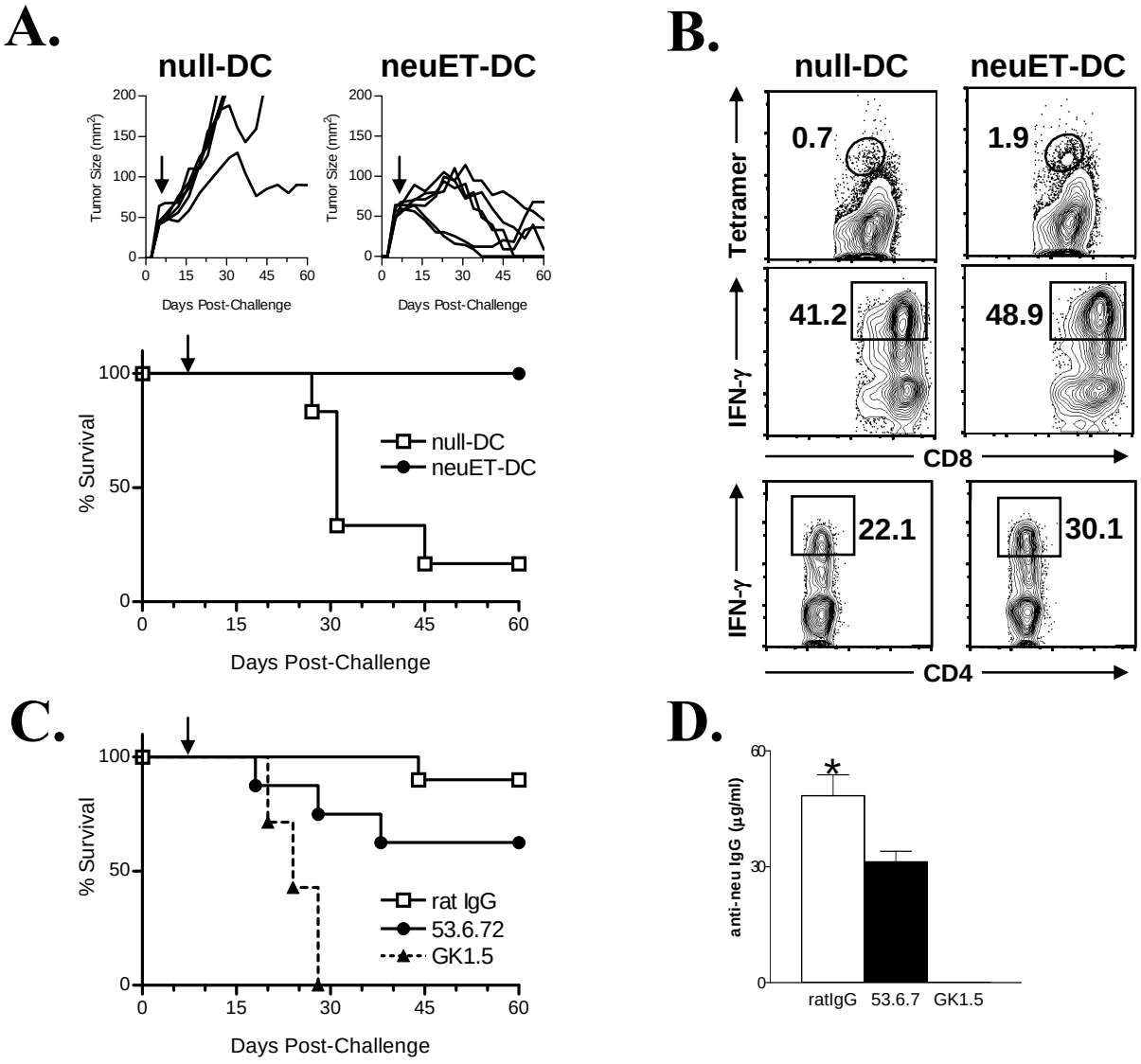

A.
null-DC
neuET-DC
Tumor Size (mm²)
Days Post-Challenge
% Survival
null-DC
neuET-DC
B.
null-DC
neuET-DC
Tetramer
IFN-γ
CD8
CD4
0.7
1.9
41.2
48.9
22.1
30.1
C.
% Survival
rat IgG
53.6.72
GK1.5
Days Post-Challenge
D.
anti-neu IgG (µg/ml)
ratIgG
53.6.7
GK1.5

Figure 3-3: Therapeutic VRP-DC vaccination of tumor-bearing FVB/N mice inhibits tumor growth and induces tumor-infiltrating effector T cells. *A.* Female FVB/N mice (n = 6 mice per group) were challenged with 2 x 10^6 NT2 tumor cells s.c. in the mammary fat pad on day 0. Seven days later, mice received a single vaccination of either 1 x 10^6 null-DCs or 1 x 10^6 neuET-DCs in the contralateral mammary fat pad (solid arrow). Tumor growth was monitored 2-3 times weekly throughout the experiment. Mice were euthanized when the tumor exceeded >2 cm^2 or became ulcerated. Mice vaccinated with neuET-DC demonstrated a significantly prolonged 60-day survival ($p = 0.0045$, Kaplan-Meier survival analysis). One of two similar experiments is shown. *B.* Tumor-infiltrating lymphocytes (TIL) were isolated at 21 days post-vaccination. neu-specific $CD8^+$ T cells were determined by staining with H2-D^q/$RNEU_{420-429}$ tetramers. For assessment of IFN-γ production, TIL were stimulated for 4 h with PMA/ionomycin prior to intracellular IFN-γ staining. *C.* Female FVB/N mice (8-10 mice per group) were treated with rat IgG, 53.6.72 or GK1.5 antibodies as described in *Materials and Methods*. Mice were then challenged with 2 x 10^6 NT2 cells and vaccinated with 1 x 10^6 neuET-DC seven days later (solid arrow). Tumor growth was monitored 2-3 times weekly, while T cell depletions were maintained by injection of antibody every 4-5 days. Cumulative data from two experiments is shown. Survival of mice receiving rat IgG was significantly increased when compared to GK1.5-treated mice or 53.6.72-treated mice ($p = 0.0001$ and 0.0002, respectively, Kaplan-Meier survival analysis). *D.* Sera was collected from mice treated with rat IgG, 53.6.72 or GK1.5 at 2 weeks post-vaccination with neuET-DCs and analyzed for the presence of neu-specific IgG as described in *Materials and Methods*. Bars represent the mean +/- SEM (n = 3-6 mice per group). *$p<0.05$, Student's t test.

Figure 3-4

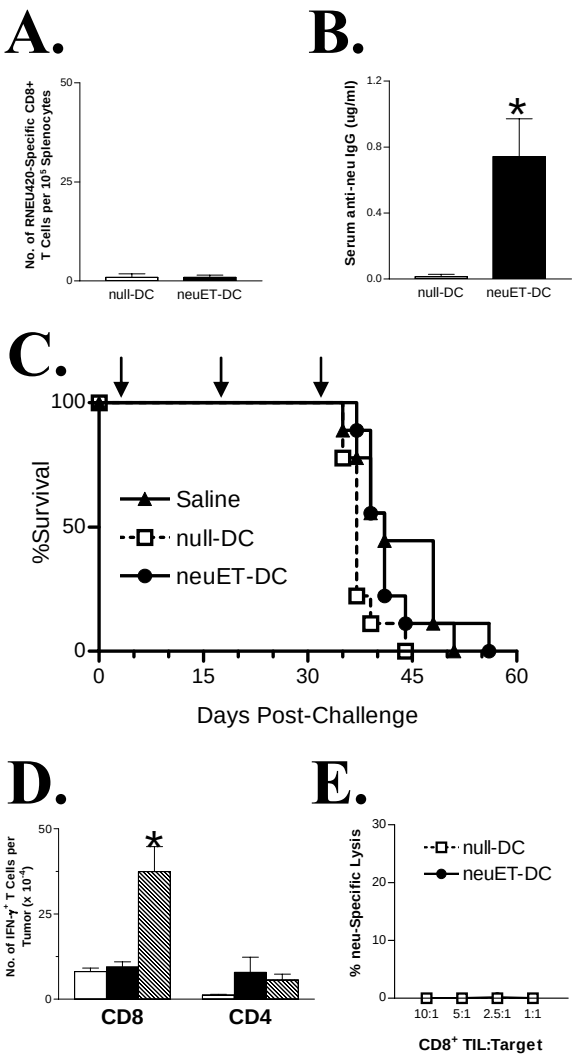

A.
No. of RNEU420-Specific CD8+ T Cells per 10^5 Splenocytes
50
25
0
null-DC
neuET-DC
B.
Serum anti-neu IgG (ug/ml)
1.2
0.8
0.4
0.0
*
null-DC
neuET-DC
C.
100
50
0
%Survival
Saline
null-DC
neuET-DC
0
15
30
45
60
Days Post-Challenge
D.
No. of IFN-γ+ T Cells per Tumor (x 10^-4)
50
25
0
*
CD8
CD4
E.
% neu-Specific Lysis
30
20
10
0
null-DC
neuET-DC
10:1
5:1
2.5:1
1:1
CD8+ TIL:Target

Figure 4: Therapeutic VRP-DC vaccination fails to inhibit tumor growth in tolerant neu-N mice, despite an accumulation of IFN-γ-producing T cells at the tumor site. *A-B.* Female neu-N mice (n = 6 mice per group) were vaccinated s.c. in the mammary fat pad with either 1 x 10^6 null-DCs or 1 x 10^6 neuET-DCs. Mice were similarly boosted two weeks later. At seven days post-boost, splenocytes and sera were harvested and evaluated for $RNEU_{420-429}$-specific $CD8^+$ T cells (*A*) or anti-neu IgG (*B*), respectively. Bars represent the mean +/- SEM. *C.* Female neu-N mice (n = 9 mice per group) were challenged with 5 x 10^4 NT tumor cells s.c. in the mammary fat pad. Three days later, mice were vaccinated with either 1 x 10^6 null-DCs, 1 x 10^6 neuET-DCs or saline only, with vaccinations repeated on days 17 and 31 post-tumor challenge (solid arrows). Tumor growth was monitored 2-3 times weekly and animals were sacrificed when tumors were >2 cm^2 or became ulcerated. Cumulative data from two experiments is depicted. *D.* TIL from mice receiving saline (white bars), null-DCs (black bars) or neuET-DCs (hatched bars) were isolated at 5-6 weeks post-tumor challenge. TIL were stimulated for 4 h with PMA/ionomycin and then analyzed by intracellular IFN-γ staining. Bars represent the mean +/- SEM (n = 3 mice per group). One of two similar experiments is depicted. *E*. TIL were isolated from vaccinated neu-N mice at 28 days post-tumor challenge. $CD8^+$ TIL were purified by magnetic selection and used as effectors cells in a standard ^{51}Cr-release assay with 3T3 or 3T3*neu* cells serving as targets. neu-specific lysis was determined by subtracting the specific lysis of 3T3 target cells from the specific lysis of 3T3*neu* target cells. *p = 0.01, Student's t test.

Figure 3-5

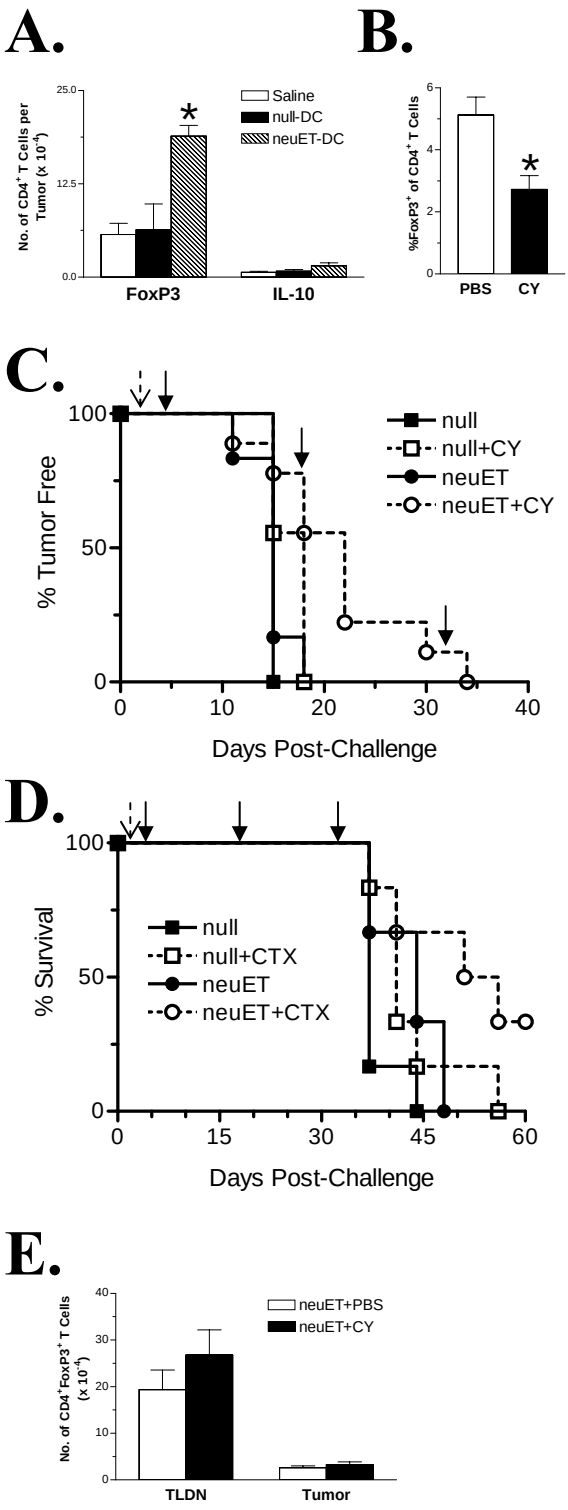

A.
B.
Saline
null-DC
neuET-DC
No. of CD4+ T Cells per Tumor (x 10-4)
FoxP3
IL-10
%FoxP3+ of CD4+ T Cells
PBS
CY
C.
null
null+CY
neuET
neuET+CY
% Tumor Free
Days Post-Challenge
D.
null
null+CTX
neuET
neuET+CTX
% Survival
Days Post-Challenge
E.
neuET+PBS
neuET+CY
No. of CD4+FoxP3+ T Cells (x 10-4)
TLDN
Tumor

Figure 3-5: Treatment of neu-N mice with cyclophosphamide enhances the efficacy of VRP-DC vaccines. *A.* $CD4^+FoxP3^+$ cells are increased within NT2 tumors in neu-N mice vaccinated with neuET-DCs. TIL were isolated from vaccinated or untreated neu-N mice at 5-6 weeks post-tumor challenge and stained for intracellular expression of either FoxP3 or IL-10. Bars represent mean +/- SEM (n = 3 mice per group). One of two similar experiments is depicted. *B.* Female neu-N mice were inoculated with 5 x 10^4 NT2 cells in the mammary fat pad. Two days later, mice received i.p. injections of either PBS (open bars) or 100 mg/kg CY (solid bars). Lymphocytes from TDLN were then harvested after 2 days and stained for intracellular expression of FoxP3. *C-D.* Female neu-N mice (n = 6-9 per group) were challenged with 5 x 10^4 NT2 tumor cells and then treated with either CY (100 mg/kg) or PBS two days later (dashed arrow). On day 4 post-tumor challenge, mice were vaccinated with either neuET-DCs or null-DCs; vaccinations were repeated on days 18 and 32 (filled arrows). Mice were monitored 2-3 times weekly for the formation of palpable tumors (>9 mm^2). Animals were sacrificed when tumors were >2 cm^2 or became ulcerated. There was a significant delay in tumor formation (*C*) for mice receiving neuET-DCs and CY in comparison to mice receiving null-DCs and CY ($p = 0.036$, Kaplan-Meier survival analysis). There was a modest trend that was not significant for improved survival (*D*) between mice receiving CY and vaccinated with either neuET-DCs or null-DCs ($p = 0.1375$, Kaplan Meier survival analysis). *E.* TDLN cells and TIL from neuET-vaccinated neu-N mice treated with either PBS (open bars) or CY (solid bars) were isolated 4 weeks post-tumor challenge and analyzed for expression of intracellular FoxP3. Bars represent the mean +/- SEM (n = 3-6 per group). *$p<0.05$, Student's t test.

Figure 3-6

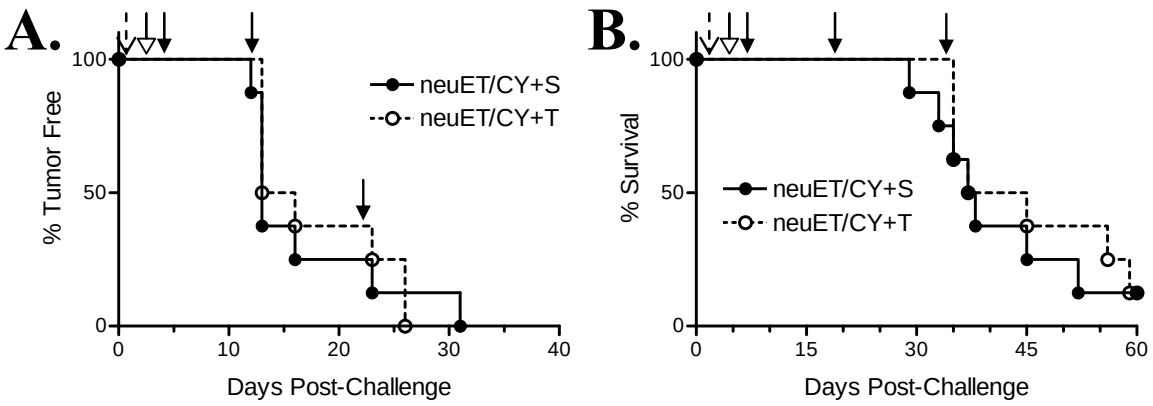
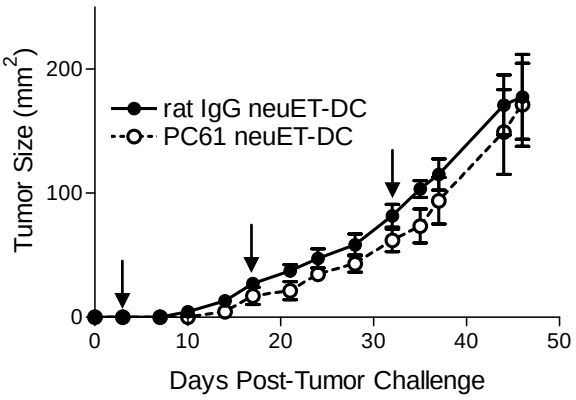

A.
100
50
0
% Tumor Free
neuET/CY+S
neuET/CY+T
0
10
20
30
40
Days Post-Challenge
B.
100
50
0
% Survival
neuET/CY+S
neuET/CY+T
0
15
30
45
60
Days Post-Challenge

Figure 3-6: Provision of tolerant mice with a neu-specific T cell repertoire does not improve VRP-DC vaccination. Female neu-N mice (n = 8 mice per group) were challenged with 5 x 10^4 NT2 tumor cells on day 0 and then treated with CY (100 mg/kg) on day 2 (dashed arrow). On day 4, mice received i.v. injections of either saline (neuET/CY+S) or 1 x 10^7 CD25-depleted splenic T cells from FVB/N mice (neuET/CY+T) as indicated by the open arrow. The following day, mice were vaccinated with 1 x 10^6 neuET-DCs, with vaccination repeated on days 19 and 33 (solid arrows). Mice were monitored 2-3 times weekly for the formation of palpable tumors (>9 mm^2). Animals were sacrificed when tumors were >2 cm^2 or became ulcerated. Cumulative data from two experiments is depicted. There was no significant difference in tumor delay (*A*) or survival (*B*) between the two groups (p = 0.234 and 0.358, respectively, Kaplan-Meier survival analysis).

Supplemental Figure 3-1



Supplemental Figure 3-1: Antibody depletion of $CD25^+$ T cells does not enhance the efficacy of VRP-DC vaccines in tolerant mice. Female neu-N mice (n = 3 per group) received i.p. injections of 0.5 mg of either rat IgG or anti-CD25 monoclonal antibody (PC61) on days -7 and -4. On day 0, mice were challenged with 5 x 10^4 NT2 tumor cells. Three days later, mice were vaccinated with 1 x 10^6 neuET-DCs and boosted on days 17 and 31 post-tumor challenge (solid arrows). Tumor growth was measured 2-3 times weekly.

CHAPTER FOUR

DISCUSSION

We hypothesized that unlike current DC vaccines, virally-transduced DCs would be capable of inducing antitumor immunity and overcoming tolerance against tumor antigens. The advantages of using viral vectors include the potential for highly efficient transduction leading to copious production of tumor antigen (5); the capacity to deliver intact antigens which can be processed into multiple epitopes and presented on all haplotypes of MHC class I and/or II molecules (8, 106); and the provision of a simultaneous activation signal resulting in optimal DC maturation through TLR-dependent or independent pathways (1, 41). Furthermore, viruses have been shown to activate signaling pathways that are required for inhibiting the activity of T_{reg}, which play an important role in maintaining tolerance against tumor antigens (103). While several viral vectors have been evaluated for *ex vivo* transduction of DCs, many of them are marred by low transduction efficiencies, interference with DC maturation and questionable safety (45 and Table 4-1). Because of these limitations, we have evaluated the feasibility of using DCs transduced with VRPs as cancer vaccines. We demonstrated that VRP could efficiently transduce human immature mono-DCs *ex vivo*, resulting in high-level transgene expression. VRP transduction induced DC maturation and the secretion of proinflammatory cytokines. Most importantly, human DCs could process and present a VRP-encoded antigen to $CD8^+$ T cells, resulting in robust T cell expansion and acquisition of effector function. Thus, VRP-transduced human DCs possessed several characteristics associated with potent DC vaccines.

Based upon the results with human DCs, we proceeded to examine the feasibility of VRP-DCs as cancer vaccines *in vivo*. As observed with human DCs, VRPs could efficiently transduce murine bone marrow-derived DCs, resulting in a similar level of maturation and proinflammatory cytokine production. Vaccination of wild-type mice with VRP-DCs

expressing a truncated neu oncoprotein induced neu-specific $CD8^+$ T cell and antibody responses. Furthermore, VRP-DC vaccines could induce regression of established neu-expressing tumors in wild-type mice, indicating that VRP-DC vaccines are highly immunogenic. However, VRP-DC vaccination of neu-N transgenic mice, which exhibit a profound immunological tolerance against neu, did not result in robust anti-neu T cell and antibody responses. Moreover, therapeutic vaccination with VRP-DCs was incapable of inhibiting the growth of existing tumors in neu-N mice. The efficacy of VRP-DC vaccines could be temporarily enhanced if mice were pretreated with CY to deplete T_{reg}. However, transfer of naïve T cells from FVB/N mice, which contain a repertoire of high-avidity T cells specific for neu, to tolerant mice was ineffective at augmenting vaccine efficacy. Taken together, these results demonstrate that while highly immunogenic in nontolerant hosts, VRP-DC vaccines cannot independently overcome T_{reg}-mediated suppression of tumor immunity in tolerant animals. These findings have broad implications for cancer immunotherapy, arguing that monotherapy with potent DC vaccines is unlikely to induce effective tumor immunity in patients with established disease. Therefore, DC vaccines should be evaluated in the context of a multimodality treatment strategy, which includes tactics for inhibiting immunoregulatory mechanisms.

Feasibility of VRP-DCs as cancer vaccines

VRP-transduced DCs appeared to possess several characteristics associated with potent DC vaccines, including high-level transgene expression, upregulation of costimulatory molecules and secretion of proinflammatory cytokines. In comparison to other vectors commonly used for DC transduction, VRPs are generally equivalent if not superior in certain

areas (Table 4-1). VRPs could transduce a mean of 37% of human immature mono-DCs at an MOI of 20 (Figure 2-2). While this transduction efficiency is relatively efficient, there are at least two different strategies for further enhancement. The first approach would involve packaging VRPs with glycoprotein coats harboring specific mutations that enhance tropism for DCs. This can be accomplished rather easily by providing the specific mutant glycoprotein gene *in trans* during the VRP packaging process. Several different VEE glycoprotein mutations have been identified in attenuated isolates or by mutagenesis (4, 7, 18, 37, 64). While these mutations have been characterized in regards to pathogenesis, their ability to confer tropism for human DCs *in vitro* remains to be fully assessed. A preliminary study has suggested that VRPs packaged in the 3014 glycoprotein coat are greater than 2-fold more efficient at transducing human DCs *in vitro* when compared to VRPs packaged in the wild-type 3000 coat (K. Davis, T. Moran, J. Serody and R. Johnston, unpublished observations). Thus, packaging VRPs in different glycoprotein coats can have dramatic effects on DC transduction efficiency. Increasing the transduction efficiency would conserve a significant amount of resources, since large-scale production of VRPs is a costly venture. A second strategy for enhancing DC transduction efficiency would be to package the VRPs in cell lines that differentially glycosylate the viral glycoproteins, such as invertebrate cell lines or mammalian cell lines that are deficient in certain glycosylation enzymes. Packaging replicon particles in these cell lines would result in preferential addition of high-mannose carbohydrate residues to the glycoproteins, which in turn results in enhanced binding to and infection of cells expressing the c-type lectin DC-SIGN. This approach has been used to increase the tropism of Sindbis virus (49) and Ross River virus (R. Shabman and M. Heise, personal correspondence) for DCs. However, enhancing the binding of VRPs to DC-SIGN

may have the deleterious repercussion of negatively modulating DC function, as interaction of pathogens with DC-SIGN can inhibit DC activation (36, 95).

A potential issue with using VRPs for tumor antigen delivery is their propensity for inducing cell death. Alphaviruses are cytopathic *in vitro* and *in vivo* (57). VRP-induced cell death of DCs was likely due to apoptosis, as infected DCs stained positive for the apoptosis marker annexin-V (Figure A-1). Although VRP-induced apoptosis could be detrimental, VRP-DCs remained predominantly viable for 12-24 hours pi *in vitro*, and it is conceivable that the half-life of DCs may be extended *in vivo* following adoptive transfer. VRP-DCs should have sufficient time to migrate to regional lymph nodes and interact with T cells, as recent studies using two-photon microscopy have shown that priming of T cells by antigen-bearing DCs occurs within the first 24 hours following vaccination (43, 68). While DC apoptosis is generally viewed as detrimental for immune induction, it is possible that apoptosis of VRP-DCs may actually enhance activation of T cells through the process of cross-presentation. In support of this idea, tumor antigens from human DCs infected with canarypox vectors can be efficiently cross-presented *in vitro* (73). Another study by Racanelli *et al.* demonstrated that DCs transfected with a cytopathic self-replicating RNA were capable of eliciting potent T cell responses by cross-presentation of DC-associated antigen by endogenous APCs (78). Moreover, apoptosis was necessary for the enhanced immunogenicity of a self-replicating DNA vaccine derived from SIN *in vivo* (54). Apoptotic vesicles released from VRP-transduced DCs appear to be loaded with VRP-encoded antigen (Figure S-1), and it is likely that they also contain double-stranded RNA associated with viral replication. This could result in enhanced cross-presentation of antigen within apoptotic vesicles via a TLR3-dependent pathway in endogenous APCs, as previously shown for cells

infected with SFV replicons (90). Regardless of the potential negative effects of VRP-induced apoptosis, it is obvious that VRP-DC vaccines have the capacity to stimulate antigen-specific T cells both *in vitro* and *in vivo* (Figures 2-6 and 3-2). Nonetheless, the DC lifespan following VRP transduction could possibly be extended by using double-promoter VRPs expressing both the tumor antigen and an antiapoptotic protein such as bcl-2, which has been shown to inhibit alphaviral-induced cell death (56).

Similar to vectors derived from Ad and SIN, VRP transduction induced phenotypic maturation of human DCs (Table 4-1 and Figure 2-4). While transduction of human DCs with retroviral or lentiviral vectors does not interfere with the potential for maturation, transduction alone rarely activates the DCs and thus requires an additional maturation stimulus. On the other hand, transduction with vectors derived from VV or HSV inhibits subsequent maturation of DCs unless certain viral genes are deleted from the vectors. VRP transduction was similar to LPS treatment at inducing expression of CD40 and further upregulating CD86 expression in human DCs, yet was inferior at inducing CD80 and CD83 expression. Because upregulation of CD80 following DC activation is relatively slow in comparison to CD86 (38), VRP-mediated inhibition of host-cell translation may prevent maximal production of CD80. Interestingly, CD80 expression has been associated with attenuation of immune responses (55, 101), and therefore reduced CD80 expression following VRP transduction could conceivably be advantageous. It may be possible to further enhance maturation by simultaneously stimulating DCs with CD40L (12), although any negative effects of this strategy upon transgene expression would need to be evaluated.

Another hallmark of VRP transduction was the copious secretion of proinflammatory cytokines, including TNF-α, IL-6 and IFN-α. These cytokines are important for activation of

both the innate and adaptive immune systems. TNF-α, IL-6 and IFN-α can induce activation of other APCs, and are likely responsible for the observed maturation of uninfected bystander DCs in the cultures (Table 2-1) (46, 60). The production of type I IFN is generally considered advantageous for tumor immunity, as these cytokines can enhance NK cell activity (40), increase cross-presentation of tumor antigen (52), augment clonal expansion of $CD4^+$ and $CD8^+$ T cells (39, 51), and directly induce apoptosis of cancer cells (92). Although type I IFN can be induced via TLR-dependent recognition of viral components, preliminary studies suggest that VRP induction of IFN-α/β is largely independent of signaling mediated by MyD88 or TLR-3 (R. Shabman and M. Heise, personal correspondence). Since VRPs replicate in the cytoplasm of infected cells, it is likely that the viral recognition proteins RIG-I and/or Mda5 are responsible for sensing VRP replication and initiating the IFN-α/β response (48). The production of IL-6 following VRP transduction may assist in circumventing T_{reg} function, as this cytokine has been shown to decrease the sensitivity of effector T cells to T_{reg}-mediated inhibition (31, 77). One potential shortcoming of VRP transduction was the limited induction of IL-12p70. IL-12p70 promotes the production of IFN-γ and development of T_H1 immune responses (50), which are often deemed necessary for effective tumor immunity. Nevertheless, studies have shown that IL-12 production by DC vaccines is not necessary for induction of antigen-specific CTL responses *in vivo* (97), which is consistent with our observations that VRP-DC vaccines could induce neu-specific $CD8^+$ T cells (Figure 3-2). Although IL-12 production by VRP-DC vaccines does not appear to be required for activation of $CD8^+$ T cells, IL-12p70 secretion could be enhanced either by codelivery of IL-12-expressing VRPs or by treatment with CD40L (13, 15).

VRP-DC vaccines had the capacity to stimulate T cell responses against foreign antigens both *in vitro* and *in vivo*. Human DCs transduced with a FMP-VRP were very potent stimulators of FMP-specific $CD8^+$ T cells, and were superior to TNF-α-matured DCs pulsed with an HLA-A2-restricted FMP peptide (Figure 2-5). Moreover, VRP-transduced DCs could stimulate both $CD8^+$ T cells and antibodies specific for neu in wild-type mice, resulting in the clearance of established tumors (Figures 3-2 and 3-3). The ability of VRP-DCs to induce antibody responses is a significant advantage over classical peptide-pulsed DC vaccines, as humoral immunity often collaborates with T cell responses for complete clearance of tumor cells (83). While the *in vitro* studies using human DCs demonstrated that VRP-DCs could stimulate antigen-specific memory T cells, the *in vivo* studies clearly show that VRP-DC vaccines can activate naïve T cells. It is likely that the elevated costimulatory molecule expression and secretion of proinflammatory cytokines associated with VRP transduction contributed to the enhanced immunogenicity of VRP-DC vaccines. However, future studies using DCs from mice deficient in various costimulatory molecules or cytokines need to be performed to support this postulation. Although it was proposed that DCs transduced with whole tumor antigens have the capacity to stimulate both $CD4^+$ and $CD8^+$ T cells, the ability of VRP-DCs to directly stimulate $CD4^+$ T cell remains to be determined. However, the observation that CD4-depleted animals fail to develop anti-neu antibodies following vaccination suggests that VRP-DCs have the capacity to activate T_H cells (Figure 3-3).

While VRP-DCs caused tumor regression in nontolerant wild-type mice, they alone were unable to induce therapeutic tumor immunity in mice tolerant to HER-2/neu (Figure 3-4). The failure to induce tumor immunity was associated with an inability to stimulate robust T

cell and antibody responses specific for neu in tolerant neu-N mice (Figure 3-4). These results are in striking contrast to results obtained in wild-type FVB/N mice, and emphasize the importance of validating vaccine efficacy in tolerant animal models. Tolerance in neu-N mice has been most thoroughly characterized by Jaffee and colleagues, and is likely mediated by a combination of central and peripheral mechanisms (28, 82). In the original paper describing tolerance in neu-N mice (82), Reilly *et al.* reported that newborn neu-N mice exhibited low expression of the neu transgene in the thymus, which could lead to central deletion of neu-specific T cells. However, thymic neu expression was not detected in fetal or adult virgin mice, suggesting that there may be a window for high-avidity neu-specific T cells to escape central deletion. Indeed, neu-specific T cells were detected following vaccination with irradiated 3T3*neu*GM cells, but at extremely low numbers. A subsequent study, though, was unable to detect $CD8^+$ T cells specific for the immunodominant epitope $RNEU_{420-429}$ in neu-N mice, suggesting that these cells are likely deleted in the thymus (29). We similarly were unable to detect $RNEU_{420-429}$-specific $CD8^+$ T cells following vaccination with VRP-DCs. To determine if tolerance in neu-N transgenic mice is primarily due to the absence of high-avidity effector T cells, we adoptively transferred CD25-depleted naïve T cells from FVB/N mice, which contain a repertoire of neu-specific T cells that have not been centrally deleted. Provision of naïve T cells from nontolerant mice to did not enhance vaccine efficacy in tolerant animals (Figure 3-6). This observation strongly argues that tolerance in neu-N mice is mediated by dominant regulatory mechanisms that actively suppress the expansion of neu-specific T cells. It is possible that regulatory mechanisms may not inhibit the effector function of primed neu-specific T cells, although studies in a melanoma model would suggest otherwise (3). We are currently planning experiments to

evaluate the ability of T_{reg} to inhibit the activity of adoptively transferred effector T cells specific for neu in neu-N mice.

There are many mechanisms that can mediate suppression of tumor immunity, including tumor-derived factors and regulatory T cells. The observation that the same tumor cell line could be rapidly rejected in nontolerant mice but not in tolerant animals suggested that NT2 tumors were not inherently immunosuppressive, and argued that suppression of tumor immunity was likely mediated by regulatory T cells. In support of this argument, Ercolini *et al.* reported that $CD8^+$ T cells specific for $RNEU_{420-429}$ could be detected in about 10-20% of vaccinated neu-N mice that had been depleted of T_{reg} with CY (28). This study indicated that high-avidity neu-specific $CD8^+$ T cells could escape thymic deletion in a minority of mice, but were actively suppressed in the periphery by T_{reg}. Adopting a similar strategy, we treated tumor-bearing neu-N mice with CY prior to vaccination with VRP-DCs. This approach resulted in inhibition of early tumor growth, as indicated by the significant delay in the formation of palpable tumors (Figure 3-5). There was also a trend for a prolonged survival, although this was not statistically significant. The temporary inhibition of tumor growth was likely due to a transient depletion of T_{reg} in the draining lymph node following CY treatment. Unfortunately, T_{reg} numbers appeared to rebound quickly, as the number of $CD4^+FoxP3^+$ T cells in the tumor and in the tumor-draining lymph node was similar in either untreated or CY-treated mice by four weeks after tumor challenge (Figure 3-5). A second treatment with CY two weeks after vaccination did not significant affect tumor growth, which is consistent with a previous report (65). Altogether, these results suggest that dominant regulatory mechanisms in tolerant hosts actively inhibit tumor immunity, and that this suppression may be difficult to overcome by vaccination alone.

We argued that *ex vivo* transduction of DCs is an ideal vaccination strategy because it delivers antigen directly to the cell responsible for initiating adaptive immunity. *Ex vivo* transduction with viral vectors is typically the preferable method for delivering antigens to DCs, since most vectors do not target DCs *in vivo*. Consistent with this notion, studies have shown that vaccination with DCs transduced *ex vivo* with Ad vectors induced superior tumor immunity compared to direct vector inoculation (84, 96). On the other hand, VRPs have been shown to target DCs *in vivo*, suggesting that direct inoculation of VRPs may not be inferior to VRP-transduced DCs at inducing tumor immunity. Indeed, a preliminary study found that vaccination of nontolerant FVB mice with 10^6 infectious units of neuET-VRP_{3000} was slightly superior to neuET-VRP_{3000}-transduced DCs at inducing neu-specific $CD8^+$ T cells, but was dramatically better at inducing neu-specific antibody responses (Figure A-2). Furthermore, tumor-bearing mice vaccinated with neu-VRP_{3000} had a similar 60-day survival compared to mice vaccinated with neuET-DCs, but tumor growth was more significantly inhibited in the former. These results may cause some to question the use of VRP-DC vaccines since direct inoculation of VRPs was better at inducing tumor immunity in nontolerant mice. However, there are certain instances where the use of VRP-transduced DCs would be preferable to direct VRP inoculation. First, virally-transduced DCs can be used in persons that have preexisting anti-vector antibodies, which can neutralize directly injected viral vectors (47, 87). While VRPs do not induce significant anti-vector immunity at low doses, vaccination with larger quantities of VRPs can result in appreciable antibody responses against the viral structural proteins, which can potentially decrease the efficacy of repeated VRP vaccinations (17). Second, direct VRP inoculation may not stimulate maximal immunity in cancer patients since many exhibit defects in DC development and function

(32). Therefore, e*x vivo* differentiation of DC precursors into functionally optimized DCs may help circumvent issues with cancer-mediated inhibition of DC activity. Regardless of their greater immunogenicity in nontolerant mice, VRPs alone could not induce regression of preexisting tumors in tolerant animals (27 and B. Long and R. Tisch, personal correspondence), which ultimately makes them no better than VRP-DC vaccines with regards to breaking tolerance.

Why do VRP-DC vaccines lack the ability to induce therapeutic tumor immunity under conditions of tolerance? The reason is likely due to a combination of factors. First, because tumor-specific $CD8^+$ T cells recognize self proteins, most that evade negative selection in the thymus are of low affinity or avidity for their cognate antigen and are thus compromised in their ability to proliferate and kill tumor cells (19, 58, 61, 72). Some studies have demonstrated that low-avidity $CD8^+$ T cells can be coaxed into mediating tumor rejection (61, 63, 72). However, most of these studies involved tumor cells expressing highly immunogenic viral antigens (i.e. influenza HA) or used adoptive transfer of TCR transgenic T cells into tolerant mice, thus artificially enhancing the tumor-specific T cell precursor frequency (63, 72, 103). Second, although deletion of high-avidity $CD8^+$ T cells specific for tumor antigens is not absolute, those that do escape into the periphery are of low frequency and are often anergic or are actively suppressed by regulatory T cells (3, 9, 21, 28, 53, 71, 75, 80). The case for active suppression is supported by our studies showing that provision of high-avidity T cells did not enhance vaccine efficacy. Third, VRP-DC vaccines may not provide an adequate amount of neu antigen for activation of low-avidity T cells. Activation of low-avidity T cells requires a significantly higher concentration of MHC-peptide complexes than needed for stimulation of high-avidity T cells (2). Increasing the VRP

transduction efficiency or the number of injected DCs may remedy this issue. Fourth, VRP-DC vaccines may not secrete the appropriate types or amounts of proinflammatory cytokines needed for optimal activation of T cells and subversion of T_{reg} activity. Fifth, it is possible that the vaccine itself may be activating regulatory T cell populations. Studies have shown that mature DCs are potent stimulators of T_{reg} expansion both *in vitro* and *in vivo* (6, 100). Furthermore, Zhou *et al.* have shown that vaccination against a model tumor antigen can activate both effector and suppressor T cells, with the suppressor T cells eventually dominating (105). Sixth, the tumors may recruit inhibitory cell populations other than T_{reg} to dampen immune responses. This is supported by our observation of large numbers of $CD11b^{+}Gr\text{-}1^{+}$ iMC within the tumors and spleens of vaccinated mice (Figure A-3). A similar finding was reported by Melani *et al.*, who described the presence of iMCs in the spleens of BALB/neu-T mice bearing spontaneous tumors (67). Immune suppression mediated by T_{reg} and iMC may not be mutually exclusive, as studies have reported that iMC may be responsible for induction of T_{reg} in the periphery (42, 101). Taken together, it is obvious that several factors may conspire against successful vaccination with VRP-DCs.

Our data suggest that virally-transduced DCs can function as potent vaccines, yet still fail to overcome tolerance. This was surprising, since studies have shown that viruses can break tolerance against self antigens and induce autoimmune disease (74, 76). Furthermore, studies by Yang *et al.* have shown that viral vectors were capable of breaking $CD8^{+}$ T cell tolerance and inducing tumor immunity, reportedly through persistent TLR stimulation (103). However, these studies were performed with highly immunogenic viral proteins used as tumor antigens. Moreover, the study by Yang *et al.* also involved adoptive transfer of high-avidity TCR transgenic T cells, which artificially inflates the precursor frequency of tumor-

specific T cells and consequently lowers the threshold for inducing tumor immunity (66). Transgenic neu-N mice are likely a much more stringent model for tolerance, since the rat neu transgene is closely related to its murine counterpart and the neu-specific T cell repertoire is of low avidity. Therefore, the mechanisms involved with maintaining tolerance in neu-N mice are likely more rigorous than those in transgenic mice expressing highly immunogenic antigens, and in turn require more than just a potent vaccine for their inhibition. Indeed, a recent study has shown that TLR stimulation combined with viral vaccination was unable to induce tumor regression in transgenic mice that develop autochthonous melanomas (93). Our studies also have an extra degree of difficulty in that we are trying to inhibit the growth of established tumors, which we feel is a more clinically relevant scenario than prophylactic vaccination. The ability of prophylactic neu vaccines to protect mice from subsequent tumor challenge or spontaneous tumor development has been demonstrated in several models (14, 15, 82, 87, 98). We were also able to demonstrate that prophylactic vaccination with VRP-DCs could significantly delay the growth of adoptively transferred tumors (Figure A-4). However, we found that tumor growth was inhibited equally by both null-DCs and neuET-DCs, indicating that protection was independent of transgene expression and was likely due to an adjuvant effect of VRP-DC vaccination on innate immunity. These observations suggest that the requirements for inducing prophylactic immunity are likely different and less stringent than those needed for inducing therapeutic immunity, which explains why vaccines in general have been unsuccessful in patients with existing disease.

In summary, transduction of DCs with VRPs is a feasible strategy for inducing immune responses against foreign antigens both *in vitro* and *in vivo*. The transduction efficiency of

human DCs with VRPs is appreciable, and there is the possibility for further enhancement. VRP transduction results in activation of DCs as illustrated by the increased expression of costimulatory molecules and the secretion of proinflammatory cytokines. Moreover, VRP-DCs expressing foreign antigens are potent stimulators of humoral and cellular immunity *in vivo*. Unfortunately, therapeutic vaccination with VRP-DCs alone is incapable of inhibiting tumor growth in tolerant mice. The reasons for this are likely multifactorial, but depend more heavily upon the dominant activity of T_{reg} rather than inhibitory factors expressed by the tumor itself. Taken together, our findings demonstrate that while potent vaccines composed of virally-activated DCs may be necessary for optimal tumor immunity, they alone are insufficient to break tolerance against tumor antigens. These findings have broad implications for tumor immunotherapy, as they imply that vaccination alone is not a practical strategy for inducing regression of established tumors in cancer patients. Altogether, effective immunotherapy will likely require: *i*) a potent vaccine capable of stimulating both $CD4^+$ T_H cells and $CD8^+$ CTLs; *ii*) a substantial number of high-avidity effector T cells; and *iii*) a strategy for inhibiting cellular mediators of tolerance.

Future directions for VRP-DC vaccines

Adjunct chemotherapy or mAb therapy. Because the immune responses generated by VRP-DC vaccines in tolerant mice are limited, it may be necessary to use adjunct chemotherapy or mAb treatment to inhibit tumor growth and allow time for the development of effective anti-tumor immunity. Several chemotherapeutic drugs have been evaluated for their ability to augment tumor immunotherapy (16, 25). Most notably, CY has been frequently combined with cancer vaccination due to its ability to deplete T_{reg} (28, 62, 85, 94) Other studies have

shown that adjunct treatment with doxorubicin or paclitaxel can enhance the efficacy of vaccination with either irradiated tumor cell vaccines (65) or VRPs encoding neu (98). Doxorubicin has the additional advantage of causing immunogenic death of tumor cells, thus resulting in enhanced presentation of tumor antigens by DCs (11). Gemcitabine has also proven effective in augmenting tumor immunotherapy, possibly due to its ability to eliminate immunosuppressive iMC in tumor-bearing animals (91). The use of mAb against neu can further augment the activity of neu-specific vaccines, possibly by inducing proteosome-dependent degradation of neu in tumor cells and consequently increasing MHC class I presentation of neu-derived epitopes (99). Taken together, these studies suggest that adjunct chemotherapy or mAb treatment may be a straightforward and efficient approach for enhancing the efficacy of VRP-DC vaccines.

Adoptive cell therapy (ACT) and VRP-DC vaccination. Because the endogenous T cell repertoire is often suboptimal for effective tumor immunity, several researchers have tried to circumvent this issue through adoptive transfer of tumor-specific T cells that had been expanded *ex vivo* (35). ACT can provide large numbers of tumor-specific effector T cells capable of inducing therapeutic regression of metastatic tumors (23). However, ACT does have some limitations including the frequent deletion of transferred cells and an inverse relationship between the *in vivo* function of T cells and the length of *ex vivo* expansion (22, 34). Furthermore, adoptively transferred cells can still be inhibited by T_{reg} *in vivo*, which likely explains why some patients have progressing tumors despite the presence of large numbers of tumor-specific T cells (86). In support of the dominant nature of T_{reg}, we found that providing tolerant mice with a naïve repertoire of T cells containing neu-specific

precursors was ineffective at overcoming T_{reg} activity and consequently enhancing vaccine efficacy (Figure 3-6). This is consistent with studies showing that tumor-specific $CD8^+$ T cells fail to inhibit tumor growth when cotransferred with 10-fold fewer T_{reg}, even when mice received concurrent injections of $CD4^+$ T_H cells (3).

Despite our failure to augment vaccine efficacy through adoptive transfer of naïve T cells, it may be possible to use VRP-DCs to enhance the efficacy of ACT using large numbers of activated neu-specific T cell clones. Concurrent DC vaccination has been shown to significantly improve the effectiveness of ACT, resulting in prolonged survival and enhanced antitumor activity of adoptively transferred $CD8^+$ T cells specific for melanoma (59). However, this study was performed with TCR transgenic T cells that had only undergone one round of stimulation. In reality, it would be extremely difficult if not impossible to generate a large number of antigen-specific T cells without multiple rounds of *ex vivo* stimulation, which can negatively affect the *in vivo* antitumor activity of these cells. Therefore, further studies are required to evaluate the effectiveness of ACT using T cell clones generated from wild-type or tolerant mice that have been expanded *ex vivo*. Incidentally, VRP-DCs may also be suitable for *ex vivo* expansion of T cell clones, as VRP-DCs were extremely efficient at expanding antigen-specific T cells *in vitro* (Figure 2-6). Because VRP-DCs could present both MHC class I- and II-restricted epitopes, they may be useful for expanding $CD4^+$ T_H and $CD8^+$ CTL. The ability of VRP-DCs to expand antigen-specific T cells *ex vivo* may be useful for the treatment of other chronic diseases, such as human immunodeficiency virus or hepatitis C virus infection.

Concluding remarks

The ability to exploit the immune system for elimination of malignant tumors remains the primary goal of tumor immunotherapy. While the immune system performs remarkably well at protecting the body from foreign invaders, its ability to attack native cells expressing self antigens is, for good reason, unsatisfactory. However, the immune system does have the capacity to mount effective and sometimes destructive immune responses against self-proteins, as evident during autoimmune diseases such as type I diabetes and multiple sclerosis. Furthermore, undeniable immune responses against tumors have been recorded in cancer patients, albeit at a very low frequency. These observations suggest that cancer immunotherapy is an achievable yet daunting goal.

Many researchers, including ourselves, have proposed that enhancing the efficacy of DC vaccines will lower the threshold for overcoming tolerance against tumors. We hypothesized that virally-transduced DCs would be an ideal vaccine platform, as viral vectors can be used to efficiently deliver tumor antigens to DCs while simultaneously providing potent immune-activating signals that may be necessary for breaking tolerance. Based on this rationale, we evaluated the use of DCs transduced with VRPs as cancer vaccines. We found that VRP-DCs possessed several putative characteristics of an ideal DC vaccine, namely high-level transgene expression, increased expression of costimulatory molecules, secretion of proinflammatory cytokines and efficient presentation of antigen to reactive T cells. Moreover, VRP-DC vaccines expressing neuET were highly immunogenic in nontolerant mice, capable of stimulating both cellular and humoral immune responses and inducing tumor regression. Despite their immunogenicity, VRP-DCs were unable to initiate effective tumor immunity against neu under conditions of tolerance. Depletion of T_{reg} prior to

vaccination enhanced the efficacy of VRP-DC vaccines in tolerant mice, suggesting that vaccination alone was incapable of overcoming tolerogenic mechanisms. Furthermore, Treg activity in tolerant mice could not be overcome through provision of high-avidity naïve T cells specific for neu. These results have significant implications for DC vaccines, as they imply that simply increasing the potency of the vaccine will be inadequate for circumventing tolerance unless additional measures are undertaken to inhibit regulatory mechanisms.

We feel that it is time to move beyond the unilateral approach of increasing DC vaccine potency for the sake of overcoming tolerance, as immunoregulatory mechanisms appear to pose a barrier too formidable for success. A paucity of high-avidity effector T cells and the dominant action of regulatory T cells collude against the induction of robust tumor immunity. These obstacles cannot be consistently surmounted by exclusively increasing the strength of DC vaccines, but also require strategies for increasing the repertoire of high-avidity T cells, such as through ACT, while concurrently inhibiting the activity of regulatory T cells through pharmacological intervention. Unfortunately, these strategies are not without risk, as broadly inhibiting T_{reg} can potentially lead to harmful autoimmunity. Therefore, extensive research needs to be performed to identify the antigenic targets of regulatory T cells so that more selective elimination of tumor-specific T_{reg} can be possible. Moreover, better methods for isolating tumor-specific T cells and expanding them *ex vivo* while maintaining their function will be necessary for effective ACT. Thus, the future directions for improving the efficacy of cancer vaccines are relatively clear, although the ability to reach the goal of curative immunity remains elusive.

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Table 4-1: Comparison of different viral vectors used for transduction of human DCs

Vector	MOI = Transduction Efficiency (Reference)	Interference with DC maturation?	Antigen presentation to T cells?	Cytopathic?
VRP	20 = 37.0%	No	Yes	Yes
Adenovirus	100 = >90% (104) 1000 = 38-100% (20) 1000 = >90% (10) 10,000 = >95% (5)	No (79, 104)	Yes (10, 20)	No (104)
Vaccinia virus	2.5 = 26-67.9% (44) 10 = 30% (102) 10 = 50-85% (8)	Yes (8, 26, 44)	Yes (8, 102)	Yes (26, 44)
Retrovirus[a]	1-10 = 22-28% (81) 1-10 = 15% (69)	No (69)	Yes (69, 81)	No (69)
Lentivirus	20 = 30-40% (24) 150 = 30% (30)	No (24, 30)	Yes (24)	No (24)
SIN replicons	50 = 18% (33)	No (33)	ND	ND
Herpesvirus	6 = >95% (88) 10 = 60% (70)	Yes (70, 88) No[b] (89)	Yes[b] (89)	Yes (88)

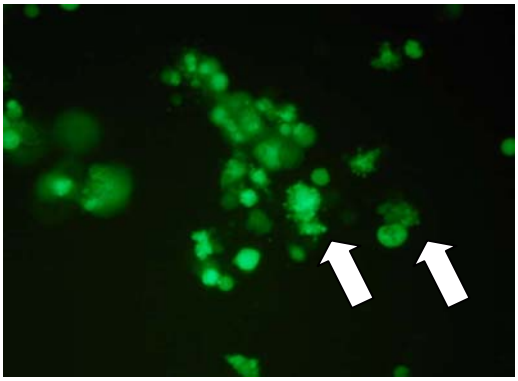
[a]Infects CD34-derived DCs only
[b]Herpes simplex virus vectors with deleted IE and *vhs* genes
ND, not determined

APPENDIX A

ADDITIONAL DATA CHARACTERIZING VRP-TRANSDUCED DC VACCINES

Figure A-1

A.



B.

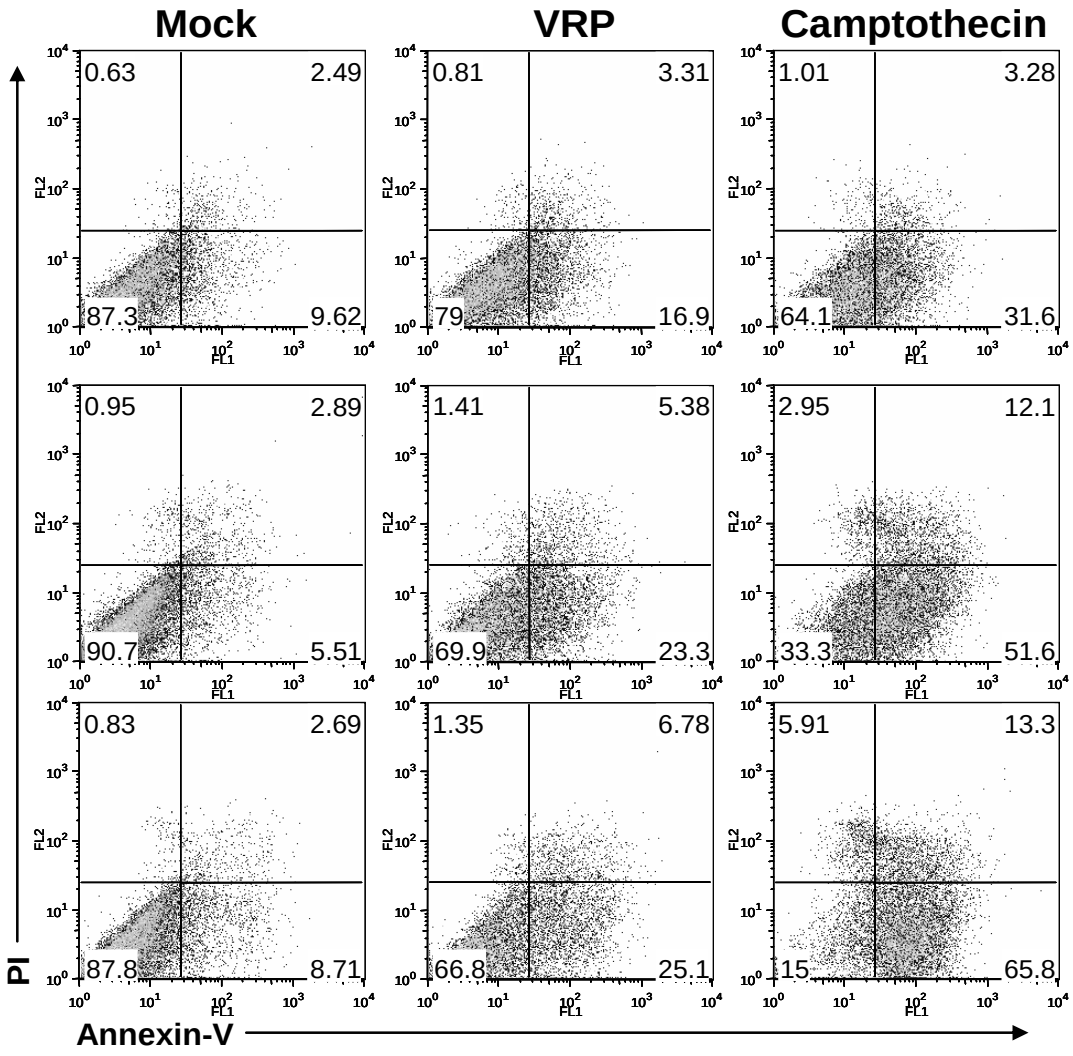

Mock
VRP
Camptothecin
0.63
2.49
87.3
9.62
0.81
3.31
79
16.9
1.01
3.28
64.1
31.6
0.95
2.89
90.7
5.51
1.41
5.38
69.9
23.3
2.95
12.1
33.3
51.6
0.83
2.69
87.8
8.71
1.35
6.78
66.8
25.1
5.91
13.3
15
65.8
PI
Annexin-V
FL1
FL2

Figure A-1: VRP infection induces apoptosis of DCs. *A.* Fluorescent micrograph of VRP-infected murine DCs at 12 hours post-infection. Arrows indicate apparent apoptosis of infected DCs as demonstrated by membrane blebbing and release of GFP-filled vesicles. *B.* Immmature DCs from neu-N mice were either mock-infected with PBS or infected with neuET-VRP (MOI = 10). Some DCs were treated with camptothecin (10uM) as a positive control for apoptosis induction. At various times post-infection, DCs were harvested and stained with FITC-conjugated annexin V and propidium iodide (PI). DCs were then gently fixed with 0.5% formaldehyde and immediately analyzed by FACS.

Figure A-2

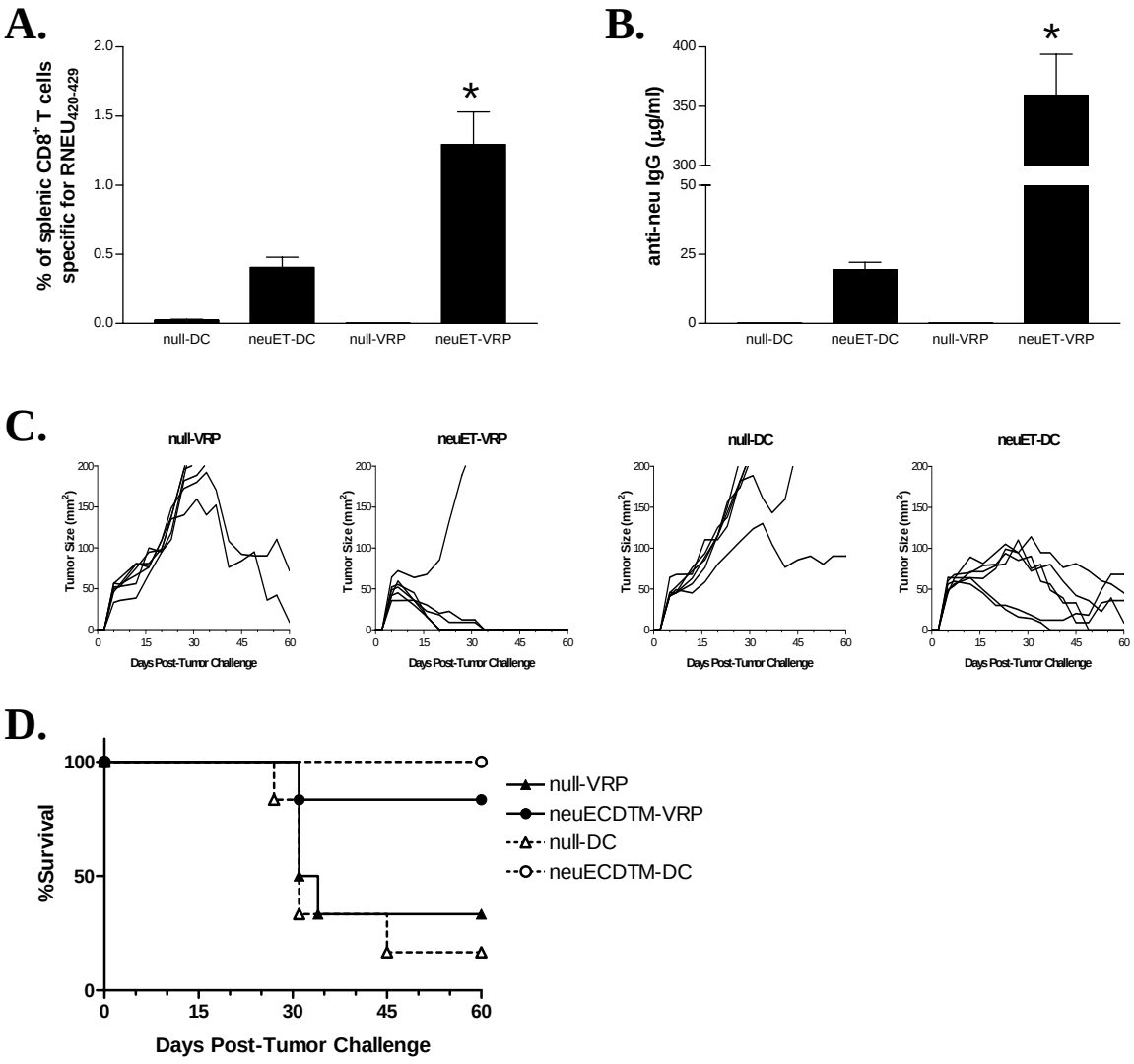

A.
% of splenic CD8+ T cells specific for RNEU420-429
2.0
1.5
1.0
0.5
0.0
*
null-DC
neuET-DC
null-VRP
neuET-VRP
B.
anti-neu IgG (µg/ml)
400
350
300
50
25
0
*
null-DC
neuET-DC
null-VRP
neuET-VRP
C.
null-VRP
neuET-VRP
null-DC
neuET-DC
Tumor Size (mm2)
200
150
100
50
0
0
15
30
45
60
Days Post-Tumor Challenge
D.
%Survival
100
50
0
0
15
30
45
60
Days Post-Tumor Challenge
null-VRP
neuECDTM-VRP
null-DC
neuECDTM-DC

Figure A-2: Direct vaccination of FVB/N mice with neuET-VRP induces more effective tumor immunity compared to vaccination with neuET-DCs. *A-B.* For vaccination with VRP-transduced DCs, female FVB/N mice were vaccinated s.c. in the mammary fat pad with 1 x 10^6 DCs that had been transduced with either null-VRP_{3000} (null-DCs) or neuET-VRP_{3000} (neuET-DCs) at an MOI of 10. For direct vaccination with VRPs, mice were inoculated in the footpad with 10^6 BHK infectious units (IU) of either null-VRP_{3000} (null-VRP) or neuET-VRP_{3000} (neuET-VRP). Mice were similarly boosted two weeks later. At seven days post-boost, splenocytes and sera were isolated and assayed for $RNEU_{420-429}$-specific T cells (*A*) or anti-neu IgG (*B*), respectively, as described in Chapter 3. Bars represent the mean +/- SEM (n = 6 mice per group). *C-D.* Female FVB/N mice (n = 6 mice per group) were challenged with 2 x 10^6 NT2 tumor cells s.c. in the mammary fat pad on day 0. Seven days later, mice received a single vaccination of either 1 x 10^6 null-DCs, 1 x 10^6 neuET-DCs, 1 x 10^6 IU of null-VRP_{3000}, or 1 x 10^6 IU of neuET-VRP_{3000}. VRP-DC vaccines were given s.c. in the contralateral mammary fat pad, while VRPs were injected into the footpad. *C.* Tumor growth of individual vaccinated mice. *D.* 60-day survival of vaccinated mice. *$p<0.05$ in comparison to neuET-DCs, Student's t test.

Figure A-3

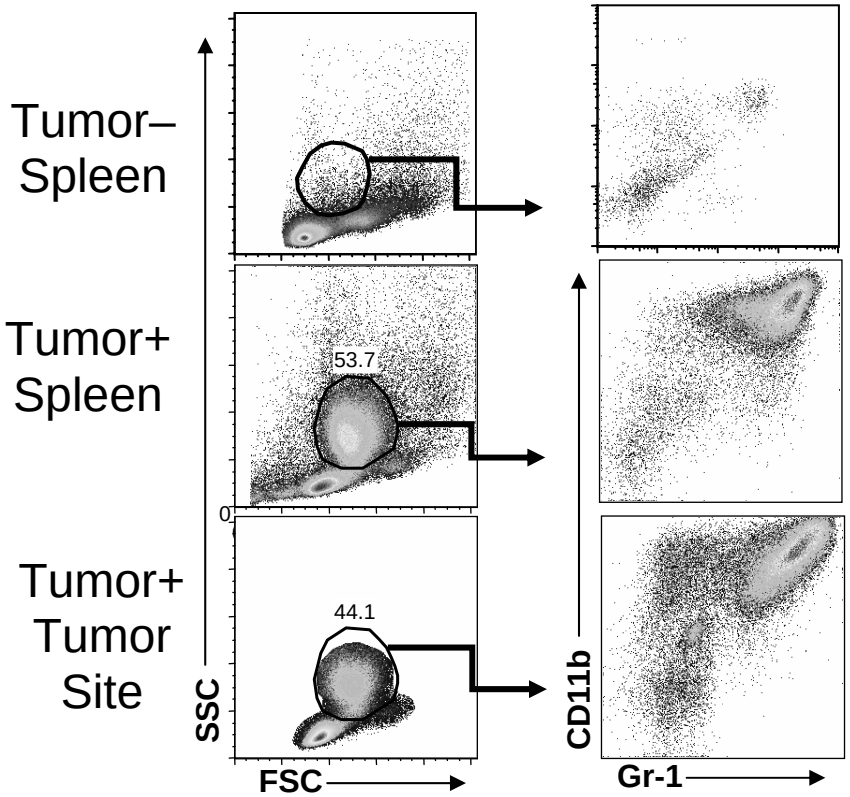

Tumor–
Spleen
Tumor+
Spleen
Tumor+
Tumor
Site
53.7
44.1
SSC
FSC
CD11b
Gr-1

Figure A-3: Transgenic neu-N mice bearing NT2 tumors have dramatically increased numbers of CD11b^{+}Gr-1^{+} immature myeloid cells in the spleen and at the tumor site. Female neu-N mice were challenged with 5 x 10^4 NT2 tumor cells. At 6-7 weeks post-tumor challenge, spleens from unchallenged mice or tumor-bearing mice were harvested, disrupted into a single-cell suspension, and analyzed for expression of CD11b and Gr-1 by FACS. Tumors were likewise excised, disrupted into a single-cell suspension, and then layered over a Percoll-Lympholyte M density gradient. After centrifuging for 30 min at 2500 rpm, 25ºC, mononuclear cells at the Percoll-Lympholyte interface were harvested and analyzed for expression of CD11b and Gr-1 by FACS. Representative data from a tumor-free (tumor-) mouse and a tumor-bearing (tumor+) mouse are depicted.

Figure A-4

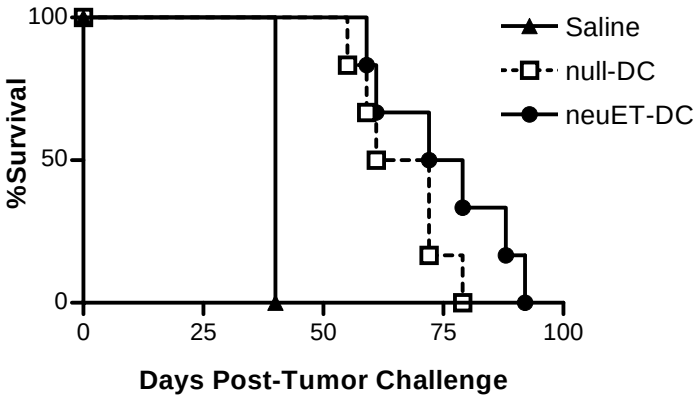

100
50
0
%Survival
0
25
50
75
100
Days Post-Tumor Challenge
Saline
null-DC
neuET-DC

Figure A-4: Prophylactic vaccination of tolerant mice with VRP-DCs nonspecifically prolongs survival following tumor challenge. Female neu-N mice (n = 6 mice per group) were vaccinated with either saline, 1 x 10^6 null-DCs, or 1 x 10^6 neuET-DCs s.c. in the mammary fat pad. Mice were boosted in a similar manner two weeks later. At seven days post-boost, mice were challenged with 5 x 10^4 NT2 tumor cells. Tumor growth was measured 2-3 times per week, and mice were euthanized when tumor area was >2 cm^2 or when tumors became ulcerated. Mice vaccinated with neuET-DCs had a significantly prolonged survival in comparison to saline-treated mice but not mice vaccinated with null-DCs ($p = 0.008$ and 0.16, respectively, Kaplan-Meier survival analysis).