

**TUMOR-INDUCED IMMUNE SUPPRESSION OF THERAPEUTIC
CANCER VACCINES**

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ABSTRACT

JOSEPH E. BURGENTS: Tumor-Induced Immune Suppression of Therapeutic
Cancer Vaccines
(Under the direction of Jonathan S. Serody)

Therapeutic cancer vaccines offer an attractive strategy for treating cancer. Not only can the immune system specifically target tumor cells, but it also provides long-term memory needed to prevent recurrent disease. Despite the therapeutic appeal of cancer vaccines, they have not been effective clinically. Thus, we set out to determine the mechanisms by which the presence of tumor inhibited vaccine efficacy. For this work, we focused on tolerant preclinical tumor models. Vaccination of tolerant neu-N mice with alphaviral replicon particles expressing rat neu (neuET-VRP) failed to induce regression of established neu-expressing tumors. The inability of VRPs to induce regression in neu-N mice was due to a dominant role of the immunosuppressive tumor environment in these animals, as opposed to central deletion of tumor-specific lymphocytes. Accordingly, transfer of neu-specific lymphocytes into neu-N mice did not inhibit tumor growth. On the other hand, we demonstrated that myeloid-derived suppressor cells (MDSC) were a main mediator of suppression in neu-N mice. Depletion of MDSCs, along with provision of neu-specific lymphocytes and neuET-VRP vaccination, induced tumor regression in the majority of neu-N mice. We also identified other immunosuppressive mechanisms that suppressed anti-tumor immunity in neu-N mice. These included suppression by T_{reg} cells, CD200 and IDO. As MDSCs were the main mediator of suppression following

therapeutic vaccination, we evaluated genes important for their function. We found that MDSCs expressed NLRP3, a member of the inflammasome complex that generates IL-1 β and IL-18. While the NLRP3 inflammasome is known to enhance immunity in response to both microbial and non-microbial stimuli, its role in anti-tumor immunity is not known. We found that NLRP3 promoted the accumulation of MDSCs in the tumor following therapeutic dendritic cell (DC) vaccination. The decreased accumulation of MDSCs in *Nlrp3*^{-/-} mice led to a fourfold improvement in survival compared to wild type mice after DC vaccination. These data establish an unexpected role for NLRP3 in impeding anti-tumor immunity and suggest novel approaches to improving cancer vaccines. In summary, our data suggest that current therapeutic vaccines are not effective due to the robust immunosuppressive mechanisms present in the tumor environment.

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LIST OF ABBREVIATIONS

Ab	Antibody
ACT	Adoptive cell therapy
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATP	Adenosine tri-phosphate
ATRA	All-trans retinoic acid
BM	Bone marrow
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen-induced arthritis
CIAS-1	Cold-induced autoinflammatory syndrome 1
CLL	Chronic lymphocytic leukemia
CpG	Cytosine-phosphate-guanine
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CY	Cyclophosphamide
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid

dsRNA	Double stranded RNA
EAE	Experimental autoimmune encephalomyelitis
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence-activated cell sorter
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage colony-stimulating factor
HER2	Human epidermal growth factor receptor 2
HPV	Human papillomavirus
HR	Hazard ratio
HUVEC	Human umbilical vein endothelial cells
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IgSF	immunoglobulin superfamily
IL	Interleukin
iNOS	Inducible nitric oxide synthase
I.P.	Intraperitoneal
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
I.V.	Intravenous
JAK	Janus kinase
LLC	Lewis lung carcinoma
LN	Lymph node

LPS	Lipopolysaccharide
mAB	Monoclonal antibody
MAP	Mitogen-activated protein
MART-1	Melanoma-associated antigen recognized by T cells
MBL	Mannan-binding lectin
MCA	Methylcholanthrene
MDSC	Myeloid-derived suppressor cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
MM	Multiple myeloma
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
MOI	Multiplicity of infection
MSR	Macrophage scavenger receptor
MT	Methyl-tryptophan
NALP3	Nacht domain, leucine-rich repeat, and PYD-containing protein 3
NF- κ B	Nuclear factor-kappa B
NIH	National Institutes of Health
NLR	Nucleotide-binding domain leucine-rich repeat containing
NLRP3	NLR family, pyrin domain containing 3
NO	Nitric oxide
NS	Not significant

OVA	Chicken ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed Death-1
PE	Phycoerythrin
PI3	Phosphoinositide 3
PKR	Protein kinase receptor
PMA	Phorbol 12-myristate 13-acetate
PSMA	Prostate-specific membrane antigen
RMANOVA	Repeated measures ANOVA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAP	Serum amyloid protein
S.C.	Subcutaneous
SEM	Standard error of the mean
shRNA	Short hairpin RNA
STAT	Signal transducer and activator of transcription
TAA	Tumor associated antigens
TCR	T cell receptor
TDLN	Tumor draining lymph node
TEM	Trans endothelial migration
TF	Tissue factor
TGF- β	Transforming growth factor beta

TIL	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
T _{reg} cell	Regulatory T cell
VEE	Venezuelan equine encephalitis
VEGF	Vascular endothelial growth factor
VRP	VEE replicon particle
V _x	Vaccine
WT	Wild type

CHAPTER ONE
INTRODUCTION

Immune Recognition of Non-Self versus Self

In order to protect the host from invading microbes, the immune system must be able to specifically recognize and react to foreign antigens, while at the same time maintaining tolerance to self-antigens. This is accomplished through the actions of both the innate and adaptive immune system. The innate immune system recognizes pathogens using receptors that are genetically fixed, while the adaptive immune system uses receptors that are generated *de novo*.

Innate immune system

The innate immune system is composed of cells with pattern recognition receptors that can recognize foreign antigens (147). These cells include macrophages, dendritic cells (DC), mast cells, neutrophils, and eosinophils. Pattern recognition receptors are intra or extracellular receptors that recognize specific motifs present on pathogens, with the classic example being Toll-like receptor (TLR) 4, which recognizes lipopolysaccharide (LPS) expressed by gram negative bacteria (306). Activation of TLRs leads to downstream activation of NF- κ B, the mitogen-activated protein (MAP) kinase pathway and phosphoinositide (PI3) kinase and results in the generation of proinflammatory cytokines such as IL-12/IL-23 and enhanced expression of co-stimulatory molecules such as CD80/CD86. Other pattern recognition receptors include mannan-binding lectin (MBL), C-reactive protein (CRP), serum amyloid protein (SAP), macrophage scavenger receptor (MSR), protein kinase receptor (PKR), and nucleotide-binding domain, leucine rich containing (NLR) proteins (147). NK cells, which are also part of the innate immune system, are unique in that they do not contain receptors for

foreign pathogens. Instead, they have inhibitory receptors specific for self-major histocompatibility complex (MHC) molecules, and are therefore activated by lack of inhibitory self proteins (219).

Adaptive immune system

The adaptive immune system is composed of T and B lymphocytes that contain antigen-specific receptors assembled from rearranged gene segments (20). This allows for a greater diversity of receptor specificity compared to the innate immune system, as well as the selection of unique, antigen-specific clones. The differentiation of antigen-specific memory cells upon antigen challenge is also a defining characteristic of adaptive immunity, and allows for a much more robust and rapid immune response upon reinfection with a previously encountered organism. With the increased diversity of receptor specificity of the adaptive immune system, exists the potential for the development of receptors specific for self-antigens. Therefore, the adaptive immune system needs to be regulated in a way to ensure that lymphocytes will not react against host cells. This is done at both the sites of lymphocyte development (central tolerance) and in the periphery (peripheral tolerance).

In central tolerance, immature T and B lymphocytes whose receptors are able to bind self-antigens with high avidity are deleted (118, 155, 160). Although central tolerance mechanisms are believed to deplete the majority of self-specific lymphocytes, cells with receptors specific for self-antigens do escape into the periphery. Therefore, further regulatory mechanisms exist to ensure that these cells do not mediate tissue pathology. These mechanisms of peripheral tolerance include induction of anergy,

suppression of adaptive immune cells by regulatory T cells of which the best characterized are thymically selected CD4⁺CD25⁺ Foxp3-expressing cells (T_{reg} cells), and induction of cell death (266, 273).

Adaptive immune responses are regulated by the innate immune system. Innate antigen presenting cells (APCs) present antigen to T cells in the context of MHC class I or class II molecules. This ensures activation of an antigen-specific immune response. Also, optimal stimulation of adaptive immunity requires upregulation of co-stimulatory molecules on innate cells, which occurs upon recognition of foreign antigen through receptors specific for PAMPs and DAMPs. This adds an additional layer of regulation to ensure that lymphocytes are only activated against foreign pathogens. Finally, activation of the innate immune system results in the production of inflammatory cytokines, which drives the polarization of naïve T cells down a number of different effector pathways (336). For example, activation of the innate immune system by viruses results in expression of type 1 cytokines such as IL-12/IL-23 which drives expression of IFN γ by T cells and promotes polarization of Th1 cells (55, 149). On the other hand, innate immune recognition of helminths results in expression of type 2 cytokines such as IL-4 and IL-13, which promote Th2 cell polarization (55). Other T cell polarization pathways also exist, including T_{reg} cells and Th17 cells, which are controlled by the cytokines TGF- β and IL-6 (336). Thus, three signals determine the nature of adaptive immune responses: (1) recognition of antigen in the context of MHC molecules, (2) expression of costimulatory molecules that promote activation of naïve T cells, and (3) expression of polarizing cytokines that determine the pathway of T cell differentiation (149).

Immune recognition of tumor cells

The progression of tumors presents a unique challenge to the immune system, in that these cells are derived from self and therefore would not be expected to express the PAMPs and DAMPs needed to activate innate immunity and promote tumor-specific adaptive immunity. Also, as the majority of antigens characterized at this time on tumor cells are derived from self, as either overexpressed self-proteins or reactivation of proteins present during development, it is unclear whether a sufficient repertoire of tumor-specific lymphocytes exists in the periphery to respond to these antigens. As discussed below, many attempts have been made to activate the immune system to treat cancer, but these have not been effective clinically as a result of the difficulty with initiating and maintaining antigen-specific responses to self-antigens present on tumor cells. The overall focus of the current work was to evaluate the mechanisms of tolerance that dominate in preventing the immune system from reacting against tumor cells. Specifically, we focused on the mechanisms of suppression induced by tumors that inhibit efficacy of therapeutic cancer vaccines.

History of Tumor Immunotherapy

The discovery that the immune could be involved in tumor clearance had its earliest origins over 200 years ago when it was first noted that infections in cancer patients were sometimes associated with cancer remissions (334). Serious investigation into this phenomenon was first conducted by William Coley in the 1890s after he observed the remission of cancer in a patient suffering from infection with *Streptococcus pyogenes*. From this observation he went on to treat cancer patients with streptococcal

cultures. His findings of tumor regression in some of these patients was published in 1893 and is the first manuscript to describe the successful use of immunotherapy (51). His bacterial preparations became known as ‘Coley’s toxin’ and were used to treat nearly 1,000 patients with a reported success rate of approximately 10% (334). Interestingly, as will be discussed later, this success rate is comparable to the best current rates achieved over 100 years later. Despite this, ‘Coley’s toxin’ was not widely accepted, and, as a result of animal studies with transplantable tumors, the subsequent years were filled with much dispute about the potential to utilize a patient’s immune system to treat cancer (338). Currently, it is believed that Coley’s toxin was effective in stimulating innate immune responses via activation of TLRs and other PAMPs present on innate immune cells.

In the 1950’s, a number of reports demonstrated that methylcholanthrene-induced tumors could be recognized by the immune system when transplanted into syngeneic mice (15, 93, 161, 247). As a result of these data, multiple different investigators began to evaluate the use of cancer immunotherapy and in the 1960’s Frank Burnet proposed that lymphocytes continually patrol tissues and eliminate transformed cells through the recognition of tumor associated antigens (TAA) (40). He labeled this process ‘immunosurveillance.’ This began an earnest search for TAAs that could be recognized by the immune system and for which cancer vaccines could be designed.

Burnet’s ‘immunosurveillance’ hypothesis appeared counter to his previous theory of immunological tolerance, in which he proposed that self-specific lymphocytes are deleted during the development of the immune system (41). Although there was much excitement with Burnet’s immunosurveillance hypothesis, for a number of reasons,

the scientific community returned to the general belief that immunotherapy was not possible. One of the main arguments against the ability of the immune system to recognize tumor cells was that T cell-deficient, athymic nude mice do not have an increased frequency of tumor incidence, although the validity of these experiments was later questioned when it was discovered that these mice do in fact contain functional T cells (190, 302-304). It was argued that, although the immune system could recognize chemically induced tumors, spontaneous tumors could not be recognized by the immune system (129). Therefore, the general opinion of the scientific community that prevailed until the 1990s was that immunotherapy was not clinically feasible.

Resurgence for tumor immunotherapy began in the 1990s with the discovery of numerous TAAs in both mice and humans (27, 29, 200, 255, 318, 321). It was also discovered at this time that some T cells could escape central tolerance, leaving open the possibility that tumor-specific T cells were present in the periphery (10, 95, 194). Also, numerous studies reported increased tumor incidence in immune deficient mice, implicating both adaptive and innate immunity in tumor immunosurveillance (60, 154, 276, 290, 291, 320). With the establishment that the immune system could recognize tumor cells and the identification of TAAs, focus shifted to evaluating the mechanisms that prevent immune-mediated clearance of tumor cells in cancer patients.

Immunosuppressive Tumor Environment

As tumors are derived from host cells, many of the mechanisms of central and peripheral tolerance discussed above that prevent immune responses against self-antigen likely prevent the induction of anti-tumor immunity. In addition, there are a number of

mechanisms of active suppression induced by tumor cells that inhibit anti-tumor immunity. These include the induction of T_{reg} cells and myeloid-derived suppressor cells (MDSC), induction of the suppressive molecule IDO, and the action of a number of immune inhibitory receptors including CD200R, CTLA-4, and PD-1 (Fig. 1-1). In the current work, we evaluated the contribution of a number of these suppressive mechanisms in inhibiting anti-tumor immunity in preclinical vaccine models. For a description of the myriad of immunosuppressive properties that are present in the tumor environment please see Figure one.

Regulatory T cells

T_{reg} cells are potent suppressors of anti-tumor immunity and are a significant contributor to peripheral tolerance (75, 266, 280, 355). T_{reg} cells were initially identified as suppressor T cells by Gershon et al. in 1970 (108, 109). Within ten years following their initial discovery, it was demonstrated that T_{reg} cells inhibited anti-tumor immunity (22, 42, 101, 230). Although there was evidence of suppressive T cells, the absence of a definitive marker for these cells left much skepticism in the field. A resurgence of interest into suppressive populations of T cells occurred in 1995 when Sakaguchi et al. identified the IL-2 receptor, CD25, as a phenotypic marker for T_{reg} cells (268). Subsequent studies demonstrated that Treg cells express a lineage-specific transcription factor, Foxp3, and T_{reg} cells are now identified by expression of CD4 and FoxP3 (94, 133, 159). The importance of FoxP3 (scurfin) was first shown with the description of the scurfy mouse by Godfrey et al. in the early 1990s, which succumb to autoimmune disease due to the unregulated proliferation and activation of T cells (114, 115). In recent years

the field of T_{reg} biology has exploded and a number of different subsets of T_{reg} cells have been described, including inducible T_{reg} cells as well as CD8⁺ regulator T cells (52, 124, 332, 333). CD4⁺CD25⁺FoxP3⁺ T_{reg} cells make up approximately 5-10% of CD4⁺ cells in normal mice and humans but increase in numbers with increased tumor burden.

The first evidence of T_{reg} cells inhibiting anti-tumor immunity came from studies in which T_{reg} cells were depleted using anti-CD25 depleting antibody (clone PC61) which resulted in decreased tumor growth (233, 282). Later, adoptive transfer experiments definitively demonstrated that T_{reg} cells suppress anti-tumor immunity in both mice and humans (9, 54, 316). Unfortunately, as CD25 is also a marker for activated T cells, use of CD25 depleting Ab is limited and is only effective when administered prior to tumor challenge (233). Future treatment targeting T_{reg} cells will likely need to inhibit specific mechanisms of T_{reg} mediated suppression.

A number of suppressive mechanisms have been proposed for T_{reg} cells. These include direct interaction with effector T cells, suppression through interaction with APCs, and production of suppressive cytokines (267, 279, 330, 355). T_{reg} cells can also directly mediate killing of effector T cells and APCs through granzyme B or perforin dependent pathways (117, 123). As mentioned above, T_{reg} cells are defined by their expression of the IL-2 receptor, CD25. T_{reg} cells can suppress effector T cells through competition for IL-2, which is an important growth cytokine for T cells (56). As T_{reg} cells make up an increased percentage of cells in tumor-bearing animals, this might be an important mechanism of T_{reg}-mediated suppression of anti-tumor immunity.

T_{reg} cells also suppress T cells through interaction with APCs. T_{reg} cells express the inhibitory molecule CTLA-4, and interaction of CTLA-4 on T_{reg} cells with CD80 or

CD86 on DCs induces expression of indoleamine 2,3-dioxygenase (IDO) in DCs. IDO, which will be discussed in more detail below, suppresses T cell activation and proliferation and appears to play a major role in T cell suppression in the tumor environment (202).

T_{reg} cells also suppress T cells through the production of the suppressive cytokines IL-10 and TGF- β (279, 333). Specifically, production of these cytokines is a proposed mechanism for induction of, as well as suppression mediated by, the subset of inducible T_{reg} cells (48, 111, 124, 240). IL-10 and TGF- β also inhibit APC function. Of note, other cells in the tumor environment besides T_{reg} cells, namely APCs, can also produce these cytokines.

Regardless of the exact mechanism of T_{reg}-mediated suppression of anti-tumor immunity, T_{reg} cells are involved in inhibiting anti-tumor effector T cells and multiple studies aimed at inhibiting T_{reg} cells have resulted in increased immunity and decreased tumor growth. Treatment with CTLA-4-specific antibody increased anti-tumor immunity (177). Treatment with antibody specific to GITR, which is expressed on the surface of T_{reg} cells and activated effector T cells, decreased the *in vitro* suppressive activity of T_{reg} cells and increased anti-tumor immunity when administered *in vivo* (163, 283, 316). Finally, treatment with low dose cyclophosphamide, which has been shown to preferentially deplete T_{reg} cells, resulted in increased anti-tumor immunity and decreased tumor growth (21, 113).

Myeloid-derived suppressor cells

Meloid-derived suppressor cells (MDSC) are immature bone marrow derived myeloid cells that accumulate in the spleen and tumor of mice and humans. Although the first evidence of induction of bone marrow-derived myeloid cells with suppressive function was over 20 years ago (350), these cells are only now being fully described (105). Under normal conditions, MDSCs give rise to mature myeloid cells (i.e. macrophages, dendritic cells, and granulocytes), but under situation of tumor growth, as well as trauma and some chronic infections, these cells expand and accumulate in the spleen and tumor where they are involved in the suppression of anti-tumor immunity.

In mice, MDSCs are defined by expression of surface markers Gr-1 and CD11b, while in humans they are defined as CD14⁻CD11b⁺ or CD33⁺ lineage (CD3,CD14,CD19,CD57) negative HLA-DR⁻ (6, 35, 173). In mice, MDSCs can be divided into two groups based on staining with antibodies specific for Ly-6G and Ly-6C (348). Using antibodies that are specific to these two genes allows identification of the two subsets of MDSCs. The first group, representing approximately 70% of splenic MDSCs in tumor-bearing animals, displays a granulocytic morphology and is identified as CD11b⁺Ly-6G^{hi}. The second group displays a monocytic morphology and is identified as CD11b⁺Ly-6G⁻Ly-6C⁺ (348). Monocytic MDSCs also express CD115 and F4/80. As discussed below, both granulocytic and monocytic MDSCs can suppress T cells by a variety of different mechanisms.

In normal, tumor-free, mice MDSCs make up at most 2% of total splenocytes, but in tumor-bearing mice they can make up close to 40% of splenocytes (348). A similar tenfold expansion is observed in the blood of cancer patients (6, 59, 231). The expansion

of MDSCs appears to be a direct result of increased production of a number of factors including cyclooxygenase 2, prostaglandins, stem-cell factor, IL-1 β , IL-6, and VEGF (38, 103, 238, 286). STAT3 is believed to be the main transcription factor responsible for MDSC expansion (49, 166, 221). Once expanded, MDSCs are activated by a number of different factors, including IFN γ , IL-4/IL-13, and TLR agonists, which activate the transcription factors STAT1, STAT6, and NF- κ b, respectively (34, 58, 105, 106, 171, 264, 287).

MDSCs suppress T cells by a variety of different mechanisms. First, MDSCs suppress T cells through enhanced metabolism of L-arginine through the production of arginase 1 and iNOS (252). Depletion of L-arginine inhibits T cell proliferation by decreasing expression of the CD3 ζ -chain and by preventing expression of cell cycle regulators cyclin D3 and cyclin-dependent kinase 4 (253, 254). Production of NO from L-arginine suppresses T cells by inhibiting JAK3/STAT5 signaling (23).

Production of ROS by MDSCs is also a main mechanism of MDSC-mediated suppression (173, 271). Production of ROS by MDSCs might be particularly important in the tumor environment as many tumor-associated factors including TGF- β , IL-3, IL-6, IL-10, and GM-CSF have been shown to induce ROS production by MDSCs (105). Also, MDSCs were unable to suppress T cells *in vitro* when ROS production by MDSCs was inhibited (173, 271).

Peroxynitrate, which is the product of NO reacting with the ROS superoxide anion, has also been shown to inhibit T cells through nitrosylation of tyrosine (33). Peroxynitrate has also been implicated in antigen-specific suppression mediated by MDSCs. Nitration of the T cell receptor on CD8⁺ T cells following direct cell contact

with MDSCs inhibited antigen specific stimulation while maintaining responsiveness to non-specific stimulation (216).

Recent data suggest that MDSCs can also suppress T cells indirectly through the induction of T_{reg} cells (138, 275, 345). A number of different mechanisms have been proposed to explain MDSC mediated induction of T_{reg} cells, which include IFN- γ dependent IL-10 production (138), interaction of CD80 with CTLA-4 (345), and presentation of TAAs to T_{reg} cells (275).

Depletion or inhibition of MDSCs has been shown to improve immunotherapy. Depletion of MDSCs with anti-Gr-1 Ab decreased tumor growth in a number of murine tumor models (179, 207, 274, 310). The chemotherapeutic agent gemcitabine also depletes MDSCs resulting in decreased tumor growth (305). Approaches to inhibit MDSC function or expansion that have resulted in increased anti-tumor immunity include treatment with the cyclooxygenase 2 (COX-2) inhibitor celecoxib (308), the phosphodiesterase 5 inhibitor sildenafil (274), the ROS inhibitor nitroaspirin (57), blocking antibody to the SCF ligand c-kit (238), the VEGF blocking antibody avastin (170), and the MMP9 inhibitor amino-biphosphonate (199). A third treatment approach targeting MDSCs is to induce their maturation. This approach is particularly appealing as maturing MDSCs into macrophages and DCs will not only eliminate a suppressive population but has the potential to increase the number of APCs that can promote anti-tumor immunity. For example, the vitamin A metabolite all-*trans* retinoic acid (ATRA) induces differentiation of MDSCs into macrophages and DCs and treatment with ATRA has been shown to enhance tumor-specific T cell responses and decrease tumor growth (169, 205, 220).

NLRP3 inflammasome

As has been discussed, it is becoming increasingly clear that the inflammation associated with the tumor environment promotes both cancer initiation and progression (16, 30, 53, 156, 191). One of the main cytokines important for the induction and maintenance of the inflammatory tumor environment is IL-1 β . There are a number of mechanisms responsible for the generation of IL-1 β including the activity of the NLR family, pyrin domain containing 3 (NLRP3; CIAS1; NALP3) inflammasome which converts pro-IL-1 β into its active form IL-1 β . The NLRP3 inflammasome is a protein complex composed of NLRP3, ASC/PYCARD/TMS/CARD5, Cardinal and pro-caspase-1 (3, 192, 193, 314). Formation of this complex results in the proteolytic maturation of caspase-1 which cleaves and activates pro-IL-1 β and pro-IL-18 to generate IL-1 β and IL-18 (192).

NLRP3 is an intracellular sensor activated by a wide variety of both microbial and nonmicrobial molecular motifs. The microbial motifs are part of the pathogen associated molecular patterns (PAMPs) and include gram positive and negative bacteria, RNA and DNA viruses, polyI:C, and LPS (193). The nonmicrobial signals include aluminum salts, asbestos, ATP, silica crystals and urate crystals (96, 97, 135, 193). Many of these nonmicrobial signals are associated with tissue injury and are referred to as damage associated molecular patterns (DAMPs) (164, 193). Little is known about whether the NLRP3 inflammasome is activated in the tumor environment. Recently, Ghiringhelli et al. found that NLRP3 was activated by ATP released from tumors cells that were exposed to specific chemotherapy drugs (110). Further, they reported that activation of IFN- γ

producing CD8⁺ T cells was dependent on activation of NLRP3 in DCs (110). These data suggested that NLRP3 played a role in the generation of anti-tumor immunity.

The role of NLRP3 in the tumor environment in the absence of chemotherapy or in the context of therapeutic vaccination is not known. Given previous work demonstrating that IL-1 β could inhibit immune responses in tumor-bearing hosts, we hypothesized that NLRP3 would inhibit anti-tumor immunity (167, 265). Chapter three of this dissertation will focus on the role of NLRP3 in the setting of therapeutic vaccination against melanoma.

CD200:CD200R

CD200 (OX-2) is a type 1 transmembrane protein with two IgSF domains but no known cytoplasmic signaling motif (19). CD200 is expressed on a variety of different cell types of both hematopoietic and non-hematopoietic origin (339). The receptor for CD200, CD200R, is restricted to myeloid cells (132, 340). CD200R is almost identical to CD200 except for the addition of a cytoplasmic ITIM motif that delivers an inhibitory signal to myeloid cells after binding with CD200 (132). CD200 is therefore believed to be involved in the regulation of myeloid cells.

Consistent with its role in the regulation of myeloid cells, CD200 knockout mice have increased numbers of macrophages and granulocytes in lymphoid organs and are more susceptible to collagen-induced arthritis (CIA) and experimental allergic encephalomyelitis (EAE) (132). In addition, CIA and EAE can be suppressed by treatment with CD200R agonists (121, 132). *In vitro*, addition of CD200R agonists suppresses T cells through decreased IL-2 and IFN- γ and increased IL-10 (119).

Our group has shown that expression of CD200 by melanoma cells suppresses T cell proliferation and cytokine production *in vitro* (244). In a humanized mouse model of CLL, treatment with anti-CD200 antibody resulted in inhibition of tumor growth (168). Blocking CD200 also improved anti-tumor immunity in a murine breast cancer model (120). These data suggest that CD200 is an important mediator of immunosuppression in the tumor environment. Neu-overexpressing breast cancer cells have high levels of expression of CD200, much more than that found from melanoma cells, and therefore this pathway could be critically important for blocking immune responses to breast cancer cells.

Indoleamine 2,3-dioxygenase

Indoleamine 2,3-dioxygenase (IDO) catalyzes the breakdown of the essential amino acid tryptophan (201). The first evidence of an immunoregulatory role for IDO came from a study by Munn et al. where they demonstrated that production of IDO protected the fetus from T cell attack (215). The proposed mechanism was decreased T cell proliferation due to IDO-dependent depletion of tryptophan (215, 325). The downstream metabolites of the kynurenine pathway initiated by IDO can also suppress T cell proliferation and induce apoptosis (83, 100).

IDO is expressed by pDCs in the tumor draining lymph node and by tumor cells (211, 213, 214, 319). IDO expression by tumor cells correlated with poor prognosis in patients with ovarian (232), endometrial (143), and colorectal (32) cancer. Furthermore, inhibiting IDO promoted tumor immunity in a number of mouse models (211, 212).

CTLA-4 and PD-1

Optimal activation of T cells is believed to require recognition of cognate antigen as well as binding to costimulatory molecules on APCs (178). The main costimulatory receptor on T cells is CD28, which binds to both B7-1 (CD80) and B7-2 (CD86) on APCs (98, 122, 128). Lack of adequate co-stimulation, particularly with CD4⁺ T cells, results in a hyporesponsive state called anergy (273). One hypothesis for the low level of anti-tumor immunity in cancer patients is lack of adequate co-stimulation. This has led to investigation evaluating the efficacy of vaccination with irradiated tumor cells expressing B7-1. Unfortunately, this has not been effective at inducing regression of established tumors in mice or in treating cancer patients (73). This is likely because, in addition to co-stimulatory receptors, T cells also express inhibitory receptors that have a much greater affinity for B7-1 and B7-2 than CD28 (13, 181).

The first such inhibitory receptor identified was CTLA-4 (37). Unlike CD28, which is constitutively expressed on the surface of T cells, CTLA-4 is induced on the surface of T cells, with maximum levels observed 2-3 days following activation (141, 329). In support of a dominant role of CTLA-4 in inhibiting immunity, CTLA-4^{-/-} mice succumb within 3-4 weeks from massive proliferation of activated lymphocytes (47). More recently, CTLA-4 was shown to be constitutively expressed on T_{reg} cells (355). In 2008, Wing et al. showed conclusively that CTLA-4 was important for T_{reg} function (337). In addition, they demonstrated that lack of CTLA-4 expression on T_{reg} cells alleviated suppression of anti-tumor immunity. They were able to induce regression of RLmale leukemia cells in 60% of mice following adoptive transfer of splenocytes containing CTLA-4 deficient T_{reg} cells (337). As a result of the clear role of CTLA-4 in

inhibiting immunity, a number of clinical trials have evaluated the efficacy of CTLA-4 blockade for the treatment of cancer (73). Although results to date have been mixed, CTLA-4 does appear to play a role in inhibiting tumor immunity (73). Future work is needed to determine the mechanisms responsible for CTLA-4 dependent suppression of anti-tumor immunity and for which specific cancers CTLA-4 targeted therapy will be most effective.

A more recently described inhibitory receptor that also appears to be involved in inhibiting anti-tumor immunity is programmed cell death-1 (PD-1; CD279) (145, 158). Like CTLA-4, PD-1 is expressed on T cells upon activation (2). The role of PD-1 as an inhibitory receptor was confirmed based on the autoimmune phenotype of PD-1^{-/-} mice (228, 229). Two ligands have been identified for PD-1, PD-L1 (B7-H1) and PD-L2 (B7-DC), which both share homology to B7-1 and B7-2 (67, 99). Upon interaction with its ligand, PD-1 transmits an inhibitory signal through phosphorylation of its ITIM and ITSM motifs (158). PD-L1 is expressed on a wide range of cells, including cells of both hematopoietic and non-hematopoietic origin, while PD-L2 expression is limited to activated DC, macrophages, and bone marrow-derived mast cells and resting B1 cells (158). PD-L1 can also be upregulated on some cells, including macrophages, DCs, and endothelial cells in response to both type I and type II IFNs (80, 158, 272). PD-L1 is also expressed by many different types of tumor cells and has been associated with decreased T cell immunity and poor prognosis (66, 127, 313). Also, expression of PD-L1 is upregulated by IFN- γ (25, 66). Murine studies have demonstrated that PD-L1 expression by tumor cells and PD-1 expression by T cells inhibit anti-tumor immunity (25, 146, 301). It appears as though expression of PD-L1 by tumor cells renders them resistant to

T cell-mediated killing (130). Two human anti-PD-1 Abs, CT-011 and MDX-1106, are currently in phase II trials (73). We are particularly interested in the role of PD-1 in our VRP vaccines as VRPs induce high levels of type I IFNs (209). Induction of PD-L1 by IFNs might be a main mechanism of tumor-induced tolerance to therapeutic VRP vaccines.

Current Types of Tumor Immunotherapy

Many different types of immunotherapy aimed at inducing anti-tumor immunity have been tested in both mice and humans. The different types of immunotherapy can be grouped into three main categories. The first involves non-specific immunotherapy. This approach uses cytokines or other immune agonists (e.g. TLR agonist) to activate the immune system. Treatment of patients with IFN α or IL-2, which are the current standards of care for metastatic melanoma, are examples of non-specific immunotherapy (185, 256, 260). Another example of non-specific immunotherapy is antibody-mediated blockade of the inhibitory molecule CTLA-4, which results in a 10-20% clinical response rate for melanoma (12, 245). Although these strategies show some efficacy there are many side effects to treatment due to systemic activation of the immune system. They also appear to work best in cancers that induce a significant endogenous immune response (e.g. as in melanoma and renal cancer).

A second type of immunotherapy that has shown increasing promise in recent years is adoptive cell therapy (ACT). This involves treatment of patients with their own tumor-specific lymphocytes that have been activated and expanded *in vitro* (258). Activation *in vitro* allows for stimulation of tumor-specific lymphocytes in the absence of

the suppressive mechanisms present in the tumor environment. This approach has been most successful for the treatment of metastatic melanoma, being pioneered by Steven Rosenberg (258). Rosenberg's first clinical trials using ACT with autologous tumor infiltrating lymphocytes (TIL) to treat 86 patients were reported in 1988 with an overall response rate of 34% (257, 261). Since these initial studies, this therapy has been optimized to reach objective response rates of 70% and complete response rates of 16% (74). It is important to note that ACT is much more effective in patients following lymphodepleting regimens, which both promote homeostatic proliferation of adoptively transferred cells and depletion of regulatory cells (258). One of the major limitations of ACT is that it is a highly specialized, labor intensive process that is specific to each patient and requires a great deal of laboratory expertise. Therefore, widespread adoption of this approach for clinical therapy has been very slow. Additionally, the magnitude or nature of the suppressive tumor environment might differ for other types of cancer and might prevent *in vitro* activated lymphocytes from functioning *in vivo*. Despite these potential limitations, there is substantial enthusiasm for the use of ACT for the treatment of patients with certain types of tumors such as malignant melanoma or renal cell cancer.

A third category of immunotherapy is the generation of therapeutic cancer vaccines. The goal of these vaccines is to specifically stimulate the patient's immune system *in vivo* against tumor cells. Unlike traditional vaccines, which are prophylactic in nature, these vaccines have been used predominantly for the treatment of established disease. This presents a much more formidable challenge as these vaccines need to overcome the mechanisms of tolerance that have already been established to allow the tumor to progress. A number of different types of vaccines have been investigated for

their ability to induce anti-tumor immunity. These include peptide based vaccines, DC vaccines, whole tumor cell vaccines, plasmid DNA, and viral vectors (70). Despite numerous attempts to utilize therapeutic cancer vaccines, the clinical response rates remain poor (259). The main focus of this dissertation was to understand why these vaccines have been so ineffective at inducing tumor regression. We used two different types of therapeutic cancer vaccines in our work: DC vaccines and VEE viral replicon particles.

Therapeutic Cancer Vaccines

DC vaccines

DCs are professional APCs first described by Ralph Steinman in 1972 (293, 295, 296, 298). DCs are able to stimulate both T and B lymphocytes (18) and appear to be the critical APCs for the stimulation of naïve T cells. Due to their ability to be effective stimulators of adaptive immunity, DC based vaccines were one of the first types of immunotherapy investigated (36, 46, 91, 195). Many different types of DC vaccines have been tested in both animal models and in humans and can be grouped based on the origin of the DC as well as the method of antigen loading (36, 44, 137, 223, 239, 297).

One of the main benefits of DC vaccines, apart from their superior ability to stimulate the immune system, is the use of *ex vivo* activated and matured DCs. This is especially important as endogenous DCs found in cancer patients are often suboptimal stimulators of immunity (246, 342). *Ex vivo* activated DCs express high levels of costimulatory molecules and proinflammatory cytokines such as IL-12 needed to induce potent Th1 responses. Therefore, isolation of DCs and activation *in vitro* offers a

compelling approach to enhancing anti-tumor immunity. DCs that are fully matured and activated *in vitro* appear resistant to immune modulating factors that likely exist *in vivo* in the tumor environment (150, 151, 324).

Ex vivo generated DC vaccines have been used to treat a number of different cancers including melanoma and renal cancer (17, 87, 112, 224, 294). In these trials, the overall objective response rates currently do not exceed 15%. Much effort has been placed on improving DC vaccines by selecting the best type of DC and by evaluating the best way to load DCs with antigen (152). Despite data indicating that these DCs are better able to stimulate immune responses, these vaccines are still not effective at inducing tumor regression, possibly due to the induction of local immunosuppressive factors (152).

Viral vector vaccines

Viral vectors offer an attractive approach for vaccination against tumor antigens. The purpose of viral vector vaccines is to deliver TAAs in the context of viral infection. Viral vectors can deliver TAAs for presentation to on both MHC I and MHC II, allowing them to activate both CD8 and CD4 T cells, respectively. Viral vectors can also elicit a strong humoral response as well as activate innate immunity (8). Using viral vectors, as opposed to peptide vaccines, allows for targeting of multiple known and unknown TAA epitopes, inducing a potentially more effective polyclonal response (8).

As mentioned above, viral vectors can also be combined with DC based vaccines by infecting DCs *in vitro* and treatment with these viral transduced DCs. Viral vectors can also be used to infect APCs *in vivo*. One benefit of direct treatment with viral

vectors, as opposed to treating with virus infected DCs, is that autologous DCs do not need to be generated, which, like ACT, is a very labor intensive, specialized process that is not easily transferable to most places of patient care.

A number of viral vector based therapeutic vaccines are being investigated for their efficacy against both infectious diseases and cancer (72, 259). The most common vectors currently being tested in late stage clinical trials utilize recombinant poxvirus and adenovirus, but other viruses are also being tested for use in cancer vaccines, including alphaviruses, vesicular stomatitis virus and herpes simplex viruses (8). These viral vectors are being used to target most types of cancer (8, 72, 259). Most of these vaccines are still in early phase trials. Unfortunately, those that have been tested in late stage trials show little efficiency, even against tumors that are known to be immunogenic, such as melanoma (259).

VEE replicon particles

A more recent type of viral vector based vaccine that gives optimal stimulation of the immune system while also addressing the main limitations of current viral vectors are Venezuelan equine encephalitis (VEE) viral replicon particles (VRP) (11, 116, 218, 248). To generate VRPs, replicon RNA, containing the TAA of interest, is co-transfected into cultured cells along with helper RNAs encoding the structural genes of the virus. The resultant VRPs, isolated from supernatant, lack structural genes and are therefore unable to replicate upon infection. Despite their inability to produce new virions, they are able to infect target cells and express TAAs at very high levels (248).

Use of VRPs, as opposed to other viral vector based systems, addresses two main limitations of current viral vaccines. First, as VRPs are propagation defective, they have significantly less safety concerns compared to other viral based vaccines (116). Second, there is limited endogenous immunity in humans to VEE in North America and vaccination with VRPs induces a low frequency of anti-vector immunity (116, 248, 328). This is a major benefit because it means that VRPs can potentially be used for multiple vaccinations without losing effectiveness.

Another main benefit of VRPs is that they have a natural tropism for DCs and B cells (187, 209, 227). Thus, VRPs given *in vivo*, which naturally infect APCs, circumvents the need to generate substantial quantities of virus and DCs for *in vitro* infection. Infection of DCs with VRPs results in increased expression of costimulatory molecules and production of proinflammatory cytokines IL-6, TNF- α , and IFN- α (209). Vaccination with VRPs induces potent cellular and humoral immunity to TAAs as demonstrated in a number of mouse models (116, 208, 222).

VRPs have been used in murine tumor models to target a number of TAAs, including Her-2/neu (174, 208, 222, 331), HPV E7 and E9 (45, 323), tyrosinase (116), and prostate-specific membrane antigen (PSMA) (76, 107), leading to prevention and even regression of antigen-expressing tumors. Early phase clinical trials are underway evaluating the use of VRPs to treat colorectal, breast, lung, pancreatic, colon, and prostate cancer (clinicaltrials.gov).

Murine Models of Cancer

One of the biggest challenges in evaluating the efficacy of cancer vaccines is the lack of stringent animal models that recapitulate the multitude of hurdles needed to achieve potent immunity clinically. The majority of vaccine studies have evaluated vaccines using tumors that express exogenous antigens in animals where that tumor does not naturally grow, or they have administered the vaccines before tumor challenge. As the antigens expressed by these tumors are not self-antigens, vaccination in this setting is not limited by central deletion of high avidity/affinity T cells that may be generated by the vaccine. Furthermore, as these vaccines are administered prior to tumor challenge, or to mice with microscopic tumors, these studies do not reflect the potent immunosuppressive environment generated by an established tumor.

We used two different murine cancer models to test both our vaccines and the nature of the immunosuppressive tumor environment. These included the MMTV neu-transgenic mouse model and the B16-F10 melanoma model. Both are extremely stringent models in which the tumors express true self-antigens, and in which the induction of tumor regression is difficult. Our vaccines were evaluated therapeutically for their ability to induce regression in mice with established tumors.

MMTV neu-transgenic mouse

The estimated lifetime risk for women developing breast cancer is 12% with a nearly 3% lifetime risk of mortality (134). There are at least 4 subtypes of breast cancer (139, 243, 292). One of these subtypes is identified by the expression of Her-2/neu. Her-2/neu is an oncogene associated with approximately 30% of breast cancers (134, 140).

Furthermore, overexpression of Her-2/neu is associated with worse prognosis (289, 292). Also, as Her-2/neu specific T cells as well as Her-2/neu-specific antibody have been identified in patients with Her-2/neu+ breast cancers, it presents an attractive target for vaccines directed against Her-2/neu (61, 63-65, 90, 144, 241, 347).

In order to investigate the role of Her-2/neu in tumor progression and to test Her-2/neu-specific vaccine strategies, a number of groups have generated neu-transgenic mice. One such mouse that has been studied extensively is the MMTV neu-transgenic mouse (neu-N) that expresses the rat protooncogene neu under control of the MMTV promoter (126). These mice express high levels of rat neu which is 85% homologous to human HER-2/neu, including at sites of immune development in the thymus and bone marrow. All female mice succumb to spontaneous mammary adenocarcinoma at 5-6 months of age (251). Neu-N mice therefore represent an extremely stringent animal model within which to test potential immunotherapy regimens as a substantial fraction of the high affinity/avidity T cells are deleted centrally.

A number of vaccine strategies have been evaluated for their ability to induce regression in neu-N mice (81, 188, 207, 211, 251, 284). Although some of these vaccines have shown efficacy in a prophylactic setting, to date no one has demonstrated complete regression of palpable tumors in these mice.

B16-F10 melanoma

Melanoma will affect 1 in 50 people during their lifetime (134). The estimates in the United States for 2009 are 68,720 cases and 8,650 deaths (134). Much effort has been undertaken to develop immunotherapy for melanoma. This is mainly because 1) other

forms of cancer treatment, i.e. chemotherapy and radiotherapy, are not very effective at treating melanoma, and 2) melanoma is considered to be more immunogenic compared to other human cancers.

The B16 melanoma cell line was originally isolated from a spontaneous tumor in C57BL/6 mice by Isaiah Fidler in the 1970s and is the most commonly used mouse model for melanoma (84, 86). The most aggressive variant of B16 is B16-F10 (86). Homologs of the five most common TAA for human melanoma (gp100/pmel17, MART-1/Melan-A, tyrosinase, TRP-1/gp75, and TRP-2) are all expressed by B16-F10 (236). Also, CTLs specific for each of these antigens have been identified in mice (26, 50, 77, 237). Unlike most human melanomas, B16-F10 is a very poorly immunogenic tumor. Although the reason for its poor immunogenicity is unknown, it is likely a result of low levels of MHC class I expression (85). Characteristic of its poor immunogenicity, treatments such as IL-2 therapy, ACT, and vaccinations with irradiated tumor cells or viral vectors that induce regression of other C57BL/6 tumors (e.g. EL-4 thymoma and MCA-induced sarcomas) and have shown some efficacy against human melanoma have little to no effect against B16 melanoma (236). The low immunogenicity of B16 melanoma, compared to human melanoma, makes B16 a stringent model for testing vaccine strategies targeting melanoma TAAs.

Dissertation Aims

While it is now clear that the immune system is capable of recognizing and killing tumor cells, attempts to design effective therapies to utilize the immune system to target cancer have not been effective. It is unclear whether the ineffectiveness of cancer

vaccines is due mainly to the central deletion of tumor-specific lymphocytes during development or due to the dominant role of the immunospressive tumor environment. Therefore, the main goal of this study was to evaluate the role of these two factors in the lack of activity of active vaccination. We examined the many mechanisms of tumor-induced immune suppression that may play a role in inhibiting vaccine efficacy. Of particular focus, we evaluated the role and mechanism for activity of myeloid-derived suppressor cells following therapeutic vaccination.

Figure 1-1

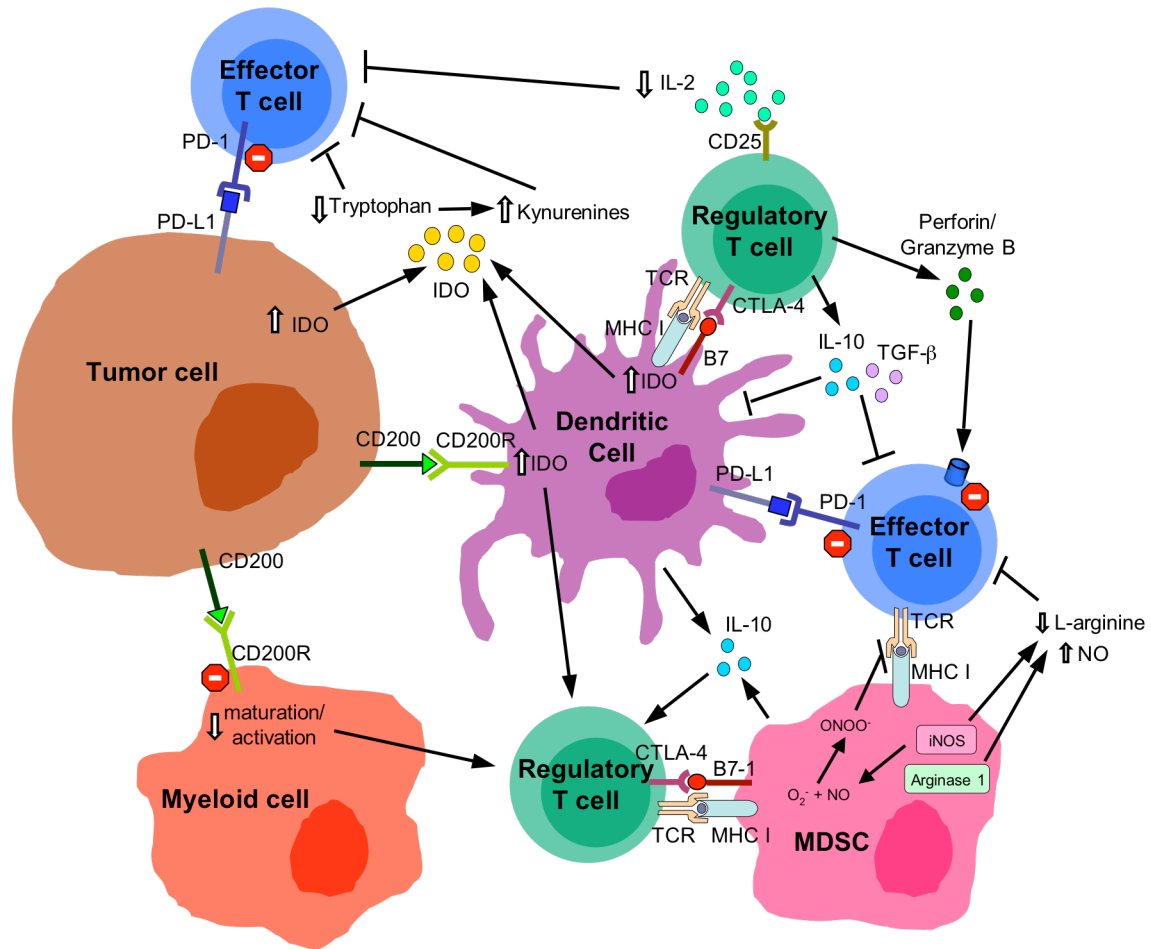


Figure 1-1. Mechanisms of tumor-induced immune suppression. A number of different mechanisms of suppression are present in the tumor environment (please see text for description). These include the induction of regulatory T cells (T_{reg} cells) and myeloid-derived suppressor cells (MDSC), induction of the suppressive molecule IDO, and the action of a number of immune inhibitory receptors including CD200R, CTLA-4, and PD-1. The main focus of the current work was to evaluate the relative contribution of these mechanisms in inhibiting the efficacy of therapeutic cancer vaccines.

CHAPTER TWO

THE IMMUNOSUPPRESSIVE TUMOR ENVIRONMENT IS THE MAJOR IMPEDIMENT TO SUCCESSFUL THERAPEUTIC VACCINATION IN NEU TRANSGENIC MICE

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Abstract

We previously demonstrated that therapeutic vaccination of FVB/N mice with alphaviral replicon particles expressing rat neu (neuET-VRP) induced regression of established neu-expressing tumors. In the current study, we evaluated the efficacy of neuET-VRPs in a tolerant mouse model using mice with transgenic expression of neu. Using the same approach that induced regression of 70 mm² tumors in FVB/N mice, we were unable to inhibit tumor growth in tolerant neu-N mice, despite demonstrating neu-specific B and T cell responses post-vaccination. As neu-N mice have a limited T cell repertoire specific to neu, we hypothesized that the absence of these T cells led to differences in the vaccine response. However, transfer of neu-specific T cells from vaccinated FVB/N mice was not effective in inducing tumor regression as these cells did not proliferate in the tumor-draining lymph node. Vaccination given with low dose cyclophosphamide to deplete regulatory T cells delayed tumor growth but did not result in tumor regression. Finally, we demonstrated that T cells given with vaccination were effective in inhibiting tumor growth if administered with approaches to deplete myeloid-derived suppressor cells. Our data demonstrate that both central deletion of lymphocytes and peripheral immunosuppressive mechanisms are present in neu-N mice. However, the major impediment to successful vaccination is the peripheral tumor induced immune suppression.

Introduction

Immunotherapy has the potential to become an essential component of a successful treatment regimen for metastatic cancer (70, 89, 258, 259, 299, 327). Not only can the immune system be utilized to specifically target tumor cells, but it also provides long term memory needed to prevent recurrent disease. Of the various types of immunotherapy, vaccination with viral vectors is particularly promising (8, 183, 204, 218, 235, 242, 262, 312, 317, 352, 354). Expression of tumor associated antigens (TAA) by viral vectors primes the immune system against multiple known and unknown epitopes specific to tumor cells. Vaccination with viral vectors has the potential to activate both humoral and cellular anti-tumor immunity. Delivery of TAAs in the context of viral infection has the capacity to activate toll-like receptors, which may be critical for overcoming mechanisms of tumor-induced tolerance (183, 312, 354).

Virus replicon particles offer an attractive viral vector based system for targeting TAAs (11, 116, 218, 248). Previously, our group and others have generated Venezuelan equine encephalitis replicon particles (VRP), which are replication-deficient vectors in which the structural genes of the parent strain are replaced by a foreign gene of interest. For packaging of VRP *in trans*, the replicon RNA is co-transfected into cultured cells along with helper RNAs encoding the structural genes of the virus. The VRPs harvested from the culture supernatant are propagation-defective vectors able to infect target cells and express TAAs at very high levels. VRPs, as compared to other viral vectors, offer the benefit of inducing a low frequency of anti-vector immunity, which allows the same VRP vaccine to be used for multiple vaccinations without decreased effectiveness (248).

VRPs have been used in mouse tumor models to target a number of TAAs, including Her-2/neu (174, 208, 222, 331), HPV E7 and E9 (45, 323), tyrosinase (116), and prostate-specific membrane antigen (76, 107), leading to prevention and even regression of antigen-expressing tumors. These studies have either evaluated the use of VRPs as prophylactic vaccines, or therapeutic vaccines in animals given tumor cell lines that are not naturally found in that host. VRPs have yet to be evaluated as therapeutic vaccines in a stringent model of tumor immunity using an animal model in which tumors arise spontaneously.

We described previously the design and efficacy of a therapeutic VRP vaccine against rat neu-overexpressing tumors (208). We found that a single vaccination with dendritic cells infected with VRPs encoding the extracellular transmembrane domain of rat neu (VRP-neu DCs) led to regression of established neu-expressing tumors in FVB/N mice. Vaccination with VRP-neu DCs induced potent cellular and humoral anti-neu immunity, and tumor regression was dependent on CD4⁺ T cells. Here, we extend these studies to tolerant neu-N mice in which the rat neu gene, driven by the MMTV promoter, is expressed as a self antigen (126). We explored the ability of neuET-VRPs to regress established tumors in neu-N mice and the roles of central and peripheral tolerance in inhibiting vaccine efficacy.

Materials and Methods

Mice, cell lines, and reagents

FVB/N and neu-N mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Charles River Laboratory (Wilmington, MA), respectively. Female mice (7-14 weeks) were used for all experiments. All experiments were conducted in accordance with protocols approved by the University of North Carolina Institutional Animal Care and Use Committee. Rat neu-expressing NT2 cells, NIH-3T3 (American Type Culture Collection), and 3T3*neu* have been described (251). 3T3-*neu*/GM cells were a gift from Dr. Marion Couch (UNC, Chapel Hill, NC). RNEU₄₂₀₋₄₂₉ (PDSLRLDSVF) peptide was purchased from New England Peptide (Gardner, MA).

Flow cytometric analysis

Monoclonal antibodies (mAbs) used for flow cytometry were purchased from eBioscience (San Diego, CA). Anti-c-ErbB2/neu (Ab4) mAb was purchased from Calbiochem (San Diego, CA). Cells were stained according to the manufacturers' instructions. Acquisition was done using BD FACS Calibur (BD Biosciences, San Jose, CA) and the resultant data analyzed using FlowJo Flow Cytometry Analysis Software (Tree Star Inc., Ashland, OR).

VRP vaccine

VRPs encoding the extracellular-transmembrane domain (AA 1-697) of rat neu (neuET-VRP) or VRPs lacking an inserted transgene (null-VRP) have been described (208, 311). VRP-DC vaccines were generated from bone marrow (BM) derived DCs as

described (208, 209, 225). DCs were infected for 2 h at 37 °C with VRPs at a multiplicity of infection (MOI) of 10. VRP-DCs (10^6) were injected subcutaneously (s.c.) in the right mammary gland adjacent to established tumors. As noted, some mice were vaccinated *in vivo* with 10^6 VRPs given into the rear footpad.

Detection of serum anti-neu IgG

Serum anti-neu IgG levels were determined as described (208). Concentration of anti-neu IgG was determined by standard curve generated with Ab4 anti-neu mAb.

Isolation of tumor-infiltrating lymphocytes (TILs) and IFN- γ staining

TILs were isolated as described (208). Cells were stained for CD4 and CD8, and intracellular cytokine staining for IFN- γ performed using the BD PharMingen reagents after activation of cells 4-6 h with PMA/ionomycin or RNEU₄₂₀₋₄₂₉ peptide.

Vaccination with neuET-VRPs and 3T3-neu/GM cells

Tolerant neu-N mice were challenged with 5×10^4 freshly prepared NT2 cells s.c. in the right mammary fat pad. FVB/N mice were challenged with 2×10^6 NT2 cells. For therapeutic vaccination, tumors were allowed to grow 4-7 days before vaccination with either VRPs given *in vivo*, VRP infected DCs, or 3T3-neu/GM cells. For prophylactic vaccination, mice were vaccinated 21 and 7 days before tumor challenge. Tumor area (L \times W) was measure 2-3 times weekly with metric calipers, and mice were sacrificed when tumors reached a maximum of 200mm².

For regulatory T cell (T_{reg} cell) depletion experiments, neu-N mice were treated intraperitoneally (i.p.) with cyclophosphamide (CY; 100mg/kg) 2-3 days prior to VRP vaccination. $CD25^{+}$ cells were depleted by i.p. injection of 0.5 mg of PC61 mAb on days -7 and -4 prior to tumor challenge. 3T3-*neu*/GM cells were irradiated at 5,000 rads and 3×10^6 cells/mouse injected as described (81).

Adoptive cell transfer

For adoptive cell transfer experiments into lethally irradiated recipients, splenocytes were isolated from naïve FVB/N or neu-N mice. $5-10 \times 10^6$ splenocytes, along with $3-5 \times 10^6$ T cell depleted syngeneic BM cells, were transferred i.v. to neu-N or FVB/N mice 18-24 h after irradiation at 850 rads (135 rads/min). NT2 tumor cells were injected s.c. at the time of splenocyte and BM cell transfer.

For T_{reg} cell transfer experiments, $CD4^{+}CD25^{+}$ cells were isolated from spleens of tumor bearing mice by positive selection using Miltenyi columns (Miltenyi Biotec, Bergisch Gladbach, Germany) as described (341). T_{reg} cells were injected i.v. one day prior to VRPs.

For FVB/N T cell adoptive transfer experiments, $CD25^{-}$ T cells were isolated from spleens of FVB/N mice 7 days after priming with 10^6 neuET-VRPs using Cedarlane total T cell columns (Cedarlane Laboratories, Nornby, ON, Canada) followed by isolation using Miltenyi columns for the depletion of $CD25$ -expressing cells (341). Cells were restimulated *in vitro* with neuET-VRP DCs (DC:T cell ratio = 1:50) 5-7 days in the presence of IL-2 (20U/ml) and then labeled with CFSE. Tumor-challenged neu-N mice

received i.v. injections of 10^7 restimulated T cells two days following CY treatment. Mice were vaccinated the next day with neuET-VRPs.

Statistical analysis

Statistical differences for T cell subsets, antibody levels and CFSE intensities were calculated by two-tailed Student's t test. Significant differences in survival were determined by Kaplan-Meier survival analysis. Significant differences in tumor growth curves were determined by two-way RM ANOVA. All statistical analyses were conducted using SigmaStat[®] 3.5 software, with a p value ≤ 0.05 considered significant.

Results

Therapeutic vaccination of tolerant neu-N mice with neuET-VRP DCs fails to inhibit tumor growth despite induction of anti-neu immunity

We previously demonstrated that a single therapeutic vaccination of non-tolerant FVB/N mice with dendritic cells infected with neuET-expressing VRPs (VRP-neu DCs) led to regression of established rat neu-expressing tumors (208). Tumor regression in FVB/N mice was completely dependent on CD4⁺ T cells and only partially dependent on CD8⁺ T cells (208). In the current study, we evaluated the efficacy of neuET-VRPs as a therapeutic vaccine in tolerant neu-N mice. Although these mice develop spontaneous focal mammary adenocarcinomas, we employed the same strategy previously used in FVB/N mice by orthotopically challenging neu-N mice with NT2 cells. This cell line was developed by Reilly et al. from a spontaneous tumor formed in a neu-N mouse and has been used in multiple studies evaluating anti-tumor immunity in neu-N mice (81, 188, 251). Using a cell line, as opposed to treatment of spontaneous tumors, allows for treatment of mice without having to wait 25-50 weeks for the development of tumors. It also allows for better control of tumor burden and the amount of antigen presentation by the tumor cells. Using the same approach that was successful previously in FVB/N mice, therapeutic vaccination of tolerant neu-N mice with VRP-neu DCs failed to inhibit growth of NT2 tumors (Fig. 2-1A).

To determine if vaccination induced an immune response in neu-N mice, cytokine and antibody production specific to neu were measured after vaccination. Vaccination of neu-N mice with VRP-neu DCs stimulated both humoral and cell mediated anti-neu immunity, demonstrated by induction of both anti-neu Ab production as well as an

increased number of IFN- γ ⁺ CD8⁺ T cells at the tumor site (Fig. 2-1B,C). However, the induction of neu-specific Ab was markedly diminished in neu-N compared to the induction seen previously in non-tolerant FVB/N mice (208).

Direct vaccination with neuET-VRPs is superior to vaccination with neuET-VRP DCs

In our initial studies, we vaccinated mice with VRP-neu DCs. Others have vaccinated mice directly with viral vectors, including VRPs (45, 76, 107, 116, 174, 222, 323, 331). In order to directly compare these two vaccination strategies, we vaccinated tumor bearing FVB/N mice with either VRP-neu DCs or directly with neuET-VRPs. Both vaccination methods induced regression of established tumors in FVB/N mice, although direct vaccination with neuET-VRPs led to complete regression of tumors at an earlier time (Fig. 2-2A). We also evaluated the induction of humoral and cell-mediated immunity. We found that direct vaccination with neuET-VRPs resulted in increased production of anti-neu Ab as well as an increased number of IFN- γ ⁺ CD8⁺ T cells compared to vaccination with VRP-neu DCs (Fig. 2-2B,C). Thus, in all subsequent experiments, mice were vaccinated directly with neuET-VRPs and not VRP-neu DCs.

Given the more robust immune response induced by neuET-VRPs, we evaluated whether direct VRP vaccination of tumor bearing neu-N mice with neuET-VRPs could induce tumor regression. Despite increased induction of anti-neu immunity, direct VRP vaccination in neu-N mice did not inhibit tumor growth (Fig. 2-2D). These data suggest that the immune response generated in neu-N mice is not sufficient to mediate tumor regression.

neuET-VRP vaccine is similar to 3T3-neu/GM vaccine when administered therapeutically

One potential hypothesis for our findings is the inferior immune activity of VRP vaccines compared to other approaches. Previous investigators have used 3T3-*neu*/GM vaccination and demonstrated tumor regression in a minority of vaccinated neu-N mice when given with CY within the first 3 days after tumor challenge (188). Thus, we were interested in determining if the lack of anti-tumor efficacy using VRPs expressing *neu* was due to impaired activity of the vaccine. For this work, we compared the efficacy of 3T3-*neu*/GM vaccination with neuET-VRPs given seven days post-tumor injection, which is an approach that allows for treatment of 30-50 mm² tumors. We found that both vaccines were unable to inhibit tumor growth when given this late after tumor challenge (Fig. 2-3). Thus, the lack of efficacy in neu-N mice was independent of the type of vaccine used.

Both central and peripheral tolerance mechanisms in neu-N mice inhibit efficacy of neuET-VRPs as a therapeutic vaccine

Our group and other investigators have shown that the T cell repertoire reactive with *neu* is significantly different in neu-N compared to FVB/N mice (284). Thus, we hypothesized that absence of high avidity T cells in the neu-N mice compromised efficacy of neuET-VRP vaccination. In order to evaluate the role of the T cell repertoire in vaccine efficacy, we lethally irradiated neu-N mice and transferred splenocytes or T cells from non-tolerant FVB/N mice. Transfer of FVB/N splenocytes into irradiated neu-N recipients did not lead to tumor regression, suggesting that tolerance in neu-N mice is not solely a result of a difference in the repertoire of *neu*-specific T cells (Fig. 2-4A). To

evaluate if infusion of T_{reg} cells might have biased these results, we transferred naïve or activated CD25⁻ T cells from FVB/N mice into non-irradiated neu-N mice. Again, there was no difference in tumor growth (data not shown).

In order to ensure that transferred FVB/N cells were functional and contained a sufficient repertoire of neu-specific lymphocytes, we transferred cells from FVB/N mice into syngeneic FVB/N recipients. When FVB/N mice were used as recipients, transfer of FVB/N splenocytes with vaccination did lead to tumor regression (Fig. 2-4B). On the other hand, transfer of neu-N splenocytes into FVB/N mice did not result in tumor regression, consistent with an altered anti-neu repertoire in neu-N mice (Fig. 2-4B). Thus, our data demonstrate that (1) infusion of lymphocytes from FVB/N mice, which are effective in FVB/N mice, is not able to induce tumor regression in neu-N mice after vaccination, and (2) that the T cell repertoire in neu-N mice is unable to mediate anti-tumor effects in a non-tolerant model. These data suggest that the impaired immune response in neu-N mice is a combination of impaired T cell function and peripheral immunosuppression, in which the peripheral suppression is dominant.

Proliferation of T cells upon neuET-VRP vaccination is preferentially suppressed in the tumor draining lymph node (TDLN)

One of the main characteristics of tumor induced tolerance is the induction of local immunosuppression at the site of tumor and the tumor draining lymph node. Thus, we sought to evaluate whether the response to neuET-VRP vaccination was locally suppressed in the TDLN compared to the spleen. We adoptively transferred activated, CFSE labeled, T cells from non-tolerant FVB/N mice into neu-N mice and evaluated the

proliferation of these cells after neuET-VRP vaccination. Approximately 5-10% of the restimulated cells were RNEU₄₂₀₋₄₂₉-antigen specific as demonstrated by tetramer staining. Proliferative responses were demonstrated by dilution of CFSE from adoptively transferred T cells isolated from the spleen and TDLN 5 days after vaccination. There was a significantly higher percentage of CFSE^{hi} cells in the TDLN compared to the spleen, consistent with localized suppression in the TDLN (Fig. 2-5). When analyzed 60 days after T cell transfer and repeated vaccination, very few of the CFSE-labeled cells isolated from the TDLN had proliferated more than two times. Thus, the TDLN in tumor-bearing neu-N mice suppresses the proliferative activity of T cells that mediate anti-tumor activity in FVB/N mice.

Depletion of CD4⁺ FoxP3⁺ T_{reg} cells with cyclophosphamide improves efficacy of neuET-VRP vaccine

The inability of therapeutic VRP vaccination to inhibit tumor growth in neu-N mice suggests that VRPs alone are insufficient to overcome tumor-induced tolerance. Previous studies have demonstrated that T_{reg} cells are involved in mediating tolerance in neu-N mice (81, 188, 284). We found increased numbers of CD4⁺ FoxP3⁺ T cells at the tumor site in mice vaccinated with neuET-VRPs compared to mice vaccinated with either null-VRPs or saline consistent with neu-specific induction of T_{reg} cells by VRPs (Fig. 2-6A). In order to evaluate the role of T_{reg} cells in the response of neu-N mice to VRP vaccination, we treated mice with low dose CY (100mg/kg), which has been shown to preferentially deplete T_{reg} cells (81, 186). Consistent with previous reports, we found that CY treatment two days prior to vaccination resulted in a significant decrease in the

relative percentage of FoxP3⁺ T_{reg} cells. The decrease in the number of T_{reg} cells was transient, lasting only 9 days. Therapeutic vaccination of neu-N mice with neuET-VRP combined with CY treatment resulted in a significant delay in tumor growth (Fig. 2-6B) but did not lead to complete regression in any of the treated mice. In contrast, treatment of neu-N mice with the anti-CD25 mAb, PC-61, did not have an impact on the growth of tumor cells post vaccination (data not shown).

In order to confirm that T_{reg} cells inhibit the effectiveness of vaccination with neuET-VRPs, we isolated T_{reg} cells from tumor bearing mice and transferred them into tumor bearing FVB/N mice. Addition of either FVB/N or neu-N T_{reg} cells inhibited the regression of tumors in FVB/N mice (Fig. 2-6C). Interestingly, there was no difference in suppression mediated by T_{reg} cells from either FVB/N or neu-N mice, demonstrating that T_{reg} cells isolated from neu-N mice are functionally equivalent to T_{reg} cells isolated from FVB/N mice (Fig. 2-6C).

Provision of FVB/N splenocytes along with depletion of myeloid-derived suppressor cells (MDSC) inhibits tumor progression in neu-N mice

One of the main characteristics of tumor bearing neu-N mice is an accumulation of CD11b⁺, Gr-1⁺ MDSCs (1), which make up approximately 40% of splenocytes, or over 1.2×10^8 cells in neu-N mice with established tumors, and are involved in the suppression of tumor-specific T cells (105, 234). In order to evaluate the role of MDSCs in neu-N mice, we depleted Gr-1⁺ cells using two different mAbs, clone RB6-8C5 or 1A8. RB6-8C5 is specific for both Ly-6G (Gr-1) and Ly-6C, while 1A8 is specific for only Ly-6G (92). We used our transplant model with transferred FVB/N splenocytes in

order to provide a sufficient repertoire of neu-specific T cells to induce regression once peripheral mechanisms of tolerance were inhibited. Tumors in mice that received isotype control Ab continued to progress once established, whereas tumors in the majority of mice treated with anti-Gr-1 mAbs did not progress (Fig. 2-7A). There was a significant difference in tumor size after day 49 between both Gr-1 depletion groups and the isotype control group ($p = 0.02$). There was no difference in tumor growth when MDSCs were depleted without transfer of FVB/N splenocytes (Fig. 2-S1).

In order to evaluate the mechanism of inhibition of tumor growth following depletion of MDSCs, we examined both the number of IFN- γ^+ T cells in the spleen as well as the amount of serum anti-neu Ab in mice surviving to day 60. There was no difference in the percentage of CD8 $^+$ IFN- γ^+ T cells in mice that received 1A8 mAb or isotype Ab. Interestingly, there were fewer CD8 $^+$ IFN- γ^+ T cells in mice receiving RB6-8C5, possibly a result of depletion of Ly-6C $^+$ T cells (Fig. 2-7B) (270). There was a significant increase in the percentage of CD4 $^+$ IFN- γ^+ T cells in the spleens of mice given 1A8 mAb compared to isotype Ab, but no increase in mice receiving RB6-8C5 mAb (Fig. 2-7C). When we looked at anti-neu Ab levels, we found that mice with non-progressing tumors following Gr-1 depletion had significantly more anti-neu Ab in their serum compared to mice with progressing tumors, consistent with increased anti-neu B-cell mediated immunity upon depletion of MDSCs (Fig. 2-7D). Thus, these data suggest that in the neu-N model peripheral MDSCs play a critical role in abrogating the immune response after active and passive immunotherapy potentially by inhibiting antibody-mediated tumor regression.

Discussion

In the current study we evaluated the efficacy of alphaviral replicon particles as a therapeutic cancer vaccine in a neu-tolerant mouse model. We previously reported regression of established tumors in non-tolerant FVB/N mice with a single neuET-VRP vaccination (208). We found that the same neuET-VRP vaccine given once or multiple times was unable to induce regression of tumors in tolerant neu-N mice, despite the production of anti-neu antibodies as well as an increase in tumor infiltrating IFN- γ ⁺ CD8⁺ T cells following vaccination. Therefore, it appears that neuET-VRPs can induce anti-neu immunity in tolerant neu-N mice, but that vaccination alone is insufficient to overcome tumor-induced tolerance.

By and large, most of the previous preclinical work suggesting a benefit for immunotherapy in the treatment of cancer has used two models, either a prophylactic approach or treatment of microscopic disease. Our data suggest that the immune response necessary to induce tumor regression or prevention in these models differs from that necessary for regression of established tumors. The environment in the TDLN and at the site of tumor growth is critically important in blunting the immune response to established tumors, which includes suppression of T cell proliferation in the TDLNs of neu-N mice. This is supported by our data showing a lack of efficacy of neuET-VRP and 3T3-*neu*/GM vaccination for the treatment of 30 mm² tumors, when both vaccines have been shown by us (data not shown) and others to be successful in preventing tumor growth and in the treatment of microscopic tumors (81, 188, 331). The inability to induce regression in tolerant mice underscores the need for using relevant animal models in a therapeutic setting when evaluating the efficacy of cancer vaccines.

T_{reg} cells play a major role in the induction and maintenance of tolerance to TAAs (353, 355). We found an increased number of intratumoral T_{reg} cells in neu-N mice vaccinated with neuET-VRPs compared to mice vaccinated with null-VRPs, consistent with neu-specific induction of T_{reg} cells by VRPs. Treatment of mice with low-dose CY has been shown to preferentially decrease the number and function of T_{reg} cells, and Ercolini et al. demonstrated that treatment of vaccinated neu-N mice with CY prevented tumor growth (81, 186). Therefore, we evaluated the effect of low dose CY on the effectiveness of therapeutic neuET-VRP vaccination. We found that CY treatment along with neuET-VRP vaccination inhibited tumor growth in neu-N mice. Unfortunately, repeated treatment with CY or anti-CD25 mAb did not improve survival, likely due to the negative effects of these treatments on effector T cells after vaccination (data not shown). Despite the inability to deplete T_{reg} cells for more than nine days, we still observed a difference in tumor growth with CY treatment. It is quite possible that persistent elimination of T_{reg} cells could induce more profound anti-tumor responses in neu-N mice after vaccination.

Although central tolerance mechanisms are clearly involved in neu-N mice, they are not the sole reason these mice do not respond to VRP vaccination. Peripheral mechanisms of tolerance are also involved since transfer of FVB/N splenocytes into lethally irradiated neu-N mice was unable to induce tumor regression. We were able to rescue a majority of neu-N mice when VRP vaccination was combined with both adoptive transfer of neu-specific lymphocytes and depletion of MDSCs. Morales et al. recently found that T cells isolated from tumor-bearing neu-N mice and expanded *ex vivo* in the presence of IL-7, IL-15 and IL-2 could mediate a substantial delay in tumor growth

if given concurrent with lymphodepleting chemotherapy and the depletion of MDSCs (207). Others have also demonstrated a decrease in tumor growth in other models after inhibiting or depleting MDSCs (169, 176, 205, 285, 305, 310). When we combined depletion of MDSCs, adoptive transfer of FVB/N splenocytes and VRP vaccination we were able to induce tumor regression in 40% of mice, regardless of which Gr-1 specific mAb they received. The inhibition of tumor growth was accompanied by a dramatic increase in anti-neu Ab levels, consistent with enhanced anti-neu immunity following MDSC depletion and suggesting that the presence of MDSCs has a direct inhibitory effect on the generation of anti-tumor B cell immunity. Interestingly, we did not observe an increase in the number of IFN γ ⁺ CD8⁺ T cells upon depletion of MDSCs. In fact, the number of IFN γ ⁺ CD8⁺ T cells was lower in mice treated with anti-Gr-1 Ab clone RB6-8C5. We previously demonstrated that the response to neuET-VRP vaccination in FVB/N mice is only partially dependent on CD8⁺ T cells, while being completely dependent on CD4⁺ T cells (208).

As we initially hypothesized that tolerance in neu-N mice was mainly due to the negative selection of neu-specific T cells in the thymus, we were somewhat surprised to find that regression of tumors in neu-N mice following adoptive cell transfer required depletion of MDSCs. These findings are somewhat at odds with previous work from Dudley et al. in which approximately 50% of patients with melanoma had a clinical response after the adoptive transfer of activated T cells following lymphodepleting chemotherapy. There are multiple reasons why tumor regression in a breast cancer model may differ from that found in patients with melanoma (28). One hypothesis for this difference is the susceptibility of the different tumors to anti-tumor immune mechanisms.

Work over the past two decades has shown that immune modulation can have a substantial effect on the growth of melanoma; unfortunately this has not been found for most carcinomas like breast cancer. It is intriguing to speculate that the role of MDSCs may be one difference between the effectiveness of anti-tumor immunity for these different tumors. In general, the number of MDSCs found post vaccination in breast cancer models is substantially greater than that found in either patients with melanoma or animal models of melanoma (7, 88, 348). Thus, it is quite possible that activation of T cells *ex vivo* can overcome T_{reg} mediated suppression in melanoma, but that the substantial number of MDSCs found at the tumor site in breast cancer poses another hurdle that needs to be cleared for effective anti-tumor immunity.

Our data underscore the need for inhibiting peripheral tolerance even if antigen-specific T cells are present. For effective vaccine approaches for the treatment of cancer, patients may require high avidity tumor-specific lymphocytes, but our data suggest that even if provided, these cells might be ineffective if not combined with strategies to inhibit the suppressive tumor microenvironment.

Figure 2-1

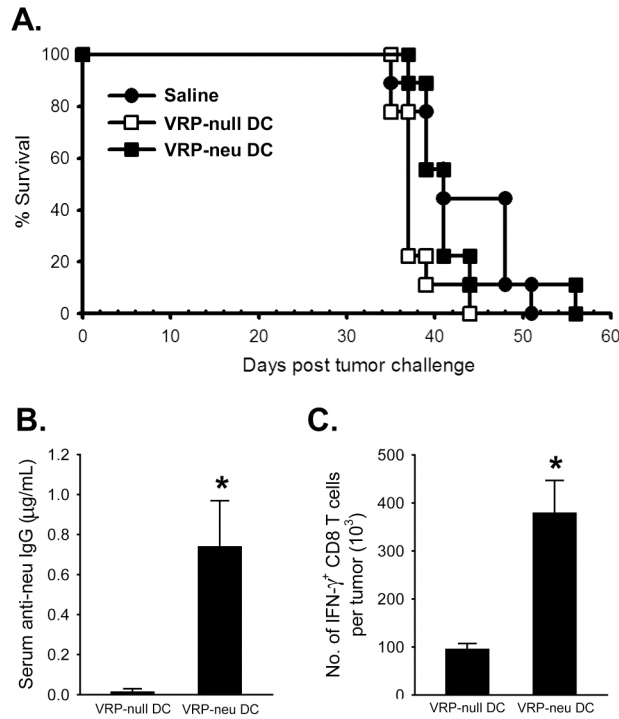


Figure 2-1. Therapeutic vaccination of tolerant neu-N mice with VRP-neu DCs does not inhibit tumor growth despite the induction of anti-neu immunity. *A*, 8-12 week old neu-N mice ($n = 9$ per group) were challenged with 5×10^4 NT2 cells and vaccinated on days 3, 17 and 31 with saline, null-VRP or neuET-VRP DCs. *B*, neu-N mice were vaccinated with either VRP-null DCs or VRP-neu DCs and similarly boosted two weeks later. At seven days post-boost, sera were evaluated for anti-neu IgG. *Columns*, mean ($n = 6$); *bars*, SEM; *, $p = 0.002$. *C*, neu-N mice, 8-12 weeks of age, were challenged with 5×10^4 NT2 cells and vaccinated with VRP-null DCs or VRP-neu DCs on days 3, 17 and 31. 5-6 weeks post-tumor challenge TILs were isolated and stimulated for 4 h with PMA/ionomycin and analyzed by intracellular IFN- γ staining. One of two similar experiments is depicted. *Columns*, mean ($n = 3$); *bars*, SEM; *, $p = 0.014$.

Figure 2-2

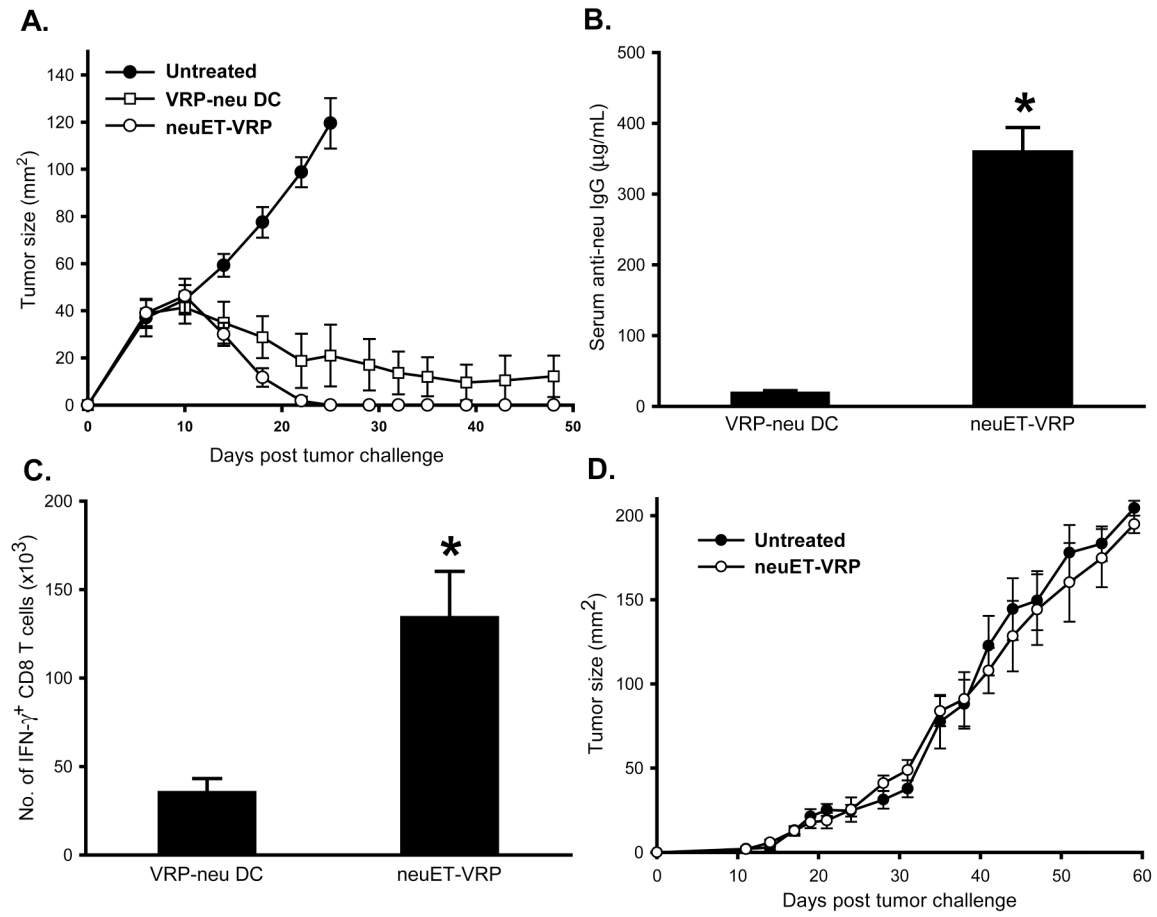


Figure 2-2. Direct vaccination with neuET-VRPs is superior to vaccination with VRP-neu DCs. *A*, Non-tolerant FVB/N mice were challenged with 2×10^6 NT2 cells and vaccinated 7 days later with VRP-neu DCs or neuET-VRPs. Vaccinations were repeated on day 21. One of two similar experiments is depicted. *Points*, mean (n = 5); *bars*, SEM. *B,C*, FVB/N mice were vaccinated with VRP-neu DCs or neuET-VRPs and boosted on day 14. Sera and spleens were harvested on day 21. *B*, Sera were analyzed for neu-specific IgG. *Columns*, mean (n = 6); *bars*, SEM; *, p = 0.002. *C*, Splenocytes were stimulated with RNEU₄₂₀₋₄₂₉ peptide and analyzed for intracellular IFN- γ . *Columns*, mean (n = 6); *bars*, SEM; *, p = 0.004. *D*, Tolerant neu-N mice, 8-12 weeks of age, were challenged with 5×10^4 NT2 cells and vaccinated on days 7, 21, and 34 with neuET-VRPs. *Points*, mean (n = 4); *bars*, SEM.

Figure 2-3

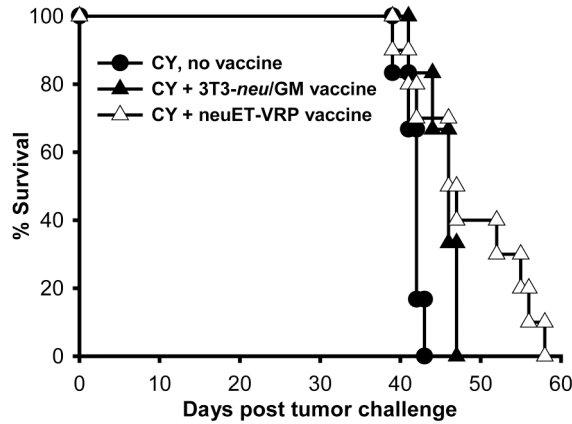


Figure 2-3. Therapeutic vaccination of neu-N mice with neuET-VRP or 3T3-*neu*/GM vaccine along with CY inhibits tumor growth but does not induce tumor regression. Neu-N mice (7-12 weeks of age, n = 22) were challenged with 5×10^4 NT2 cells. On day 5 post tumor challenge, mice were injected i.p. with 100 mg/kg CY. On day 7, mice were vaccinated with 3T3-*neu*/GM cells (solid triangles) or neuET-VRPs (open triangles) or left untreated (solid circle). Vaccines were repeated every 14 days. There was a significant difference in survival between both vaccination groups compared to the no vaccine control group ($p = 0.007$, Kaplan Meier survival analysis), but no difference between the vaccines ($p = 0.25$).

Figure 2-4

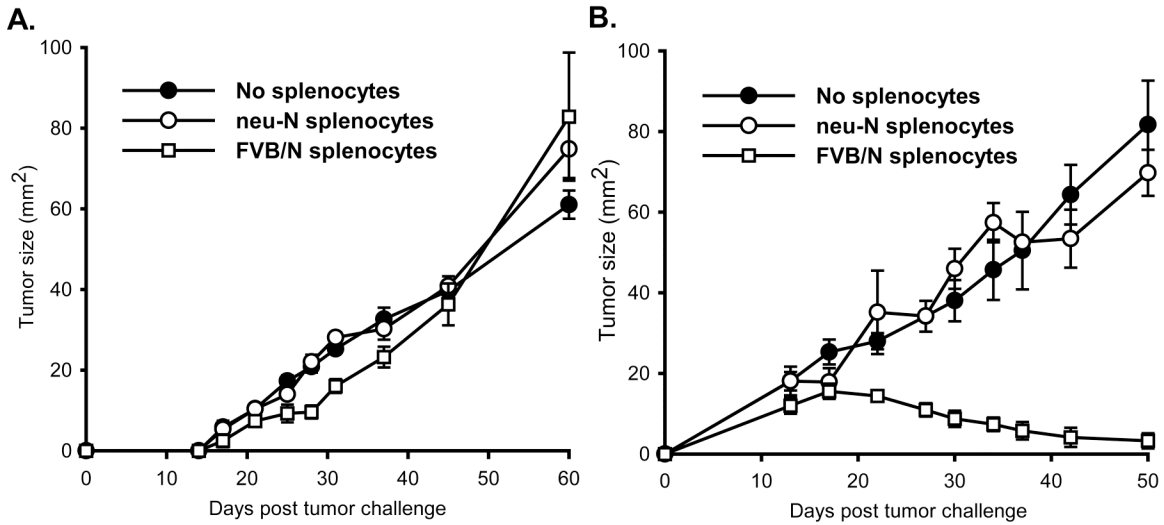


Figure 2-4. Both central and peripheral tolerance mechanisms inhibit efficacy of neuET-VRP vaccine. *A.* 5×10^6 splenocytes from either FVB/N or neu-N mice were injected i.v. along with 5×10^6 T cell-depleted BM cells into lethally irradiated neu-N mice, 8-12 weeks of age, followed by tumor challenge with 5×10^4 NT2 cells. Mice were vaccinated with neuET-VRPs on day 4 post-tumor challenge. *Points*, mean ($n = 3-5$); *Bars*, SEM. *B.* Same as (A) except that splenocytes were transferred into FVB/N mice challenged with 2×10^6 NT2 cells. *Points*, mean ($n = 3-5$); *Bars*, SEM.

Figure 2-5

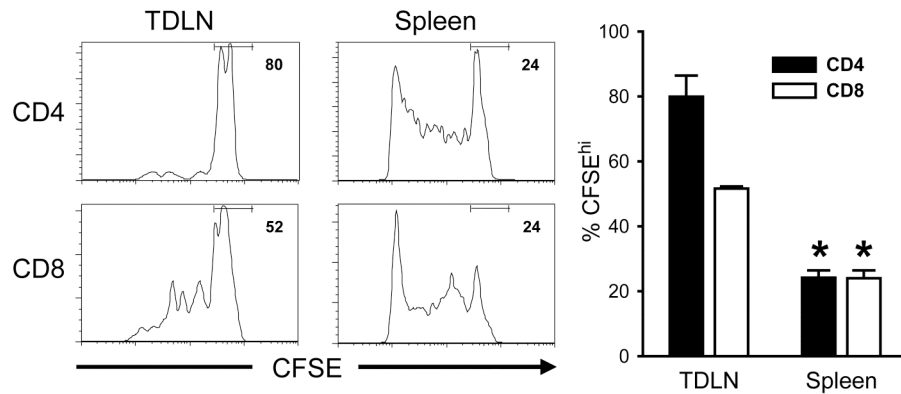


Figure 2-5. Proliferation of T cells upon neuET-VRP vaccination is suppressed at the tumor draining lymph node (TDLN). 10^7 CFSE labeled *in vitro* stimulated T cells were transferred i.v. into NT2 tumor bearing neu-N mice followed by vaccination with neuET-VRPs. 5 days later, cells were harvested from the TDLN and spleen and analyzed for CFSE dilution. One of three similar experiments is depicted. *Left*, CFSE staining of CD4⁺ and CD8⁺ T cells isolated from the TDLN and spleen. *Right*, % of transferred cells that failed to proliferate (CFSE^{hi}). *Columns*, mean (n = 4); *Bars*, SEM; *, p < 0.001.

Figure 2-6

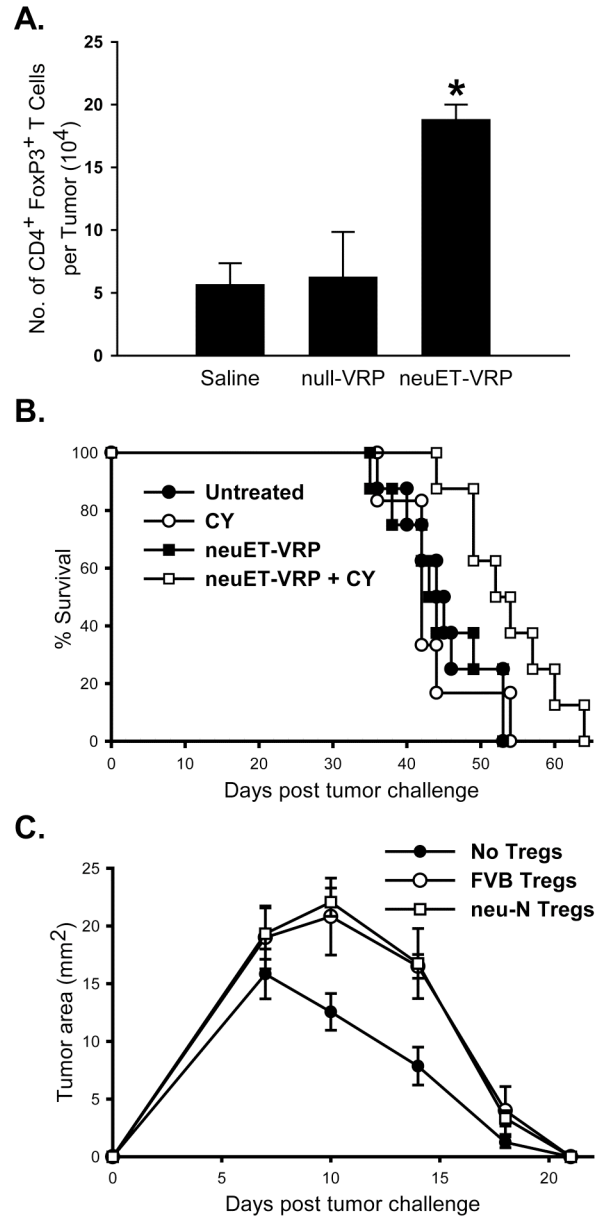


Figure 2-6. Depletion of CD4⁺ FoxP3⁺ T_{reg} cells with CY improves efficacy of neuET-VRP vaccine. *A*, neu-N mice were vaccinated with null-VRPs or neuET-VRPs three days post-tumor challenge with 5 x 10⁴ NT2 cells. At 5-6 weeks, TIL were isolated and analyzed for CD4 and FoxP3 expression by FACS. *Columns*, mean (n = 3); *bars*, SEM; *, p = 0.014. *B*, neu-N mice (n = 8 per group) were treated with CY (100 mg/kg) two days post-tumor challenge. On day 4, mice were vaccinated with neuET-VRPs and similarly boosted on days 18 and 32. One of three similar experiments is depicted. There was a significant improvement in survival in mice receiving CY with neuET-VRP vaccine (p = 0.030, Kaplan Meier survival analysis). *C*, On day 6 post-tumor challenge CD4⁺, CD25⁺ T_{reg} cells from tumor bearing FVB/N mice or neu-N mice were transferred into FVB/N mice treated with CY. The next day, mice were vaccinated with neuET-VRPs. *Points*, mean (n = 3-5); *bars*, SEM.

Figure 2-7

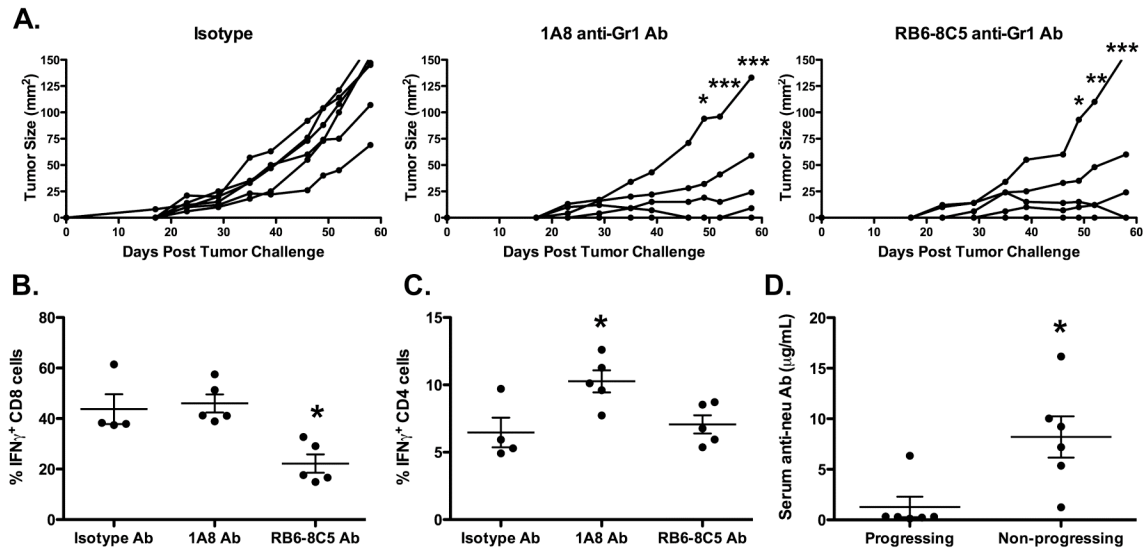


Figure 2-7. Depletion of MDSCs inhibits tumor progression in neu-N mice. *A*, 10^7 naïve FVB/N splenocytes were injected i.v. along with 5×10^6 T cell-depleted BM cells into lethally irradiated neu-N mice followed by tumor challenge with 5×10^4 NT2 cells. Mice were vaccinated with neuET-VRPs 4 days later. Mice were injected i.p. with 300µg anti-Gr-1 mAb (RB6-8C5 or 1A8) or rat IgG control every 3 days starting on day 7 post-tumor challenge. Tumor growth curves of individual mice are depicted. Tumor growth of both Gr-1 Ab groups were significantly different than isotype controls ($p = 0.024$, two-way RM ANOVA; Bonferroni posttests compared to isotype control, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *B,C*, Percentage of CD8⁺ (*B*) and CD4⁺ (*C*) T cells in the spleen at day 60 that were IFN- γ ⁺ following 6 h stimulation with PMA/ionomycin. *Points*, individual mice; *line*, mean; *bars*, SEM, *, $p < 0.05$. *D*, Serum anti-neu Ab levels on day 60 in mice with progressing or non-progressing tumors (tumors $< 25\text{mm}^2$ from both Gr-1 mAb groups). *Points*, individual mice; *line*, mean; *bars*, SEM, *, $p = 0.01$.

Figure 2-S1

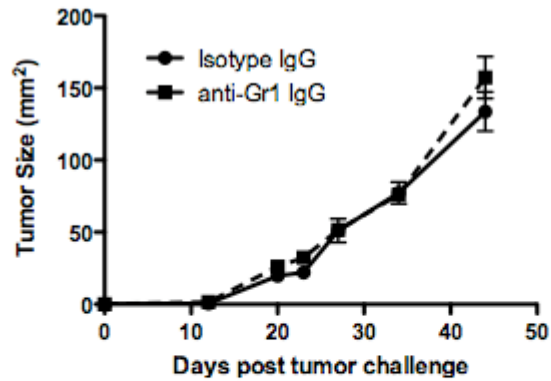


Figure 2-S1. No difference in tumor growth following depletion of MDSCs without adoptive transfer of neu-specific lymphocytes from FVB/N mice. Neu-N mice, 10-12 weeks of age, were challenged with 5×10^4 NT2 cells. On day 4 post-tumor challenge, mice were vaccinated with 1×10^6 neuET-VRPs and similarly boosted on days 18 and 32. Mice were injected i.p. with 300 μ g anti-Gr-1 mAb (RB6-8C5) or rat IgG control mAb every 3 days starting on day 7 post-tumor challenge. *Points*, mean ($n = 4$); *bars*, SEM.

CHAPTER THREE

THE INFLAMMASOME COMPONENT, NLRP3, IMPAIRS ANTITUMOR VACCINE BY ENHANCING ACCUMULATION OF PERITUMORAL MYELOID-DERIVED SUPPRESSOR CELLS

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Abstract

The inflammasome is a proinflammatory complex that generates IL-1 β and IL-18. It is activated by a subgroup of NLR proteins in response to microbial and nonmicrobial stimuli. Among NLRs, NLRP3 senses the widest array of stimuli and enhances adaptive immunity. However, its role in antitumor immunity is unknown. Therefore, we evaluated the function of the NLRP3 inflammasome in the immune response using dendritic cell vaccination against the poorly immunogenic melanoma cell line B16-F10. Vaccination led to a fourfold improvement in survival for *Nlrp3*^{-/-} over WT mice. This outcome was confirmed using vaccination against E.G7-OVA tumor cells. Immunity was dependent on CD8⁺ T cells and exhibited immune specificity and memory. Increased vaccine efficacy in *Nlrp3*^{-/-} mice was not due to differences in dendritic cells but rather to differences in myeloid-derived suppressor cells (MDSCs). Though MDSCs expressed NLRP3, the absence of NLRP3 did not alter the morphology of MDSCs or their functional capacity to inhibit T cells. The number of MDSCs in peripheral lymphoid tissues was similar. However, the number of peritumoral MDSCs was reduced fivefold in *Nlrp3*^{-/-} mice. Adoptive transfer experiments demonstrated that *Nlrp3*^{-/-} MDSCs were significantly less efficient in reaching the tumor site. Depletion of MDSCs with an anti-Gr-1 antibody increased survival of tumor-bearing WT mice but not *Nlrp3*^{-/-} mice. We conclude that NLRP3 increases accumulation of MDSCs in the tumor and inhibits antitumor T cell immunity post DC vaccination. This work establishes an unexpected role for NLRP3 in impeding antitumor response and suggests novel approaches to improving antitumor vaccines.

Introduction

NLR family, pyrin domain containing 3 (NLRP3) is a member of the nucleotide-binding domain and leucine-rich repeat containing gene family of intracellular sensors. When activated, NLRP3 forms a protein complex called the inflammasome (192, 193, 314). The inflammasome combines NLRP3 with the adaptor molecule, ASC/PYCARD/TMS/CARD5, Cardinal and pro-caspase-1 to form a multimer (3). The result is the proteolytic maturation of caspase-1 that cleaves and activates proIL-1 β and proIL-18 to generate IL-1 β and IL-18 (192).

NLRP3 is activated by a wide variety of both microbial and nonmicrobial molecular motifs. The microbial signals are part of the pathogen associated molecular patterns or PAMPs and include gram positive and negative bacteria, RNA and DNA viruses, polyI:C, and LPS (193). The nonmicrobial signals of NLRP3 activation include exogenous compounds such as asbestos and endogenous signals such as urate crystals (96, 97, 193). Many of these nonmicrobial stimuli are included in the group of signals referred to as damage associated molecular patterns or DAMPs (164). Furthermore, increasing evidence indicates a role for inflammasome products in enhancing Th1, Th2 and Th17 responses (125). These mechanisms allow the inflammasome to respond to many pathological environments (281).

In contrast to the extensive literature on PAMPs and DAMPs, there have been few studies of NLRP3 in the tumor microenvironment. However, work on caspase-1 activated cytokines suggests a potential tumor promoting role. For example, IL-1 β promotes tumor growth in several mouse models (167, 265). Conversely, reduction of

IL-1 β diminishes metastases in the lung (175) and knocking-out IL-1 β inhibited local and metastatic growth in a murine melanoma model (326).

Human studies have been less definitive but remain consistent with the murine data. For example, the risk of gastric cancer is associated with genetic polymorphisms linked to enhanced IL-1 β expression (79). Similar studies on IL-18 polymorphisms have demonstrated an increased risk for other epithelial cancers (226). More directly, serum IL-18 concentrations are inversely correlated with survival in hepatocellular cancer (309).

These cytokines can contribute to tumorigenesis by a variety of mechanisms; however, an emerging interest is the increased activity of myeloid derived suppressor cells (MDSCs) (234, 315). MDSCs are a heterogeneous population of immature myeloid cells that are most readily identified in the mouse by their expression of Gr-1 and CD11b (104). These cells suppress T cell responses directly by a variety of mechanisms (307). Furthermore, MDSCs also contribute to tumorigenesis indirectly by inducing regulatory T cells (138), skewing towards a Th2 immune response (285), suppressing NK cells (131), and increasing angiogenesis (343). Clinical studies have documented these cells in several human cancers (59) including head and neck (349), renal cell (351), and hepatocellular (131) cancers. Both murine and human studies have found the number of MDSCs increases with tumor burden (59, 198).

These data led us to test the hypothesis that activation of NLRP3 could inhibit the antitumor immune response required for cancer vaccine efficacy by activating cytokines and recruiting MDSCs. This premise implies that blockade of NLRP3 would result in an improved vaccine response. We tested this hypothesis using a dendritic cell vaccine in the B16-F10 and E.G7-OVA tumor models.

Materials and Methods

Mice

Nlrp3^{-/-} mice were generated as described (335) and were backcrossed for a total of nine generations to C57BL/6J mice. All other mice were purchased from Jackson Laboratories (Bar Harbor, ME). All experiments were conducted using protocols approved by Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Dendritic Cell Vaccination Model

Tumor lines were purchased from the American Type Culture Collection (ATCC, Rockland, MD). Subcutaneous tumors were formed by injecting either 10,000 cells (B16-F10, Lewis Lung) or 5x10⁵ cells (E.G7-OVA) in the leg. The leg diameter was measured three times a week and mice were euthanized when this diameter reached 6 mm.

DCs were isolated from bone marrow cultures treated with GM-CSF and IL-4 (Peprotech Inc, Rocky Hill, NJ) (208). The DCs were pulsed with either B16-F10 lysates or with class I and class II peptides (SIINFEKL and ISQAVHAAHAEINEAGR) and matured with LPS. 1x10⁶ cells were subcutaneously injected on day three and ten after tumor injection.

Cell depletion was accomplished by the intraperitoneal injection of 200 mcg of PK136 (NK cell) and RB6-8C5 (MDSC) and 500 mcg of 2.43 (CD8 T cell) and GK1.5 (CD4 T cell) mAb (BioExpress, West Lebanon, NH). The schedule for lymphoid cell

depletion was day -1, day 0 and then biweekly. MDSC depletion was also biweekly starting on day 6. Efficacy of cell depletion was confirmed by FACS analysis.

Flow Cytometry and Fluorescent Microscopy

Flow cytometry was performed as previously described (322). Cryosections were obtained after perfusing mice with 4% paraformaldehyde. The slides were fixed with acetone and blocked with 10% goat serum. Primary antibody signals were amplified using anti FITC and anti PE antibodies.

Gr-1⁺, CD11b⁺ cells were counted by making a “digital slide” from a low power photomicrograph using ImageJ software. Fluorescent images were optimized with “Brightness/Contrast” adjustments and then converted with the “Binary” command. Background noise was reduced with one application of the “Despeckle” command. The digital slide was completed by the “Analyze Particles” command. The fidelity of these digital images was confirmed by comparing them to the original photomicrograph. Double staining cells were then counted using the “Colocalization” plug-in.

MDSC Assay

MDSCs for the *in-vitro* suppression assays were harvested by FACS sorting from the lungs of WT and *Nlrp3*^{-/-} mice two weeks after intravenous injection with 1x10⁶ B16-F10 melanoma cells. Immunosuppression was evaluated by adding these cells to a mixed lymphocyte reaction. Stimulator cells were taken from the adherent fraction of BALB/cJ splenocytes and responder cells were harvested from the non-adherent fraction of C57BL/6J splenocytes. Stimulators and CD11b⁺ Gr-1⁺ cells were treated with mitomycin

C and responders were labeled with CFSE. For the migration assays, MDSCs were harvested from the spleens of EGFP transgenic C57BL/6 or *Nlrp3*^{-/-} mice by immunomagnetic bead separation (Miltenyi, Bergisch Gladbach, Germany) two weeks after intravenous tumor injection.

Real time PCR

PCR amplification was performed using *Nlrp3* specific primers and probe: 5'-CTCCCGCATCTCCATTTGT-3', 5'-GCGTGTAGCGACTGTTGA-3', and FAM-CCCACTCTCACCTAGACGCGC-TAMRA with TaqMan PCR reagents and 7900HT Thermocycler (Applied Biosystems). Expression values normalized to cell number are reported.

Statistical Analysis

Data are reported as a mean \pm standard error of the mean (SEM). Results were considered significant if $p \leq 0.05$ as determined by the Mann Whitney test. Comparisons in survival were done by the Cox proportional hazard regression method.

Results

Dendritic cell vaccination improves survival in $Nlrp3^{-/-}$ mice but not WT mice

We examined the function of NLRP3 in a tumor model by comparing the survival of WT and $Nlrp3^{-/-}$ mice after the subcutaneous injection of B16-F10 melanoma cells. In this model, none of the WT or $Nlrp3^{-/-}$ mice survived (Fig. 3-1A). The median survival was also not significantly different (15.3 vs. 16.6 days, $p = NS$). Therefore, the presence or absence of NLRP3 is not critical for the growth of B16-F10 tumor cells.

The survival of $Nlrp3^{-/-}$ mice could be improved by treatment with a dendritic cell (DC) vaccine. In these experiments, mice were given an inoculation of 1×10^6 WT DCs pulsed with B16-F10 tumor cell lysate. To mimic treatment of human disease, these inoculations were given three and ten days *after* tumor injection. With this treatment, only 9.1% of the WT mice demonstrated long term survival while $Nlrp3^{-/-}$ mice showed nearly a 4-fold increase in survival (35%). The hazard ratio favoring survival in the $Nlrp3^{-/-}$ mice was 2.4 [1.2 – 4.8] ($p = 0.017$) (Fig. 3-1B).

The survival benefit was not limited to $Nlrp3^{-/-}$ mice in the B16-F10 model system. To demonstrate its broader applicability, WT and $Nlrp3^{-/-}$ mice were subcutaneously injected with 5×10^5 E.G7-OVA tumor cells. The mice were then treated with 1×10^6 peptide pulsed DCs using the same schedule. As before, $Nlrp3^{-/-}$ mice had a substantial improvement in survival compared to WT mice (28.6% vs. 62.5%) (Fig. 3-S1).

NLRP3 expression by the host limits the effectiveness of the dendritic cell vaccine

Next, we determined if this survival advantage was dependent on the expression of NLRP3 by the DCs used for vaccination. In these experiments, *Nlrp3*^{-/-} tumor-bearing mice were treated with DC vaccines from WT or *Nlrp3*^{-/-} mice. Thirty three percent of the *Nlrp3*^{-/-} mice injected with *Nlrp3*^{-/-} DC vaccine survived compared to 40% injected with the WT vaccine ($p = NS$) (Fig. 3-1C). Thus, the *Nlrp3*^{-/-} DC vaccine produced survival that was comparable to WT vaccines in *Nlrp3*^{-/-} mice.

Subsequently, we evaluated the effectiveness of vaccination using *Nlrp3*^{-/-} and WT DCs in WT tumor-bearing mice. None of the WT mice survived in these experiments. WT mice treated with a WT vaccine had a median survival of 21.8 days versus 16.1 days with an *Nlrp3*^{-/-} vaccine ($p=NS$) (Fig. 3-1D). From these data, we conclude that the poor outcome in WT mice was due to NLRP3 expression by the host and not the vaccine.

*The benefit of the dendritic cell vaccine in *Nlrp3*^{-/-} mice is CD8 dependent*

Having established a survival advantage for vaccinated *Nlrp3*^{-/-} mice, their immunological memory was tested by rechallenging these mice three months after their initial tumor exposure. Eleven *Nlrp3*^{-/-} mice received a second tumor injection and ten survived (90.9%) (Fig. 3-2A). Two of the surviving WT mice were also rechallenged and one survived (50%). Due to the low number of WT mice surviving the first tumor challenge, we were unable to establish any meaningful statistical comparisons. Nevertheless, these experiments demonstrate an antitumor memory response in the *Nlrp3*^{-/-}

^{-/-} mice and imply the improved outcome was due to an enhanced immune response to the vaccine.

The specificity of this tumor protection was examined by challenging surviving *Nlrp3^{-/-}* mice with an unrelated tumor. In these experiments, eight *Nlrp3^{-/-}* mice received 1×10^4 Lewis lung carcinoma cells (LLCa). Two of these mice survived (25%) (Fig. 3-2A). This result was significantly less than those rechallenged with B16-F10 cells ($p = 0.003$) but not different than naive *Nlrp3^{-/-}* mice injected LLCa cells (*survival* = 20%). Therefore, the immunological memory generated after vaccination in *Nlrp3^{-/-}* mice was specific only for the tumor used in the vaccine.

The efficacy of the adaptive immune response was further tested by examining the survival of *Nlrp3^{-/-}* mice after depletion of CD4⁺, CD8⁺, or NK cells (Fig. 3-S2). The survival of the cell depleted mice was compared to control *Nlrp3^{-/-}* mice by calculating a hazard ratio using Cox regression analysis. In this analysis, a hazard ratio greater than one signifies a poorer outcome for the cell-depleted *Nlrp3^{-/-}* mice. As shown in Fig. 3-2B, the survival of all three cohorts of cell-depleted *Nlrp3^{-/-}* mice was decreased compared to the *Nlrp3^{-/-}* mice. However, this finding was only statistically significant for *Nlrp3^{-/-}* mice after the depletion of CD8⁺ T cells ($HR = 2.06, p = 0.028$). Therefore, the enhanced activity of tumor vaccination given to *Nlrp3^{-/-}* mice required the presence of CD8⁺ T cells.

Nlrp3^{-/-} MDSCs are morphologically and functionally equivalent to WT suppressor cells in vitro

The vaccine's dependence on CD8⁺ cells is consistent with our hypothesis that MDSCs are differentially suppressing T cell immunity (172). This premise was further strengthened by gene expression studies of peritumoral cells. In these experiments, cells from subcutaneous B16-F10 tumors were sorted by their expression of Gr-1 and CD11b. Both Gr-1⁺, CD11b⁺ and Gr-1⁻, CD11b⁺ cells expressed NLRP3 at transcript numbers significantly greater than found in CD11b⁻ cells (4.30 ± 0.13 , 4.69 ± 0.94 vs. 0.00 ± 0.0 , $p = 0.033$) (Fig. 3-3). These results demonstrate that NLRP3 is expressed by both MDSCs (Gr-1⁺, CD11b⁺) and other myeloid cells (Gr-1⁻, CD11b⁺). It was not expressed by CD11b⁻ cells including the tumor cells and tumor infiltrating lymphocytes. It was also not expressed in cells from *Nlrp3^{-/-}* mice as expected.

Given the expression of NLRP3 in MDSCs, we compared the morphology and functional activity of these cells from *Nlrp3^{-/-}* and WT tumor-bearing mice. Since the numbers of MDSCs within the tumor are limited, we isolated these cells from the lungs of WT and *Nlrp3^{-/-}* mice with B16-F10 metastasis. MDSCs from both mice could be further divided into two subpopulations based on their expression of Gr-1 and CD11b. The Gr-1⁺⁺, CD11b⁺ cells had a neutrophil morphology and the Gr-1⁺, CD11b⁺ cells had a monocytic morphology. These subpopulations correspond to the granulocytic and monocytic MDSCs described by Youn *et al.* (348). However, there were no morphologic differences between *Nlrp3^{-/-}* or WT MDSCs within each subpopulation (Fig. 3-4A).

Both populations of Gr-1, CD11b double positive cells were then tested for their ability to suppress a mixed lymphocyte reaction (MLR). The monocytic MDSCs

displayed a greater suppressive capacity compared to the granulocytic MDSCs.

However, we did not find a difference in the ability of WT compared to *Nlrp3*^{-/-} MDSCs to suppress T cell proliferation *in vitro* (Fig. 3-4B).

Regardless of the differences between Gr-1⁺⁺ and Gr-1⁺ MDSCs, the central observation from these experiments is that no differences were detected when comparing cells from WT and *Nlrp3*^{-/-} mice. These data suggest that the disparity in the vaccine response between the WT and *Nlrp3*^{-/-} mice cannot be explained by differences in the intrinsic suppressive capacity of the MDSCs.

Nlrp3^{-/-} mice have fewer MDSCs at the tumor site

Since the survival advantage of the *Nlrp3*^{-/-} mouse could not be explained by functional differences in MDSCs, we speculated that this advantage was due to a reduction in the number of MDSCs. In the first series of experiments, splenic and tumor draining lymph node MDSCs were measured by flow cytometry 14 days after tumor injection. The average leg size was 4.6 ± 0.3 mm in the WT mouse and 3.8 ± 0.1 mm in the *Nlrp3*^{-/-} mouse ($p = NS$). We found no significant differences in the number of Gr-1, CD11b double positive cells isolated from WT and *Nlrp3*^{-/-} mice. There was a trend to a lower percentage of splenic MDSCs in the *Nlrp3*^{-/-} mice but this finding was not statistically significant ($1.61 \pm 0.24\%$ vs. $2.67 \pm 0.40\%$, $p = 0.11$) (Fig. 3-5B). No such trend was noted in the tumor draining lymph node ($5.9 \pm 1.6 \times 10^4$ cells vs. $5.7 \pm 1.1 \times 10^4$ cells, $p = 0.78$). This observation suggests that MDSCs are mobilized to a similar degree in the spleen and lymph nodes of each mouse.

We next evaluated the number of MDSCs within the tumor mass by immunofluorescent microscopy. Mice were included in this analysis only if their tumor was visible by light microscopy. This approach revealed a five fold increase in the number of CD11b/Gr-1⁺ cells in the peritumoral area of WT mice compared to *Nlrp3*^{-/-} mice ($18.6 \pm 3.0/LPF$ vs. $3.5 \pm 0.5/LPF$, $p = 0.02$) (Fig. 3-5A).

Since immunohistochemistry cannot distinguish between Gr-1⁺⁺ and Gr-1⁺ cells, the percentage of these two populations was measured using flow cytometry. In these experiments, tumors were harvested when palpable; mice without tumor were excluded. Gating on the Gr-1⁺, CD11b⁺ cells revealed a 4.7 fold increase of the monocytic MDSCs in WT mice compared to *Nlrp3*^{-/-} mice ($2.1 \pm 0.5\%$ vs. $0.45 \pm 0.24\%$, $p = 0.003$) (Fig. 3-5C), while a six fold increase was noted in the granulocytic MDSCs ($0.54 \pm 0.18\%$ vs. 0.09% , $p = 0.003$). No difference was observed in the number of MDSCs in the tumor with out vaccination (Table 3-S1). Thus, both flow cytometry and immunohistochemistry demonstrated a significant reduction in the number of peritumoral MDSCs in *Nlrp3*^{-/-} mice compared to WT mice following DC vaccine.

Depletion of MDSCs improves survival in vaccinated WT but not Nlrp3^{-/-} mice

To establish if the decrease in peritumoral MDSCs accounted for the improved survival in *Nlrp3*^{-/-} mice, we measured survival in WT and *Nlrp3*^{-/-} mice following MDSC depletion. Anti-Gr-1 antibody was injected twice a week beginning on day 6 post-tumor challenge. This treatment produced a 2.6 fold decrease in the number of MDSCs in the spleen (Fig. 3-S3). As shown in Fig. 3-6, depletion of MDSCs eliminated the survival advantage of the vaccinated *Nlrp3*^{-/-} mice over WT mice ($HR = 1.15$, $p =$

0.75). When compared to our earlier experiments, we found this change was exclusively due to an improvement in overall survival in the WT mice from 9.1% to 38.9% ($HR = 2.06, p < 0.05$). There was no difference in overall survival of the *Nlrp3*^{-/-} mice after MDSC depletion (40.0% vs. 35.0%, $p = 0.82$). This result strongly suggests that the decreased number of peritumoral MDSCs accounts for the increased efficacy of the dendritic cell vaccine in *Nlrp3*^{-/-} mice.

NLRP3 expression promotes migration of MDSCs into the tumor

The number of peritumoral MDSCs could be increased by enhanced migration. To assess the effect of NLRP3 expression on migration, WT or *Nlrp3*^{-/-} EGFP⁺ MDSCs were intravenously injected into tumor bearing *Nlrp3*^{-/-} mice and detected by flow cytometry. 83.4 ± 0.7% of the EGFP⁺ cells expressed Gr-1 and CD11b at the time of the injection (Fig. 3-7A). This approach revealed that significantly fewer *Nlrp3*^{-/-} MDSCs migrated into the tumor compared to WT MDSCs (178.1 ± 91.0 cells/tumor vs. 448.0 ± 36.1 cells/tumor, $p < 0.05$) (Fig. 3-7B). These results were not biased by tumor size since no difference in tumor size was detected at the time of injection (4.1 ± 0.06 mm vs. 4.2 ± 0.25 mm, $p = NS$).

Discussion

DC vaccines represent a promising therapy for cancer; however, their efficacy is frequently suboptimal. This inefficiency was substantiated in our model by the vaccine's inability to improve survival in WT mice. This outcome is not surprising since we used a poorly immunogenic tumor and administered the vaccine after tumor initiation. Interestingly, we found that the efficacy of DC vaccination could be markedly improved by inactivating NLRP3.

Further analysis implicated differences in MDSCs as the reason for the survival advantage in *Nlrp3^{-/-}* mice. NLRP3 does not appear to affect the ability of MDSCs to suppress T cells. Instead, the *Nlrp3^{-/-}* mice had a significant reduction in the number of MDSCs at the tumor site. Such a reduction would result in a decrease in MDSC inhibition of cytotoxic CD8⁺ T cells and prompt a more effective antitumor response. This interpretation explains the loss of effectiveness of the vaccine in a CD8 depleted animal. It also explains why MDSC depletion by anti-Gr-1 antibodies restored the efficacy of the vaccine in WT mice to a level comparable to *Nlrp3^{-/-}* mice.

Our findings further show that the differences in MDSC number are likely due to differences in the migration of these cells to the tumor site. These cells were present in the spleen and tumor draining lymph node in equal numbers suggesting that MDSCs are mobilized to the peripheral lymphoid organs independently of NLRP3. However, when EGFP MDSCs were injected into tumor bearing mice, WT cells were 2.5 times more efficient than *Nlrp3^{-/-}* cells in their migration into tumors. This difference was not due to tumor size since both WT and knockout mice had similar size tumors at the time of

injection. This observation suggests a critically important role for NLRP3 in the migration of MDSCs to the tumor microenvironment.

Inflammasome activation has been associated with infiltration of other myeloid cells including monocytes, macrophages, and granulocytes (165, 203, 217). One potential mechanism for this migration is the activation of IL-1 β by the inflammasome. Over expression of IL-1 β leads to the enhanced migration of monocytes (24) and MDSCs (315). Conversely, IL-1 receptor blockade delays MDSC recruitment (38). Despite the appeal of this mechanism, we have been unable to document differences in IL-1 β in the tumors of WT and *Nlrp3*^{-/-} mice. IL-1 β was undetectable in western blotting and ELISAs on tumor homogenates, *ex-vivo* tumor isolates, and *in-vitro* tumor/macrophage co-cultures (not shown). Though not conclusive, these results suggest the possibility that MDSC recruitment may be IL-1 β independent. Such a conclusion is possible since the activation of IL-1 β only accounts for a portion of inflammasome function. This complex also leads to the generation of IL-18 (192), IL-33 (180), and chemotactic factors (335).

We believe our interpretation is more consistent with the literature than several alternative hypotheses. One such hypothesis is that WT MDSCs had a survival advantage over *Nlrp3*^{-/-} MDSCs. However, NLRP3 is more likely to induce cell death than cell survival. For example, monocytes that are hypersensitive to NLRP3 activation are prone to cell death in a cathepsin B dependent manner (102). Furthermore, myeloid cells from *Nlrp3*^{-/-} mice are resistant to cell death induced bacterial pathogens (335).

Wild type MDSCs are also not likely to have a proliferative advantage over *Nlrp3*^{-/-} cells. Though multiple mechanisms are involved, MDSC expansion appears to take place in niches distant from the tumor. The primary sites of proliferation of MDSCs

and their precursors are the spleen, bone marrow, and liver (1, 142, 172, 182). Finding no significant differences in the percentage of MDSCs in the spleens of WT and *Nlrp3*^{-/-} mice strongly argue against proliferation as a reason for differences in peritumoral MDSC number.

At first glance, our result stands in contrast to recent findings that NLRP3 might enhance adaptive immunity. Several reports have shown that NLRP3 is required for alum adjuvant function in enhancing IgE production and Th2 priming, although there are conflicting reports regarding the effect of NLRP3 on IgG production (78, 165, 180, 196). More recently, Ghiringhelli *et al.* demonstrated that the NLRP3 inflammasome was critically important in the P2X7R-dependent activation of DCs to generate IFN- γ producing CD8⁺ T cells (110). This was mediated by the release of ATP from dying tumor cell lines in the presence of the chemotherapeutic drugs oxaliplatin, doxorubicin or mitoxantrone. These data suggest that NLRP3 plays a role in preventing tumor growth after administration of chemotherapy, which is somewhat at odds with our findings as well as earlier findings by others on the role of IL-1 β in tumors (167). However, a major difference between the two studies is the inclusion of chemotherapy induced apoptosis. Furthermore, the requirement for the NLRP3 inflammasome was bypassed by the addition of IL-12, which we have found is significantly generated by the DCs used by our group. Thus, our findings suggest that in the presence of IL-12, which alone can generate Th1 polarized T cells, activation of the NLRP3 inflammasome enhances the accumulation of MDSCs and therefore suppresses peritumoral immune responses. Thus, the function of NLRP3 in cancer may be quite complex and different depending on the stimuli used for activation of the inflammasome.

In summary, the expression of NLRP3 in the tumor microenvironment diminishes antitumor immunity and vaccine efficacy by facilitating the migration of MDSCs to the site of tumor growth. Since the MDSCs also express NLRP3, their influx becomes part of a positive feedback loop leading to further expansion of these cells. These findings support a novel role for NLRP3 in cancer progression.

Figure 3-1

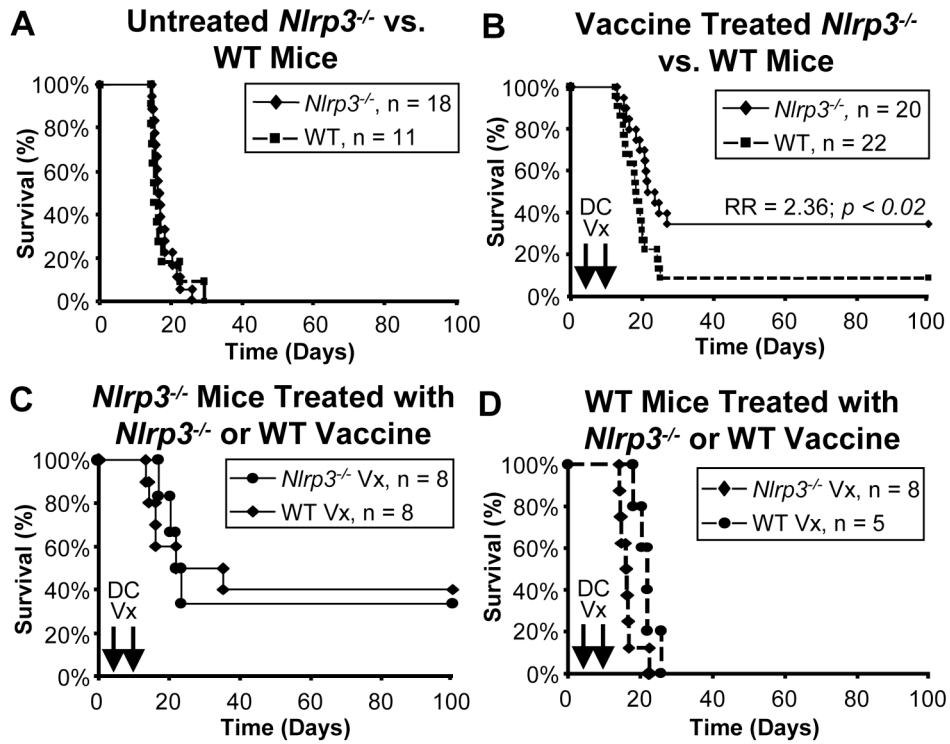


Figure 3-1. Dendritic cell vaccine improves survival in *Nlrp3*^{-/-} mice but not WT mice. *A.* Survival curves of WT and *Nlrp3*^{-/-} mice after receiving a subcutaneous injection with 10,000 B16-F10 melanoma cells. *B.* Survival of *Nlrp3*^{-/-} mice was significantly improved after receiving 1x10⁶ tumor-lysate pulsed DCs (DC Vx) on day 3 and day 10 after tumor injection. No improvement was seen in WT mice. *C.* *Nlrp3*^{-/-} mice show improved survival after receiving vaccines from either *Nlrp3*^{-/-} or WT mice. *D.* WT mice show no improvement in survival after receiving vaccines from WT or *Nlrp3*^{-/-} mice.

Figure 3-2

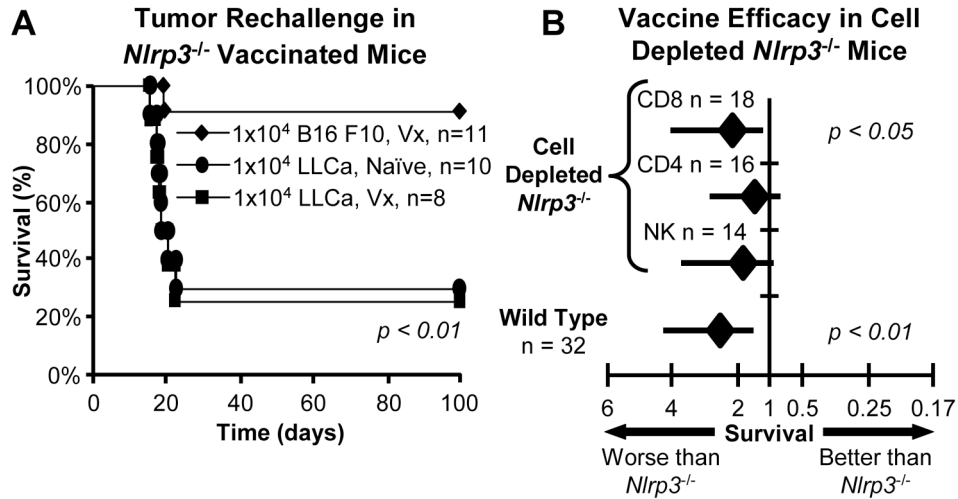


Figure 3-2. Vaccinated *Nlrp3*^{-/-} mice demonstrate an antitumor memory response.

A. Survival curves of vaccinated or naïve *Nlrp3*^{-/-} mice after rechallenge with B16-F10 cells or Lewis Lung cancer (LLCa) cells. *B.* Survival of vaccinated *Nlrp3*^{-/-} mice following depletion of CD8⁺, CD4⁺, and NK cells. The graph shows the hazard ratio and confidence interval comparing the survival of the cell depleted animals with *Nlrp3*^{-/-} mice. Values greater than 1 imply decreased survival compared to untreated *Nlrp3*^{-/-} mice.

Figure 3-3

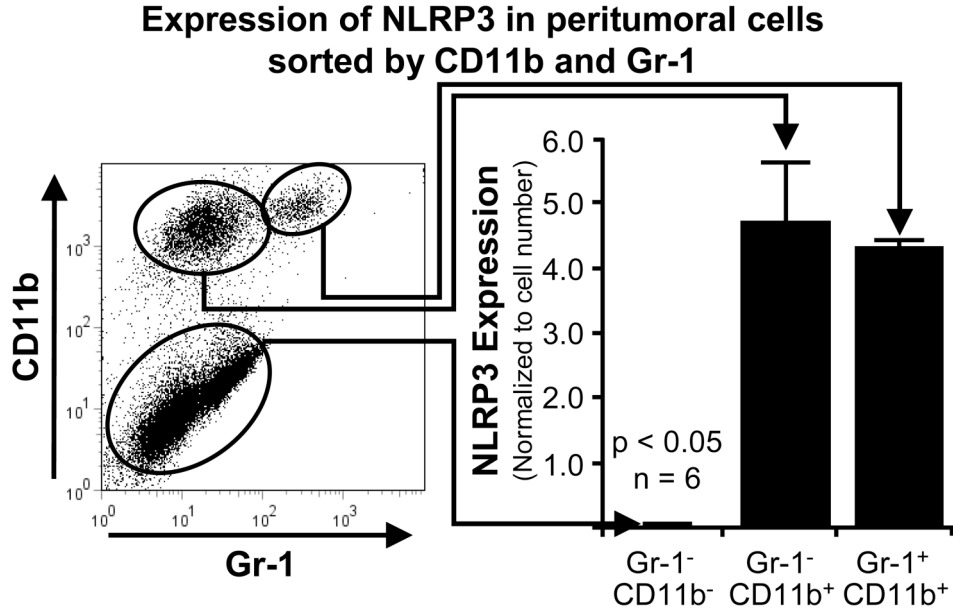


Figure 3-3. Nlrp3 is expressed by peritumoral myeloid cells. Expression of Nlrp3 by real time PCR in (1) Gr-1⁺, CD11b⁺ (2) Gr-1⁻, CD11b⁺ and (3) Gr-1⁻, CD11b⁻ sorted cells. The panel on left is a representative dot plot of sorted cells from a subcutaneous B16-F10 tumor. The panel on the right is a bar graph of Nlrp3 transcripts normalized to cell number.

Figure 3-4

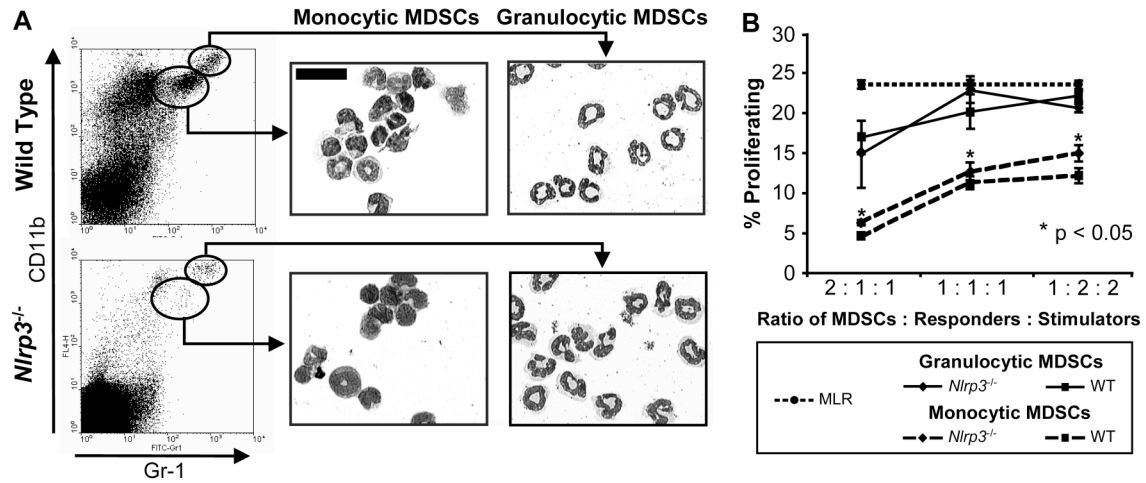


Figure 3-4. WT and *Nlrp3*^{-/-} MDSCs have a similar morphology. *A.* Flow cytometry dot plots for cells isolated from WT and *Nlrp3*^{-/-} mice after intravenous injection with B16-F10 tumor cells. Cells were sorted by the gates shown and then examined under cytopsin with Wright Giemsa staining. Lens magnification is 500X; scale bar is 20 μm. *B.* Line graph showing suppression of a MLR reaction by granulocytic (solid line) and monocytic (dashed line) MDSCs from WT (squares) and *Nlrp3*^{-/-} (diamonds) mice. Significance was determined by comparing proliferation with control MLR response (dotted line). Results are averaged from three separate experiments.

Figure 3-5

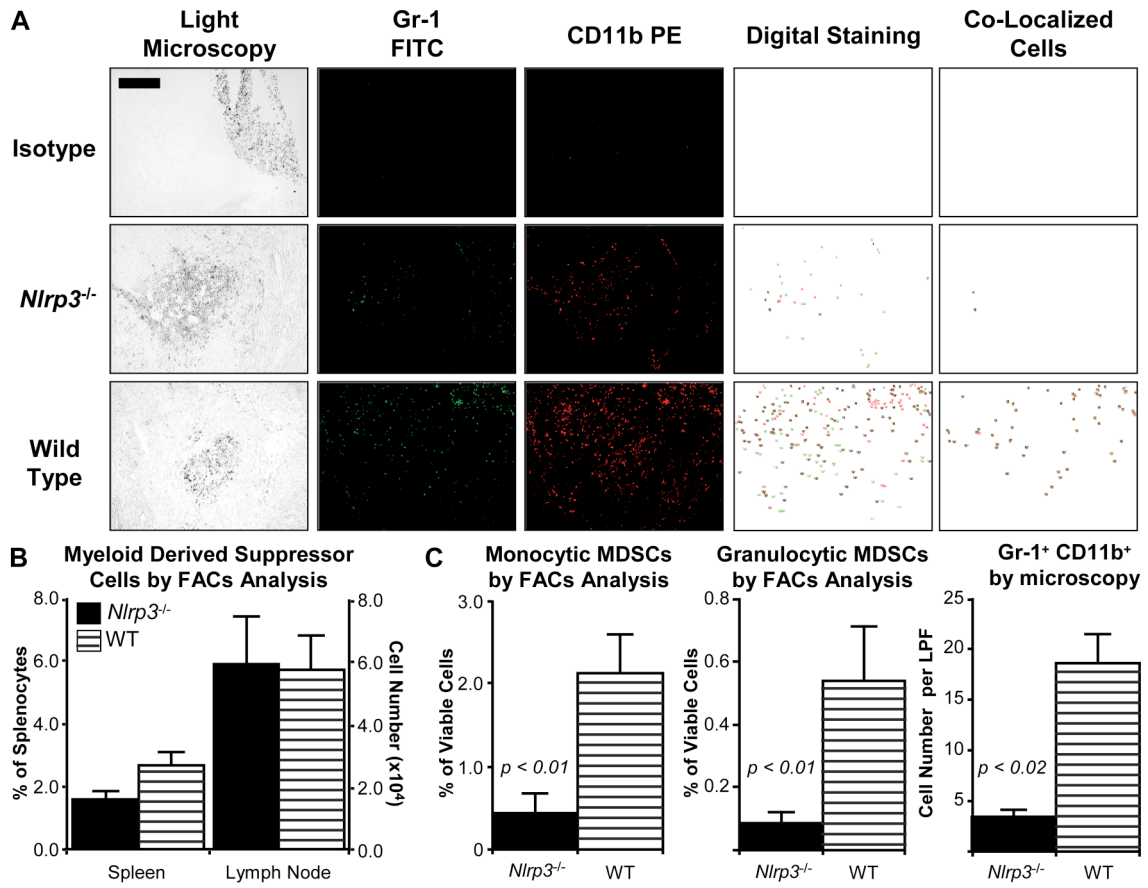


Figure 3-5. *Nlrp3*^{-/-} mice have fewer myeloid derived suppressor cells at the tumor site. *A.* Representative immunofluorescent images for Gr-1 and CD11b at the tumor site in *Nlrp3*^{-/-} (middle row) and WT (bottom row) mice. First column: Light microscopic images of melanoma. Second column: Immunofluorescence for Gr-1. Third Column: Immunofluorescence for CD11b. Fourth Column: Digital representations. Fifth Column: Double positive cells by co-localizing software. Lens magnification is 40X; scale bar is 500 μ m. *B.* Percentages of MDSCs from the spleen and total number of MDSCs in the lymph node of WT (striped) and *Nlrp3*^{-/-} (solid) mice determined by flow cytometry 14 days after tumor injection. *C.* Increased percentage of monocytic MDSCs (right) and granulocytic MDSCs (center) from the tumor in WT (striped) and *Nlrp3* (solid) mice determined by flow cytometry. The graph on the left shows the increased number of Gr-1⁺, CD11b⁺ cells in WT mice determined by microscopy

Figure 3-6

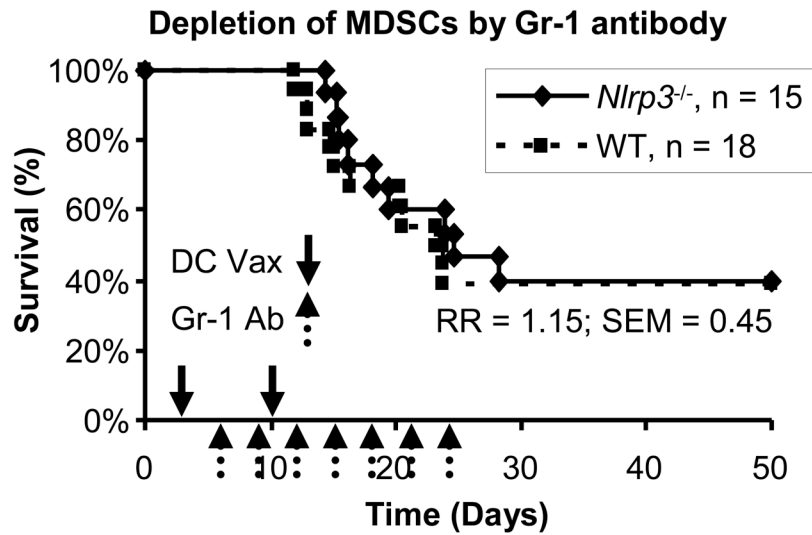


Figure 3-6. Depletion of myeloid derived suppressor cells improves vaccine response in WT but not $Nlrp3^{-/-}$ mice. Survival curves WT and $Nlrp3^{-/-}$ mice treated with anti-Gr-1 antibody (dotted arrows) and dendritic cell vaccine (solid arrows).

Figure 3-7

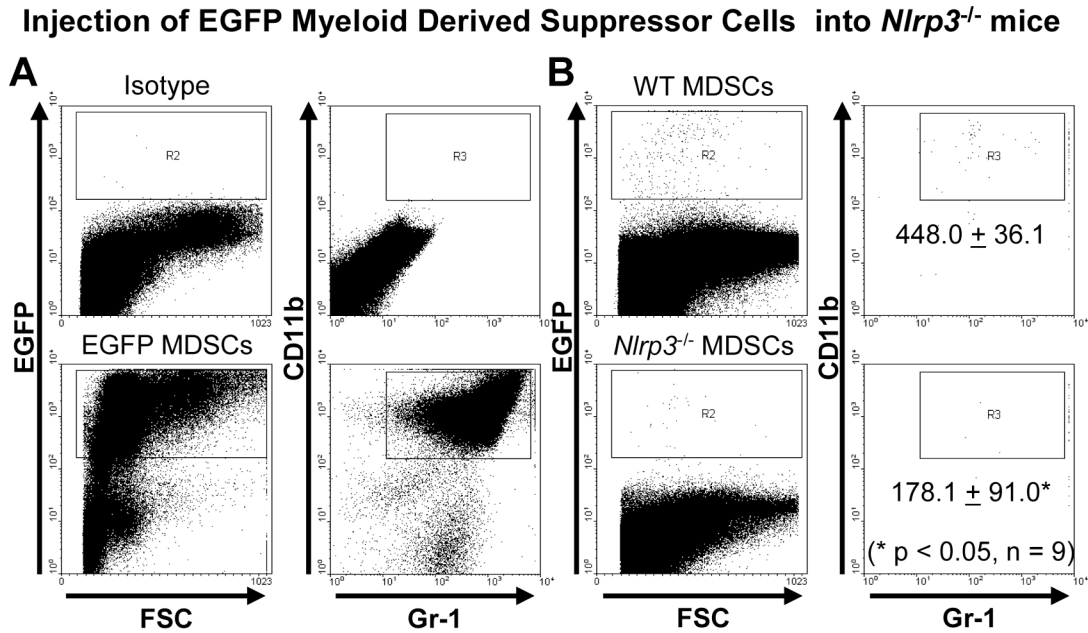


Figure 3-7. Migration of myeloid derived suppressor cells is enhanced by NLRP3.

A. Upper dot plots show gates for detecting EGFP⁺, Gr-1⁺, CD11b⁺ cells using isotype stained, EGFP⁻ control cells. Lower dot plots are EGFP⁺ splenic MDSCs prior to injection into *Nlrp3*^{-/-} mice. *B.* Representative dot plots of cells recovered from B16 tumors in *Nlrp3*^{-/-} mice. Upper panels are WT EGFP⁺ MDSCs and lower panels are *Nlrp3*^{-/-} EGFP⁺ MDSCs. Values represent the average number of cells recovered from each tumor.

Table 3-S1**Accumulation of MDSCs in WT and *Nlrp3*^{-/-} mice with or without DC vaccination**

Treatment	Mouse	n	Tumor Diameter	Tumor-derived MDSCs (% of total cells)			Splenic MDSCs
				All	Monocytic	Granulocytic	All
-	WT	5	6.8 ± 0.6	2.2 ± 1.0	1.9 ± 0.8	0.32 ± 0.21	3.4 ± 0.6
-	<i>Nlrp3</i> ^{-/-}	4	7.1 ± 1.1	1.7 ± 0.5	1.5 ± 0.5	0.11 ± 0.06	3.7 ± 1.1
DC Vaccine	WT	11	5.1 ± 0.3	3.0 ± 0.5	2.1 ± 0.4	0.50 ± 0.14	2.9 ± 0.5
DC Vaccine	<i>Nlrp3</i> ^{-/-}	11	3.9 ± 0.2	0.5 ± 0.3*	0.5 ± 0.2*	0.09 ± 0.04*	1.8 ± 0.4

* p < 0.001 (Kruskal-Wallis with Bonferroni Correction)

Figure 3-S1

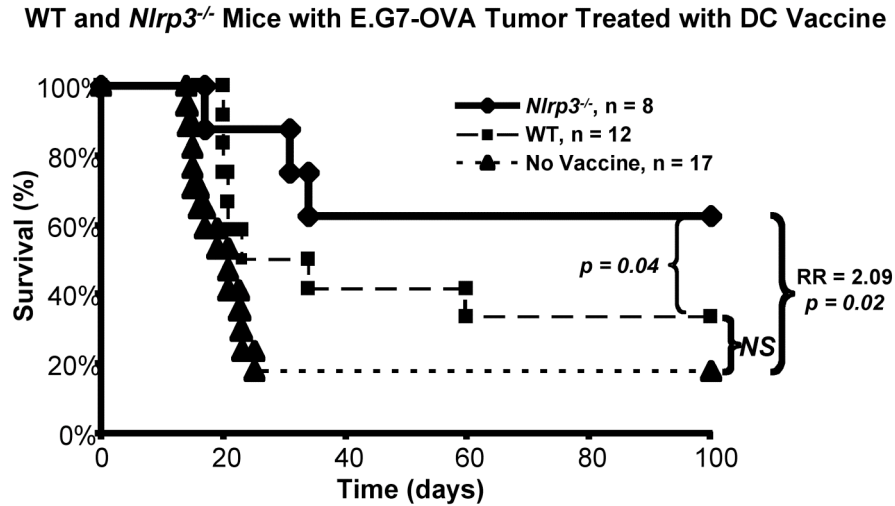


Figure 3-S1. Dendritic cell vaccine improves survival in *Nlrp3*^{-/-} mice but not WT mice using an E.G7-Ova model. Survival curves of WT and *Nlrp3*^{-/-} mice after receiving a subcutaneous injection with 500,000 E.G7-Ova tumor cells. WT (squares) and *Nlrp3*^{-/-} (diamonds) received a 1×10^6 DCs pulsed with Ova peptide days 3 and day 10. The survival of *Nlrp3*^{-/-} mice was significantly improved by this vaccination. Survival of vaccinated WT mice (squares) was not significantly better than unvaccinated WT mice (triangles).

Figure 3-S2

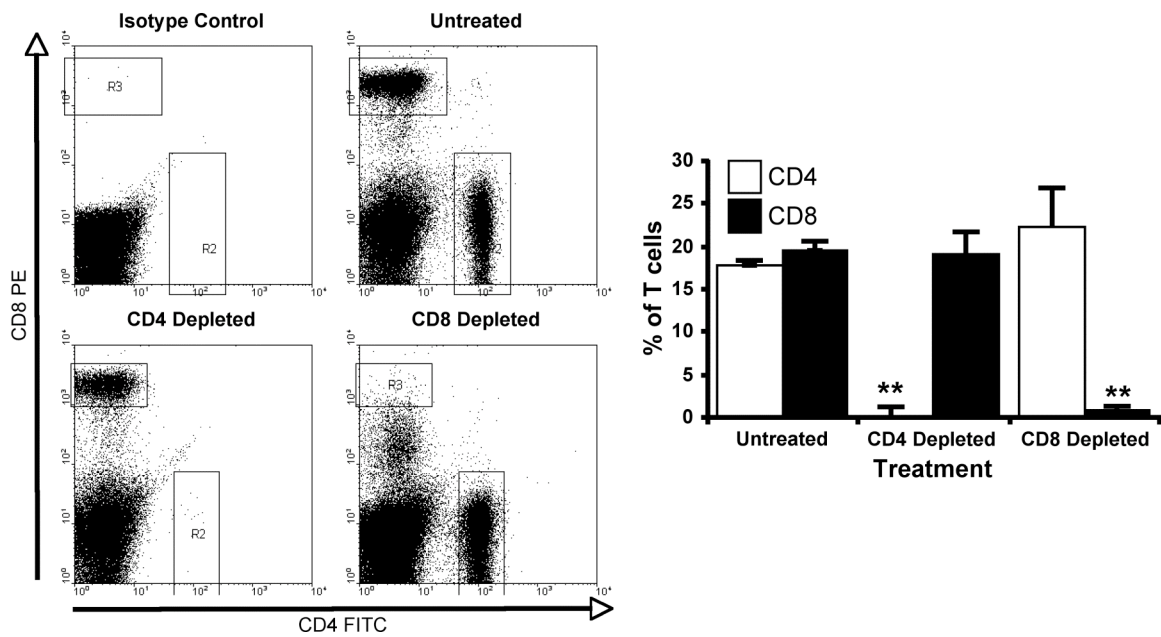


Figure 3-S2. Antibody depletion of CD4⁺ and CD8⁺ cells. Representative dot plots of splenocytes from WT and *Nlpr3*^{-/-} mice that are either untreated or treated with anti-CD4 (lower left) or anti-CD8 (lower right) antibody. Combined data from both strains of mice are given in graphic form on right. All mice received B16-F10 melanoma and DC vaccines. The efficiency of depletion was the same in both strains.

Figure 3-S3

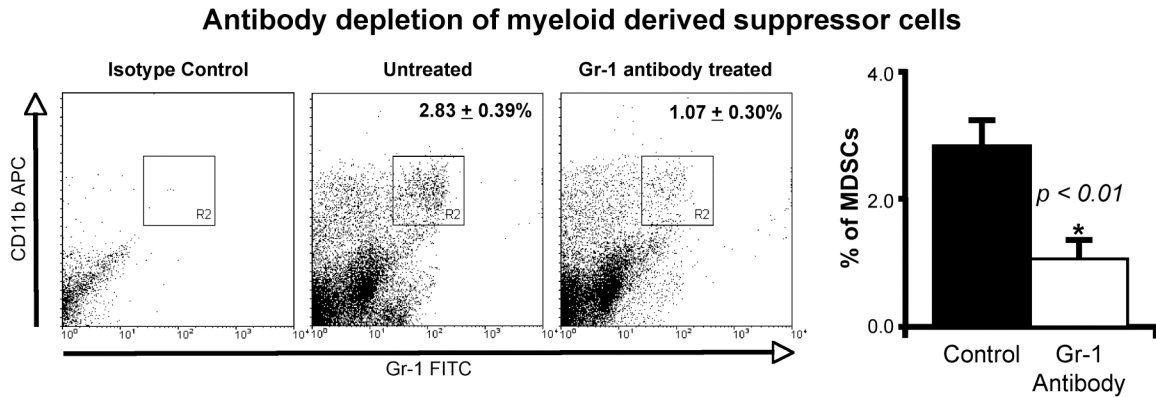


Figure 3-S3. Depletion of MDSCs with Gr-1 antibody. Representative dot plots of splenocytes from WT mice that are either untreated or treated with anti-Gr-1 antibody. All mice received B16-F10 melanoma and DC vaccine on day 3 and 10. Splenocytes were harvested on day 14. Bar graphs of combined data are on right.

CHAPTER FOUR

EVALUATION OF MULTIPLE IMMUNE SUPPRESSIVE MECHANISMS THAT INHIBIT THERAPEUTIC CANCER VACCINES

Abstract

We previously demonstrated that the immunosuppressive tumor environment was the major impediment to successful therapeutic VRP vaccination of neu⁺ tumors in tolerant neu-N mice. Two major components of this suppressive environment were T_{reg} cells and MDSCs. Depleting either of these cells improved efficacy of our VRP vaccine. Here, we extend these studies to evaluate the involvement of other potential mechanisms of suppression in neu-N mice. These included the inhibitory molecules CD200, indolamine 2-3 dioxygenase (IDO), CTLA-4, and PD-1. We found that blocking either CD200 or IDO improved efficacy of neuET-VRP vaccination, whereas blocking CTAL-4 or PD-1 had no effect on tumor growth. Additionally, we report that intratumoral vaccination with VRPs inhibited tumor growth in neu-N mice, likely due to innate immune activation via TLR stimulation. Finally, as we previously identified MDSCs as a major suppressor of anti-tumor immunity in neu-N mice, we examined the effect of TF expression on MDSC function. TF has been shown to promote migration of macrophages. Therefore, we hypothesized that TF suppresses anti-tumor immunity by promoting the migration of MDSCs. Although we found that TF promoted migration of MDSCs *in vitro*, it did not promote migration of MDSCs *in vivo* and the absence of TF had no impact on tumor growth. In summary, these data suggest that multiple mechanisms of suppression are present in the tumor environment. Future studies evaluating the effect of combination therapies targeting multiple mechanisms of immune suppression are warranted in order to determine the optimal treatment regimens for cancer patients.

Introduction

We previously identified the immunosuppressive tumor environment as the major impediment to successful therapeutic vaccination of neu⁺ tumors in neu-transgenic (neu-N) mice (39). Depleting either T_{reg} cells or MDSCs improved the efficacy of therapeutic neuET-VRP vaccination. However, there are multiple other mechanisms that can impact on the immune response after tumor vaccination. Here, we will evaluate whether blocking the activity of CD200, IDO, CTLA-4 or PD-1 impacts on tumor growth after active immunotherapy.

CD200 (OX-2) is a type 1 transmembrane protein with two IgSF domains but no known cytoplasmic signaling motif (19). CD200 is expressed on a variety of different cell types of both hematopoietic and non-hematopoietic origin (339). The receptor for CD200, CD200R, is restricted to myeloid cells (132, 340). CD200R is almost identical to CD200 except for the addition of a cytoplasmic ITIM motif which delivers an inhibitory signal to myeloid cells after binding with CD200 (132). Therefore, CD200 is proposed to be important for the regulation of myeloid cells. Expression of CD200 by tumor cells suppressed T cells *in vitro* (197, 244), and blocking CD200 improved anti-tumor immunity in a humanized mouse model of CLL and in a murine breast cancer model (120, 168).

Indoleamine 2,3-dioxygenase (IDO) catalyzes the breakdown of the essential amino acid tryptophan and is involved in the suppression of T cells through both the depletion of tryptophan and the production of metabolites of the kynurenine pathway (212). IDO is expressed by both pDCs in the tumor draining lymph node and by tumor cells and has been associated with poor prognosis in patients with a number of different

types of cancer (32, 143, 211, 213, 214, 232, 319). In addition, IDO has been proposed to increase the function of T_{reg} cells (14, 277). Furthermore, inhibiting IDO promoted anti-tumor immunity in a number of mouse models (211, 212).

CTLA-4 is an inhibitory receptor expressed on the surface of activated T cells (37, 141, 329). In support of an inhibitory role for CTLA-4, CTLA-4^{-/-} mice die by four weeks of age due to massive proliferation of activated lymphocytes (47). More recently, CTLA-4 was shown to be constitutively expressed on T_{reg} cells and to be vital for their function (337, 355). In addition, lack of CTLA-4 expression on T_{reg} cells alleviated suppression of anti-tumor immunity (337). A number of clinical trials have evaluated the efficacy of CTLA-4 blockade for the treatment of cancer (73). Although results to date have been mixed, CTLA-4 does appear to play a role in inhibiting anti-tumor immunity (73). Future work is needed to determine the mechanisms responsible for CTLA-4 dependent suppression of anti-tumor immunity and for which specific types of cancer CTLA-4 targeted therapy will be most effective. .

A more recently described inhibitory receptor that also appears to be involved in inhibiting anti-tumor immunity is programmed cell death-1 (PD-1; CD279) (145, 158). Like CTLA-4, PD-1 is expressed on T cells upon activation (2). Also like CTLA-4, PD-1^{-/-} mice suffer from autoimmune disease (228, 229). Two ligands have been identified for PD-1, PD-L1 (B7-H1) and PD-L2 (B7-DC) (67, 99). PD-L1 is expressed on a wide range of cells, including those of both hematopoietic and non-hematopoietic origin, while PD-L2 expression is limited to activated DC, macrophages, and bone marrow-derived mast cells and resting B1 cells (158). PD-L1 can also be upregulated on some cells, including macrophages, DCs, and endothelial cells in response to both type I and type II

interferons (80, 158, 272). PD-L1 is also expressed by many different types of tumor cells and has been associated with decreased T cell immunity and poor prognosis (66, 127, 313). Murine studies have demonstrated that PD-L1 expression by tumor cells and PD-1 expression by T cells inhibit anti-tumor immunity (25, 146, 301). Mechanistically, expression of PD-L1 by tumor cells renders them resistant to T cell-mediated killing (130). We are particularly interested in the role of PD-1 in our VRP vaccines as VRPs induce high levels of type I IFNs (209). Induction of PD-L1 by IFNs might be a main mechanism of tumor-induced suppression of therapeutic VRP vaccines.

In chapter two we identified MDSCs as the major suppressive cells that inhibited VRP vaccine efficacy in neu-N mice. Depletion of MDSCs resulted in regression of 40% of neu-N mice when combined with VRP vaccination and adoptive transfer of neu-specific lymphocytes (39). Therefore, we were interested in identifying genes important for the function of MDSCs. One such gene that was identified by microarray analysis of MDSCs from tumor-bearing neu-N mice was tissue factor (TF) (Burgents, Serody, unpublished). TF (coagulation factor III) plays a key role in the initiation of coagulation upon binding to factor VII (43, 189). TF is mainly expressed by cells shielded from contact with blood, e.g. vascular adventitia, organ capsules, epidermis, and mucosal epithelium, thereby being distributed in a way to activate coagulation when vascular integrity is compromised (71). More recently, TF expression has been reported on activated myeloid cells in response to inflammatory cytokines (31). TF is also expressed on tumor cells and has been associated with increased growth and metastasis (184, 263, 269). This is believed to be because TF promotes the migration of both tumor cells and myeloid cells (68, 148, 249). As we found TF expressed by MDSCs in the tumor

environment, we were interested in evaluating whether TF promoted MDSC-mediated suppression of anti-tumor immunity by enhancing the migration of MDSCs to the tumor.

Thus, here we have taken a systematic approach to evaluating the role of multiple inhibitory pathways in the activity of DC or VRP vaccination in several preclinical models.

Materials and Methods

Mice, cell lines, and reagents

FVB/N and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MMTV-neu (neu-N) mice were purchased from Charles River Laboratory (Wilmington, MA). Female mice (7-14 weeks) were used for all experiments. All experiments were conducted in accordance with protocols approved by the University of North Carolina Institutional Animal Care and Use Committee. B16-F10 and HUVEC cells were purchased from ATCC (Rockland, MD). Rat neu-expressing NT2 cells have been described (251). Anti-CD200 Ab was provided by Trillium Therapeutics (Toronto, ON). PD-1 (J43), CTLA-4 (UC10-4F10-11), CD4 (GK1.5), CD8 (53.6.72), and isotype control Abs for *in vivo* experiments were purchased from Bio X Cell (West Lebanon, NH). 1MT was purchased from Sigma-Aldrich (St. Louis, MO).

Flow cytometric analysis

All monoclonal antibodies (mAbs) used for flow cytometry were purchased from eBioscience (San Diego, CA). Cells were stained according to the manufacturers' instructions. Acquisition was done using BD FACS Calibur (BD Biosciences, San Jose, CA) or MACSQuant Analyzer (Bergisch Gladbach, Germany). Resultant data were analyzed using FlowJo Flow Cytometry Analysis Software (Tree Star Inc., Ashland, OR).

VRP & DC vaccines

VRPs encoding the extracellular-transmembrane domain (AA 1-697) of rat neu (neuET-VRP), the melanoma antigen gp100 (gp100-VRP) or VRPs lacking an inserted

transgene (null-VRP) have been described (208, 311). Tolerant neu-N mice were challenged with 5×10^4 freshly prepared NT2 cells s.c. in the right mammary fat pad. FVB/N mice were challenged with 2×10^6 NT2 cells. C57BL/6 mice were challenged with 1×10^4 B16-F10 cells or 1×10^6 E.G7-OVA cells s.c. in the right rear leg flank. For therapeutic VRP vaccination, tumors were allowed to grow 4-7 days before vaccination with $2 \times 10^5 - 1 \times 10^6$ VRPs injected in the footpad. For intratumoral VRP vaccines, 1×10^5 null-VRP or neuET-VRP were injected into tumors greater than 10 mm^2 . For DC vaccines, DCs were isolated from bone marrow cultures treated with GM-CSF and IL-4 (Peprotech Inc, Rocky Hill, NJ) (208). The DCs were pulsed with either B16-F10 lysates or OVA class I peptide (SIINFEKL) and matured with LPS. 1×10^6 cells were injected s.c. adjacent to the tumor site.

Adoptive cell transfer experiments

For adoptive cell transfer experiments into lethally irradiated recipients, splenocytes were isolated from naïve FVB/N or neu-N mice. 1×10^7 splenocytes, along with $3-5 \times 10^6$ T cell depleted syngeneic BM cells, were transferred i.v. to neu-N or FVB/N mice 18-24 h after irradiation at 850 rads (135 rads/min). NT2 tumor cells were injected s.c. on the same day as splenocyte and BM cell transfer.

Treatment with IDO inhibitor 1MT

The 1MT solution was prepared as described (136). Briefly, 1 g of 1MT (Sigma) was added to a 15 mL conical tube with 7.8mL Methocel/Tween solution (0.5% Tween 80/0.5% Methylcellulose in water). This mixture was bead milled overnight by adding 1-

2 mL of 3 mm glass beads and mixing by inversion. 4 mL Methocel/Tween solution was added the next day to bring the concentration to approximately 85 mg/mL. 100µL of this solution (8.5 mg) was administered to mice twice per day by oral gavage for the five consecutive days following each VRP vaccine.

TFshRNA and CD200shRNA NT2 cell lines

Mission lentiviral transduction particles (Sigma, St. Louis, MO) containing shRNA specific for CD200 (CCGGGCCCATAGTACACCTTCACTACTCGAGTAGTGAAGGTGTACTATGGGC TTTTTG) and TF (CCGGGCACAAATGCTTTAGATTGTACTCGAGTACAATCTAAAGCATTGTGCTTTTTG) or non-target control transduction particles (product #SHC002V) were used to stably transduce NT2 tumor cells. NT2 cells were thawed into T75 flasks in NT2 media (RPMI 1640 supplemented with 20% FBS, 2mM L-glutamine, 12mM HEPES, 0.1mM NEAA, 1mM Na Pyruvate, 1% Pen/Strep, 50µM 2-ME, 0.2U/mL Novolin R-Insulin). The next day, cells were plated in a 96 well plate at 1.6×10^4 cells per well in 120 µL and incubated overnight. 4µg/mL Polybrene was added to each well followed by addition of lentiviral particles at an MOI of 5. Lentiviral particles were removed the next day and fresh media was added. On the following day, media was replaced with NT2 media containing 2µg/mL puromycin to select for transduced cells. Cells were expanded for 5-7 days and tested for expression of either CD200 or TF. Limited dilution cloning was used to generate cell lines. Cell lines were maintained with puromycin at a concentration of 0.5 µg/mL.

In vitro trans endothelial migration (TEM) assay

On day -2, 5×10^4 HUVEC cells were plated onto matrigel coated filters of 24-well transwell migration plates. The following day the cells were activated for 24 hours with addition of 1 ng/mL IL-1 β . 5×10^5 MDSCs from the spleens of either WT or TF^{fllox}LysM^{cre} tumor-bearing mice were added to the upper chamber. MDSCs were isolated by magnetic separation using miltenyi beads for Gr-1⁺ cells. Cells were greater than 95% Gr-1⁺CD11b⁺. 100 ng/mL rTF was added to wells as indicated. The number of cells in the lower chamber was counted after 24 hours.

In vivo migration of MDSCs

On day -1 C57BL/6 mice were lethally irradiated at 950 rads (135 rads/min). The next day BM was isolated from eGFP-B6, TF^{fllox}LysM^{cre}, and B6 mice. 5×10^6 total BM cells were injected i.v. to irradiated recipients. The experimental group received a 1:1 ratio of eGFP-B6 BM to TF^{fllox}LysM^{cre} BM, while the control group received a 1:1 ratio of eGFP-B6 to B6 BM. 8 weeks later, mice were challenged with 2×10^6 E.G7-OVA tumor cells. On day 14 post-tumor challenge tumors were harvested and digested with collagenase A. The percent GFP⁺ MDSCs was determined by FACS.

Statistical analysis

Statistical differences between groups was determined by two-tailed Student's t test or ANOVA analysis. Significant differences in survival were determined by Kaplan-Meier survival analysis. All statistical analyses were conducted using SigmaStat[®] 3.5 software, with a p value ≤ 0.05 considered significant.

Results

Blocking CD200 improves efficacy of neuET-VRP vaccine

We previously demonstrated that the immunosuppressive tumor environment is the major impediment to successful therapeutic VRP vaccination of neu-N mice (39). In the current report, we evaluated a number of potential mechanisms of immune suppression that might be involved in inhibiting vaccination in neu-N mice. One such mechanism that has been proposed to inhibit tumor immunity is the inhibitory molecule CD200 (120, 210). We found that CD200 was expressed by our neu⁺ NT2 tumor cells (data not shown). Therefore, we sought to determine whether inhibition of CD200 with an anti-CD200 blocking Ab would improve the efficacy of therapeutic neuET-VRP vaccination. Treatment with anti-CD200 Ab inhibited tumor growth following vaccination with neuET-VRP suggesting that CD200 is involved in promoting tumor growth of neu-expressing tumors (Fig. 4-1A).

In our previous study we found that neu-N mice lacked a sufficient repertoire of neu-specific lymphocytes to induce regression of neu-expressing tumors. Therefore, we hypothesized that adoptive transfer of neu-specific lymphocytes from FVB/N mice along with treatment with CD200 blocking Ab would result in a further decrease in tumor growth compared to CD200 Ab treatment without adoptive cell transfer. While treatment with CD200 blocking Ab improved efficacy of neuET-VRP vaccination, we did not observe an increased effect of CD200 Ab treatment when combined with transfer of FVB splenocytes (Fig. 4-1B). These data indicate that although CD200 inhibits tumor growth it is not the main or dominant mechanisms of suppression of tumor-specific lymphocytes.

We next sought to further investigate the mechanism of increased vaccine efficacy with CD200 Ab treatment. We depleted mice of either CD4⁺ cells or CD8⁺ cells and measured tumor growth following CD200 Ab treatment and neuET-VRP vaccination. When CD200 Ab treatment was combined with depletion of CD4⁺ cells, there was no longer a significant difference between groups based on CD200 Ab treatment, consistent with CD4⁺ cells being involved in the improved efficacy of vaccination following CD200 Ab treatment (Fig. 4-2). On the other hand, the effect of CD200 Ab treatment was not dependent on CD8⁺ cells, as these mice still had decreased tumor growth following CD200 Ab treatment (Fig. 4-2).

One of the main sources of CD200 in the tumor environment is production by tumor cells. In fact, we have shown that NT2 tumor cells express high levels of CD200 (data not shown). Therefore, we wanted to determine whether CD200 expressed by the tumor cells was responsible for the observed CD200-dependent inhibition of tumor growth. To do this, we transduced NT2 cells with lentivirus expressing shRNA for either CD200 or a control shRNA that is not specific for any known mouse gene. NT2 cells were stably transduced to express each shRNA. Transduction with CD200 shRNA resulted in a greater than 95% knockdown of C200 compared to NT2 cells transduced with the control shRNA (data not shown). We challenged neu-N mice with either CD200 shRNA or control shRNA NT2 cells followed by vaccination with neuET-VRPs. There was a trend, that was not statistically significant, for smaller tumors in mice given NT2-CD200 ShRNA tumors compared to those given the control tumors ($p = 0.08$, Fig. 4-3A). To ensure that CD200 remained knocked down *in vivo* in the population of CD200 shRNA NT2 cells, we harvested the tumors on day 46 and measured CD200 surface

levels by FACS. We found that CD200 remained knocked down *in vivo* in CD200 shRNA tumors (Fig. 4-3B).

These data are consistent with CD200 being involved in inhibiting anti-neu immunity following therapeutic VRP vaccine. The effect of CD200 is clearly not dependent on CD8⁺ lymphocytes but possibly dependent of CD4⁺ T cells. More work will be needed to address whether the effect of CD200 is dependent on CD4⁺ T cells and whether CD200 expressed by the tumor cells promotes tumor growth.

Inhibiting IDO improves efficacy of neuET-VRP vaccine

Another mechanism of suppression in the tumor environment that has been shown to inhibit T cells as well as increase the activity of T_{reg} cells, is the production of IDO (212). DCs in the TDLN are a main producer of IDO in the tumor environment. We found increased IDO production at the TDLN compared to the spleen (Fig. 4-4A). In order to examine the role of IDO in the induction of tolerance in neu-N mice we treated mice with the IDO inhibitor 1 methyl tryptophan (1MT). 1MT was administered by oral gavage twice a day for the first 5 days after each vaccination. Mice treated with 1MT along with neuET-VRP had a significant delay in tumor growth which resulted in a modest improvement in median survival compared to control treated mice (Fig. 4-4B). These data suggest that production of IDO does inhibit anti-tumor immunity in neu-N mice.

Treatment with blocking antibodies to the inhibitory receptors CTLA-4 or PD-1 does not improve neuET-VRP vaccine efficacy

CTLA-4 and PD-1 are inhibitory receptors expressed by T cells that have been shown to be involved in suppression of anti-tumor immunity (158). We were interested in whether either of these inhibitory receptors were involved in the suppression of anti-neu immunity in neu-N mice. For these experiments, tumor-bearing neu-N mice were treated with blocking Abs specific for either CTLA-4 or PD-1. Treatment with either blocking Ab did not inhibit tumor growth in neu-N mice (Fig. 4-5A). We went on to evaluate the effect of these blocking Ab in our transplant model with transfer of FVB/N splenocytes and again did not see a difference in tumor growth (Fig. 4-5B). Therefore, it appears as though neither of these inhibitory receptors plays a major role in suppression of anti-tumor immunity in our VRP model.

Intratumoral VRP vaccination improves efficacy of neuET-VRP vaccine

One approach that investigators have used to overcome the local immune suppression in the tumor is the activation of TLR ligands (278, 344). VRPs can potentially activate innate immune cells through TLR3 due to the production of dsRNA intermediates upon infection (4, 250, 300). Since we previously observed a specific suppression of T cells in the TDLN (39), we examined the effect of intratumoral VRP vaccination on tumor growth in neu-N mice. Mice were challenged with 5×10^4 NT2 cells and either vaccinated with neuET-VRPs in the footpad every 2 weeks or left untreated. Mice were also injected twice a week with 1×10^5 neuET-VRPs, 1×10^5 null-VRP, or saline into the tumor. We found that intratumoral injection of VRPs inhibited

tumor growth (Fig. 4-6). Interestingly, the inhibition of tumor growth by intratumoral VRP vaccination was not dependent on the expression of neu by VRPs and did not require footpad vaccination. Thus, activation of the innate immune response, perhaps via TLR3 stimulation, was sufficient to diminish tumor growth.

Tissue factor expression by MDSCs promotes migration in vitro but does not inhibit tumor growth

As we previously found that MDSCs were a dominant suppressor of anti-tumor immunity in neu-N mice, we were interesting in evaluating genes that might be important for their function in the tumor environment (39). One such gene that we found to be expressed by MDSCs in tumor-bearing mice was tissue factor (TF) (data not shown). TF is mainly known for its role in the initiation of coagulation upon interaction with factor VII, but it has also been shown to play a role in the progression of tumor growth as well as metastasis by promoting migration (189, 269). TF also promoted migration of macrophages (249). Therefore, we evaluated whether TF promoted the migration of MDSCs.

In order to specifically address the role of TF on MDSCs we used $TF^{fllox}LysM^{cre}$ mice in which TF is selectively knocked out on myeloid cells based on expression of LysM. As $TF^{fllox}LysM^{cre}$ mice are on the C57BL/6 background we challenged these mice with B16F10 melanoma cells. MDSCs isolated from tumor-bearing $TF^{fllox}LysM^{cre}$ mice had decreased migration to TF *in vitro* compared to MDSCs from WT mice (Fig. 4-7A). Addition of rTF enhanced migration of MDSCs (Fig 4-7A). These data suggest that TF promotes migration of MDSCs. We also addressed whether TF expression by MDSCs

promoted their accumulation in the tumor. For these studies, we transferred 1:1 ratios of either TF^{fllox}LysM^{cre} BM to eGFP BM or WT BM to eGFP BM into lethally irradiated C57BL/6 recipients. Eight weeks later, mice were challenge with EG.7 tumor cells and the percentage GFP⁺ MDSCs in the tumor was determined by FACS. We found equal percentages of GFP⁺ and GFP⁻ MDSCs in both groups suggesting that TF^{fllox}LysM^{cre} MDSCs were able to effectively migrate and accumulate in the tumors compared to WT MDSCs (Fig. 4-7B). We also injected TF^{fllox}LysM^{cre} mice or WT mice with B16-F10 melanoma with or without gp100-VRP vaccine and did not observe any difference in the number of MDSCs in the tumor (data not shown).

Although we were not able to demonstrate a role for TF in the migration of MDSC *in vivo*, it was still possible that TF expression by MDSCs promoted tumor growth (269). Therefore, we challenged TF^{fllox}LysM^{cre} or WT mice with B16-F10 followed by vaccination with gp100-VRP. There was no difference in survival between TF^{fllox}LysM^{cre} and WT mice, suggesting that TF expression by MDSCs did not promote tumor growth or inhibit vaccine efficacy (Fig. 4-8).

MDSCs are not the main source of TF in the tumor environment as tumor cells also express TF. Therefore, we sought to investigate whether expression of TF by tumor cells promoted tumor growth or the migration of MDSCs. To do this, we knocked down TF expression in our neu-expressing NT2 cells by stably transducing these cells with lentiviral vectors expressing TF shRNA. Transduction with TF shRNA resulted in a 95% knockdown of TF expression. Mice were challenged with TF shRNA NT2 cells or control shRNA NT2 cells followed by neuET-VRP vaccination. There was no difference in tumor growth based on TF expression by the tumor cells (Fig. 4-9A). Also TF

expression by the tumor cells did not promote migration of MDSCs to the tumor (Fig. 4-9B). Actually, there were significantly more MDSCs in the tumors of mice challenged with TF shRNA NT2 cells, but this was likely because of slightly larger tumors in these mice (Fig. 4-9). Nevertheless, TF does not appear to play a role in promoting tumor growth or in inhibiting vaccine efficacy in our tumor vaccine models.

Discussion

We previously identified the tumor suppressive environment as the major impediment to successful VRP vaccination (39). We identified T_{reg} cells and MDSCs as having a dominant role in the suppression of VRP vaccines (39). In the current report, we extended these studies to the investigation of other possible mechanisms of tumor-induced suppression that might play a role in inhibiting VRP vaccines. We found that CD200 and IDO both promoted growth of neu-expressing tumor in VRP vaccinated neu-N mice. We also found that intratumoral vaccination with VRPs was able to inhibit tumor growth regardless of antigen expression by the VRPs. Surprisingly, CTLA-4 and PD-1 did not appear to play a role in inhibiting anti-tumor immunity in our VRP model. Finally, although TF appeared to enhance migration of MDSCs *in vitro* it was not a major factor in their migration to the tumor *in vivo* and blocking the expression of TF by either MDSCs or tumor cells did not diminish tumor growth.

We found that treatment with CD200 blocking Ab inhibited tumor growth in neu-N mice vaccinated with neuET-VRPs. The effect of CD200 Ab treatment was not dependent on CD8⁺ cells but appeared to be dependent on CD4⁺ cells. However, confirmation of this finding will require additional groups being treated with anti-CD-200 mAb and the inclusion of a group of mice that undergoes depletion of CD4⁺ T cells without anti-CD200 treatment. Future work will focus on confirming our initial findings and evaluating mechanisms by which the blockade of CD200 promotes the efficacy of vaccine therapy.

Our data with CD200 Ab treatment are consistent with other reports demonstrating a role for CD200 in suppressing anti-tumor immunity (120, 168, 210, 244,

288). The first attempt to use CD200 Ab as a treatment to enhance anti-tumor immunity was performed in a humanized B-CLL model in which treatment with CD200 Ab resulted in an almost complete inhibition of tumor growth by adoptively transferred human PBMCs (168). At the onset of our study, the efficacy of CD200 Ab treatment had not been evaluated in a solid tumor model or in mice in which tumors arise spontaneously (i.e. tolerant animals). Recently, Gorczynski et al. reported decreased tumor growth of transplanted breast cancer cells in mice treated with anti-CD200 Ab (120).

We also demonstrated that IDO is involved in promoting tumor growth in neu-N mice. Inhibiting IDO improved survival of mice vaccinated with neuET-VRPs. Others have also inhibited tumor growth by treatment with 1MT (136). Further study is needed to address the exact mechanism of IDO-dependent suppression of anti-tumor immunity. Both pDCs and tumor cells express IDO and the role of these cells in the production of IDO in the growth of MMTV-Neu tumors cells needs to be evaluated. As we have already demonstrated that T_{reg} cells play a major role in suppressing anti-neu immunity, it will be important to determine if the T_{reg} -dependent suppression that we observe is dependent on IDO production by DCs induced by T_{reg} cells. In addition, IDO has also been proposed to increase the function of T_{reg} cells (14, 277), and future work should determine if blocking IDO decreases the number or function of T_{reg} cells in the tumor environment.

The effects that we see with CD200 Ab treatment and IDO may be related. pDCs, as well as myeloid cells (e.g. MDSC, personal observation) express CD200R and CD200 induces expression of IDO on these cells (82). Therefore, it is possible that the similar effects that we observe with CD200 Ab treatment and IDO inhibition involve the same

mechanism. This conclusion is even more compelling given our CTLA-4 Ab results in which blocking CTLA-4 did not have an effect on tumor growth. CTLA-4 expression on T_{reg} cells is thought to be a main inducer of IDO (355). As we did not observe an effect of CTLA-4 treatment it is possible that a main source of IDO in our tumor model is through CD200 and not CTLA-4 induction of pDCs.

A number of studies as well as many current clinical trials address the role of the inhibitory receptors CTLA-4 and PD-1 on inhibiting anti-tumor immunity (73).

Therefore, we were surprised to find that blocking these receptors did not improve efficacy of our neuET-VRP vaccines. Our data demonstrate that CTLA-4 and PD-1 are not the dominant mechanisms of suppression of anti-neu immunity in neu-N mice.

Although we demonstrated previously that T_{reg} cells are involved in suppression in neu-N mice, T_{reg} cells can suppress independent of CTLA-4 (157, 355). This appears to be the case in neu-N mice.

Treatment of tumor-bearing neu-N mice with PD-1 blocking Ab also did not inhibit tumor growth. PD-L1 expression by tumor cells is thought to render these cells resistant to T cell-mediated killing (130). This might be the case in our model, but since this mode of immune escape is a last line of defense, other mechanisms of suppression are likely dominant, e.g. MDSCs, T_{reg} cells, and IDO. It is possible that combination therapy with PD-1 blocking Ab and MDSCs depletion would result in a synergistic effect due to the alleviation of suppressive mechanisms upstream of PD-1. The same might be the case with combination therapies to deplete T_{reg} cells or inhibit IDO. The presence of multiple mechanisms of tumor-induced immune suppression needs to be taken into

account when designing clinical trials aimed at inhibiting the suppressive peritumoral environment.

We also demonstrated that intratumoral injection of VRPs inhibited tumor growth in neu-N mice. The effect of intratumoral VRP injection could be the result of signaling through TLR3 by dsRNA produced upon VRP infection. Intratumor injection of the TLR agonists induced anti-tumor immunity and decreased tumor growth in colon cancer, melanoma, and breast cancer mouse models (278, 344). Upon infection, VRPs produce dsRNA intermediates that can potentially activate innate immune cells through TLR3 (4, 250, 300). Inhibition of tumor growth by intratumoral VRP vaccination was not dependent on the expression of neu by VRPs and did not require footpad vaccination. It is possible that activation of the innate immunity was sufficient to diminish tumor growth. Alternatively, it is possible that the effect of intratumoral VRP vaccination induced adaptive immunity specific to neu and that neu expression by VRPs was not needed because neu is already expressed at significantly by tumor cells and cross-presented by infected DCs.

As we previously found that MDSCs were a dominant suppressor of anti-tumor immunity in neu-N mice, we were interesting in evaluating genes that might be important for their function in the tumor environment (39). We reported here that TF is expressed by MDSCs and promotes TEM *in vitro*. We were unable to demonstrate a role for TF in the migration of MDSCs *in vivo* on in the promotion of tumor growth. TF is expressed by both myeloid cells as well as tumor cells, with tumor cells being the main source in the tumor environment. We also knocked down TF in our tumor lines to evaluate the role of TF expression by tumor cells and still did not observe any difference in tumor growth

or migration of MDSCs. Therefore, although TF is expressed by MDSCs it does not appear to be vital for their function in the tumor environment. Others have reported TF dependent promotion of tumor growth and metastasis (184, 263, 269). We were unable to knock down total TF levels at the tumor site by more than 50% (data not shown). It is possible that further knockdown of TF would result in significant inhibition of tumor growth.

In summary, we report here the evaluation of a number of suppressive mechanisms that inhibit VRP vaccination in neu-N mice. The identification of multiple suppressive mechanisms induced by the tumor further illustrates the great challenge of designing successful therapeutic cancer vaccine regimens. Successful therapy will likely need to address multiple mechanisms of suppression, as there are likely many layers of suppression utilized by the tumor to suppress anti-tumor immunity.

Figure 4-1

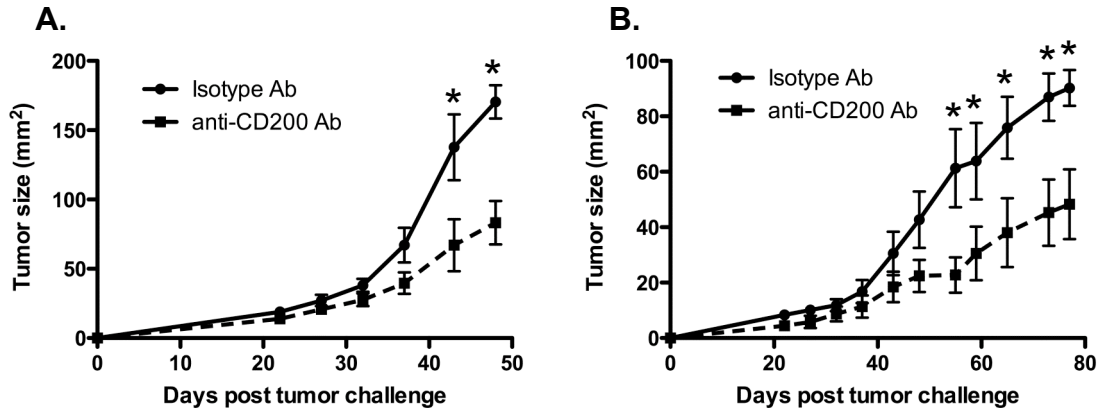


Figure 4-1. Blocking CD200 improves efficacy of neuET-VRP vaccine in neu-transgenic mice. *A*, neu-N mice were treated with anti-CD200 Ab (200 μ g) i.p. twice per week starting three days post-tumor challenge with 5×10^4 NT2 cells. On day four post tumor challenge, mice were vaccinated with neuET-VRPs and similarly boosted on days 18 and 32. *B*, 1×10^7 splenocytes from FVB/N mice were injected i.v. along with 5×10^6 syngeneic T cell-depleted BM cells into lethally irradiated neu-N mice, followed by tumor challenge with 5×10^4 NT2 cells. Mice were treated with 200 μ g anti-CD200 Ab twice per week starting on day 3 post tumor challenge. Mice were vaccinated with neuET-VRPs on day four post-tumor challenge and vaccine was repeated every 14 days. *Points*, mean (n = 5); *Bars*, SEM; *, p < 0.05.

Figure 4-2

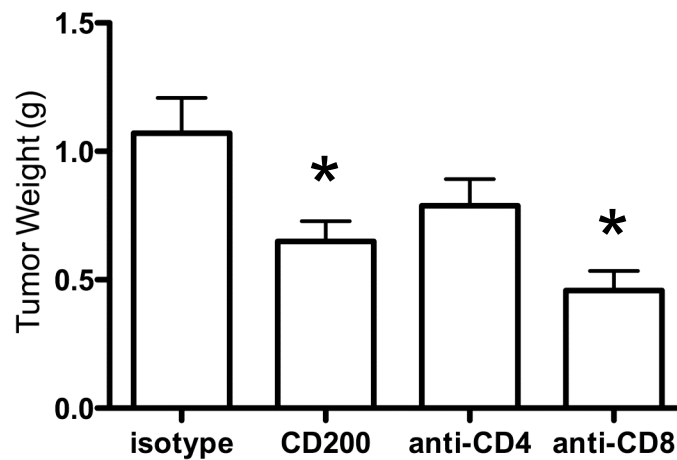


Figure 4-2. Decreased tumor growth following CD200 Ab treatment is not dependent on CD8⁺ T cells, but possibly dependent on CD4⁺ T cells. neu-N mice were treated with anti-CD200 Ab (200 μ g) i.p. twice per week starting three days post-tumor challenge with 5×10^4 NT2 cells. On day four post tumor challenge, mice were vaccinated with neuET-VRPs and similarly boosted on days 18 and 32. Mice were also treated i.p. with 500 μ g of either isotype, anti-CD4, or anti-CD8 Ab twice per week as indicated. Tumor weight was determined on day 50 post tumor challenge. *Columns*, mean (n = 4); *Bars*, SEM; *, p < 0.05 compared to isotype.

Figure 4-3

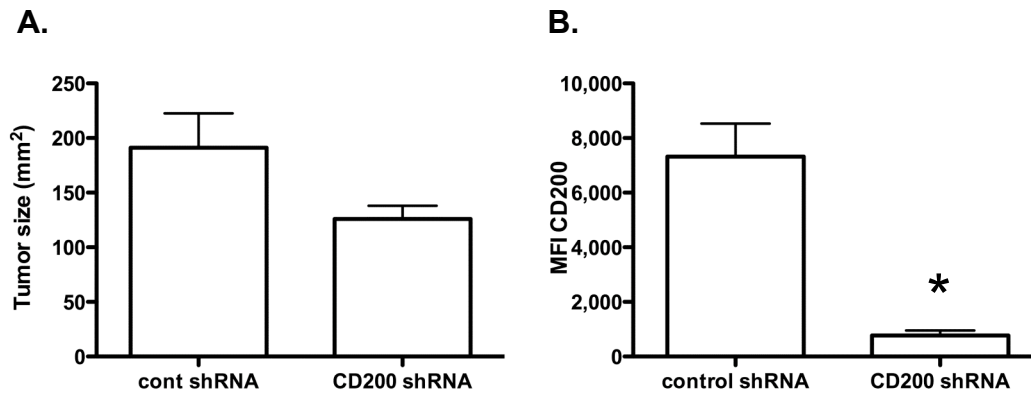


Figure 4-3. CD200 expression by tumors cells does not promote tumor growth following neuET-VRP vaccine. Neu-N mice were challenged with 5×10^4 cont shRNA or CD200 shRNA NT2 cells. On day four and every two weeks thereafter mice were vaccinated with neuET-VRPs. *A.* Tumor size at day 46 post tumor challenge. *Columns*, mean (n = 4); *Bars*, SEM; p = 0.08. *B.* Expression of CD200 on CD45⁺ tumor cells isolated from mice on day 46 post tumor challenge. *Columns*, mean (n = 4); *Bars*, SEM; *, p = 0.001.

Figure 4-4

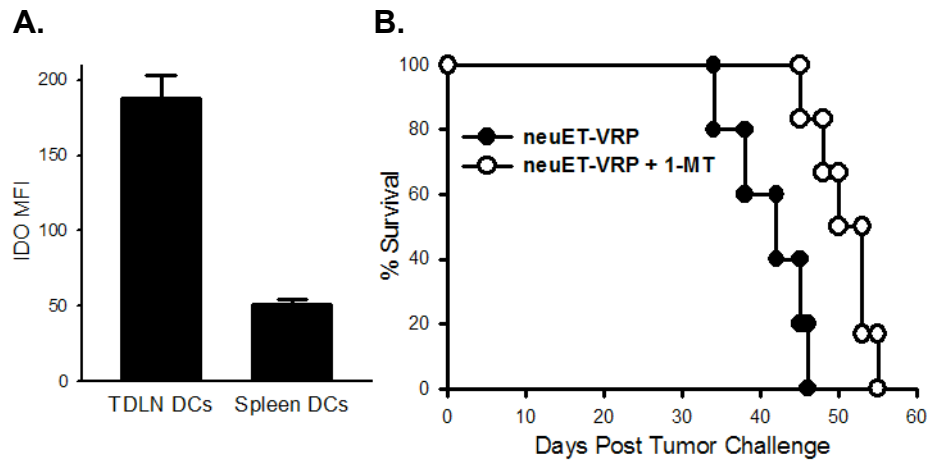


Figure 4-4. Inhibition of IDO with 1MT improves efficacy of neuET-VRP vaccine in neu-N mice. *A.* MFI of IDO from CD11c⁺ cells isolated from either the TDLN or spleen of tumor bearing neu-N mice. *Columns*, mean (n = 5); *Bars*, SEM; p = 0.008. *B.* neu-N mice were challenged with 5 x 10⁴ NT2 cells followed by neuET-VRP vaccine on day 4, 18, and 32. Mice were treated with the IDO inhibitor 1 methyl tryptophan (1 MT) or saline by oral gavage twice a day for the first 5 days after each vaccination. There was a significant improvement in survival in mice receiving 1MT (p < 0.05, Kaplan Meier survival analysis).

Figure 4-5

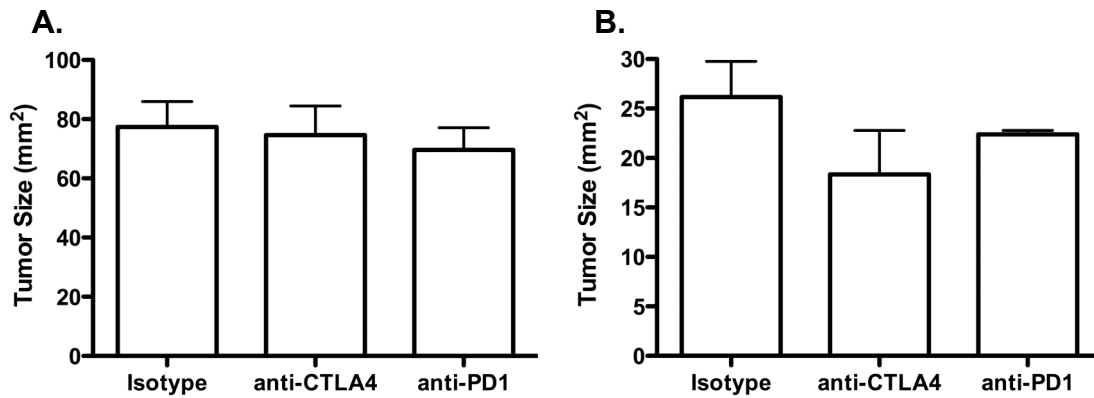


Figure 4-5. Treatment with CTLA-4 or PD-1 blocking Ab does not improve efficacy of neuET-VRP vaccine in neu-N mice. *A.* neu-N mice were challenged with 5×10^4 NT2 tumor cells followed by vaccination with neuET-VRP on day 4 and 18. Mice were injected i.p. with 200 μ g hamster IgG isotype Ab, anti-CTLA-4 Ab (UC10-4F10-11), or anti-PD-1 Ab (J43) twice per week. Mean tumor size \pm SEM on day 35 is reported ($p = 0.86$, One-way ANOVA). *B.* 7.5×10^6 naïve FVB/N splenocytes were injected i.v. along with 2.5×10^6 T cell-depleted BM cells into lethally irradiated neu-N mice followed by tumor challenge with 5×10^4 NT2 cells. Mice were vaccinated with neuET-VRPs on day 4 and 18 post-tumor challenge. Mice were injected i.p. with 200 μ g hamster IgG isotype Ab, anti-CTLA-4 Ab (UC10-4F10-11), or anti-PD-1 Ab (J43) twice per week. Mean tumor size \pm SEM on day 35 is reported ($p = 0.55$, One-way ANOVA).

Figure 4-6

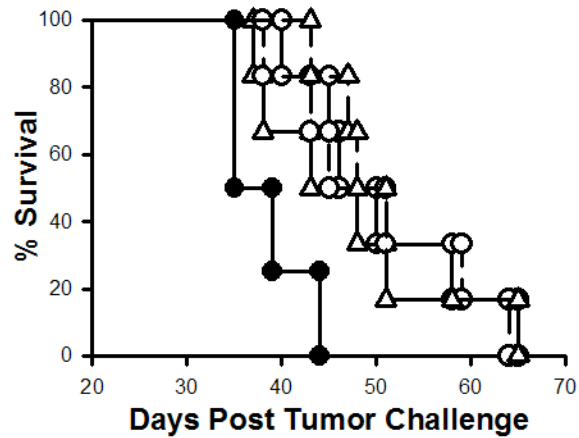


Figure 4-6. Intratumoral injections of VRP inhibits tumor growth in neu-N mice.

Neu-N mice (n = 28) were challenged with 5×10^4 NT2 tumor cells. On day 4 post tumor challenge, mice were either vaccinated with 1×10^6 neuET-VRP (dashed lines) or left untreated (solid lines). Mice received intratumor injections every 3-4 days with either 1×10^5 null-VRP (open circles) or 1×10^5 neuET-VRP (open triangles). Control mice received intratumor injections with PBS (filled circles). There was a significant difference in survival upon intratumor injection with either null-VRP or neuET-VRP compared to PBS ($p = 0.010$, Kaplan Meier survival analysis).

Figure 4-7

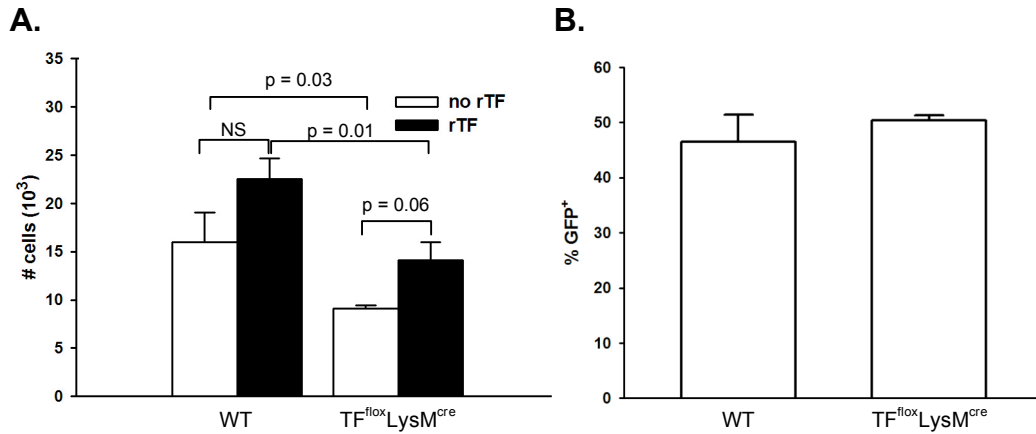


Figure 4-7. TF promotes migration of MDSCs *in vitro* but not *in vivo*. *A.* Trans endothelial migration of splenic Gr-1⁺ MDSCs with or without the addition of 100 ng/mL recombinant TF. *Columns*, mean (n = 8); *Bars*, SEM. *B.* On day -1 C57BL/6 mice were lethally irradiated at 950 rads (135 rads/min). 5×10^6 total BM cells from either eGFP-B6, $TF^{fllox}LysM^{cre}$, or B6 mice were injected i.v. into lethally irradiated B6 recipients. The WT control group received a 1:1 ratio of eGFP-B6 to B6 BM while the experimental $TF^{fllox}LysM^{cre}$ group received a 1:1 ratio of eGFP-B6 to $TF^{fllox}LysM^{cre}$ BM. 8 weeks later, mice were challenged with 2×10^6 E.G7-OVA tumor cells. On day 14 post-tumor challenge tumors were harvested and digested with collagenase A. Mean percent GFP⁺ MDSCs \pm SEM is reported.

Figure 4-8

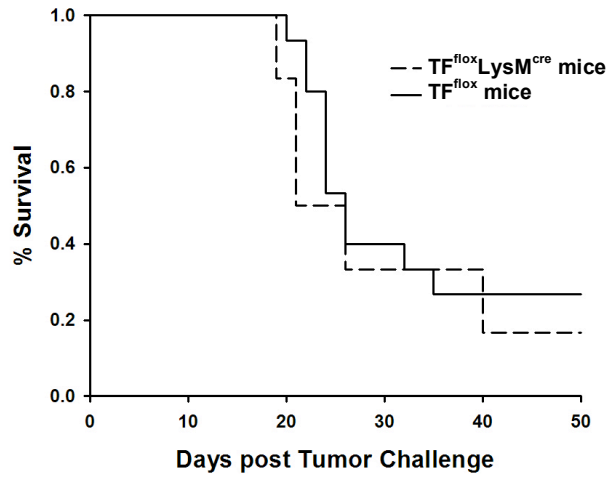


Figure 4-8. TF expression by myeloid cells does not promote tumor growth.

TF^{lox}LysM^{cre} or TF^{lox} control mice were challenged with 1×10^4 B16-F10 cells and vaccinated on days 3 and 10 with 1×10^6 gp100-VRPs. Mice were sacrificed when tumor size reached 200mm^2 .

Figure 4-9

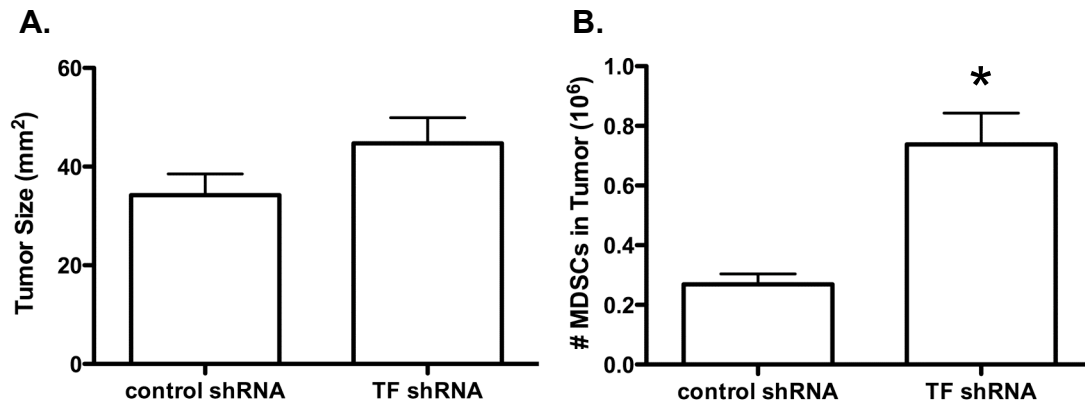


Figure 4-9. TF expression by tumor cells does not promote tumor growth or the accumulation of MDSCs. Neu-N mice were challenged with 5×10^4 control shRNA or TF shRNA NT2 cells and vaccinated with neuET-VRP on day 4 and 18. *A.* Mean tumor size on day 35 post-tumor challenge \pm SEM. *B.* Mean number of Gr-1⁺CD11b⁺ MDSCs in the tumor of mice on day 35 post-tumor challenge \pm SEM ($p < 0.01$, student's t test).

CHAPTER FIVE

DISCUSSION

In our previous work we demonstrated that therapeutic vaccination with neuET-VRPs induced regression of neu-expressing tumors in WT mice (208). These same VRPs were unable to inhibit tumor growth in transgenic neu-N mice. We evaluated whether the inability of VRPs to induce regression in neu-N mice was due to a dominant role of active suppression of anti-tumor immunity in these animals, as opposed to central deletion of neu-specific lymphocytes. Transfer of neu-specific lymphocytes into neu-N mice did not inhibit tumor growth, suggesting that the lack of a sufficient repertoire of neu-specific lymphocytes was not the reason why these mice did not respond to neuET-VRP vaccination. We demonstrated that MDSCs were a major mediator of suppression in neu-N mice. Depletion MDSCs, along with provision of neu-specific lymphocytes and neuET-VRP vaccination, resulted in tumor regression in the majority of mice. We went on to identify a number of other immunosuppressive mechanisms that play a role in suppressing anti-tumor immunity in neu-N mice, including T_{reg} cells, CD200 expression, and the generation of IDO.

As MDSCs appeared to be one of the major impediments to successful immunity following therapeutic VRP vaccination, we went on to investigate the function of these cells. We found that activation of the NLRP3 inflammasome mediated the accumulation of MDSCs in the tumor following therapeutic DC vaccination. The decreased accumulation of MDSCs in *Nlrp3*^{-/-} mice improved the efficacy of our DC vaccine. Therefore, we were able to demonstrate that immunotherapy aimed at augmenting MDSCs function was able to improve efficacy of therapeutic vaccines.

VRP Vaccines

We were somewhat surprised with the degree to which the immunosuppressive tumor environment was involved in suppression of our neuET-VRP vaccine in neu-N mice. Although we hypothesized that the immunosuppressive tumor environment was involved in suppression of anti-neu immunity in neu-N mice, we had anticipated that central deletion of high avidity neu-specific T cells would be the major hurdle to successful vaccine therapy. This is because neu-N mice are known to lack high avidity neu-specific CD8⁺ T cells compared to FVB/N mice (284). Surprisingly, we did not observe any difference in tumor growth following adoptive transfer of FVB/N splenocytes. These data have important implications for current clinical trials utilizing VRPs as therapeutic cancer vaccines.

There are a number of clinical trials using VRPs currently being proposed or in early phase trials for treatment of cancer patients (Table 5-1). The farthest along is a phase I/II study using VRPs expressing the carcinoembryonic antigen (CEA). CEA is a TAA expressed by a variety of cancers including colorectal, breast, lung, pancreatic, and colon cancers. The purpose of this early phase trial is to determine both the safety of VRP vaccination as well as the effect of the vaccine on CEA-specific immune responses (NCT00529984; ClinicalTrials.gov). Other VRP vaccines are being designed for treatment of prostate and breast cancer (Table 5-1). Based on our pre-clinical results reported here, we would predict these trials to show little efficacy when measuring overall response rates. VRP vaccines might be able to induce tumor-specific lymphocytes, similar to what we observed in neu-N mice, but without addressing the highly immunosuppressive peritumoral environment, clinical efficacy would be unlikely.

This is even more likely as many of these early phase trials enroll patients with advanced disease, which would have an even more advanced immunosuppressive environment than what we observed in our studies or what would be expected in cancer patients with early disease.

While clinical trials evaluating the efficacy of VRP vaccines are still in early phases, other viral vaccines have been evaluated for their efficacy in cancer patients. The clinical response rates from these trials have been quite modest. For example, the cumulative overall objective response rate using the viral vaccines Fowlpox, Vaccinia, and Adenovirus for treatment of metastatic melanoma was only 1.9% (259). Our findings would support that active vaccination is unlikely to be effective without approaches to diminish the peritumoral immunosuppressive environment.

We have identified a number of immunosuppressive mechanisms that inhibit VRP vaccines. Although we found MDSCs to be the main suppressors of anti-tumor immunity in neu-N mice, not all mice were rescued following depletion of MDSCs. This is likely because there are multiple mechanisms of immune suppression in neu-N mice.

Although regression in neu-N mice required adoptive transfer of neu-specific lymphocytes from FVB/N mice, provision of tumor-specific lymphocytes might not be a requirement in cancer patients. The neu-N mouse is an extremely stringent model in regards to central deletion of tumor-specific lymphocytes as neu is expressed at very high levels in the thymus (251). Cancer patients, including those with Her-2/neu⁺ breast cancer, do contain Her-2/neu-specific lymphocytes that can be expanded post vaccination (29, 61, 62, 162). Thus, it is conceivable that vaccination may be more effective in expanding antigen-specific T cells in clinical trials as compared to that found using

MMTV-Neu mice. Alternatively, an optimal vaccine strategy may require ACT which would allow for a rapid expansion of antigen-specific T cells, which is found in the lymphopenic environment after stem cell transplantation.

NLRP3 Inflammasome

We report here that the NLRP3 inflammasome has a negative regulatory role in anti-tumor immunity. Others have demonstrated that activation of the inflammasome promotes infiltration of other myeloid cells including monocytes, macrophages, and granulocytes (165, 203, 217). As MDSCs are the main myeloid cell in the tumor environment, the dominant role of NLRP3 in this context is one of immune suppression. This goes against the prevailing notion that NLRP3 promotes immunity, but our data is consistent with the role of immature myeloid cells in the context of the suppressive tumor environment (78, 165, 180, 196).

We were unable to determine the mechanism for NLRP3-dependent accumulation of MDSCs in the tumor. As discussed in chapter three, the decreased number of MDSCs in *Nlrp3^{-/-}* mice could be due to effects on migration, proliferation, or maturation of MDSCs. One potential hypothesis that was not directly addressed by our work is that NLRP3 is important for preventing the maturation of immature myeloid cells in the peritumoral environment. This could be mediated through STAT3, which is the main transcription factor for MDSCs (105). STAT3 is specifically activated in immature myeloid cells (MDSCs) from tumor-bearing mice compared to immature myeloid cells isolated from mice without tumors (221). The two main cytokines in the tumor environment known to activate STAT3 are IL-1 β and IL-6. As has already been

discussed, NLRP3 is involved in the conversion of pro-IL-1 β to its active form.

Therefore, it is possible that NLRP3 dependent accumulation of MDSCs is a result of IL-1 β dependent activation of STAT3.

We were also unable to determine what activated NLRP3 in the tumor environment. To date, no one has demonstrated that tumor cells stimulate NLRP3 during tumor progression. Recently, Ghiringhelli et al. reported that NLRP3 was activated by ATP released from chemotherapeutically killed tumors cells (110). Although it is possible that dying tumor cells activate NLRP3 in our model we have been unable to demonstrate this either *in vitro* with tumor lysate, or *ex vivo* in isolated MDSCs (Burgents, Van Deventer, Woodford, McElvania-Tekippe, Serody, Ting, unpublished). Another possibility is that NLRP3 is activated by our DC vaccine. Unfortunately, we have also been unable to demonstrate DC-dependent activation of NLRP3 *in vitro* in bone marrow-derived macrophages. Current studies are aimed at determining the method of activation of NLRP3 in our DC vaccine model.

It is interesting to note that we did not observe a survival advantage in *Nlrp3*^{-/-} mice when vaccinated with a melanoma-specific, gp100-VRP vaccine (Fig. 5-1). This suggests that the effect we see with NLRP3 is specific to our DC vaccine. This also supports a role for the vaccine and not the tumor in activating NLRP3. There are two possible explanations for why we do not observe a survival advantage in *Nlrp3*^{-/-} mice with our VRP vaccine. First, the VRPs were injected in the footpad of mice whereas the DCs were injected in the peritumoral region. Activation of NLRP3 could be a result of injection of DC into the tumor area as opposed to some intrinsic difference between DC and VRP vaccination. It is quite possible that injection of VRPs into the tumor would

have the same effect as our DC vaccine, especially considering that RNA viruses are a known activator of the NLRP3 inflammasome (193). Although there was not a significant difference in survival between *Nlrp3*^{-/-} and WT mice with our VRP vaccine, the trend suggested decreased survival in *Nlrp3*^{-/-} compared to WT mice (Fig. 5-1). It is tempting to speculate that this could be due to the NLRP3-dependent promotion of anti-tumor immunity when NLRP3 is activated at a site other than the tumor environment, i.e. the draining lymph node following VRP vaccination in the footpad.

The second possible explanation for why there is not a survival advantage in *Nlrp3*^{-/-} mice following VRP vaccination is the difference in the immune response elicited by each vaccine. The DCs used in our vaccine, which are activated and matured by LPS, express high levels of IL-12 but low levels of type I IFNs. On the other hand, VRP infected DCs express high levels of type I IFN and IL-6, but very little IL-12 (209). Also, as a RNA virus based vaccine, VRPs might induce anti-tumor immunity in a NLRP3-dependent manner, whereas our DC vaccine can likely stimulate the immune system independent of NLRP3 (5, 110). Again, the trend towards decreased survival in *Nlrp3*^{-/-} mice following VRP vaccination would support his conclusion. Further research is needed to evaluate the scope to which NLRP3 affects anti-tumor immunity. This will greatly affect the design of therapies aimed at modulating the NLRP3 inflammasome.

Role of B cells in neu-VRP Vaccines

Most of the work by our group and others has focused on using vaccines to generate T cell specific tumor immunity. One of the main benefits of VRP vaccines, compared to other vaccines, is that they are strong activators of both cellular and humoral

immunity (116, 208, 222). Our initial studies investigating the efficacy of neuET-VRP vaccination in FVB/N mice demonstrated that CD4⁺ T cells were critical for VRP-induced regression of neu-expressing tumors (208). Surprisingly, there was only a partial dependence on CD8⁺ T cells, with the majority of mice still able to regress tumors following depletion of CD8⁺ T cells (208). We have not been able to show specifically that B cells are critical for the immune response after VRP vaccination although our data support a correlation between antibody titers and vaccine efficacy (Fig. 5-2). B cells could be important in anti-tumor immunity either due to (1) infection of B cells with presentation of antigen by these cells or (2) generation of anti-neu antibody (Ab). Future studies should investigate the role of B cells in FVB/N or neu-N mice by either depletion of B cells with anti-CD20 Ab or by transfer of B cell-depleted splenocytes in our transplant model.

If B cells and anti-neu Ab production do promote anti-tumor immunity in neu-N mice following VRP vaccination, strategies to augment Ab production in these mice might improve vaccine efficacy. One such strategy that can be employed to increase Ab production in neu-N mice is treatment with recombinant BAFF. BAFF is a cytokine important for the growth and survival of B cells (153). Also, BAFF has been shown to increase the production of autoantibodies, and blocking BAFF appears to be a viable strategy to treat autoimmune disease (69, 153, 206). Therefore, we are interested in whether treatment with BAFF will increase production of anti-neu Abs in neu-N mice. To date, BAFF has not been investigated for its use in augmenting tumor immunotherapy. We have preliminary data suggesting that treatment of mice with recombinant BAFF can improve efficacy of our neuET-VRP vaccine in neu-N mice (Fig.

5-3). Future studies are aimed at further evaluating the role of B cells in neu-N mice, and whether treatment with BAFF will improve efficacy of VRP vaccines. Also, we are interested in determining whether vaccination with BAFF expressing VRPs will increase anti-neu Ab levels and induce regression of tumors in neu-N mice.

Concluding Remarks

Therapeutic cancer vaccines offer an attractive strategy to treat cancer. They can be used to specifically target tumor cells without the negative side effects of other treatment regimens. Also, as they utilize the patient's immune system, these patients should be protected from recurrent disease. Despite the elegance and simplicity of design, vaccine therapy has not been successful clinically in the overwhelming majority of the patients treated. Objective response rates for cancer vaccines are dismal, with approximately 2- 5% of patients responding clinically to this therapy. The data presented here offer a compelling rationale for the role of tumor-induced immune suppression in inhibiting the efficacy of therapeutic cancer vaccines. Our data underscore the need for inhibiting peripheral tolerance even if tumor-specific T cells are present. For effective vaccine approaches for the treatment of cancer, patients may require high avidity tumor-specific lymphocytes, but our data suggest that even if provided, these cells might be ineffective if not combined with strategies to inhibit the suppressive tumor environment. We also presented novel data implicating a role of the NLRP3 inflammasome in inhibiting anti-tumor immunity. This is counter to the prevailing notion that NLRP3 promotes immune response, but again these data underscore the dominant nature of the suppressive tumor environment. Despite their lack of success so far, we believe that

cancer vaccines still warrant significant focus due to the huge potential that they represent, but further work is clearly needed to evaluate the many mechanisms of suppression that prevent these vaccines from being effective.

Table 5-1. Current VRP vaccines in clinical trials for treatment of cancer

Cancer	TAA	Status	Sponsor	Preclinical Refs.
Colorectal, Breast, Lung, Pancreatic, Colon	CEA	Phase I/II	AlphaVax	(346)
Prostate	PSMA	Proposed/Phase I	AlphaVax/ Sloan-Kettering	(76)
Breast	Her-2/neu	Proposed	AlphaVax	(174, 208, 222)

Figure 5-1

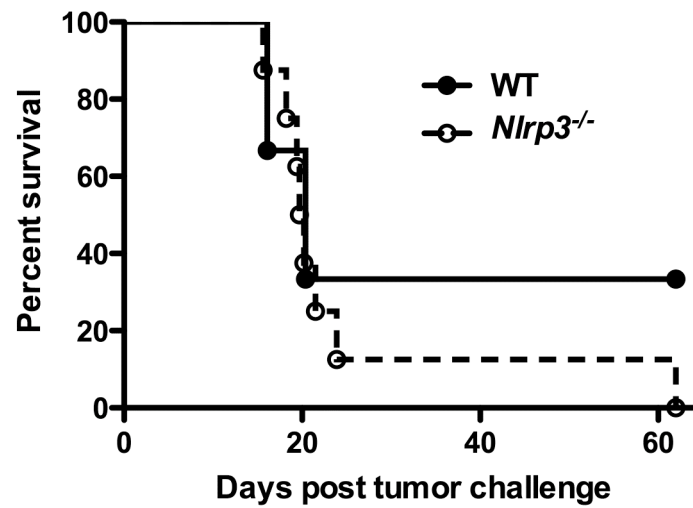


Figure 5-1. No difference in survival of B16 challenged WT and *Nlrp3*^{-/-} mice following gp100-VRP vaccine. WT (C57BL/6) (n = 3) or *Nlrp3*^{-/-} (n = 8) mice were challenged with 1×10^4 B16-F10 melanoma cells followed by vaccination with 1×10^6 gp100-VRPs on days 3 and 10 post-tumor challenge. Mice were sacrificed when tumor diameter reached 6mm. (p = 0.43, Log-rank, Mantel-Cox, test)

Figure 5-2

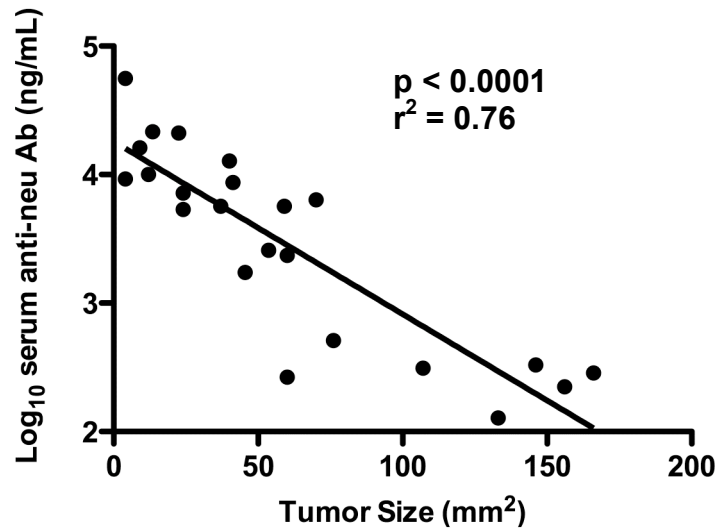


Figure 5-2. Serum anti-neu Ab levels are inversely correlated with tumor size in neu-N mice following therapeutic neuET-VRP vaccination. 1×10^7 FVB/N splenocytes and 5×10^6 syngeneic T cell depleted BM cells were injected i.v. into lethally irradiated neu-N mice. On the same day, mice were challenged with 5×10^4 NT2 cells. Mice were vaccinated with neuET-VRPs on day 4 post-tumor challenge and boosted every 14 days. Anti-neu antibody levels in the serum were determined when mice were sacrificed. Data reported are combined from two experiments, one in which mice were treated with Gr-1 depleting Ab (200 μ g 2x/wk) and the other in which mice were treated with recombinant human BAFF (1 μ g/day for the 3 consecutive days following each vaccine and 2x/wk on the weeks without vaccine). Significant regressions were also obtained from each individual experiments. *Points*, individual mice; *line*, linear regression; $p < 0.0001$.

Figure 5-3

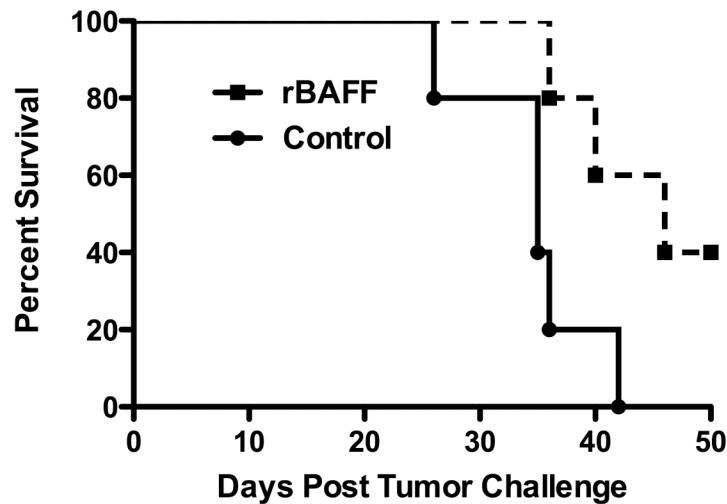


Figure 5-3. Treatment with rBAFF improves survival in neu-N mice following adoptive transfer of FVB/N splenocytes and neuET-VRP vaccine. 1×10^7 FVB/NJ splenocytes and 5×10^6 syngeneic T cell depleted BM cells were injected i.v. into lethally irradiated neu-N mice. On the same day, mice were challenged with 5×10^4 NT2 cells. Mice were vaccinated with neuET-VRPs on day 4 post-tumor challenge and boosted every 14 days. Mice were treated with recombinant human BAFF ($1 \mu\text{g}$) s.c. on the three consecutive days following each vaccine and twice on the weeks without vaccine. Survival to 50mm² is reported (n = 5 per group, p = 0.0288, Log-rank, Mantel-Cox, test).

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