MS-275 ADMINISTRATION IN THE CONTEXT OF

BOOSTING ONCOLYTIC IMMUNOTHERAPY

MS-275 (ENTINOSTAT) PROMOTES SUSTAINED TUMOR REGRESSION IN THE CONTEXT OF BOOSTING ONCOLYTIC IMMUNOTHERAPY

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Abstract

We showed previously that histone deacetylase (HDAC) inhibition with MS-275 in the context of boosting oncolytic immunotherapy can drive heightened antitumor responses, leading to increased survival in mouse intracranial melanoma models. However, it is currently unclear how the co-administration of MS-275 directly impacts tumor growth. Here, we investigated the role of MS-275 in preventing the outgrowth of antigen-deficient tumor variants as a result of suboptimal treatment protocols. By adoptively transferring tumor antigen-specific memory T cells (Tm) that were expanded *in vivo* with recombinant Vesicular Stomatitis Virus (VSV-gp33), we observed complete regression of 5-day old, intradermal B16-gp33 tumors (B16-F10 overexpressing the LCMV GP33-41 epitope); however, the tumors relapsed within a month of treatment. Relapsing tumor explants were able to grow in mice that were prophylactically immunized with recombinant Adenovirus (Ad-gp33), indicating that the tumor could no longer be recognized. Strikingly however, there was zero tumor recurrence if MS-275 was co-administered with Tm and VSV-gp33, suggesting that MS-275 may prevent the emergence and/or escape of antigen loss variants. Such a benefit is lost if the administration of the drug is delayed as little as five days post VSV treatment, suggesting that its synergistic effects coincide with early immune responses and oncolytic activity. Furthermore, transplantation studies of relapsing tumor explants showed that combination treatment was unable to provide tumor protection, confirming that the mechanisms by which MS-275 prevents tumor recurrence are unlikely through direct up-regulation of antigen presentation in low- or non-antigen-expressing variants *in vivo*. Indeed, CD4 depletion in the absence of MS-275 resulted in sustained tumor regression, implying that immunoregulatory cells such as CD4+ Treg play a prominent role in sustaining tumor regression. Moreover, MS-275 modulates the phenotypic status of tumor-

infiltrating MDSCs toward the differentiation of inflammatory macrophages. Taken together, the data suggests that combination therapy with HDACi with oncolytic immunotherapy mediates a synergized immune attack against the tumor through subversion of immunomodulatory mechanisms.

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ABBREVIATIONS:

ACT: adoptive cell transfer therapy
Ad: adenovirus
CTL: cytotoxic lymphocyte
DC: dendritic cell
DCT: dopachrome tautomerase
FACs: flow cytometry
GP1: glycoprotein 1
HAT: histone acetylase
HDAC: histone deacetylase
HDACi: histone deacetylase inhibitor
id: intradermal
IFN: interferon
ip: intraperitoneal
iv: intravenous
LCMV: lymphocytic choriomeningitis virus
mAb: monoclonal antibody
MDSC: myeloid-derived suppressor cell
MHC: major histocompatibility complex
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OV: oncolytic virus
pfu: plaque-forming units
PCR: polymerase chain reaction
qRT-PCR: quantitative reverse transcription polymerase chain reaction
TAA: tumor-associated antigen
TIL: tumor-infiltrating lymphocyte
Tm: memory T cell
T_R1: adaptive regulatory T cell
Treg: regulatory T cell
TSA: tumor-specific antigen
VSV: vesicular stomatitis virus

Chapter 1: Background

1.1 Introduction and Rationale

The design of clinically effective anticancer immune therapies faces significant challenges. Vaccination platforms many not be sufficiently immunogenic as monotherapy [1, 2]; furthermore, immunogenicity may not necessarily correlate with objective responses in clinical trials (ex. TeloVac phase III trial, DERMA phase III trial, etc.). This is not surprising considering tumors have evolved mechanisms to successfully evade and suppress the antitumor immune response. While traditional paradigms of cancer vaccine design have been pre-occupied with optimizing the magnitude and potency of antitumor immune effector cells, it is apparent that in order to elicit a more comprehensive attack on the tumor, the elimination of inhibitory factors and prevention of immunoresistant phenotypes should also be considered.

To address this issue, our group developed a novel approach, termed *oncolytic vaccination,* whereby recombinant oncolytic viruses (OV) that express tumor antigens are used to bolster pre-existing antitumor immunity. We hypothesized that oncolytic vaccines could provide a powerful stimulus to tumor-specific memory T cells while retaining their inherent ability to directly infect and debulk the tumor and reverse the immunosuppressive tumor microenvironment. Indeed, our studies demonstrated that recombinant vesicular stomatitis virus (VSV) could elicit rapid secondary expansion of antitumor T cells and robust antitumor effects, resulting from synergistic interactions between viral oncolysis and cancer immunotherapy [3].

Histone deacetylase inhibitors (HDACi) have been shown to extend the replicative capacity of oncolytic viruses [4-6]. By incorporating HDACi into our therapy, we wanted to investigate if improved viral oncolysis could further potentiate the effects of cancer

immunotherapy. Interestingly, combination therapy induced significantly enhanced therapeutic outcomes; however, viral replication was only minimally improved [7]. MS-275 has anti-inflammatory [8-11] and antitumor capabilities and is currently being tested in patients with advanced leukemia and solid tumors as monotherapy [12-15]. Since the drug alone had little therapeutic efficacy in our murine tumor model, we postulate that our enhanced therapeutic outcomes were derived from its immunomodulatory properties. Consequently, there is investigative value in determining how tumor growth is directly impacted by MS-275 co-administration and how MS-275 manipulates host immunity to potentiate the antitumor response.

Since our previous studies and observations were conducted in intracranially challenged mice, we were unable to directly observe tumor growth. Moreover, the aggressiveness of tumor development and lengthy prime-boost vaccination schedule created only a small window in which therapeutic efficacy was observed. This limits the size of the initial tumor challenge and in turn hinders intratumoral analyses. To overcome these limitations, we utilized adoptive cell transfer therapy (ACT) protocols to introduce pre-existing tumor antigen-specific memory T cells into the recipient before stimulating secondary expansion with VSV-boosting. We postulate that rapid expansion of the antitumor response may decrease the length of the vaccination schedule, increase the therapeutic window, and allow for more intensive tumor challenges. Furthermore, rapid expansion may be able to overwhelm adaptive tumor-induced suppressive mechanisms normally induced in response to immune attack [16]. Lastly, by utilizing ACT therapy, we are extending the applicability of VSV-boosting to other promising therapeutic approaches.

In summary, we predict that concomitant administration of MS-275 in the context of adoptive memory T cell transfer and VSV-boosting will facilitate enhanced tumor regression in a

challenge model that is conducive to observable tumor growth. To analyze how MS-275 potentiates immune attack on the tumor, we plan to closely monitor the immunological events that may be occurring within the periphery and tumor as a result of treatment.

1-2 The Threat of Cancer

Cancer malignancies encompass some of the most life-threatening and prevalent diseases across the globe and are a driving force in medical research. The World Health Organization (WHO) has reported that cancer is the second leading cause of death in industrialized countries and third worldwide [17]. While established therapies such as surgical resection of primary tumors, radiation therapy, and chemotherapy have improved throughout the years, cancer is still responsible for 25% of mortalities [18]. Approximately 1% of patients diagnosed with cancer will die on an annual basis [18]. Five-year survival rates range from 10–20% for lung, esophagus and stomach cancer, to 40–60% for colon, bladder and cervix cancer, and 60–80% for breast and prostate cancer [18]. Consequently, the relative ineffectiveness of current therapeutic methods underscores an ever-increasing need for clinically promising treatments against cancer.

1-3 Tumorigenesis and the Hallmarks of Cancer

The tumorigenic process that defines the transformation of a normal cell into a cancer cell can be divided into three distinct stages: tumor initiation, tumor promotion, and tumor progression. During tumor initiation, growth-regulatory genes undergo genetic or epigenetic mutations. This results in the activation of oncogenes (such a Ras and Myc) or inactivation of tumor-suppressors (such as p53 and Rb). The second stage of tumorigenesis, tumor promotion, is dependent on the clonal expansion of tumor initiated cells as a result of increased proliferative capacity or decreased ability to undergo cell death. Lastly, tumor progression is characterized by an increased rate of growth and invasiveness due to acquisition of a malignant phenotype. Traditionally, the characterization of malignant tumor cells was relegated to six specific criteria [19]: 1) self-sufficiency in growth signals, 2) insensitivity to anti-growth signals, 3) evasion of

apoptosis 4) limitless replicative potential 5) sustained angiogenesis, and 6) tissue invasiveness and metastasis. However, Schreiber and colleagues proposed that the avoidance of immunosurveillance could be the seventh hallmark of cancer [20].

1-3.1 Cancer Immunosurveillance

Paul Ehrlich was the first to suggest that the immune system could repress the majority of carcinomas [21], but the idea of immunological control of neoplastic diseases was not further explored until the mid-twentieth century. Thomas and Burnett were responsible for coining the term "immunosurveillance" and proposing the idea that the immune system protects the host against cancer development from a non-viral origin [22]. This was based on early work showing that mice could be immunized against syngeneic transplants of tumors induced by chemical carcinogens, viruses, and other means [23, 24]. While caveated studies with immunocompromised athymic nude mice by several investigators dampened enthusiasm to the idea [25-27], key observations by Schreiber *et al* renewed interest in the concept of immunosurveillance[28-30]. He further suggested that host immunity could modulate the immunogenicity of developing tumors by a process in which he termed, "immunoediting" [31].

1-3.2 Cancer Immunoediting

Given that immunosurveillance can actively prevent tumor formation, it is relatively unclear why cancer can occur in immunocompetent individuals. Schreiber suggests that the immune system may select for variants that are better suited to survive in an immunologically intact environment, resulting in an outgrowing population that possesses low immunogenicity [31]. He proposes a dual role of the immune system in host-protecting and tumor-sculpting

which he described as "immunoediting". This is envisaged as a result of three distinct processes: 1) elimination, 2) equilibrium, and 3) escape.

The elimination process encompasses the original concept of cancer immunosurveillance, whereby the host immune response attempts to successfully delete the developing tumor. Minor disruption in surrounding tissue induces inflammatory signals, leading to innate cell recruitment (natural killer (NK), NKT, γδ T cells, macrophages, and dendritic cells (DCs)) to the tumor site [32-34]. Tumor cell recognition by infiltrating lymphocytes (NKT, NK or γδ T cells) through danger-associated molecular patterns (DAMPs), dying/damaged tissues (ex. HMGB1), and expressed stress ligands (ex. natural killer cell protein group 2D (NKG2D) ligands such as major histocompatibility class I (MHC class I) chain-related molecules A/B (MICA/B)) induces the production and secretion of interferon γ (IFNγ) [35-37], which may facilitate tumor death by anti-proliferative [38] and apoptotic [39] mechanisms. Early tumor cell death by (non)immunologic mechanisms can release debris which is taken up by dendritic cells, leading to the activation and recruitment of tumor-specific CD4+ and CD8+ T cells [40, 41].

Any tumor cell variant that has survived elimination enters into a dynamic equilibrium. At this stage, the adaptive immune system prevents immediate outgrowth of the tumor, but is insufficient for complete elimination. Darwinian selection dictates that while much of the original tumor population is destroyed, tumor cells with genetic and epigenetic traits conferring heightened immune resistance are granted a growth and survival advantage. At the escape stage of cancer immunoediting, tumor cells that can escape immunological detection and/or elimination due to genetic or epigenetic changes begin to grow rapidly, resulting in disease that is clinically observable.

Avoidance of immunosurveillance through successful immunoediting forms the crux of Schreiber's seventh hallmark of cancer. Host immunity can thus play an active role in shaping the development of immune-resistant tumor phenotypes during outgrowth and malignancy. However, it is also apparent that the immune system plays a fundamental role in controlling tumor growth. This may suggest that strategies which aim to stimulate antitumor immunity may be therapeutically viable and should undergo further investigation.

1-4 Cancer Immunotherapy as a Platform for Tumor Control

Utilizing host immunity for the purpose of combating cancer is not a novel concept. In 1891, New York surgeon William Coley treated sarcoma patients by vaccinating them intratumorally with a mixture of attenuated *Streptococcus pyogenes* and *Serratie marcescens* which became known as Coley's toxin [42, 43]. Similarly, it was found that intravesical injection of live bacillus Calmette-Guéin after surgical resection of superficial bladder cancer was able to extend the survival of patients [44-46]. In the 1950s, Burnett demonstrated that the immune system could mount antitumor responses and suggested that transplantation antigens expressed on tumor cells could elicit the generation of protective immunity [47, 48].

Cancer immunotherapy aims to restore the reactivity of the host's immune system to combat cancer in a non-specific (Coley's toxin) or tumor-specific manner. Tumor-targeted therapeutic designs elicit immune responses that are specific for cancer antigens expressed on tumors. Tumor-specific antigens (TSA) are a small group exemplified by cancer-testis antigens (ex. melanoma antigen gene, MAGE) [49, 50]. These are silent in normal tissue but are expressed in cancer cells. Tumor-associated antigens (TAA) are expressed by normal cells but are overexpressed in cancer (ex. MART-1 [51], gp100 [52], TRP-2 [53]). Mutational antigens

can arise from point mutations of growth-regulatory genes such as the p53 oncogene that render the tumor cell immunogenically distinct from normal cells [54, 55]. Lastly, certain viruses have an oncogenic capacity (HPV Type 16) and the gene products encoded by these viruses (E6 and E7 proteins) are distinct from normal cells and are immunogenic [56]. Cancer immunotherapy can be broadly divided into: 1) non-specific immune activation 2) adoptive cell transfer therapy (ACT) 3) passive immunotherapy and 4) active immunotherapy. Each strategy offers a unique method of propagating immune attack on the tumor; however, it is apparent that pervasive side effects and limited clinical efficacy emphasize an ever urgent need to adopt alternative modalities or to create novel strategies altogether.

1-4.1 Non-specific Immune Activation

The non-specific activation of endogenous tumor-reactive T cells can be catalyzed through the use of adjuvants. For instance, cytokines can exert their effects by binding to their respective receptors on target cells. Therapeutic administration of cytokines such as interleukin 2 (IL-2) [57, 58], granulocyte macrophage colony-stimulating factor (GM-CSF) [59, 60], and IFNα [61, 62] may promote cytotoxic immunity by inducing T cell proliferation, up-regulating DC recruitment and activation, and increasing tumor immunogenicity through MHC I up-regulation, respectively. In particular, IL-2 can reproducibly lead to the regression of several solid tumors in humans [63-65]. This led to approval by the U.S. Food and Drug Administration (FDA) of IL-2 (Proleukin) for the treatment of metastatic renal cancer and metastatic melanoma. Unfortunately, cytokine infusions have been associated with significant side effects including the exacerbation of inflammatory conditions and autoimmunity [66]. Furthermore, only modest therapeutic benefits have been demonstrated [67-69]. This may be attributed to failures in recapitulating the

repertoire or synchronized function of cytokines in the context of antigen uptake and presentation.

1-4.2 Passive Cancer Immunotherapy

Passive cancer immunotherapy is dictated by a passive transfer of short-lived effector molecules that do not directly engage the host adaptive immune system to attack the tumor. Antibody-based immunotherapy establishes a physical linkage between TAAs expressed on cancer cells and the host immune system through tumor antigen-specific immunoglobulin (Ig) and an Fc region which interacts with the immune system. Administered antibodies have multiple biological effects including agglutination, neutralization of signaling proteins, blocking receptor binding sites of growth factor molecules, modulation of signaling pathways, complement activation, antibody-dependent cellular cytotoxicity (ADCC)/complement-dependent cytotoxicity (CDC), and the delivery of covalently linked cytotoxic agents [70, 71]. While mAb-based cancer therapies have shown clinical responses and FDA approval for several hematological malignancies and solid tumors [72-78], passive cancer immunotherapy can be limited by practical, toxicological, and biological concerns. Since mAbs provide only short-term protection, successful therapy requires high amounts of tumor antigen-specific antibodies. If the target is expressed by both normal and malignant cells (ex. Rituximab targets human CD20), treatment-induced autoimmunity may occur [79]. Even mAbs targeting tumor-specific structures (ex. Herceptin targets HER-2/neu, which is expressed in 20% of breast cancers) may only induce modest clinical outcomes (24% reduction in the risk of death after one year) [80].

1-4.3 Adoptive Cell Transfer Therapy

Adoptive cell therapy begins with the identification of autologous or allogenic lymphocytes with antitumor activity (from peripheral blood, tumor-draining lymph nodes, or directly from the tumor mass). These cells are then expanded *in vitro* and re-infused into the tumor-bearing patient. ACT is normally preceded by a preparative, non-myeloablative lymphodepleting regimen (total body irradiation or cytotoxic drugs) to eliminate regulatory T cells (Tregs) and normal endogenous lymphocytes that compete with the transferred cells for homeostatic cytokines (IL-7, IL-15). T cell growth factors such as IL-2 are administered during ACT to stimulate the survival and expansion of transferred cells *in vivo* [81-83]. Studies have shown that the differentiation state of adoptively transferred T cells can affect the success of therapy. Cells that are less differentiated (ex. memory T cells) have been shown to have greater proliferative capacity and antitumor efficacy [84-88]. Furthermore, lymphocytes can be gene-modified prior to adoptive transfer to confer properties that will enhance their therapeutic efficacy. This includes the insertion of genes that confer antigen reactivity/specificity [89], enhance co-stimulation [90], prevent apoptosis [91], induce inflammation or homeostatic proliferation [92, 93], and/or promote T cell migration to the tumor site [94].

While objective clinical response rates to autologous tumor-infiltrating lymphocytes (TIL) were encouraging in patients with stage IV melanoma, a large number of patients did not respond favorably to treatment [95-97]. Furthermore, the efficacy of naturally occurring TILs appears restricted to melanoma for unknown reasons. The use of gene-engineered T cells may induce off-target toxicities as a result of recognition of unintended structures [98-102]. Also, toxicities have been associated with the use of nonspecific preconditioning regimens based on chemotherapy and radiation [83]. On a practical side, ACT is a highly personalized treatment, is labor-intensive, is expensive, and requires laboratory expertise [81].

1-4.4 Active immunotherapy (Cancer Vaccines)

The aim of active cancer immunotherapy is to induce an endogenous, robust, and long-lasting tumor antigen-specific immune response. Since some cancers originate from chronic infections, therapies that prevent the infection prior to tumor formation are defined as prophylactic (ex. HBV and HPV/16/18 vaccines against liver and cervical cancers) [103]. The development of therapeutic cancer vaccines, which aim to clear tumors once they have been established, is much more challenging. This vaccination approach requires defined TAAs or material obtained from direct tumor biopsies rather than a foreign antigen expressed by the infectious agent. Therefore, the efficacy of treatment is dependent on the ability of the vaccine to prime an immune response that can overcome host immune tolerance for the TAA, which is a self-antigen. While many approaches have been implemented, they have a commonality shared across all active immunotherapeutics in that a priming response is initiated against the tumor antigen.

CD8+ effector T cells have a central role in the elimination of tumors. Immature DCs mature after being exposed to inflammatory signals (ex. TNFα, IL-1β, IL-6, Type I IFN), DAMPs (eg, HMGB1, heat-shock proteins (HSPs)), or pathogen-associated molecular patterns (PAMPs) (ex. LPS, dsRNA). Resulting mature DCs have improved antigen-presenting abilities, increased expression of co-stimulatory molecules, and acquire migratory potential to secondary lymphoid tissue. Presentation of antigen on MHC class II to cognate CD4+ T cells and CD40-CD40L interaction completes DC maturation in a process known as *licensing* [104].

Naïve CD8+ T cells express T-cell receptors (TCRs) which interact with mature DCs via 8-10 amino acid long peptides buried in the antigen-presenting groove of MHC class I

molecules. This interaction initiates the priming of the naïve CD8+ T cell and provides the first signal for T cell activation. However, CD8+ T cell-mediated tumor rejection requires additional DC-CD8+ T cell signals. CD28 co-stimulatory receptors expressed on T cells interact with CD80/CD86 ligands on DCs to produce a second signal, the absence of which would provoke T cell anergy. Additional co-stimulatory receptor-ligand interactions that are crucial for optimal T cell activation include ICOSL-ICOS, OX40L-OX40, and CD137L-CD137 among others. Lastly, the presence of IL-12 and/or IFNα/β during T cell priming provides the third signal for optimal CD8+ T cell activation, leading to the differentiation and expansion of tumor-specific cytotoxic T lymphocytes (CTLs) [105].

CTL infiltration into the tumor microenvironment is followed by tumor killing through various mechanisms. Recognition of target tumor cells leads to the release of apoptosis-inducing cytotoxins (perforin, granzymes, granulysin). Also, cell-surface interactions may also lead to surface expression of Fas ligand (FasL). Fas-FasL interaction can induce apoptosis of the tumor cell through recruitment of death-induced signaling complex (DISC) and Fas-associated death domain (FADD) [106, 107]. Therefore, it is clear that cytotoxic T cells play a major role in the clearance of established tumors. Several strategies will be described which attempt to elicit high quality tumor-specific CD8+ T cell responses but, like previously described strategies, have had limited success in terms of therapeutic efficacy and positive clinical responses.

1-4.4a DC-based Vaccines

Dendritic cells have potent immune-stimulating capacity and have been explored intensely as a platform for vaccination. Owing to their properties as professional antigen-presenting cells, DCs are considered 'nature's adjuvants' and are viewed as natural targets for antigen delivery. This can be accomplished *ex vivo* by culturing DCs derived from patients with

an adjuvant that can induce DC maturation (ex. GM-CSF, IL-4, IL-15) [108] and the tumor antigen (provided in the form of peptides, proteins, tumor lysate). DCs can also be transfected/transduced with recombinant viral vectors or nucleic acids that encode the tumor antigen [109]. Sipuleucel-T is a clinically successful example of a DC-based vaccine for the treatment of hormone-refractory prostate cancer in which immature DCs are incubated with PA2024 fusion protein consisting of tumor antigen, prostatic acid phosphatase (PAP), and GM-CSF [110, 111]. However, it confers a survival benefit of only 4.1 months and is unable to mediate tumor regression or long-term durable responses. While pre-clinical data suggest otherwise [112], it may be possible that concurrent therapeutic modalities (glucocorticoid administration or cytotoxic chemotherapy) could interfere with the cellular immune response in prostate cancer patients [113]. Furthermore, treatment may induce immune tolerance as a result of faulty antigen presentation [114]. Lastly, the cost intensity and time-intensive nature of production creates logistical difficulties in generating sufficient cells for DC vaccination.

1-4.4b Whole Tumor Cell Vaccines

A vaccine consisting from the whole tumors could allow host antigen-presenting cells to take up, process, and present the entire tumor antigen repertoire, inducing a broad antitumor immune response. Tumor cell-based vaccines can be generated from autologous or allogenic tumor cells removed during surgery that are cultured, inactivated (irradiated/lysed), and infused into the patient. Most approaches require coupling the vaccine with a strong adjuvant (ex. BCG [115], QS-21 [116]) or cytokines (IL-2, GM-CSF or IL-12) [117-122] to elicit a strong immune response. Autologous whole tumor vaccines may also be engineered to express cytokines [117, 118, 121, 122] or growth factors [123-126] to prevent tolerization and elicit danger signals necessary for the activation of antigen-presenting cells. Overall, several autologous whole cell

vaccines (ex. OncoVAX with BCG adjuvant) have reached phase II/III clinical trials for a variety of malignancies including colorectal cancer [127-130], melanoma [131, 132], and renal cell cancer [133]. The modest clinical outcomes derived from these autologous tumor vaccines were dependent on adjuvants; however, BCG induces ulcers at vaccination sites and thus non-toxic alternatives must be considered. Another limitation of whole tumor vaccines is that its antigen repertoire, which includes self-antigens, may induce autoimmunity [134]. Similarly, it is difficult to quantify the overall antitumor response since the immune attack is so broad. Lastly, the patient's tumor must be resectable in order to manufacture the vaccine.

1-4.4c Nucleic Acid Vaccines

Nucleic acid vaccines are primarily composed of naked plasma DNA which encodes the tumor antigen of interest and are injected intramuscularly into the tumor-bearing host. They are highly flexible and can encode a number of immunological components, are associated with lower cytotoxicity, are relatively stable, and are potentially more cost-effective for manufacture and storage [135, 136]. However, results obtained from clinical trials indicate poor immunogenicity relative to small animal studies, indicating a need to improve antigen presentation and delivery methods to activate effective immunity against tumor antigens [135, 137-139]. A more refined version of this technology was described previously with the transfection of autologous dendritic cells with nucleic acids encoding tumor antigens.

1-4.4d Recombinant Protein Vaccines

As previously mentioned, several TSAs have been identified; as a result, several lines of inquiry have been made as to whether the administration of purified recombinant tumor antigens can serve as a viable vaccination platform. Indeed, using recombinant proteins is an attractive

option because it enable's the body's own immune system to cleave and bind peptides. This may result in the presentation of both CD8+ and CD4+ epitopes and activation of CD8+ killer T cells and CD4+ helper T cells [140]. Furthermore, since the cleaved peptides are host-derived, there is no need for HLA selection and broader patient populations can be treated. The manufacture of recombinant proteins is also well controlled and such products are easy to administer [141]. Since this approach requires the characterization of specific targets on tumor cells, it may be limited by the relative shortage of known TSAs [2]. Recombinant protein vaccines also require optimized adjuvant selection in order to improve antitumor immune responses. Clinical trials with various vaccine-adjuvant combinations (ex. carcino-embryonic antigen (CEA) with GM-CSF [142], NY-ESO-1 with ISCOMatrix [143], MAGE-A3 with AS15 [144, 145]) showed promising cellular and humoral responses. It should be mentioned that the effectiveness of recombinant protein vaccines is currently restricted to well-defined protein patient populations that are still at risk for relapse after conventional surgical treatment. Furthermore, even at minimal disease burdens, clinically advanced recombinant protein vaccines may not meet primary endpoints (MAGE-A3 with AS15 failed to significantly extend disease-free survival relative to placebo controls).

1-4.4e Peptide Vaccines

Peptide vaccines generally incorporate short amino acid sequences as tumor antigens combined with a vaccine adjuvant. They are easy and cheap to manufacture, do not require immunological processing, and allow ready control of the dose and route of administration [146]. However, used on their own, it is apparent that they do not elicit measurable immune responses [147]. Peptide vaccines targeting from gp100, NA17, MART-1, or tyrosinase [148-151] could not induce tumor regression until adjuvants such as GM-CSF [152], IL-2 [151], or IL-12 [150]

were incorporated, suggesting that peptide vaccines can induce significant immunological and therapeutic responses only when coupled with a strong immunostimulating agent. However, even clinically advanced peptide vaccines (ex. Stimuvax [153], GV1001 [154]) were unable to induce significant survival advantages in Phase III testing. This may be attributed to their short *in vivo* half-life in the circulation (~30 min) [147] and potential for non-specific binding to MHC that is expressed on non-antigen presenting cells [155]. In addition, since the peptides are human leukocyte antigen (HLA)-restricted, the target patient population is narrowed to patients that express adequate HLA molecules [141, 156].

1-4.4f Viral Vector Vaccines

Viral vectors are considered an attractive choice as an antigen delivery system for cancer immunotherapy. They are able to mimic natural infection and provide potent danger signals which are necessary for the activation of innate immune responses [157]. Furthermore, many types of recombinant viruses have been shown to infect professional APCs and express various transgenes [158-163], leading to enhanced tumor antigen presentation and increased frequency and avidity of the antitumor CTL response. Recombinant viruses are also produced more easily compared to whole tumor vaccines and DC vaccines due to ease of production, purification, and storage [135]. On the other hand, multiple injections of the same recombinant virus can promote host-induced neutralizing antibodies to the vector itself, severely limiting its continued use [164]. The existence of pre-existing immunity towards many commonly used viral vectors poses a similar challenge due to the production of neutralizing antibodies [164]. Numerous recombinant viral vector systems (ex. vaccinia, avipox, adenovirus) have been developed and encode a diverse array of cytokine/co-stimulatory molecules and/or TAAs (ex. CEA, gp100, MART-1)

[164, 165]. Unfortunately, monotherapies with recombinant viral vectors have not been conducive to significant objective response rates during clinical investigation [166].

1-5 The Challenges of Cancer Vaccine Design

It is apparent that the design of clinically effective anticancer immune therapies faces significant challenges. Until recently, researchers were pre-occupied with increasing the magnitude and potency of antitumor immune effector cells in order to optimally stimulate extrinsic tumor suppressor mechanisms. In the case of cancer vaccines, this was mediated through the provision of immune activating signals, optimal antigen processing and presentation, and generation of significant antitumor responses. However, it was found that sufficient numbers of tumor-specific immune effector cells may not necessarily correlate with tumor regression in pre-clinical studies or objective responses in clinical trials.

What may not be entirely surprising is that tumors have evolved mechanisms to inherently evade and suppress antitumor immune responses. It is apparent that current paradigms of cancer vaccine design should now also consider the elimination of inhibitory factors and prevention of immunoresistant phenotypes. Mechanisms of self-tolerance, tumor-induced immunosuppression, and immune selection and escape will now be described.

1-5.1 Central and Peripheral Tolerance

If the tumor antigen is self-derived (ex. TAA), the generation of effective antitumor responses requires the breaking of self-tolerance. These are mechanisms by which the host prevents the immune system from attacking self-tissue. Tolerance is generally divided into two broad categories: central tolerance and peripheral tolerance. Central tolerance is conducted in two stages (positive and negative selection) during the differentiation of immature lymphocytes

in primary lymphoid organs. If maturing T cells can bind to surface MHC molecules expressed by thymic epithelial cells, it does not undergo programmed cell death (positive); however, if thymic epithelial cells display self-antigens on MHC to developing T cells, the ones with high-affinity TCRs for self-antigens are removed from the T cell repertoire (negative) [167]. Any self-reactive T cells that escaped central tolerance are regulated in the periphery through several mechanisms including anergy, activation-induced cell death (AICD), and peripheral suppression by natural thymus-derived Tregs [168].

1-5.2 Tumor-induced Immunosuppression

Tumors can subvert endogenously- or exogenously-induced antitumor immunity by a variety of immunosuppressive mechanisms. Secretion of paracrine mediators such as VEGF-A [169, 170], adenosine [171], prostaglandin E2 (PGE2) [172, 173], IL-10 [174, 175], and TGFβ [176-178] can indirectly inhibit T-cell penetration into the tumor bed, suppress effector T cell activation, and promote Treg function. Furthermore, these factors may inhibit DC differentiation and maturation through suppression of nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) signaling [179, 180]. Cancer-associated fibroblasts can also promote the recruitment of immunosuppressive cells through the secretion of CCL2 and CXCL2 and suppress effector T cell function through TGFβ [181-183].

Tumor cells may also directly up-regulate surface ligands which can mediate T-cell anergy by binding to inhibitory T-cell receptors. Programmed death 1 (PD-1) protein is such a receptor with distinct biological function and ligand specificity and is expressed on activated T cells. It has two known ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), the former being selectively expressed on many tumors and cells within the tumor microenvironment in response

to inflammation. PD-1 ligation inhibits cytokine production and cytolytic activity from tumor-infiltrating antigen-specific CD4+ and CD8+ T cells [16, 184-189]. Though controversial, another mechanism of tumor-induced immunosuppression is the expression of death receptor ligands by tumor cells. A variety of cancer cells (lung carcinoma, melanoma, colon carcinoma, and hepatocellular carcinoma) have been shown to express FasL, which induces apoptosis of Fas-susceptible target cells including activated T cells [190-197].

Tumors can also potentiate the infiltration and activity of immunomodulatory leukocyte subsets in order to suppress tumor antigen-specific T-cell responses. While IL-10-producing B cells, B regulatory cells, Type II NKT cells, NK cells, and γδ T cells have been implicated in the down-regulation of antitumor activity [181], Treg cells and myeloid lineage cells have received the most consideration in the design of effective cancer immunotherapeutics.

1-5.2a Regulatory T Cells

Previous observations have shown that the onset of cancer can often be correlated with an accumulation of Tregs in tumor-bearing hosts. Questions have arisen whether or not this could be attributed as a host response to endogenous antitumor immunity which is sensed as an auto-reactive immune response, or if the tumor is actively manipulating and accumulating Tregs to orchestrate its own defence against host immune surveillance. Studies indicating that there may be two populations of CD4+ regulatory T cells suggest the latter [198]. In contrast to natural thymus-derived Tregs which arise under homeostatic conditions as a safeguard against autoimmunity, adaptive Tregs (T_R1 cells) are induced during inflammatory processes like infection or cancer. Interestingly, while natural Tregs are characterized as CD4+ CD25+ FOXP3+ [198], T_R1 cells are characterized as CD4+ IL-10+ FOXP3- [199].

The tumor microenvironment can promote the accumulation of regulatory T cells through several mechanisms, including: trafficking, differentiation, expansion, and conversion. Tumor cells and cells within the tumor microenvironment express CC-chemokine ligand 22 (CCL22), which facilitates the migration of natural Tregs from the thymus, lymph node, bone marrow, and periphery to the tumor bed via CC-chemokine receptor 4 (CCR4) [200-202]. Furthermore, the secretion of various cytokines and growth factors (ex. IL-10, TGFβ, VEGF) can suppress the differentiation/activation of DCs, which in turn induces the differentiation and expansion of regulatory T cells [203, 204]. Lastly, the secretion of TGFβ may be responsible for the conversion of conventional CD4+ CD25+ T cells into CD4+ CD25+ FOXP3+ Tregs [205-207]. It should also be mentioned that the presence of tumor-infiltrating plasmacytoid DCs as well as IL-10 in the tumor microenvironment can play a contributing role towards the induction of T_R1 cells [203, 208, 209].

All things considered, the tumor microenvironment might contain natural and converted Tregs as well as T_R1 cells. These regulatory T cells require TCR triggering to become functional; however, once activated, they suppress T cells in a non-specific manner. Tregs can induce immunosuppression by a variety of mechanisms, including: secretion of immunosuppressive cytokines [210-213], competitive consumption of IL-2 [214-216], direct killing via perforin and granzyme pathways [217, 218], and direct subversion of antigen-presenting cell (APC) function through down-regulation of co-stimulatory molecules [200, 219-221]. T_R1 cells primarily suppress immune activity through the production of IL-10 [198].

Interestingly, active vaccination of patients with cancer may induce TAA-specific Tregs. This may not be surprising since Tregs can be considered as another type of antigen-specific T cell elicited during an immune response. It has been suggested that dysfunctional DCs that

express TAA might induce regulatory T-cell differentiation within the tumor microenvironment or within tumor draining lymph nodes [222-224]. Furthermore, while natural Tregs express FOXP3 but not IL-10 and TGFβ, and T_R1 cells express IL-10 but not FOXP3, TAA-specific Tregs may express both FOXP3 and IL-10 [198]. The expression of IL-10 from these cells has been hypothesized to play a profound role in suppressing APC and T cell function [225].

1-5.2b Myeloid-Derived Suppressor Cells (MDSCs)

Early studies focused on the characterization of immature myeloid cells (IMCs), which were comprised of immature macrophages, granulocytes, DCs and other myeloid cells at early differentiation stages. In mice, they are phenotypically defined as Gr-1+ CD11b+ cells [226]. These cells are normally present in the bone marrow and spleen of healthy mice where they eventually undergo differentiation into mature myeloid cells. However, during cancer, differentiation is partially inhibited and they accumulate at secondary lymphoid tissues as well as the tumor site [227-231]. They suppress antigen-specific T cell activity through a variety of mechanisms, including inhibition of IFNγ production by CD8+ T cells through direct cell-cell contact [230, 232], secretion of immune suppressive factors [182], induction of T cell anergy [233, 234], and promotion of Treg development [234]. Furthermore, Gr-1+ IMCs can differentiate into F4/80+ tumor-associated macrophages (TAM), which are able to inhibit T-cell-mediated immune responses by apoptosis (arginase 1 (ARG1) and NO) and suppression (signal transducer and activator of transcription 1 (STAT1)) [235]. More interestingly, the release of immune suppressive factors within the tumor microenvironment may expand another IMC population with suppressive capacity: myeloid-derived suppressor cells.

Recent studies demonstrate that MDSCs consist of two main subsets: polymorphonuclear (PMN-MDSCs) and monocytic MDSCs (M-MDSCs). M-MDSCs are characterized by CD11b+ Ly6G-/Ly6C+ in mice and HLA-DR-, CD11b+, CD33+, CD14+ in humans. PMN-MDSCs are characterized by CD11b+ Ly6G+/Ly6C^{lo} in mice and HLA-DR-, CD11b+, CD33+, CD15+ in humans. In tumor-free mice, these subsets are referred to as polymorphonuclear neutrophils and inflammatory monocytes, respectively. PMN-MDSCs comprise the majority of MDSCs in cancer despite M-MDSCs having a longer lifespan and higher proliferative capacity. Since the expansion of M-MDSCs was barely detectable in cancer, it was postulated that PMN-MDSCs may be replenished from M-MDSCs. It has been shown that monocytes differentiate into DCs and macrophages in non-pathological conditions, but preferentially differentiate into PMN-MDSCs in a tumor environment. This suggests that during cancer, regular monocyte differentiation is subverted in order to generate PMN-MDSCs from M-MDSCs. Extensive investigation correlated this process with the loss of retinoblastoma protein (Rb1) in MDSCs, which is coupled with the recruitment of histone deacetylase 2 (HDAC2) to the Rb1 promoter. [236-238]

MDSCs migrate to the tumor site via CCL2, CXCL12 and CXCL5 [239] and orchestrate a variety of immunosuppressive processes to inhibit tumor-specific immune attack [240]. Activated MDSCs produce high levels of inducible nitric oxide synthase (iNOS) and ARG1. This in turn increases the production of urea and accelerates the depletion of essential amino acids in the tumor microenvironment [241]. For instance, a deficit of L-arginine and cysteine can inhibit T cell proliferation and activation, respectively [242]. MDSCs also increase intratumoral levels of NO and ROS [243]. NO inhibits E-selectin expression on endothelial cells and thus obstructs T cell recruitment to the tumor [244]. In addition, NO and ROS are associated with

peroxynitrite production, which in turn causes nitration of TCRs and suppression of CD8+ T cell responses [245, 246]. PGE2 is the main receptor found in MDSCs and up-regulates ARG1 production as well as MDSC recruitment to the tumor [247-250]. Lastly, MDSCs are also a major source of TGFβ production, which promotes tumor cell invasion/metastasis and induces anergy in immune effector cells (via membrane-bound TGFβ1) [251, 252].

1-5.3 Immune Selection and Escape

As previously described, tumor cells can evade host immunosurveillance through successful immunoediting. The process of Darwinian selection and tumor escape does not only occur during endogenous host immune responses against increasingly malignant tumors. It can be applied to suboptimal or partially successful antitumor immunotherapies as well. In the context of cancer, natural selection is a process by which the survival of individual tumor cells is dependent on genetic and epigenetic traits that can confer a survival advantage. The outcome of this selection process is determined by multiple factors, including growth factors, nutrient supply, and immune pressure [253]. Several of these outcomes will be described below.

1-5.3a Loss or Down-Regulation of MHC Class I Antigens

Descriptions of MHC loss have had a great deal of intuitive appeal [254], but were correlative and indirect. At present time, there is little controlled evidence in humans or animals that a loss of MHC class I molecules can lead to immunoresistance and increased incidence of spontaneous tumors in unmanipulated hosts. However, studies in mice (with pre-existing immunity induced by immunization) [255] and humans (with partial responses to immunotherapies) [256] have shown that recurring tumors can down-regulate MHC class I expression. Dysfunctional antigen processing machinery has been implicated with a loss of

MHC. For instance, defects in antigen processing components (ex. proteosome multicatalytic complex subunits, low molecular mass protein (LMP) 2 and 7) or peptide transporters (ex. transporter associated with antigen processing (TAP) 1 and 2) were shown to induce MHC class I down-regulation [257-260]. Additionally, mutations in one copy of the β2-microglobulin in association with loss of heterozygosity (LOH) involving the second allele on chromosome 6 have resulted in loss of the MHC class I haplotype [261].

1-5.3b Loss of Tumor Antigens and Immunodominance

Loss of surface antigen expression can occur independently from MHC class I dysfunction. Due to the heterogeneity of tumor antigen expression within the same tumor, immunological pressures may promote the proliferation of non- or low-antigen expressing tumor cells and lead to disease progression. Unfortunately, the exact mechanisms of tumor antigen down-regulation are not known; however, outgrowth of antigen loss variants may be facilitated by epitope immunodominance, which is defined as the preferential detection of one or a few epitopes among many on a given target [262]. Antigen loss variants within a tumor are shielded from immune pressure because parental tumor cells carry the immunodominant epitope and thus divert immune attack away from variant cells. Elimination of the parental cell establishes a new hierarchy of immunodominant epitopes among the remaining subpopulations.

1-5.3c Defective Death Receptor Signaling

The expression of death receptor ligands FasL and TRAIL plays a large role in immunosurveillance [107, 263-265]. Death receptor ligation can engage cytoplasmic sequences known as "death domains" that transmit apoptotic signals via caspase cascades. Down-regulation or loss of Fas receptors, which are expressed on tumors, may contribute to their resistance to

apoptosis. Missense mutations and loss of gene mutations [266-268] as well as inactivating mutations of downstream Fas signaling [269, 270] have been implicated as potential causes. In the case of TRAIL-mediated apoptosis, chromosomal loss may lead to a loss of caspase-8 expression, while Fas-associated protein with death domain (FADD) mutation may result in lack of signaling from DISCs [271]. Lastly, low expression of death receptors by post-transcriptional regulation can be associated with tumor resistance to TRAIL-mediated apoptosis [271].

1-6 The Prospects of Combination Therapy

The scientific rationale behind most cancer immunotherapeutic strategies fails to account fully for the seventh hallmark of cancer: immune escape. Tumors employ a myriad of mechanisms in order to suppress, subvert, and evade immune attack. As previously stated, optimal cancer immunotherapy should thus account for these mechanisms while inducing sufficient stimulation of tumor-specific effector responses. More recent vaccination modalities have begun to take advantage of this concept. Some pleiotropic chemotherapeutic agents such as cyclophosphamide have been combined with cancer vaccines to induce a tumor-specific immune attack with multiple contingencies. For instance, cyclophosphamide has been shown to induce direct cytotoxicity, deplete immunosuppressive Tregs, activate and mediate the proliferation of T and B cells, and promotes T cell infiltration into the tumor [272-275]. Monoclonal antibodies to CTLA4 and PD-1 as immune checkpoint blockers have been combined with vaccines to induce antitumor responses in many poorly immunogenic tumor models [276-279]. Lastly, chemotherapeutics such aspaclitaxel (TAX), cisplatin (CIS), and doxorubicin (DOX), in combination with several cancer vaccines and even adoptive T cell transfer approaches have resulted in the sensitization of tumor cells to tumor antigen-specific immune attack [280] and even resulted in bystander killing of non-targeted tumor cells [281]. Consequently, there is

therapeutic value in a combinatorial approach. Based on what was previously discussed, there is now also increasing interest in utilizing oncolytic viruses in combination with cancer immunotherapeutic modalities to synergize antitumor attack.

1-7 The Therapeutic Potential of Oncolytic Viruses

Oncolytic viruses (OV) can selectively infect, replicate in, and kill tumor cells (proliferating and noncycling) with minimal impact on normal tissue. They encompass human (ex. herpes simplex virus (HSV), adenovirus (Ad), measles virus (MV)) and veterinary (vesicular stomatitis virus (VSV), Newcastle disease virus (NDV) myxomavirus (MYXV)) viruses, are inherently or artificially oncotropic, and induce minimal pathology [282]. Viruses with inherent oncotropism express surface receptors for binding and entry which may be aspecific or specific to a malignant phenotype [283, 284]. In order for the oncotropic virus to persist, the tumor must be replication-permissive. This can occur through defective IFN and dsRNA-activated protein kinase (PKR) response pathways [285, 286], aberrant cell cycle control [287], resistance to apoptosis [288], or constitutive activation of Ras or Akt [289] .

OVs can mediate tumor killing by a variety of documented mechanisms. Many cancer cells have undergone adaptation, typified by uncontrolled entry into S-phase, disruption of apoptotic and p53 pathways, loss of the ability to produce and/or respond to innate immune effectors, and evasion of cell-mediated immunity [19]. The cellular changes induced by viral infection are often similar to the cellular changes acquired during carcinogenesis [290]. Since OV can encode proteins that induce these processes or acquire improved replicative capacity as a result of these processes, tumor cell death may thus be a direct by-product of the lytic viral replication cycle [291]. Conversely, OVs may also produce apoptosis- or necrosis-inducing viral

proteins (ex. adenoviral protein E3, 11.6K, and E4ORF4) as well as induce autophagic cell death to facilitate its own replication and persistence [292, 293]. Lastly, oncolysis can indirectly induce tumor necrosis through the induction of tumor vasculature shutdown [294, 295].

More often, genetic engineering of OVs may facilitate improved oncotropism, allowing for improved viral targeting to tumor cells (coat protein alteration, masking of ligands, redirecting), reduced virulence in normal tissues (virulence factors, genes required for replication in normal tissues), and insertion of regulatory elements in viral genes (suicide genes) [291]. OVs may also be engineered/armed to further improve their killing capacity through direct bystander mechanisms. The most common approaches either incorporate a toxic transgene or pro-drug converting enzyme (ex. HSV-thymidine kinase (TK), cytosine deaminase-5-fluorocytosine (CD/5-FC)) [291]. Engineered OVs may also express pro-apoptotic proteins (TRAIL, IL-24) [296-298], tumor-suppressors (p53, p16, SOCS3) [299-301], small hairpin RNA targeting factors involved in cell survival or proliferation (ex. Ki67, survivin) [302, 303], and anti-angiogenic proteins [282]. Interestingly, the amenability of OVs toward genetic engineering and their inherent capacity to target, replicate in, and kill tumor cells can be utilized to drive extensive antitumor immune responses.

1-8 Oncolytic Immunotherapy: The Marriage between Oncolytic Virotherapy and Cancer Immunotherapy

Since cancer cells are not detected or are tolerated by host immunity, the induction of local inflammation promotes a microenvironment that favors the activation of immune cells and breaking of immune tolerance. Early studies proved that tumor cell transfection with genes encoding cytokines, chemokines, or interferons can lead to aggressive immune-mediated tumor

rejection [117, 304-306]. Accordingly, oncolytic viral infection and replication can create an inflammatory storm that arouses both innate and adaptive immune responses against the tumor. OVs are also a source of immunogenic danger signals such as PAMPs (dsRNA, unmethylated CpG motif DNA) and DAMPs (HMGB1, HSP27/70) which play a role in the initiation of DC-mediated antigen uptake and presentation [282]. Consequently, intratumoral injection of potently immunogenic replication-competent viruses may be seen as an effective method of inducing multiple inflammatory cytokines and signals at the tumor site.

To further propagate these effects and promote antitumor immunity, OVs have been engineered to express immunostimulatory cytokines/chemokines to enhance their potential for eliciting antitumor responses while retaining their ability to selectively replicate within the tumor. In clinically advanced OVs (ex. JX-594 [307], Oncovex [308]), GM-CSF is the gene that has been inserted most successfully which allows for the recruitment and differentiation of activating DCs in the tumor microenvironment. IFNα/β, though implicated in antiviral immune responses, has also shown to support antitumor immune activation when expressed as an OV transgene [309-312]. Alternative attempts to potentiate OV-mediated antitumor immunity in a non-specific manner include co-injection of OVs with immature DCs [313] and OVs with anti-CTLA4 antibodies [279].

As was previously alluded to, OVs can contribute to the induction of adaptive antitumor immune responses. The process of viral oncolysis may lead to the release of TAA/TSAs from dying tumor cells. Virally-induced inflammatory cytokines and signaling may recruit immunostimulatory cells to the tumor bed, including DCs, which can take up TAA/TSA and cross-present them to adaptive immune cells, inducing a tumor-specific CTL response. These cells may be able to recognize and destroy any remaining tumor cells not already killed by the

OV and may provide long-term tumor protection. The broad applicability of this process is evident in the sheer range of oncolytic viruses that have been reported to induce adaptive antitumor immunity [282, 291, 314].

It has been well documented that one of the most efficient ways to prime a T cell response towards a tumor antigen is to engineer a viral vector to express that particular tumor antigen. To take full advantage of oncolytic capacity of OVs as well as their ability to elicit tumor-specific antitumor immunity, OVs have been utilized as viral vectors for cancer immunotherapy. In this system, expression of the target antigen occurs at the time and site of the inflammatory reaction. Furthermore, the OV can spread from the tumor bed through infected antigen presenting cells to express tumor antigen in secondary lymphoid tissue. Oncolysis may also facilitate significant tumor debulking, allowing antitumor responses to be more effective due to the reduction of the tumor burden and breaking of tolerance [315]. While multiple parameters are involved in the selection of immunogenic tumor antigens, OVs have been engineered to express artificial tumor antigens (ex. β-galactosidase) [316], foreign tumor antigens (ex. ovalbumin (OVA)) [317], and even tumor-associated xenogeneic antigens (ex. human dopachrome tautomerase (hDCT)) [318]. Emergence of oncolytic viral vectors as a means of cancer vaccination formed the basis for what would be known as "oncolytic immunotherapy".

1-9 Prime-Boost Strategy for Oncolytic Immunotherapy

One of the challenges facing oncolytic immunotherapy is the competitive immunogenicity of transgenes encoding the tumor antigen versus viral antigens specific to the oncolytic vector. This competition is inherently biased in cases where the transgene is an autologous tumor antigen against which the host is fully tolerized; by contrast, viral antigens

expressed in the oncolytic vector are highly immunogenic. As such, there is a higher precursor frequency of cells specific for viral epitopes, and virus-specific T cells will possess higher affinity TCRs which will allow them greater opportunity to interact with APCs [319]. In previous studies, systemic vaccination with recombinant VSV encoding the xenogeneic TAA, hDCT, was not able to induce robust CD8+ T cell responses against the transgene [318]. It was postulated that the immunodominant epitopes from the transgene ($DCT_{180-188}$) and the virus (RGYVYQGL) shared the same K^b allele, resulting in clonal competition of antitransgene and antiviral CD8+ T cells [318, 320]. Unsurprisingly, VSV-hDCT induced a greater CD8+ T cell response towards the viral peptide [320].

Interestingly, the authors created a novel system whereby vector-biased immune attack could be subverted through a treatment modality known as heterologous "prime-boost". In a highly aggressive, intracranial, murine melanoma (B16) challenge model, mice were initially vaccinated ("primed") with recombinant Ad expressing hDCT (Ad-hDCT). After several days, a heterologous ("boost") was given through systemic delivery of recombinant VSV expressing hDCT (VSV-hDCT). The boosting response elicited massive expansion of hDCT-specific effector T cells, resulting in some durable cures [3]. Furthermore, substantive immunity was generated against additional antigens (epitope spreading) and the immune response to VSV was dampened [3].

It is apparent that Ad-hDCT priming established an early population of hDCT-specific memory T cells within the host [320]. Administration of VSV-hDCT as a boosting agent then induced a secondary response against the transgene while eliciting a primary response against the oncolytic vector [3]. Consequently, the subsequent induction of massive and rapid expansion of the hDCT-specific memory T cell population inverted the polarity of competitive

immunogenicity to favor a tumor-specific immune response [320]. As a therapeutic platform for anticancer therapy, its effects are multifold: 1) The OV can mediate direct and indirect tumor killing; 2) OV replication can alter the tumor microenvironment in a pro-inflammatory manner 3) Systemic administration of the OV is likely to be effective against metastatic disease; 4) OV expression of the tumor transgene can mediate adaptive antitumor immunity; 5) Heterologous prime-boost expression systems concentrate immune attack on the tumor and not the vector. Since oncolytic virotherapy and cancer immunotherapy are amenable to synergistic therapeutic outcomes, there may be investigative and clinical value to re-investigating other anticancer therapies in the context of combination therapy.

1-10 Altering IFN Responsiveness during Oncolytic Immunotherapy

Although oncolytic immunotherapy in the context of prime-boosting can demonstrate synergistic interplay between direct oncolysis and induction of antitumor immunity, one important caveat needs to be addressed. As previously mentioned, one of the mechanisms which define the tumor selectivity of OVs is the permissiveness of cancer cells towards viral replication. This can be acquired through defects in innate antiviral responses, such as the Type I IFN pathway [285, 286]. Thus, IFN-sensitive viruses such as VSV are capable of selectively replicating in cancer cells while sparing normal tissue [321, 322]. Unfortunately, IFN responsiveness is variable across cancer cell lines and patient tumors, suggesting that the replicative efficiency and oncolytic capacity of OVs is case-dependent [323, 324]. Since OVs have also been shown to induce larger boosting responses in tumor-bearing animals, the persistence of virus within the tumor may correlate with successful antigen presentation and recruitment of tumor-specific T cells. As a result, the IFN responsiveness of tumor cells may also impact the efficacy of OV-induced immune responses.

Recent studies have shown that histone deacetylases (HDACi) can play a modulatory role in the expression of innate antiviral genes. Consequently, it may be possible to increase the susceptibility of tumor cells to viral oncolysis in order to potentiate cancer immunotherapy.

1-11 Histone Acetyltransferases (HATs) and Histone Deacetylases (HDACs) as Targets for Gene Regulation

Histone proteins organize DNA into repeating structures of chromatin called nucleosomes. The acetylation status of histones alters chromatin structure and regulates gene expression on an epigenetic level [325, 326]. Two classes of enzymes can affect histone acetylation status: histone acetyltransferases (HATs) and HDACs [327].

Nucelosomes contain 146 base pairs of DNA wrapped around a core histone octamer which is composed of an H3-H4 tetramer and two H2A-H2B dimers. These proteins are highly conserved and each contains a lysine rich amino (N)-terminal tail which is the site of post-translational modification. The N-terminal histone tail is enveloped by the DNA double helix and modification of these structures by acetylation or deacetylation affects the interaction of DNA with transcription-regulatory non-nucleosomal protein complexes. [328]

HATs can be divided into several families on the basis of highly conserved structural motifs (ex. Gcn5-related *N*-acetyl transferase (GNAT) family [329-333]). These families can be further subdivided into Type A HATs, which are involved in the regulation of gene expression, and Type B HATs, which are involved in the assembly of nascent histones into chromosomes. HATs engage in complex association patterns with protein complexes that can include other HATs, transcriptional co-activators and co-repressors in order to regulate gene expression [328]. This results in the acetylation of specific histone lysine substrates by transfer of an acetyl group

from acetyl CoA to form ε-*N*-acetyllysine. The process neutralizes the positively charged lysine residues and reduces their affinity for DNA, unwinding the nucleosomal array and allowing for gene transcription to occur [334]. HATs may also target non-histone protein substrates, including transcription factors and are termed, factor acetyltransferases (FATs) [335].

The acetylation status of chromatin is also dependent on HDAC activity. Four classes of HDAC have been described depending on yeast homology; however, Class I and II are considered "classical" HDACs due to their mechanism of action. Class I human HDACs (HDAC 1, HDAC2, HDAC3, and HDAC8) are found within the nucleus and seem to be ubiquitously expressed in human tissues [327]. Class II human HDACs (IIa: HDAC4, HDAC5, HDAC7, HDAC9; IIb: HDAC6, HDAC10) have been shown to localize either in the nucleus or cytoplasm, suggesting a role in the deacetylation of nonhistone proteins [328]. As with HATs, Class I HDACs are also constituents of multiprotein transcriptional complexes which include nuclear-hormone corepressors (NCOR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) [336, 337]. Class I and II HDACs possess highly conserved catalytic domains and deacetylate histone lysine substrates by activating a water molecule with a divalent cation in cooperation with histidine-aspartate residues. The removal of charge-neutralizing acetyl groups from histone lysine tails results in the compaction of chromatin structure and repression of gene transcription [336].

Tumorigenic mutations can modify the expression of genes (ex. Ras, p53) that are normally controlled by epigenetic modification. Genetic abnormalities can also impact HATs/HDACs directly and affect their targeting to certain loci [338]. Since cell development and differentiation is governed by sequential gene activation, disruptions in chromatin remodelling can induce the proliferation of undifferentiated cells and cancer. It has been

proposed that restoration of epigenetic control over endogenous differentiation and apoptotic programmes can be achieved using histone deacetylase inhibitors (HDACi) [328, 338]. Consequently, the original application of HDACi was in transcription-based anticancer therapy.

1-12 The Therapeutic Potential of Histone Deacetylase Inhibitors (HDACi)

HDACi have shown much promise as direct anticancer agents and many have progressed to clinical development [339-349]. HDACi can impact several cellular processes that are dysregulated in neoplastic cells. Induction of cell cycle arrest (G1/S, G2/M) results in a disruption of normal differentiation programmes and leads to cytostatic effects [345, 350, 351]. Direct treatment with HDACi can directly induce tumor cell death through the activation of death-receptor and intrinsic (ex. mitochondrial death pathway [352]) apoptotic pathways and activation of caspase cascades. Furthermore, HDACi have anti-angiogenic (ex. down-regulation of VEGF, basic fibroblast growth factor (bFGF), hypoxia-inducible factor 1 (HIF-1), etc.) and anti-invasive (ex. transcriptional repression of matrix metalloproteinases (MMP) 2 and 9) effects *in vitro* and *in vivo* can obstruct tumor development [353-358]. Finally, HDACi can induce apoptosis through indirect regulation of gene expression by modulating the activity of transcriptional factors (E2F1, p53, STAT1/3, and NFκB) [359-363] as well as expression-independent mechanisms. Taken together, it is apparent that the effects conferred by HDACi are as varied as the types of HDACi known, suggesting that there is a correlation between HDACi type and their function.

HDACi can be broadly characterized by a common pharmacophore which includes key elements of inhibitor-enzyme interactions [364]. This includes a hydrophobic cap that blocks the entrance to active site, a polar site, and a hydroxamic acid type zinc-binding active site separated

by a hydrophobic spacer spanning the hydrophobic pocket on the enzyme [365, 366]. The common mechanism of these drugs is to bind a critical Zn^{2+} ion required for the catalytic function of HDACs [367, 368]. With a few exceptions, HDACi can be divided into specific structural classes, including: carboxylates/short-chain fatty acids (valproic acid, sodium butyrate, 4-phenylbutyrate), small-molecule hydroxyaminic acids (suberoylanilide hydroxamic acid (SAHA), pyroxamide, trichostatin A (TsA), oxamflatin, and cyclic hydroxamic acid-containing peptides (CHAPS)), electrophilic ketones (epoxides), cyclic tetrapeptides (trapoxin, apicidin, and depsipeptide), benzamides (MS-275 and CI-994), and other hybrid compounds. The important clinical implication of these structural variants is their unique specificity and potency for HDAC isoenzymes and effects on the acetylation of nonhistone substrates, resulting in broad efficacies, toxicities, and therapeutic uses [364]. In particular, synthetic benzamides have shown significant promise for anticancer therapy and have been explored further due to its antiviral and anti-inflammatory properties.

1-12.1 Benzamides and MS-275 (Entinostat)

These compounds consist of structurally diverse agents that possess a benzamide moiety and inhibit HDAC activity at a micromolar range [369]. As with other HDACi, it is postulated to enter the catalytic site and bind the active zinc; however, it is unclear whether or not this binding is reversible (ex. SAHA) or irreversible (epoxides). Diaminophenyl groups expressed on benzamide HDACi may be essential for optimum activity and have been suggested as potential chelators of the metal ion in the catalytic site [370]. Several compounds have been described as members of this group (MS-275, CI-994, etc.) and are currently in clinical trials for the treatment of several cancers [14, 371-373].

MS-275 (2-aminophenyl-4-[n-pyrydin-3-metyloxycarbonyl]-(aminomethyl)-[benzamide]) is a newly synthesized benzamide derivative that preferentially inhibits HDAC1 with a median inhibitory concentration (IC50) of 0.3 μM, HDAC3 with an IC50 of ~ 8 μM, and has no inhibitory effect against HDAC8 [343, 374]. It has been clinically evaluated in phase I clinical trials as treatment against refractory solid tumors, leukemias, and lymphomas [13-15, 375, 376]. Oral administration of the drug showed attractive safety/efficacy profiles and a long half-life (39-80 hours).

It is the first HDACi to be discovered with oral anticancer activity (associated with increased expression of CDKI $p21_{CIP1/WAF1}$ and accumulation of cells in G1-phase in preclinical models) [377]. It has been found to inhibit tumor proliferation in several cancer lines including breast, colorectal leukemia, lung, ovary, and pancreas [377], and is associated with an extensive gene induction ($p21_{WAF1}$, gelsolin, metallothionein, histone H2B) and repression (thymidylate synthase, importin-b, c-myc) profile [351]. In human breast cancer and pediatric solid tumor cell lines, it has been postulated that HDACi-mediated antitumor activity is dependent on the induction of TGFβ-receptor expression and tumor suppressor activity [378, 379]. In hematological malignancies, MS-275 is associated with the activation of death receptor pathways through induction of TNF-related apoptosis-inducing ligand (TRAIL) and FasL as well as up-regulation of co-stimulatory molecules such as 4-1BBL [12, 380].

Aside from direct tumor control, HDACi can mediate extensive anti-inflammatory processes. Pre-clinical studies have shown that HDACi therapy can ameliorate inflammatory/autoimmune diseases, enhance allograft survival, and induce immune tolerance in graft-versus-host disease [8-11]. MS-275 is currently under investigation as an immunosuppressive drug. In rodent models of rheumatoid arthritis (RA), MS-275 was able to

mediate growth arrest of RA synovial fibroblasts, inhibit pro-inflammatory cytokines and NFκB signaling, and down-regulate angiogenesis and matrix metalloproteinases [381].

HDACi have also been shown to suppress innate cellular antiviral responses by down-regulating Type I IFN and IFN-stimulated genes [4-6]. It was postulated that these molecules could thus decrease the IFN responsiveness of tumor cells towards OV replication, giving rationale to combine HDACi with oncolytic virotherapy. Indeed, the combination of oncolytic HSV with TsA or valproic acid enhanced HSV oncolysis in squamous cell carcinoma cells and human glioma cells, respectively [382, 383]. Also, the antitumor effect of telomerase-specific, replication-selective adenovirus (OBP-301) in human lung cancer cells was enhanced by a lesser known HDACi known as FR901228 [384]. HDACi have been combined with VV and semliki forest virus to induced heightened replication as well [385]. MS-275 was also able to dampen cellular IFN responses and augment OV-induced apoptosis. VSV-Δ51, an attenuated oncolytic VSV mutant which is incapable of blocking IFN production was able to synergize with MS-275 to induce heightened cell death and increased viral output [5]. Consequently, we postulated whether the addition of MS-275 to boosting oncolytic immunotherapy could further synergize the immune-potentiating roles of viral oncolysis to promote heightened tumor regression.

1-13 Combination Oncolytic Immunotherapy with MS-275

We proposed that MS-275 could provide an attractive solution to increase the amenability of IFN-responsive tumor lines to oncolytic immunotherapy; however, due to the pleiotropic effects of HDACi, it is also possible that the immunosuppressiveness of MS-275 could actually dampen the tumor-specific immune response. Therefore, in a highly aggressive, intracranial, murine melanoma model, it is unclear whether the addition of MS-275 in the context of prime-

boosting using recombinant viral vectors expressing the tumor antigen (Ad-hDCT and VSV-hDCT, respectively) will enhance viral oncolysis at the expense of optimal development of antitumor immunity and whether or not combination therapy will favor enhanced tumor clearance.

The study allowed us to make some very interesting observations: 1) MS-275 given alone does not prolong survival relative to control mice; 2) MS-275 modestly prolonged VSV replication in the tumor; 3) Priming responses were significantly abrogated if MS-275 was co-administered with Ad-hDCT 4) Boosting responses were unaffected if MS-275 was co-administered with VSV-hDCT 5) Oncolytic immunotherapy in the context of prime-boost and MS-275 led to dramatically enhanced tumor-free survival compared to scenarios where the drug was not given [7].

It has been documented that DCs mature after being exposed to inflammatory signals such as Type I IFN [386-388]. Resulting mature DCs have improved antigen-presenting abilities, increased expression of co-stimulatory molecules, and acquire migratory potential to secondary lymphoid tissue. While unproven, it is possible that the administration of MS-275 during Ad-hDCT may have dampened the production of Type I IFN within the tumor microenvironment, resulting in a compromised priming response due to the inability of immature DCs to present antigen in the context of proper co-stimulation. Surprisingly, the boosting response was not abrogated, suggesting that the re-activation of hDCT-specific memory CD8+ T cells could occur in the absence of proper co-stimulatory signals. However, while early studies support the idea of secondary expansion in the absence of co-stimulation [389-393], more recent reports suggest that CD28 co-stimulation is essential [394-398]. Regardless, in this study we were able to generate a

"have your cake and eat it too" scenario whereby antitumor immune responses were not sacrificed in favor of oncolysis.

Interestingly, the combination of VSV and MS-275 did not significantly enhance viral replication as expected, but modestly prolonged its persistence within the tumor [7]. While it is clear that MS-275 had a synergistic role in mediating viral oncolysis in this situation, the extent of oncolysis was unclear and its effect on tumor debulking was also not elucidated further. The administration of MS-275 alone also did not seem to impact on overall survival [7], despite having direct anticancer benefits in other models. It may be that its direct effects could not be observed (intracranial model and thus tumor growth cannot be observed), or was negligible.

In this model, MS-275 was able to extend VSV-induced lymphopenia, resulting in the selective removal of non-hDCT specific lymphocytes such as naïve lymphocytes and Treg cells [7]. We postulate that removal of these cells creates an immunological niche for the expansion of tumor-specific effector T cells, which is a similar concept utilized in adoptive cell transfer therapy. A subsequent increase in homeostatic cytokines may allow for the generation of effector T cells with higher killing capacity. Furthermore, removal of Treg cells abrogates one of the main mechanisms by which cancer cells can suppress and escape the antitumor immune response. This corresponds with the seventh hallmark of cancer that Schreiber proposed.

Taken together, we have shown that the administration of MS-275 in the context of boosting oncolytic immunotherapy was able to extensively improve therapeutic outcomes despite only a minimal improvement in oncolytic viral replication. Since it is apparent that MS-275 did not drastically alter the magnitude of the antitumor response, our observations suggest a significant immunomodulatory role for MS-275 in the direct control of tumor growth.

1-14 Potentiation of Immunotherapy through HDACi-mediated Immunomodulation

The immunomodulatory properties of HDACi have been documented in the literature. They can transcriptionally activate MHC class I and II proteins, co-stimulatory molecules (CD40, CD80, and CD86), and intracellular adhesion molecule (ICAM1) to augment immune cell recognition and activation [399, 400]. It has also been demonstrated that HDACi induce the expression of MICA and MICB on tumor cells, which in turn can induce NKG2D-restricted cytotoxicity from NK cells [401, 402]. Furthermore, direct histone hyperacetylation may alter the activity of STAT1 [6], STAT3 [362], and NFκB [363], which are considered "master immune regulatory transcription factors". In the context of boosting oncolytic immunotherapy however, it is relatively unclear by which mechanism(s) MS-275 improves therapy and how tumor growth is affected.

Unfortunately, our current model is incapable of addressing the questions we would like to ask. Intracranial challenge models do not allow for direct observation of how MS-275 impacts tumor growth. The prime-boost regimen also carries technical limitations because the timing of vaccine administration (VSV-boosting 14 days post-Ad treatment) creates only a small treatment window in which the treatment is effective. Due to the length of treatment and aggressiveness of the tumor, the initial tumor burden must be small. This reveals a significant limitation regarding the potency of our treatment and hinders intratumoral analyses conducted at multiple time points. Furthermore, it will be difficult to conduct prime-boost vaccination in gene-deficient mice (ex. $Rag2^{-/-}\gamma c^{-/-}$ mice) for mechanistic studies (ex. elucidating the impact of lymphopenia on tumor control). Lastly, T-cell priming may induce adaptive tumor-induced mechanisms that may actively suppress the boosting response [16].

In our previous studies, we have demonstrated that oncolytic viruses such as VSV could be effectively used as a boosting agent for the expansion of tumor antigen-specific memory T cells derived from initial priming injections. In order to elicit rapid expansion of the secondary T cell response, it may be possible to extend the application of oncolytic boosting to adoptive cell transfer protocols. It has been previously reported that the differentiation state of transferred cells is inversely related to their capacity to proliferate, persist, and mediate antitumor effector responses [81]. Furthermore, additional vaccination in the context of ACT may provide acute activation of transferred T cells to further improve their antitumor efficacy and prevent tumor adaptation [81]. Taken together, treating an intradermal or subcutaneous challenge model with adoptively transferred memory T cells, VSV-boosting, and MS-275 administration not only allows us to examine the immunomodulatory effects of MS-275 on a visual and intratumoral level, but extends the usefulness of this treatment strategy to ACT therapies as well.

1-15 Initial Hypothesis:

Concomitant delivery of MS-275 in the context of boosting oncolytic immunotherapy can significantly enhance the magnitude of acute tumor regression. Furthermore, MS-275 may have immunomodulatory effects that can potentiate tumor antigen-specific immune responses.

1-16 Specific Aims:

1. To establish an intradermal tumor challenge model as well as a vaccination schedule that can elicit significant antitumor responses through VSV-mediated expansion of adoptively transferred memory T cells.

2. To observe the direct impact of MS-275 on tumor growth when administered concomitantly with adoptively transferred memory T cells and VSV-boosting.

3. To analyze the peripheral and intratumoral immunologic events that occur as a result of therapy in order to dissect the immunomodulatory mechanisms of MS-275

Chapter 2: Materials and Methods

2-1 Animals

Age-matched female C57BL/6 mice (6-8 weeks old) were purchased from Charles River Laboratory (Wilmington, MA) and housed in a specific pathogen-free facility. Male IL-15$^{-/-}$ mice (Taconic) were obtained internally through Dr. Ali Ashkar. Mice were caged and maintained under controlled lighting (12:12 L:D) and temperature (22°C) with *ad libitum* access to a low fat irradiated chow diet containing 18.6% protein, 6.2% fat, and 3.5% fiber (2918, Tekland Global Diets, Indianapolis, IN) and water. All animal studies complied with the Canadian Council on Animal Care guidelines and were approved by McMaster University's Animal Research Ethics Board.

2-2 Viruses

The Armstrong CA1371 strain of lymphocytic choriomeningitis (LCMV-Armstrong) which was used in this study was described previously [403]. Recombinant vesicular stomatitis virus (rVSV) of the Indiana serotype rVSV-ΔM51 possesses a deletion mutation in the coding region for the matrix (M) protein [3, 321]. rVSV-gp33 was constructed from a rVSV-ΔM51 vector and engineered to express the Db-restricted immunodominant CD8+ T cell epitope of LCMV GP33-41 [404]. The recombinant rAd5-GP33-ER (Ad-gp33) is an E1/E3-deleted human type 5 Adenovirus vector engineered to express the immunodominant CD8+ T cell epitope of LCMV-GP33-41 [405].

2-3 Peptides

The H-2D^b-restricted peptide of LCMV-GP (GP_{33-41}; KAVYNFATM) was purchased from the Dalton Chemical Laboratory (Toronto, Canada). Peptides were dissolved in distilled water and stored at −20°C.

2-4 Cells and Culture Conditions

B16-F10$_{gp33}$ (B16-gp33) cells were generated from the transfection of B16-F10 cells with a LCMV minigene corresponding to the LCMV GP33 epitope (M-KAVYNFATM) in the *Pin*AI and *Bam*HI restriction sites of the β-actin-driven expression vector pActin-IRES-TK-Neo (NTS) as described previously [406]. B16-gp33, B16-F10 and relapsed tumor (B16-relapse) cells were grown at 37 °C in a humidified atmosphere with 5% CO2 in F11-minimum essential medium containing 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 5 ml sodium pyruvate, 5 ml minimum essential medium nonessential amino acids, 5 ml vitamin solution, 55 μmol/l 2-mercaptoethanol, 100 U/mL penicillin, 100 ng/mL streptomycin, and (all cell culture reagents from Invitrogen, Grand Island, NY). G418 (800 μg/ml) was used to maintain gp33 expression in the B16-gp33 line.

2-5 Memory T (Tm) cells

C57BL/6 mice were infected with 10^5 plaque-forming units (pfu) of LCMV-Armstrong for one month. Spleens were collected and gently ground between microscope slides then filtered through a 0.22 μm filter to create a single cell suspension. Red blood cells were lysed using ACK lysis buffer (0.15 mol/L NH_4Cl, 10.0 m mol/L $KHCO_3$, 0.1 m mol/L Na_2EDTA, PH 7.2-7.4). The remaining bulk splenocytes were washed with phosphate buffered saline (PBS), centrifuged at 1500 rpm for 5 minutes, and resuspended in PBS. Tm was initially quantified in the bulk splenocyte population by flow cytometry using H-2Db-GP33 tetramer. Subsequent experiments

did not require quantification prior to injection and 10^5 Tm cells per spleen per mouse was assumed.

2-6 *In Vivo* Tumor Model

Mice were challenged intradermally with 10^5 B16-gp33 cells **(Appendix 1)**. Tumor growth was monitored daily and measured with calipers every other day. Tumor volume was calculated as width x length x depth. Tumor endpoint was defined as 10mm in at least two dimensions.

2-7 Vaccination Protocol

Anaesthetized mice were injected intravenously (i.v.) with 10^4 Tm cells in 30 µL of PBS on Day 4 post-tumor challenge. 24 hours later, $2x10^8$ pfu of VSV-gp33 was injected i.v. in 200 µL of PBS. After VSV injection, MS-275 was delivered intraperitoneally (i.p.) at 100 µg/mouse in 50 µL of PBS on a daily basis for five days **(Appendix 1)**. In some experiments, mice were depleted of lymphocyte populations using monoclonal antibodies (mAb) specific for CD8+ T cells (clone 53-6.72), CD4+ T cells (clone GK1.5), CD25+ T cells (clone PC61), or NK cells (clone PK136). Mice were injected with 250 µg of mAb in 500 µL of PBS on Day 4 and 6 post-tumor challenge and bi-weekly (every two weeks) afterwards.

2-8 Isolation of Tumor-infiltrating Leukocytes

Tumors were excised and digested in a mixture of 0.5 mg/mL collagenase Type I (Gibco), 0.2 mg/mL DNase (Roche) and 0.02 mg/mL hyalorunidase (Sigma) prepared in Hanks buffered saline (10ml/250mg of tumor) at 37°C for 1 hr. The digested material was filtered successfully through 40µM and 70µM nylon strainers and leukocytes were purified using

CD45.2 positive selection kits by magnetic selection according to the manufacturer's instructions (EasySep, Stemcell Technologies).

2-9 Intracellular Cytokine Staining (ICS)

Tumor-infiltrating leukocyte collection was described above, while peripheral blood mononuclear cell collection was obtained from blood obtained from the periorbital sinus. Red blood cells were lysed with ACK lysis buffer. Mononuclear cells from blood and tumor-infiltrating leukocytes from the tumors were stimulated with gp33 peptides (1 µg/ml) in the presence of 5 µg/mL brefeldin A (GolgiPlug; BD Pharmingen, 1 µg/ml) to prevent cytokine release. Cells were treated with 1:200 Fc block (αCD16/CD32 antibody, BD Biosciences) and stained with fluorescently labelled surface marker antibodies. Cells were then permeabilized and fixed with Cytofix/Cytoperm (BD Pharmingen) and stained with labelled antibodies with specificity for intracellular cytokines. Data were acquired using a FACS Canto flow cytometer with FACS Diva 5.0.2 software (BD Pharmingen) and analyzed with FlowJo Mac, version 6.3.4 software (Treestar, Ashland, OR). All antibodies used for flow cytometry were described in **Appendix Table 1**.

2-10 Tumor Transplantation

Relapsing tumors (B16-relapse) were excised and suspended in ethylenediaminetetraacetic acid (EDTA, Sigma) at 3 mL/g of tumor before being digested in a mixture of 3 mg/mL collagenase A (Roche) and 0.1% trypsin prepared in complete Roswell Park Memorial Institute 1640 medium (RPMI, Gibco; 10% FBS, 100 U/mL penicillin, 100 ng/mL streptomycin) (7 mL/g of tumor) at 37°C for 1 hr. The digested material was filtered through a 40µM strainer, washed and suspended in PBS for injection.

2-11 Polymerase Chain Reaction (PCR) and Gel Electrophoresis

Relapsing tumors (B16-relapse) were digested to form single cell suspensions and cultured *in vitro*. Genomic DNA was extracted from B16-relapse, B16-F10, and B16-gp33 cells using Purelink Genomic DNA Extraction Kits (Life Technologies) according to the manufacturer's instructions. PCR was carried out using Taq DNA polymerase with ThermoPol Buffer (New England Biolabs) on a T3000 Thermocycler (Biometra). The gp33 primer sequence is as follows: FWD – GTCCTTTGGGCGCTAACTGPCR, REV – GTGGCGAAATTGTACACAGC. The amplification product was run on a 1% UltraPure agarose gel (Life Technologies) with EZ-Vision loading buffer/dye (Amresco) and imaged on a UV transilluminator.

2-12 Tumor RNA Extraction and Quantitative Reverse-Transcription PCR (qRT-PCR)

Tumors were excised and snap-frozen in liquid nitrogen and stored at -80°C. Samples were then homogenized in Trizol (Invitrogen) using a Polytron PT 1200C (Kinematica). RNA was extracted and purified using an RNeasy mini kit (Qiagen) and treated with Ambion's DNA-free kit according to the manufacturer's instructions. Reverse transcription was performed with Superscript II First-Strand reverse transcriptase according to the manufacturer's instructions. Quantitative PCR was carried out on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using Perfecta SYBR Green Supermix, ROX (Quanta Biosciences). Data were analyzed via the ΔΔCT method using the Sequence Detector Software version 2.2 (Applied Biosystems). The primer sequences are as follows:

GZMB:

FWD – GGCCCACAACATCAAAGAAC,

REV – CCAGCCACATAGCACACATC;

HPRT:

FWD – ACACCTGCTAATTTTACTGGCAACA,

REV – TGGAAAAGCCAAATACAAAGCCTA (endogenous control).

2-13 Statistics

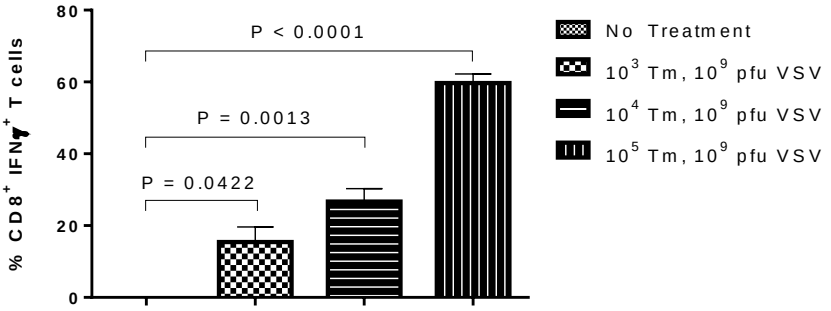
GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA) was used for graphing and statistical analyses. One-way and two-way analysis of variance (ANOVA) was used to query immune response data. All data were presented as means ± SE and differences between means were considered significant at $p < 0.05$. Error bars indicate 95% confidence intervals throughout. Survival data were analyzed using the Kaplan-Meier method and differences between groups were investigated using the log-rank test.

Chapter 3: Results

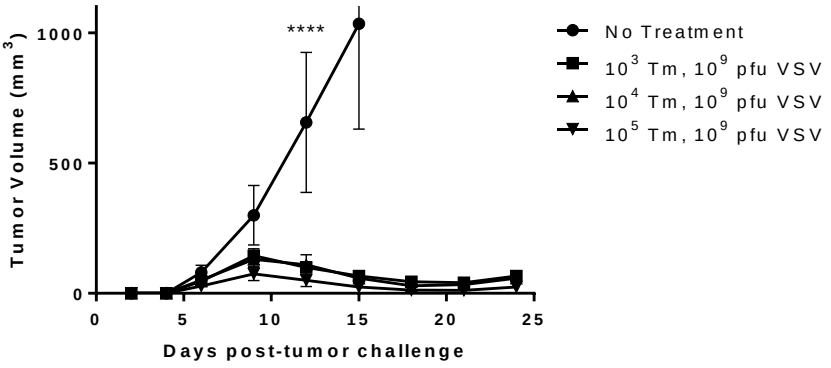
3-1 Gp33-specific CD8+ T cell Responses and Tumor Regression are Tm and VSV Dose-Dependent

To generate donor memory CD8+ T cells (Tm) with specificity for LCMV GP1 (H-2Db restricted epitope: gp33-41), C57BL/6 mice were immunized with 10^5 pfu LCMV-Armstrong. Bulk splenocytes were derived after approximately one month because we previously observed that the majority of gp33-specific effector T cells had differentiated into memory cells by that time (unpublished data). Tm cells were injected intravenously (i.v.) as a percentage of bulk splenocytes as determined by gp33-specific tetramer staining. In mice that were challenged intradermally (i.d.) with 5-day old B16-gp33 tumors and treated with Tm transfer and i.v. VSV-boosting, we were able to observe significant gp33-specific CD8+ T cell responses as early as five days post-treatment. The magnitude of the response could further be manipulated depending on the dose of transferred Tm (10^3-10^5 cells) and the dose of VSV-gp33 (10^6-10^9 pfu) **(Figure 1A, B)**. Indeed, through manipulation of these parameters, the antitumor response could be sufficient to induce significant, if not complete, tumor regression **(Figure 1C, D)**. Interestingly, very few transferred cells (as low as 10^3 cells) were sufficient to produce such an effect. As such, for our subsequent studies we opted to use a dose of Tm (10^4 cells) and VSV-gp33 ($2x10^8$ pfu) that would allow us to consistently observe acute regression, even at higher tumor burdens **(Figure 2A)**. Using depletion studies, we were able to demonstrate that CD8+ T cells were the primary mediator of acute tumor regression and that CD4 and NK1.1 depletion did not compromise our therapeutic efficacy **(Figure 2B)**. However, none of the mice showed sustained tumor regression. Within one month post-treatment, early tumor clearance was followed by a period of tumor equilibrium (where observable growth was not seen) and eventual tumor relapse

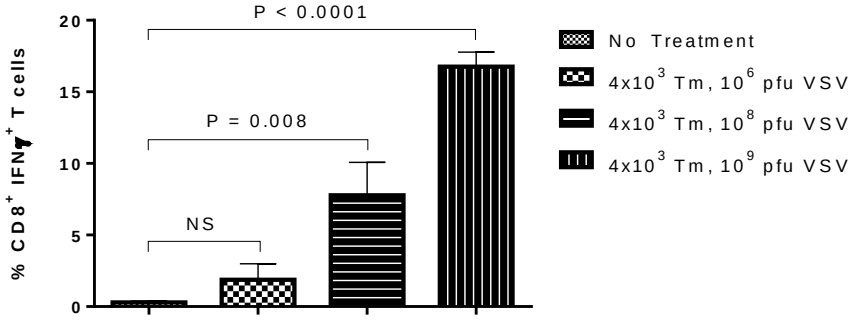
A


80
60
40
20
0
% CD8+ IFNγ+ T cells
P < 0.0001
P = 0.0013
P = 0.0422
No Treatment
10^3 Tm, 10^9 pfu VSV
10^4 Tm, 10^9 pfu VSV
10^5 Tm, 10^9 pfu VSV

B


1000
500
0
Tumor Volume (mm3)
****
0
5
10
15
20
25
Days post-tumor challenge
No Treatment
10^3 Tm, 10^9 pfu VSV
10^4 Tm, 10^9 pfu VSV
10^5 Tm, 10^9 pfu VSV

C



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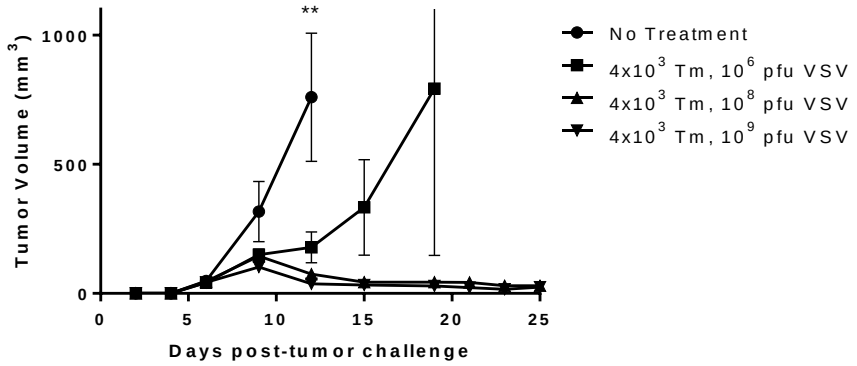
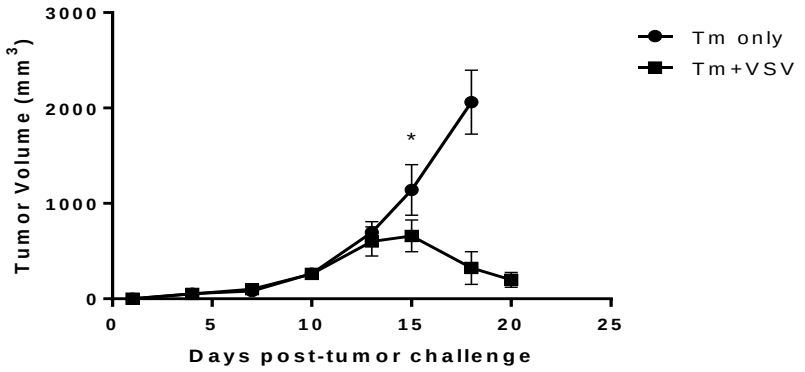


Figure 1: The magnitude of the gp33-specific antitumor response and the extent of vaccine-induced tumor regression are dependent on the dose of Tm and VSV-gp33. C57BL/6 mice (n=3 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. After varying the dose of Tm **(A)** and VSV-gp33 **(B)**, the frequency of antigen-specific CD8+ T cells was measured 5 days post-VSV-boosting in the peripheral blood of tumor-bearing mice by *ex vivo* peptide stimulation and FACs staining. The percentages represent the frequency of antigen-specific T cells relative to total CD8+ T cells. Tumor growth in Tm+VSV-treated mice was evaluated in mice that were given variable doses of Tm **(C)** and VSV-gp33 **(D)**. Tumor volumes were calculated based on height, width, and length. **** $p<0.0001$, ** $p<0.01$; Tm, memory T cell; VSV, vesicular stomatitis virus; FACs, flow cytometry; NS, not significant

A



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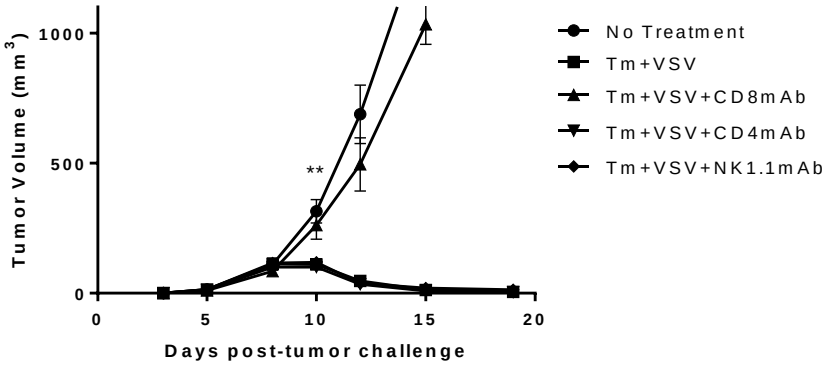


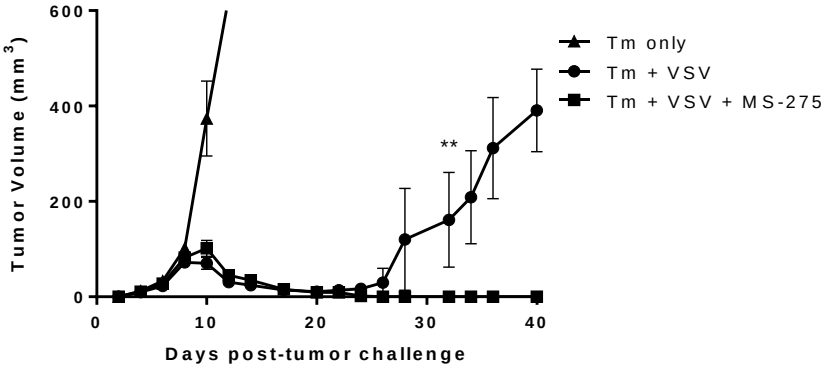
Figure 2: Tm+VSV can control higher tumor burdens in a CD8+ T cell dependent manner. **(A)** C57BL/6 mice (n=4 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 8 (i.v.) and VSV-gp33 (i.v.) injection at Day 9 post-challenge. **(B)** C57BL/6 mice (n=3-6 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. Depletion antibodies specific to CD8, CD4, and NK1.1 were injected (i.p.) one day before and after VSV-boosting (250 µg/mouse) and bi-weekly afterwards (150 µg/mouse). Tumor volumes were calculated based on height, width, and length. * $p<0.05$, ** $p<0.01$; Tm, memory T cell; VSV, vesicular stomatitis virus

(rapid growth of the tumor leading to endpoint) **(Figure 3A)**. In future studies, it may be interesting to determine if optimizing the antitumor immune response could prevent tumor relapse. By maximizing the dose of Tm and VSV-gp33, it may be possible to overwhelm tumor-induced immunosuppressive mechanisms that limit the efficacy of therapy. FACs analysis of tumor-infiltrating lymphocytes may also confirm whether increasing the magnitude of the gp33-specific response in the periphery translates into increased T cell migration into the tumor microenvironment.

3-2 Concomitant Administration of MS-275 to Boosting Oncolytic Immunotherapy Mediates Sustained Tumor Regression

Previous studies using the intracranial challenge model showed that concomitant administration of histone deacetylase inhibitor MS-275 with boosting oncolytic immunotherapy was able to significantly increase the extent of tumor protection[7]. We were able to recapitulate these results using an intradermal challenge model. In C57BL/6 mice, 5-day old established B16-gp33 tumors were subsequently treated with Tm (10^4 cells) at Day 4 post-challenge and VSV-gp33 ($2x10^8$ pfu) at Day 5 post-challenge. MS-275 (100 µg/mouse) was delivered intraperitoneally (i.p.) on the same day as VSV infection and further given on a daily basis for five days. With Tm+VSV alone the tumor regressed significantly before relapsing within one month post-treatment. Conversely, the addition of MS-275 promoted sustained tumor regression **(Figure 3A)**, resulting in 100% tumor-free survival for at least two months post-treatment **(Figure 3B)**. We were unable to assess if MS-275 could potentiate the magnitude or kinetics of acute tumor regression, because Tm+VSV alone regressed the tumor so significantly that it was difficult to observe improved early tumor killing. To determine the extent to which MS-275 enhances early tumor regression, we may adopt a sub-optimal dose of VSV-gp33 (ex. 10^6 pfu) to

A



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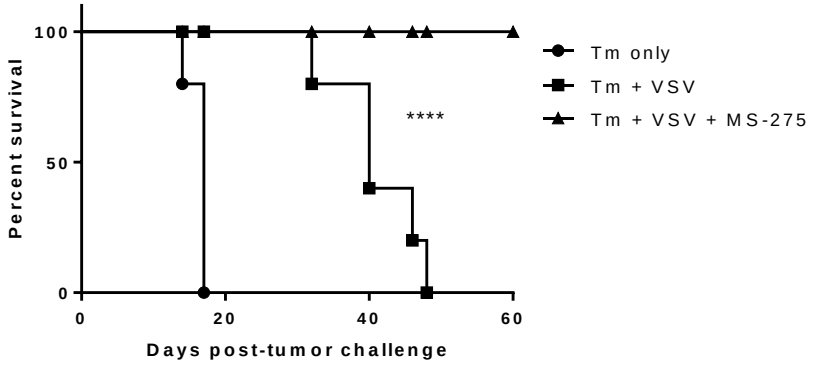

Figure 3: Concomitant MS-275 administration can inhibit tumor recurrence resulting from Tm+VSV treatment. C57BL/6 mice (n=5 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. MS-275 (100 µg/mouse) was injected (i.p.) daily for five days starting on Day 5 post-challenge. **(A)** Tumor volumes were calculated based on height, width, and length. **(B)** Survival over time was calculated for each treatment group where n=5 mice per group. ** $p = 0.057$, **** $p < 0.0001$; Tm, memory T cell; VSV, vesicular stomatitis virus

first elicit partial tumor regression and then utilize the drug to examine if the extent of acute regression can be improved.

3-3 Relapsing Tumors in Tm+VSV-treated Mice Display Characteristics of Antigen-loss Variance

The genetic heterogeneity of cancer cells and their susceptibility to mutation can often promote the growth of sub-populations that no longer express the target antigen. Immunotherapy often fails because the antitumor response selects for these immunoresistant variants which then proliferate and repopulate the tumor microenvironment [253]. While MS-275 prevented instances of tumor recurrence, we wanted to see if Tm+VSV alone induced sufficient selective immunological pressure to induce phenotypic changes in the tumor population to facilitate immune escape. In order to confirm this, we isolated relapsing tumors from Tm+VSV-treated mice and transplanted them into mice that had been previously immunized with Adenovirus (Ad)-gp33. In earlier studies, we showed that recombinant Ad expressing a tumor transgene was ineffective at controlling tumor growth post-challenge; however, it was highly effective at controlling tumor growth when administered prophylactically. In untreated mice, both the relapsed tumor cells (B16-relapse) and parental B16-gp33 were able to engraft. Conversely, in mice that were Ad-gp33 pre-treated, the B16-relapse was able to grow while the parental B16-gp33 was not **(Figure 4A)**. The gp33-specific response elicited by Ad immunization was thus unable to control tumor growth, suggesting that the tumor could no longer be recognized by the antitumor immune response.

We were then interested in learning if MS-275 could restore immune recognition of these resistant tumor populations. Previously untreated mice were challenged with B16-relapse or

parental B16-gp33 to determine if our combination therapy could mediate the sustained regression of relapsed tumor cells. We observed that, while Tm+VSV+MS-275 was able to control the growth of B16-gp33 cells, B16-relapse-challenged tumors were unaffected by treatment and their growth was comparable to untreated controls. Similar to Ad-gp33 immunization, combination therapy-induced gp33-specific immune responses were unable to mediate tumor control. Furthermore, MS-275 was not able to restore tumor recognition **(Figure 4B)**, suggesting that if the drug has a productive role in promoting tumor antigenicity it cannot be in a situation where immune recognition of the tumor has been completely lost. Taken together, the data suggest that the relapsed tumors from Tm+VSV therapy no longer expressed the gp33 epitope.

We utilized genomic DNA derived from relapsed tumors that were enzymatically digested and cultured briefly *in vitro* in order to detect if the gp33 gene sequence was still present. The PCR amplification product was generated using forward (5'-GTCCTTTGGGCGCTAACTG-3') and reverse (5'-GTGGCGAAATTGTACACAGC-3') primers specific for the gp33 gene sequence as well as a portion of the promoter region from the β-actin-driven expression vector pActin-IRES-TK-Neo (original cloning vector). Compared to genomic DNA obtained from parental B16-gp33 (positive control) and B16-F10 (negative control), PCR analysis of B16-relapse genomic DNA did not show any amplification product **(Figure 5)**, suggesting that the relapsing tumor population does not contain the target antigen. The gene amplification product of the positive control (B16-gp33) will be sequenced in order to confirm that it is in fact the gene of interest. While the PCR data suggests otherwise, future studies may explore the possibility that the tumor antigen is not lost but down-regulated. To ascertain if relapsed tumors down-regulate antigen expression globally or in a gp33-specific

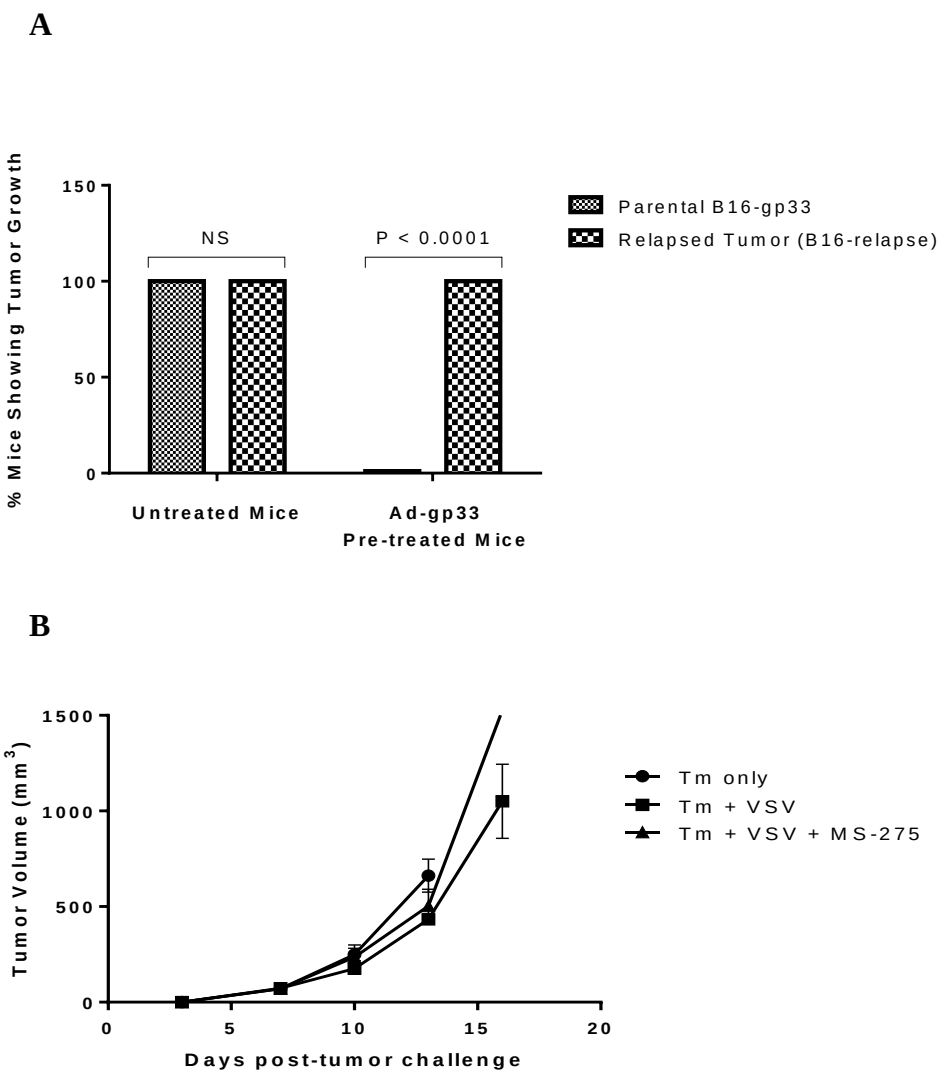

Figure 4: gp33-specific antitumor immunity is unable to control the growth of relapsed tumors, even in the presence of MS-275. (A) Previously untreated or Ad-pre-treated (10^8 pfu, i.m.) C57BL/6 mice (n=3 per group) were challenged with 10^5 B16-relapse cells (i.d.) that were derived from Tm+VSV-treated mice. Tumor growth was defined by visual assessment and palpation. **(B)** C57BL/6 mice (n=3 per group) were challenged with 10^5 B16-relapse cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. MS-275 (100 µg/mouse) was injected (i.p.) daily for five days starting on Day 5 post-challenge. Tumor volumes were calculated based on height, width, and length.
Ad, adenovirus; Tm, memory T cells; VSV, vesicular stomatitis virus; NS, not significant

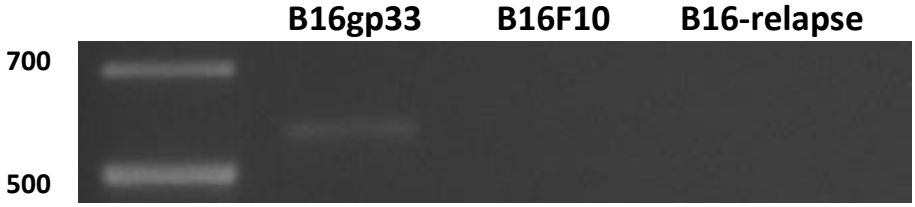

Figure 5: gp33-specific transgene can no longer be detected in B16-relapse genomic DNA. Relapsed tumors from Tm+VSV-treated mice were digested with Type I collagenase and cultured briefly. Genomic DNA was extracted from B16-relapse tumor-derived cells as well as B16-gp33 and B16F10 cell lines. Primers specific for the gp33 transgene were used to amplify genomic DNA for PCR analysis. Tm, memory T cells; VSV, vesicular stomatitis virus; PCR, polymerase chain reaction

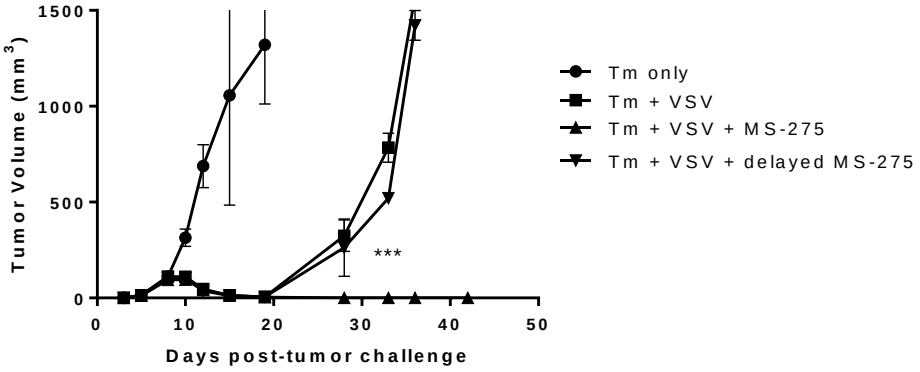

Figure 6: Delayed administration of MS-275 abrogates sustained tumor regression. C57BL/6 mice (n=3-6 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. MS-275 (100 µg/mouse) was injected (i.p.) daily for five days starting on Day 10 post-challenge. Tumor volumes were calculated based on height, width, and length. *** p = 0.0009; Tm, memory T cells; VSV, vesicular stomatitis virus; FACs, flow cytometry

manner, it is worth investigating if vaccination of these relapsed tumors with recombinant viral vectors expressing another TAA (ex. Ad-hDCT) will induce antigen-specific immune responses and tumor control.

3-4 The Effects of MS-275 are Time and Context Dependent

HDACis have been implicated in the immunosurveillance of cancer through the up-regulation of specific molecules (ex. MHC), leading to the enhancement of tumour antigenicity and/or targeted immune-mediated cytotoxicity. We initially wanted to determine if MS-275 directly acted on the tumor to potentiate antitumor immune attack and prevent tumor recurrence. However, in the previous experiment, we demonstrated that administering Tm+VSV+MS-275 to mice that were challenged with B16-relapse could not rescue the clinical and therapeutic benefits that were seen with parental B16-gp33 melanoma challenge. If MS-275 could not improve the susceptibility of antigen non-expressing relapsed tumors to immune killing, then what is the proper timeframe in which MS-275 administration is effective? To that end, we treated B16-gp33-challenged mice with Tm+VSV and delayed the delivery of MS-275 by five days (Day 10 post-challenge). Surprisingly, we found that tumor growth mimicked Tm+VSV treatment in that there was significant regression followed by a period of equilibrium and rapid relapse **(Figure 6)**. Consequently, even in the presence of primarily gp33-expressing tumor cells, the efficacy of MS-275 is temporally and contextually dependent on early immunological events that are induced by Tm transfer and VSV-boosting. While time limitations inhibit further exploration, several assays can be conducted to examine if MS-275 has direct immunopotentiating effects on the tumor. Using *in vitro* killing assays, we can determine if MS-275 can sensitize Tm+VSV-treated tumors to CTL killing. Tumors will be excised from Tm+VSV-treated mice and digested using Type I collagenase. CD8+ T cells can be isolated using negative selection CD8+ T cell

enrichment kits and co-cultured with B16-gp33 cells that have been incubated with MS-275 and plated on 96-well plates. Using incubation conditions that have already been optimized, the level of killing can be assessed according to colorimetric standards using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and compared to tumor cells that were not exposed to the drug.

3-5 MS-275 May Improve the Cytolytic Activity of gp33-specific CD8+ T Cells

To determine the primary cell types involved in vaccine-mediated, sustained tumor regression, we independently depleted CD8+, CD4+, and NK1.1+ cells in B16-gp33-challenged mice using monoclonal Abs before treating them with Tm+VSV+MS-275. Mice that did not receive CD8+ T cell depletion showed sustained tumor regression and 100% survival. Conversely, CD8+ T cell-depleted mice were unable to control tumor growth and displayed similar growth kinetics to that of untreated control mice **(Figure 7A)**. Consequently, it is apparent that MS-275 does not alter host dependency on CD8+ T cells for acute regression of the tumor. Furthermore, CD4+ T cells and NK cells do not seem to actively promote sustained tumor regression in the context of HDAC inhibition. Interestingly, we found that the frequency of the gp33-specific response over time did not differ significantly between +/- MS-275 groups **(Figure 7B)**. This suggests that, with drug administration, it may be unnecessary to enhance the magnitude of the peripheral antitumor immune response in order to elicit durable tumor control.

Since antigen-specific CD8+ T cells play such a critical role in controlling tumor growth, we questioned whether or not MS-275 directly or indirectly impacts on the ability of gp33-specific CTLs to kill the tumor. In treatment situations where suboptimal therapeutic conditions can promote immune escape and tumor relapse, it is plausible that MS-275 can improve the

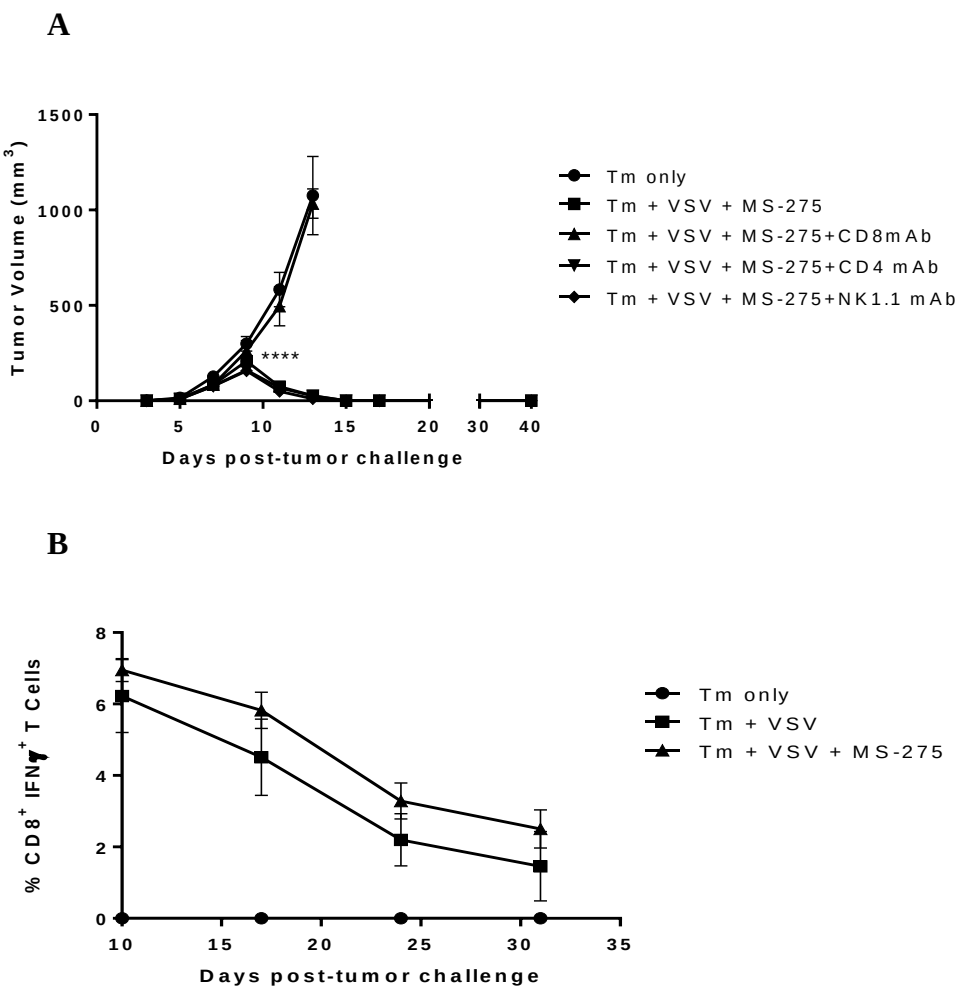

Figure 7: Concomitant MS-275 delivery does not alter host dependency on gp33-specific CD8+ T cells nor modulates magnitude of the antitumor response. (A) C57BL/6 mice (n=3-6 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. Depletion antibodies specific to CD8, CD4, and NK1.1 were injected (i.p.) one day before and after VSV-boosting (250 µg/mouse) and bi-weekly afterwards (150 µg/mouse). MS-275 (100 µg/mouse) was injected (i.p.) daily for five days starting on Day 5 post-challenge. Tumor volumes were calculated based on height, width, and length. **(B)** The frequency of antigen-specific CD8+ T cells was assessed by FACs analysis over several time points from the peripheral blood of tumor-bearing mice. The percentages represent the frequency of antigen-specific T cells relative to total CD8+ T cells. **** $p < 0.0001$; Tm, memory T cells; VSV, vesicular stomatitis virus

potency and quality of CTL killers so that the tumor is killed off before outgrowth of antigen-loss variants can occur. Since peripheral response levels may not be fully indicative of what may be occurring within the tumor microenvironment, we first wanted to confirm that MS-275 did not up-regulate gp33-specific CD8+ T cell infiltration into the tumor. To address this, tumor samples were excised from treated mice and digested with Type I collagenase. Biotinylated CD45.2 antibodies were used for positive selection of leukocytes, which were then stimulated with gp33 peptide and analyzed by flow cytometry. Indeed, we found that there was little difference in the number of infiltrating IFNγ-secreting CD8+ T cells when MS-275 was co-administered with VSV-gp33 **(Figure 8A)**. Furthermore, tumor-infiltrating CD8+ T cells were unable to secrete TNFα in treated mice, regardless of MS-275 administration **(Figure 8A)**. Lastly, these cells showed similar expression of classical exhaustion markers, namely PD-1 and KLRG1, even when MS-275 was withheld **(Figure 8B)**. Consequently, the presence of MS-275 does not seem to modulate CD8+ T cell migration to the tumor nor impact their quality or level of senescence. Interestingly however, the expression level of GZMB was highly up-regulated **(Figure 9)**, suggesting that MS-275 can improve the cytolytic activity of tumor-specific T cells through increased production of granzyme B.

To determine if MS-275 can improve the cytolytic activity of tumor-infiltrating CTLs, we can monitor their killing capacity using *in vitro* killing assays. Tumors will be excised from Tm+VSV +/- MS-275 treated mice and digested using Type I collagenase. CD8+ T cells can be isolated and co-cultured with B16-gp33 cells that have been plated on 96-well plates. The extent of tumor cell killing will then be assessed using colorimetric MTT assays. If a difference in killing is observed, we may examine if T cell activity is directly enhanced by MS-275. CD8+ T cells will be enriched from tumors derived from Tm+VSV-treated mice. These cells will be

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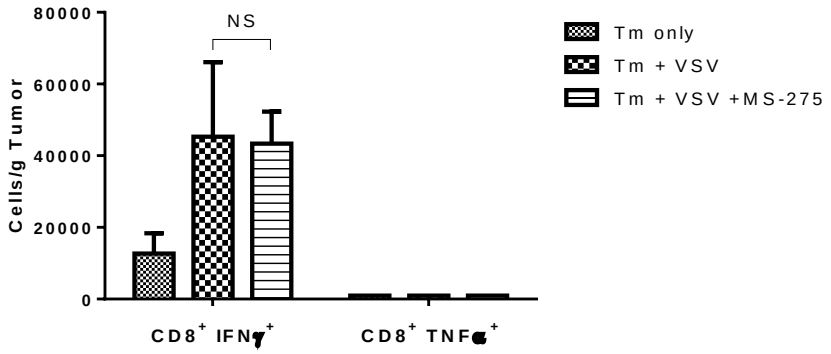

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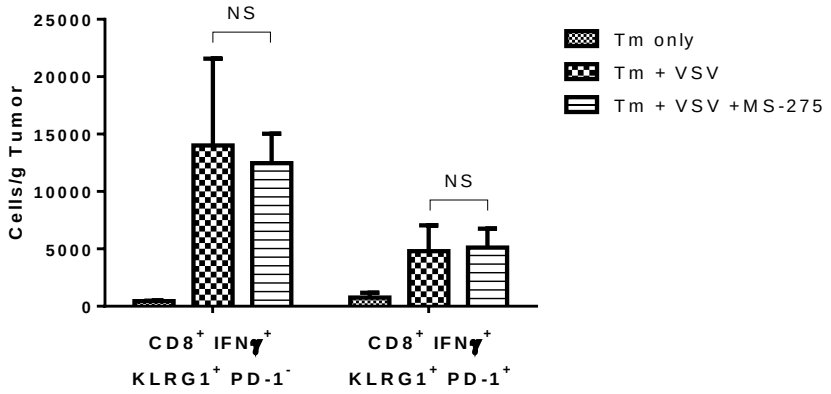

Figure 8: Tm+VSV+MS-275 treatment does not improve CD8+ T cell infiltration into the tumor, cytokine secretion, and T cell senescence/exhaustion. C57BL/6 mice (n=3-5 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. MS-275 (100 µg/mouse) was injected (i.p.) daily for five days starting on Day 5 post-challenge. After 5 days post-VSV-boosting, tumors were digested and enriched for CD45.2+ cells. **(A)** Antigen-specific CD8+ T cells were quantified by FACs analysis by gating on CD8, IFNγ, and TNFα. **(B)** Quantification of exhaustion markers was conducted by gating KLRG1 and PD-1 on CD8+ IFNγ+ cells.
Tm, memory T cells; VSV, vesicular stomatitis virus; FACs, flow cytometry; NS, not significant

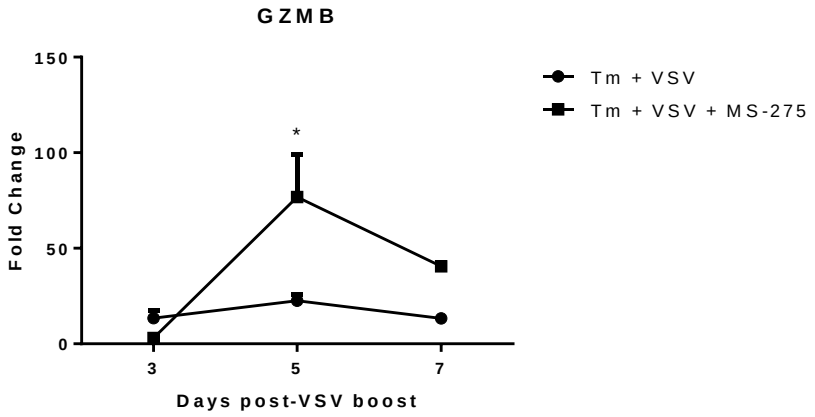

Figure 9: MS-275 co-administration may improve the cytolytic capacity of tumor-infiltrating lymphocytes. C57BL/6 mice (n=3 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. MS-275 (100 µg/mouse) was injected (i.p.) daily for five days starting on Day 5 post-challenge. After 3, 5, and 7 days post-VSV-boosting, tumors were excised and homogenized for qRT-PCR analysis. GZMB expression was quantified as a fold change relative to HPRT housekeeping gene expression. * p = 0.0114; Tm, memory T cells; VSV, vesicular stomatitis virus; qRT-PCR, quantitative reverse transcription polymerase chain reaction

cultured briefly in the presence or absence of MS-275 before introducing them into the B16gp33 monolayer. Again, the level of killing will be assessed by MTT. Lastly, if direct incubation with the drug improves tumor killing, we can determine if the acquisition of enhanced cytolytic capacity is mediated through HDAC inhibition. Using CD8+ T cells derived from Tm+VSV+MS-275 mice or CD8+ T cells derived from Tm+VSV mice cultured in the presence of MS-275, we can determine the acetylation status of these cells by flow cytometry using fluorochrome-conjugated Acetyl-Lysine antibodies.

3-6 Selective Removal of Regulatory CD4+ T Cells Mediates Sustained Regression in the Absence of MS-275

To determine if immunoregulatory cells could functionally impair the gp33-specific CD8+ T cell response, we independently depleted CD4+ and CD25+ cells in B16-gp33-challenged mice using monoclonal Abs before treating them with Tm+VSV without MS-275. We hypothesized that the depletion of regulatory T cells may promote sustained tumor regression in the absence of MS-275. Indeed, we observed that CD4+ T cell depletion in the context of therapy resulted in sustained tumor regression and durable cures relative to CD4-replete mice **(Figure 10A)**. Interestingly, CD25+ T cell depletion induced acute tumor regression and eventual relapse similar to Tm+VSV without depletion **(Figure 10A)**. To reconcile why CD25+ depletion did not also mediate sustained tumor regression, it is well-documented that PC61 antibody mediates partial or incomplete depletion of CD4+ CD25+ Tregs [407-410]. This may be insufficient for abrogating the negative regulatory effects placed on the antitumor immune response. Flow cytometric analysis of peripheral blood indicates that the magnitude of the gp33-specific CD8+ T cell response was relatively unchanged across treatment groups

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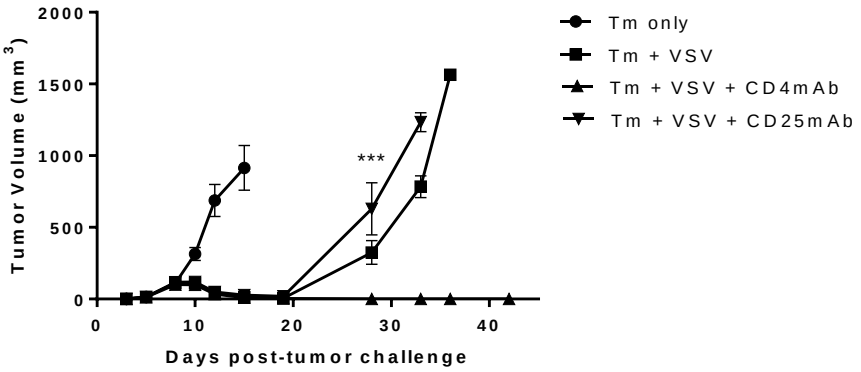
Tumor Volume (mm3)
2000
1500
1000
500
0
0
10
20
30
40
Days post-tumor challenge
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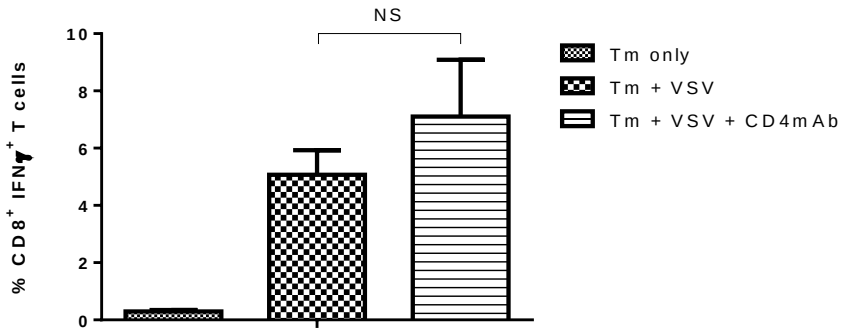
Tm only
Tm + VSV
Tm + VSV + CD4mAb
Tm + VSV + CD25mAb

B


% CD8+ IFNγ+ T cells
10
8
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4
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Tm only
Tm + VSV
Tm + VSV + CD4mAb

C

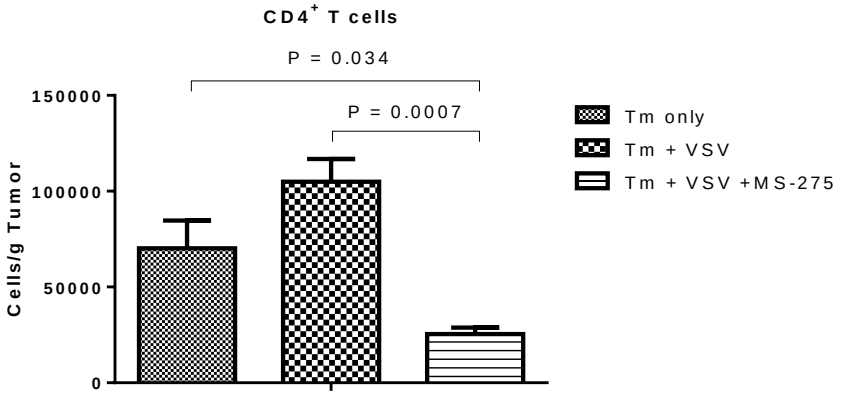

Figure 10: CD4+ T cells may have a regulatory role and their depletion in the context of αCD4 mAbs or MS-275 correlates with sustained tumor regression. C57BL/6 mice (n=3-6 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. Depletion antibodies specific to CD4 and CD25 were injected (i.p.) one day before and after VSV-boosting (250 µg/mouse) and bi-weekly afterwards (150 µg/mouse). **(A)** Tumor volumes were calculated based on height, width, and length. **(B)** The frequency of antigen-specific CD8+ T cells was measured 5 days post-VSV-boosting in the peripheral blood of tumor-bearing mice by *ex vivo* peptide stimulation and FACs staining. The percentages represent the frequency of antigen-specific T cells relative to total CD8+ T cells. **(C)** C57BL/6 mice (n=5 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. MS-275 (100 µg/mouse) was injected (i.p.) daily for three days starting on Day 5 post-challenge. After 3 days post-VSV-boost, tumors were digested and enriched for CD45.2+ cells. CD4+ T cells were quantified by FACs analysis. *** $p < 0.0004$; Tm, memory T cells; VSV, vesicular stomatitis virus; FACs, flow cytometry; NS, not significant

regardless of CD4 depletion status **(Figure 10B)**. Taken together, this would suggest that CD4+ T cells do not promote nor attenuate the secondary expansion of gp33-specific CD8+ T cells, but may play a regulatory role in suppressing their function at the tumor site. Furthermore, MS-275 can serve to overcome Treg-mediated immunosuppression through the removal of CD4+ T cells. In support of this, we observed that in Tm+VSV+MS-275-treated mice, tumor-infiltrating CD4+ T cell populations were severely reduced compared to Tm+VSV alone **(Figure 10C)**.

To follow up on these observations, we plan to selectively deplete regulatory T cells (rather than CD4+ T cells) in the context of Tm+VSV in order to associate the removal of immunosuppressive cell subsets with improved therapeutic outcomes. We are currently in the process of setting up a collaborative study with Dr. Tim Sparwasser, the Director of the Institute for Infection Immunology at TWINCORE, in order to utilize a DEREG (DEpletion of REGulatory T cells) mouse model to conduct our tumor challenge and vaccination regime in the absence of regulatory T cells. DEREG mice carry a DTR-eGFP transgene under the control of an additional FoxP3 promoter which allows us to selectively deplete Treg cells by application of diphtheria toxin (DT). In tumor-challenged DEREG mice that have been given DT and treated with Tm+VSV, we will be able to determine if Treg depletion can mediate sustained tumor regression without MS-275. Furthermore, the expression of eGFP transgene under the FoxP3 promoter allows us to sort out Treg cells and introduce them into tumor-challenged mice post-Tm+VSV+MS-275 treatment to determine if sustained tumor regression can be abrogated.

3-7 MS-275-induced Therapy Mediates Myeloid Cell Changes within the Tumor Microenvironment to Promote a Pro-inflammatory Phenotype

Immunomodulatory cell types that have recently attracted our interest are tumor-infiltrating myeloid cell populations that may alter the inflammatory status of the tumor microenvironment. Gabrilovich's group states that under physiological conditions, inflammatory monocytes differentiate into macrophages and dendritic cells; however, during infection and cancer, monocytic MDSCs (the counterpart to monocytes) differentiate into granulocytic MDSCs which then accumulate at the tumor site [236]. Surprisingly, preliminary analyses of tumor-infiltrating populations suggest that our combination therapy alters myeloid cell differentiation back towards the physiological norm.

In our current model, tumor samples were derived from mice treated with Tm+VSV +/- MS-275 and subsequently digested with Type I collagenase. Biotinylated CD45.2 antibodies were then used to positively select for tumor-infiltrating leukocyte populations. To gain a better sense of the phenotypic changes that occurred as a result of MS-275, conventional myeloid markers such as CD11b, F4/80, Ly6C, and Ly6G were used to stain the enriched tumor-derived leukocytes. We observed that the total number of myeloid cells in the tumor did not vary significantly as a result of treatment **(Figure 11A)**. In mice that only received Tm, the majority of tumor-infiltrating myeloid cells were CD11b+ F4/80+, Ly6G-, $Ly6C^{hi}$ or $Ly6C^{lo}$ which may correspond to classical inflammatory macrophages (M1 macrophages) and non-classical resident macrophages (M2 macrophages) respectively **(Figure 11B)**. Interestingly, in Tm+VSV-treated mice, the tumor-infiltrating myeloid cells were primarily CD11b+ F4/80- Ly6G-, $Ly6C^{hi}$, suggesting that they were immunosuppressive monocytic MDSCs **(Figure 11B)**. Lastly, Tm+VSV+MS-275 treatment produced a myeloid cell phenotype that was primarily CD11b+ F4/80+ Ly6G-, $Ly6C^{hi}$, which once again corresponds to the M1 macrophage **(Figure 11B)**. Although preliminary, the data suggests that Tm+VSV treatment affects myeloid cell

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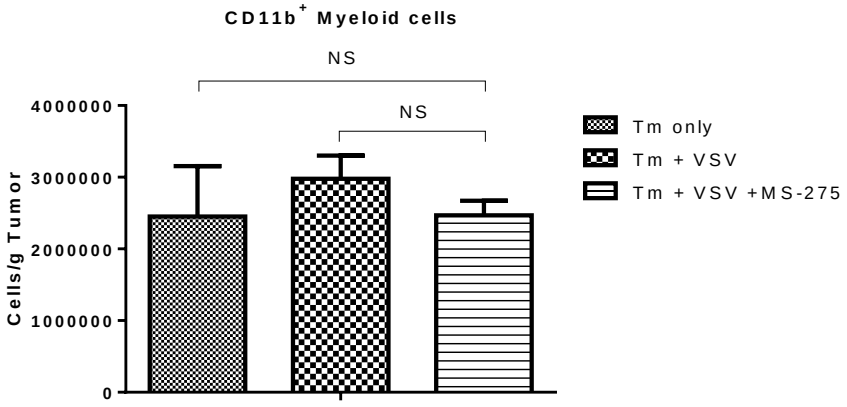
CD11b+ Myeloid cells
NS
NS
4000000
3000000
2000000
1000000
0
Cells/g Tumor
Tm only
Tm + VSV
Tm + VSV +MS-275

B

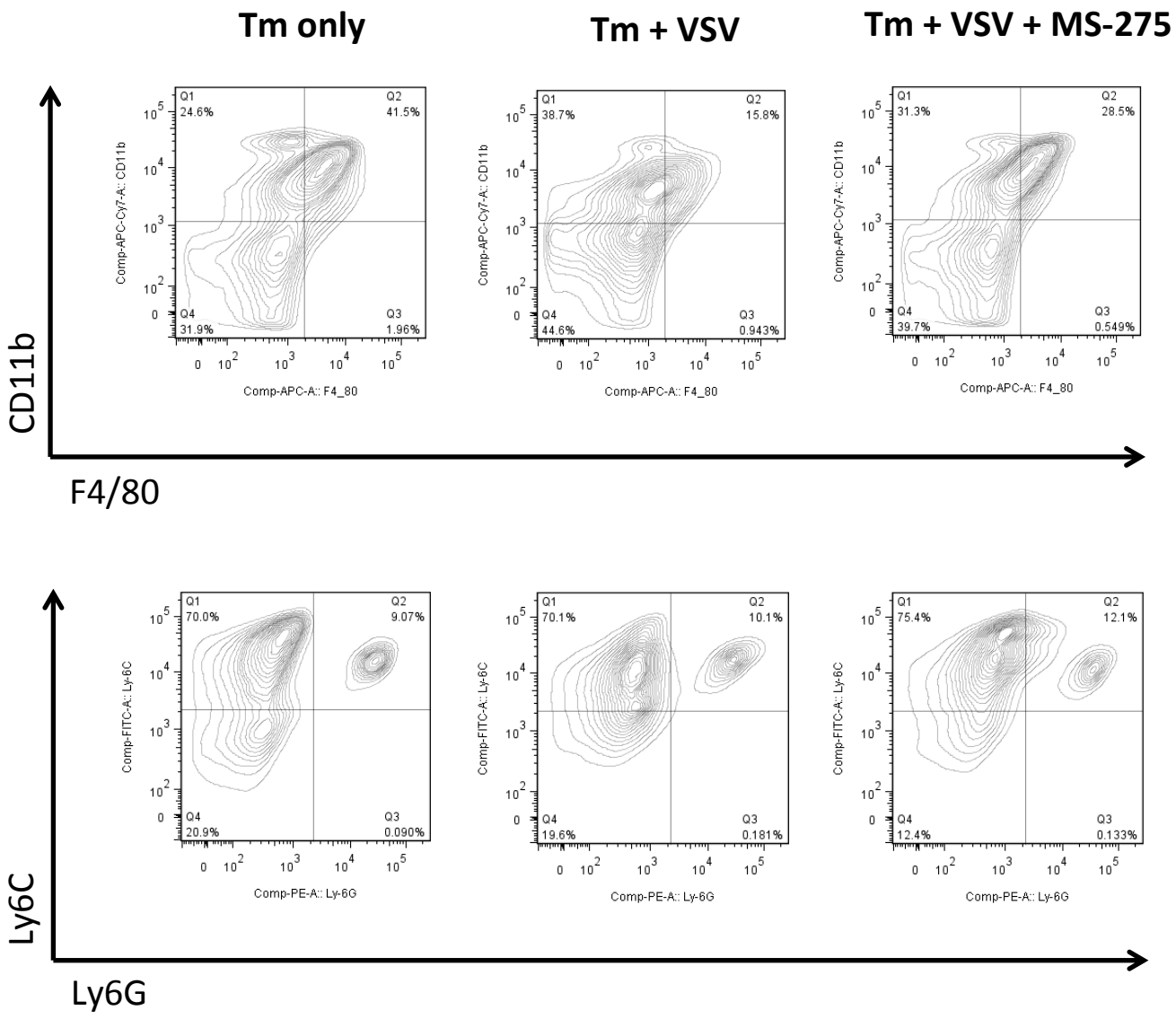

Figure 11: MS-275 administration in the context of Tm+VSV does not improve myeloid cell infiltration into the tumor but alters their phenotype. C57BL/6 mice (n=3-5 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. MS-275 (100 µg/mouse) was injected (i.p.) daily for five days starting on Day 5 post-challenge. After 5 days post-VSV-boosting, tumors were digested and enriched for CD45.2+ cells. **(A)** CD11b+ T cells were quantified by FACs analysis. **(B)** Myeloid cell morphologies were determined using staining markers specific for CD11b, F4/80, Ly6G, and Ly6C. Tm, memory T cells; VSV, vesicular stomatitis virus; FACs, flow cytometry; NS, not significant

differentiation and promotes the infiltration of monocytic MDSCs to the tumor. The addition of MS-275 may correct this aberrant differentiation and promote the migration and/or differentiation of inflammatory macrophages. These cells in turn may utilize pro-inflammatory mechanisms to mediate non-specific killing of the tumor. However, functional roles have yet to be assigned to each of these observed phenotypes.

To investigate this further, we would first like to confirm that MS-275's effects on myeloid cell differentiation and infiltration plays a significant role in mediating tumor regression. By selectively removing myeloid cells from the host, we hope to induce tumor regression through Tm+VSV treatment. There are two methods that can be utilized: depletion or neutralizing mAbs (monoclonal antibodies) or KO (knock-out) mice. We considered purchasing Gr-1-specific (clone: RB6-8C5) antibodies which would lead to elimination of Ly6G+ and Ly6C+ expressing cell types; however, since it has been documented that memory T cells also express Ly6C, we would risk eliminating the very cells we hoped to expand with our vaccine.

We are currently considering using CD11b-specific (Mac-1-specific) mAbs to inhibit the recruitment of myeloid cells to established tumors. There has been documented success using the aforementioned antibody so we may set up a collaborative study in order to receive the hybridomas necessary for generating our neutralizing antibodies. Along with CD11b KO mice, we are also considering purchasing CCL2 KO mice in order to set up a model in which myeloid cells cannot migrate to the tumor. CCL2 KO mice have been used extensively by Gabrilovich *et al* and Kroemer *et al* to inhibit myeloid cell infiltration to great success.

To confirm that MS-275 up-regulates the infiltration of inflammatory macrophages into the tumor on a gene expression level, we will be conducting qRT-PCR on existing tumor RNA

samples derived from Tm+VSV and Tm+VSV+MS-275-treated mice. By observing the gene expression levels of iNOS, IL-12, and TNFα for instance, it may indicate that MS-275 increases the inflammatory status within the tumor through macrophage recruitment, leading to enhanced non-specific killing of tumor cells.

Lastly, we would like to prove the inverse by showing that myeloid cells in Tm+VSV-treated mice are more immunosuppressive than Tm+VSV+MS-275-treted mice. To do so, myeloid cells will be sorted by CD11b+ Ly6G- $Ly6C^{hi}$ and co-cultured with conventional CD8+ T cells derived and enriched from bulk splenocytes from untreated mice. MTT will be used as an appropriate readout of suppressive activity.

3-8 Depletion of Homeostatic Cytokines in the Context of MS-275 Abrogates Sustained Tumor Regression

Previous studies have shown that successful adoptive transfer therapy is accompanied by lymphoablative preconditioning [81]. Since the size of the lymphoid compartment is tightly controlled by homeostatic factors such as competition for cytokines, a systemic decrease of conventional lymphocyte populations would therefore increase the systemic availability of cytokines and improve the effector function of adoptively transferred cells [81]. As we have previously shown, concomitant MS-275 administration prolongs a state of severe lymphopenia that was induced by VSV-boosting [7]. It is thus plausible that Tm+VSV+MS-275 can mediate sufficient lymphodepletive pre-conditioning to eliminate cytokine sinks and improve therapeutic outcomes. In this study, we utilized IL-15KO mice to show that IL-15 is a required cytokine for sustaining tumor regression. When treating C57BL/6 wild-type mice versus IL-15KO mice with Tm+VSV+MS-275, the latter was able to mediate significant tumor regression but, after a period

of tumor equilibrium, the tumor relapsed **(Figure 12A)**. Consequently, the therapeutic efficacy seen in wild-type mice was lost in IL-15KO mice.

Since the peripheral gp33-specific antitumor response was attenuated in IL-15KO mice, it is apparent that IL-15 may assist in the secondary expansion of antigen-specific memory T cells **(Figure 12B)**. However, it is unlikely that increased availability of IL-15 as a result of MS-275 potentiates immunotherapy by improving the magnitude of the antitumor response. In IL-15KO mice, Tm+VSV+MS-275 treatment was still able to facilitate significant tumor regression. Furthermore, in wild type mice, the addition of the drug did not enhance the magnitude of the antitumor response relative to Tm+VSV alone. Studies have shown that endogenously or exogenously introduced IL-15 is capable of enhancing the *in vivo* antitumor activity of pmel-1 cells that were adoptively transferred into sub-lethally irradiated mice [411]. Therefore, we postulate that MS-275 increases the systemic availability of IL-15 which in turn improves the antitumor activity of adoptively transferred Tm in order to promote sustained tumor regression.

To provide evidence to support this, we may need to confirm that VSV+MS-275-induced lymphopenia actually up-regulates systemic amounts of IL-15. Commercial enzyme-linked immunosorbent assay (ELISA) kits may thus be used to quantify IL-15 from sera in drug treated and drug untreated mice. We may also decide to exogenously introduce recombinant IL-15 into Tm+VSV-treated mice to determine if increased IL-15 in the absence of MS-275 could recapitulate sustained regression. We would also like to adopt a mouse model of lymphopenia to determine if MS-275 creates a physical niche for the expansion of adoptively transferred Tm cells and increases the availability of homeostatic cytokines. Rag1 KO mice produce no mature T cells or B cells and can be utilized in the context of Tm+VSV treatment to see if inherent lymphopenia can mediate sustained tumor regression in the absence of MS-275.

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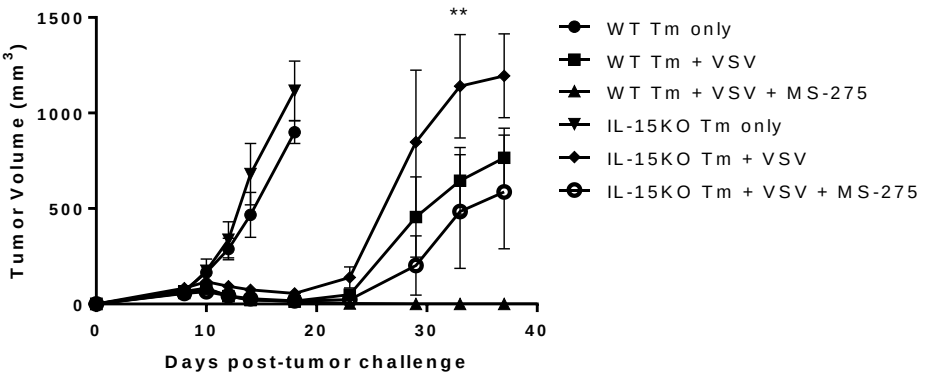

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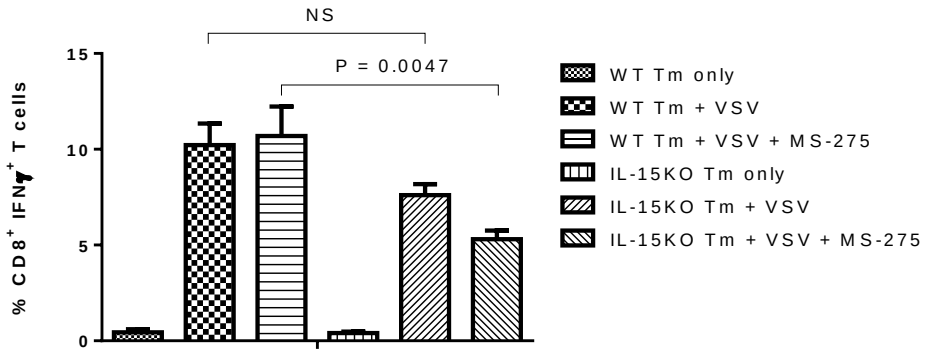


Figure 12: Depletion of IL-15 abrogates sustained regression from Tm+VSV+MS-275 treatment and modestly attenuates the antitumor response. C57BL/6 and IL-15KO mice (n=4-6 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. MS-275 (100 µg/mouse) was injected (i.p.) daily for five days starting on Day 5 post-challenge. **(A)** Tumor volumes were calculated based on height, width, and length. **(B)** The frequency of antigen-specific CD8+ T cells was measured 5 days post-VSV-boosting in the peripheral blood of tumor-bearing mice by *ex vivo* peptide stimulation and FACs staining. The percentages represent the frequency of antigen-specific T cells relative to total CD8+ T cells. After 5 days post-VSV-boosting, tumors were digested and enriched for CD45.2+ cells. ** $p < 0.005$; Tm, memory T cells; VSV, vesicular stomatitis virus; FACs, flow cytometry; NS, not significant

Chapter 4: Discussion

While we initially postulated that concomitant delivery of MS-275 could enhance the magnitude of acute tumor regression, we were surprised to discover that VSV-boosting alone was sufficient to elicit significant, if not complete, regression of the established tumor. It is apparent that our current treatment modality was too potent to observe whether or not MS-275 could enhance tumor cell killing. However, we observed that VSV-boosting alone could not prevent eventual regrowth of a tumor variant which no longer expressed the target antigen; interestingly, MS-275 co-administration did not result in tumor recurrence and led to durable tumor regression. As was earlier explained, the tumor effectively utilizes immune self-tolerance, immunosuppressive mechanisms, and genetic heterogeneity in order to evade, suppress, and escape immune attack [253]. Since the tumor antigen was derived from a viral protein, endogenous immune self-tolerance is unlikely to occur. However, tumor-induced immunosuppressive mechanisms may have rendered therapy to be suboptimal, leading to the selection of escape variants. The challenge is to understand the means by which the tumor becomes refractory to immune attack and how MS-275 subverts these processes.

PCR analysis of genomic DNA derived from the tumor would suggest that the sequence encoding the tumor antigen could not be amplified due to mutations that prevented the PCR primers from binding (the sequence of the tumor antigen overlaps with the primer sequence) or mutations that removed the sequence completely. Alternatively, gene-negative variants may have already been present in the initial challenge. Random genome integration of plasmid DNA in the transduced cell line could have conferred resistance to selection antibiotics without the presence of the actual tumor transgene. Regardless of the mechanism, the amplification product derived from the parental cell line still needs to be sequenced to confirm that the tumor antigen was lost

in recurrent tumors. While the data suggests that antigen loss variance was mediated by mutational loss of the foreign insert, other tumor escape processes (loss of MHC class I expression, epigenetic down-regulation of gene expression, etc.) are still a distinct possibility [253]. Therefore, it may be interesting to examine if relapsed tumors are susceptible to immune responses specific for other tumor-associated antigens, such as DCT. This would allow us to determine if it is exclusively resistant to gp33-specific responses or if it can avoid immune recognition as a whole.

Interestingly, concomitant delivery of MS-275 was able to coordinate an antitumor response that was sufficient enough to completely inhibit the growth of variant populations. However, if the drug is delayed by as little as five days, sustained regression is lost and the tumor relapses completely. It should be noted that the tumor still regressed significantly after delayed MS-275 delivery, indicating that the immunodominant gp33 epitope was still being expressed by the majority of tumor cells. It is unclear then why a small delay in MS-275 administration compromised the efficacy of our current therapy. We postulate that the immunological and intratumoral landscape had been altered drastically in that period of time, rendering MS-275 ineffective. Indeed, by the time MS-275 was delivered, the tumor had already peaked in size and begun to regress as a result of VSV-boosting. Furthermore, the secondary expansion and tumor infiltration of gp33-specific effector T cells had also peaked by the time MS-275 treatment began. Consequently, if MS-275 improves therapy by increasing tumor antigenicity and/or targeted immune-mediated cytotoxicity, its effects may be contextually and temporally dependent on immunological events that occur early during VSV-gp33 vaccination. Aside from direct tumor sensitization, it may also be entirely possible that other mechanisms are involved that are context and timing dependent.

One possibility is that MS-275 facilitates the destruction of both antigen and non-antigen presenting tumor cells by means of "bystander killing". Uptake of highly-expressed, soluble tumor antigen by the surrounding tumor stroma may result in their cross-presentation via MHC class I [412]. Since stromal cells are essential for the survival and growth of cancer cells, stromal cell targeting by the antitumor response can mediate bystander elimination of antigen-loss variants as well as parental cancer cells [413]. In our hands, we have shown that a tumor challenge mixture consisting of 10^5 B16-gp33 cells and 10^3 B16-F10 cells (gp33 non-expressing) could not be controlled by our therapy in the presence of MS-275; however, it was able to visibly delay tumor regrowth compared to mice not given MS-275 **(Appendix 2)**.

It has been previously shown that MS-275 can promote the replication and oncolytic capacity of VSV within tumor cells [5]. This may lead to the increased release of soluble tumor antigen from lysed cells. Furthermore, studies using SIY-2Lo and SIY-2Hi cancer cell lines indicate that higher levels of antigen expression from parental tumors promote antigen spreading to stromal cells while lower levels of antigen expression promote the outgrowth of immune-evaded cells [412]. Since we previously hypothesized that MS-275 increases tumor antigenicity, in this context, it is possible that enhanced antigen expression in low antigen-expressing cells might further contribute to bystander killing. While the literature shows that stromal IFNγ signaling and cross-presentation are necessary elements for stromal-targeted bystander killing [413, 414], it has also been suggested that TNFα signaling plays a role in this process [413]. Others have also argued that CD4+ T cells cooperate with CD8+ T cells during the effector response to directly kill stromal cells and/or release inflammatory cytokines that act on the stroma [415]. However, if the durable regression conferred by MS-275 is mediated through bystander killing, our data suggest that it occurs through CD4+-independent and TNFα-

independent mechanisms. This is because we were able to recapitulate sustained tumor regression using α-CD4 mAbs; furthermore, infiltrating CD8+ T cell populations were unable to express TNFα with or without drug administration. Interestingly, another group proposed that expression of IL-15 in the tumor microenvironment allows for the elimination of antigen-negative cells in a non-cognate T cell receptor-dependent manner [416]. Since boosting vaccination in the context of MS-275 was ineffective in IL-15KO mice and tumors relapsed, it is possible that the increased systemic bioavailability of IL-15 as a result of VSV+MS-275-induced lymphopenia can promote non-specific tumor killing; however, since IL-15 is implicated with several immunological processes, other roles for IL-15 may be just as relevant.

Another school of thought suggests that, rather than initiate a non-specific immune attack against the tumor microenvironment, a massive antigen-targeted response will eliminate large established tumors before antigen loss variants can develop. Allen *et al.* proposes that the failure of immune-mediated tumor clearance and emergence of antigen loss variants is dependent on four factors: 1) lack or insufficient tumor-rejection antigen recognized by the CTL that are generated, 2) inhibition of CTL activity by the tumor microenvironment, 3) failure of CTL to localize to the tumor site, and 4) failure of CTL to be sustained at the tumor site [417]. To overcome (and perhaps overwhelm) these obstacles, Allen *et al.* injected as many T cells as possible, as rapidly as possible. While our current therapy was not designed to elicit the largest possible tumor-specific CD8+ T cell response, we may do so by modulating the dose of memory T cells and oncolytic virus. It is evident however, that MS-275 can subvert the proposed limitations to induce sustained regression without magnifying the antitumor immune response. Since efficacious delivery of MS-275 is timing- and context-dependent, its effects must coincide with viral and immunologic events that occur within the same timeframe. As was previously

suggested, MS-275 may increase tumor antigenicity, leading to increased CTL recognition in the tumor microenvironment. Furthermore, VSV+MS-275-induced lymphopenia may increase the bioavailability of homeostatic cytokines (ex. IL-15) and allow for enhanced antitumor efficacy as a result of increased tumor-reactive T cell functionality. While we did not observe significant changes in the magnitude, quality, and/or senescence of tumor-specific CD8+ T cells, heightened GZMB expression may suggest enhanced cytolytic capacity. Interestingly, the literature puts forth the possibility of a reverse correlation between GZMB expression in effector CD8+ T cells and regulatory T cell activity [418, 419]. Consequently, this may suggest that MS-275's immunomodulatory effects can potentiate the killing capacity of CTLs in our model.

As was previously described, Tregs play a major role in the suppression of induced antitumor immune responses. We showed that CD4+ T cell depletion could mediate sustained tumor regression in the absence of MS-275, suggesting that some CD4+ T cell subset plays a distinct negative regulatory role during the induction of antitumor immunity and that MS-275 abrogates this effect. We attempted to recapitulate our results using α-CD25 mAbs to deplete Treg cells; however, there was no difference in tumor growth relative to Tm+VSV mice. As previously mentioned, this may be an issue with the antibody and not an experimental outcome. Consequently, we are pursuing alternate means of depleting regulatory T cells from the tumor-bearing host. Intratumoral analysis was similarly inconclusive. Gene expression analysis did not indicate a difference in FoxP3 expression at day 5 post-VSV; however, at day 3 there seemed to be decreased FoxP3 expression in mice treated with MS-275 **(Appendix 3)**. This coincides with our previous study where we showed that MS-275 down-regulates FoxP3-expressing Tregs in the periphery. However, intracellular staining and flow analysis did not reinforce this observation

(Appendix 3). We still strongly suspect that Tregs may suppress CD8+ T cell responses within the tumor microenvironment and our experimental data may suggest certain possibilities.

Since CD4+ CD25+ cells did not make up a large percentage of the total tumor-infiltrating CD4+ T cell population and did not change significantly in number during MS-275 treatment, we postulate whether regulatory T cell activity can be primarily mediated in a CD25-FoxP3-independent manner. Indeed, regulatory T cells such as tumor-induced T_R1 cells can play a dominant suppressive role [198] and warrant further investigation in this challenge and treatment model.

If Tregs indeed play a suppressive role in our immunotherapy, then MS-275 may improve therapeutic outcomes through the removal of these cells. We have previously shown that co-administration of MS-275 in the context of VSV-boosting extends a state of severe lymphopenia that results in significant reduction of CD4+ T cell numbers [7]. If these cells correspond to tumor-infiltrating CD4+ regulatory T cells in our model, it may explain why delayed administration of MS-275 did not result in durable tumor regression: If MS-275 is not given concomitantly at the time of boosting, VSV-induced lymphopenia is transient and the reconstitution of lymphocyte compartments quickly occurs. The delay in MS-275 may thus allow for resurgences in Treg numbers and suppression of the antitumor response.

The proliferation and activity of MDSCs within the tumor bed provides an additional source of immunosuppression. As was stated previously, MDSCs are a heterogeneous population of myeloid precursors that can mediate a variety of immunosuppressive mechanisms including production of iNOS, ARG1, ROS, TGFβ, and IL-10 [420]. Based on FACs analysis of tumor-infiltrating myeloid cells, it is apparent that significant phenotypic changes were induced during

therapy. However, without additional functional/suppression assays, it will difficult to associate myeloid cells with their functional role within the tumor microenvironment. In mice that received only Tm transfer, tumor-infiltrating myeloid cells expressed marker phenotypes that correspond to both classical inflammatory macrophages (M1 macrophages) and non-classical resident macrophages (M2 macrophages) [421]. Interestingly, the majority of tumor-infiltrating myeloid cells in Tm+VSV mice did not express conventional macrophage markers, indicating that they may be monocytic MDSCs as proposed by Gabrilovich's group [422]. To reinforce this notion, it has been suggested by some that increased local inflammation as a consequence of viral replication increases the frequency of immature myeloid cells within the tumor microenvironment [423]. With concomitant administration of MS-275, the majority of intratumoral myeloid cells adopted a phenotypic profile corresponding to classic M1 macrophages with slightly heightened expression of Ly6G+. MS-275 also seems to down-regulate M2 macrophage differentiation, a cell type which promotes tumor development and an immunosuppressive environment [424]. Interestingly, one of the most promising approaches for therapeutic targeting of MDSCs is to promote their differentiation into a mature myeloid cell subset that no longer has suppressive function [425]. Though entirely preliminary, we would like to investigate the possibility that MS-275 can restore the differentiation of MDSCs to provoke non-specific inflammatory effector mechanisms and mediate complete elimination of the tumor.

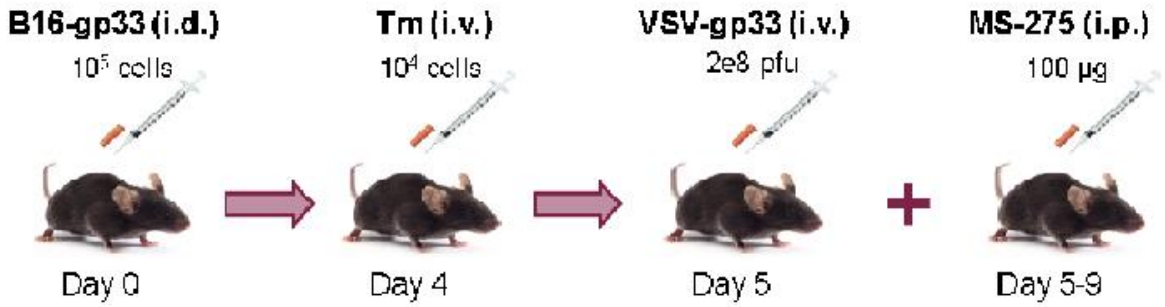
While it is interesting that the immunomodulatory effects of MS-275 extend to both Tregs and MDSCs, it may not be surprising considering their reciprocal relationship. It is unclear whether Tregs can directly promote MDSC differentiation; however, it is well documented that they can significantly impair DC function. Tregs secrete various cytokines (ex. IL-10, TGFβ) that suppress the differentiation/activation of DCs [198]. MDSCs in can turn promote Treg

differentiation and recruitment through cytokine secretion (ex. IFNγ, IL-10), NO/Arg1 production, expression of CTLA4, and/or direct cell-cell interactions [234, 426]. MDSCs have also been found to express high levels of chemokines comprising the CCR5 ligands CCL3, CCL4, and CCL5, which up-regulate Treg recruitment into the tumor microenvironment [427]. Consequently, our drug can subvert the immunosuppressive repertoire through one of several scenarios: MS-275 can induce broad biological effects that can affect Tregs and MS-275 through independent mechanisms, specific effects that influence a common mechanism shared by both cell subsets, or directly affect one particular subset which indirectly attenuates the differentiation, activation, and recruitment of the second.

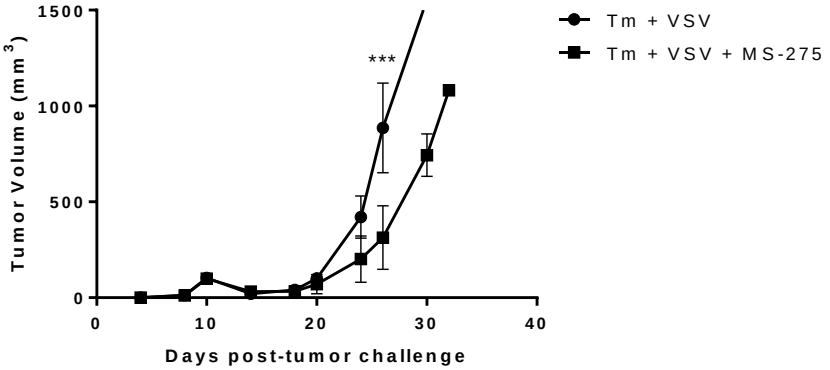
It is apparent that MS-275 can potentiate the synergy between oncolytic virotherapy and cancer immunotherapy to promote sustained tumor regression in a very aggressive cancer model. We postulated that its therapeutic effects were weighted towards immunomodulation and enhancement of the antitumor immune response. While our earlier studies have shown that the drug alone has no therapeutic benefit in the B16 tumor model, it is important to remember that MS-275 is also being investigated as a monotherapy for several advanced leukemias and solid tumors. Therefore, it would be interesting to explore the potential synergy between all three therapeutic strategies in a tumor model that is amenable to direct killing by MS-275, viral oncolysis, and antigen-specific immunity. The addition of HDACi-mediated chemotherapy may create dynamic interplay between all three therapeutic strategies which enhances the potency of therapy even further. For instance, is MS-275 administration conducive to immunologic cancer cell death? Ultimately, the prevalent failure of mono and dual therapies in clinical trials reinforces the notion that sub-optimal antitumor responses may simply induce selective immunological pressure which actually promote its escape; however, a balanced interplay

between three complementary treatment methods can act as a 'therapeutic tri-force' and induce a comprehensive attack that can prevent the natural selection of tumor cell variants.

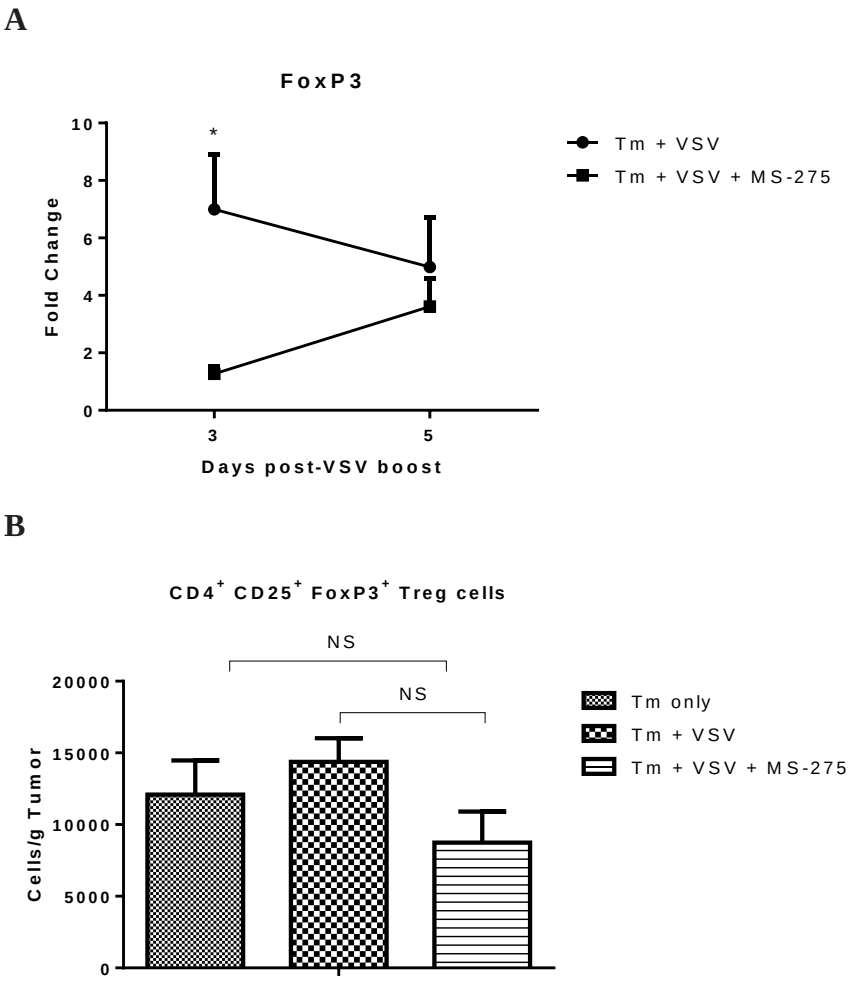
Chapter 5: Appendix



Appendix 1: Schematic of the Therapeutic Tumor Model. Tumor challenge was conducted intradermally (i.d.) while adoptive T cell transfer and vaccine delivery was performed by intravenous injection (i.v.). MS-275 was administered daily for five days by intraperitoneal injection (i.p.). This challenge and treatment model was used for all experiments unless otherwise stated.



Appendix 2: Tm+VSV+MS-275 treatment delays the regrowth of mixed tumor challenges (B16-gp33, B16F10; 10:1). C57BL/6 mice (n=3 per group) were challenged with 10^5 B16-gp33 and 10^3 B16F10 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. MS-275 (100 µg/mouse) was injected (i.p.) daily for five days starting on Day 5 post-challenge. Tumor volumes were calculated based on height, width, and length. ** $p < 0.005$; Tm, memory T cells; VSV, vesicular stomatitis virus



Appendix 3: MS-275 co-administration in the context of Tm+VSV has ambiguous effects on the infiltration of CD4+ CD25+ FoxP3+ T cells in the tumor. C57BL/6 mice (n=5 per group) were challenged with 10^5 B16-gp33 cells (i.d.) and treated with adoptively transferred Tm at Day 4 (i.v.) and VSV-gp33 (i.v.) injection at Day 5 post-challenge. MS-275 (100 µg/mouse) was injected (i.p.) daily for five days starting on Day 5 post-challenge. After 3 days post-VSV-boosting, tumors were excised and homogenized for qRT-PCR analysis and FACs staining. **(A)** FoxP3 expression was quantified as a fold change relative to HPRT housekeeping gene expression. **(B)** Digested tumors were enriched for CD45.2+ cells and stained for CD4, CD25, and FoxP3. * $p < 0.05$; Tm, memory T cells; VSV, vesicular stomatitis virus; qRT-PCR, quantitative reverse transcription polymerase chain reaction; FACs, flow cytometry; NS, not significant

Appendix Table 1: Flow Cytometry Antibodies

Antibody Name	Fluorochrome	Dilution	Clone	Company
CD4	PerCP-Cy5.5	1:800	RM4-5	BD Biosciences
CD8	PE	1:400	53-6.7	BD Biosciences
CD11b	APC-Cy7	1:100	M1/70	BD Biosciences
F4/80	APC	1:100	BM8	eBioscience
FoxP3	FITC	1:25	FJK-16s	eBioscience
IFNγ	APC	1:100	XMG1.2	BD Biosciences
KLRG1	PerCP-eFluor710	1:100	2F1	eBioscience
Ly6C	FITC	1:100	AL-21	BD Biosciences
Ly6G	PE	1:100	1A8	BD Biosciences
PD-1	PE	1:100	J43	BD Biosciences
TNFα	FITC	1:300	MP6-XT22	BD Biosciences

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