IMPROVING ADOPTIVE CELL THERAPY TO OVERCOME TUMOR RESISTANCE

MS-275 ENHANCES ANTITUMOR IMMUNITY DURING ADOPTIVE CELL THERAPY TO OVERCOME TUMOR RESISTANCE

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TITLE: MS-275 enhances antitumor immunity during adoptive cell therapy to overcome tumor resistance

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Lay Abstract:

The host immune system has the ability to recognize and destroy tumor cells. Therapeutic platforms that leverage antitumor immune cells, specifically T cells, have shown potency in the elimination of cancer. In the clinic, cancer immunotherapies have demonstrated early success against hematological malignancies; however, are unreliable in the treatment of solid tumors. Solid tumors utilize intrinsic and adapted mechanisms of resistance to mitigate the effectiveness of cancer immunotherapy. This thesis pursues research questions aimed at understanding how tumors resist immunotherapy, what mechanisms are utilized, and how to overcome these obstacles. We anticipate that these results will contribute to the development and incorporation of strategies to subvert tumor resistance and potentiate T cells against solid tumors.

Abstract:

Cancer immunotherapy has gained attention in recent years for its successes in potentiating immune responses that can elicit tumor control. In particular, adoptive cell therapy (ACT), which involves the autologous/allogeneic transplant of *ex vivo*-cultivated tumor-specific T lymphocytes, can mediate potent tumor recognition and killing; however, durable clinical responses are often difficult to obtain in solid tumors. Solid tumors and their unique microenvironments have the capacity to evade and suppress antitumor immune responses and represent significant hurdles for effective ACT. Recently, we have discovered that chemical inhibition of histone deacetylases via MS-275 (Entinostat) during ACT can subvert tumor resistance to foster potent, broad-spectrum antitumor immunity. Overall, the work described supports the efficacy of ACT in the treatment of immunosuppressive, solid tumors; however, consistency in durable clinical outcomes can only be achieved through the concurrent therapeutic targeting of tumor resistance mechanisms.

This thesis uses pre-clinical models to describe how tumor resistance to ACT can manifest, and demonstrates that concurrent MS-275 delivery drives extensive immunomodulation to promote sustained tumor clearance. This includes:

1) The polarization of tumor-infiltrating myeloid cells into cytotoxic effectors with the ability to reject immune escape variants
2) The inflammatory remodeling of the tumor microenvironment to potentiate epitope spreading against secondary tumor antigens
3) The transcriptional reprogramming of adoptively transferred T cells to overcome tumor-burden-dependent exhaustion

We expect that the results will help facilitate the development of next-generation ACT platforms that will feature strategies for multi-mechanistic perturbation of tumor resistance.

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To say that this experience has been not fraught with emotional hardship would be to undersell the effort my mentors have undertaken to guide me to where I am today. The anxiety, the self-doubt, the frustration… in spite of all these feelings that persist to this day, at this moment, I am filled with nothing but gratitude.

Thank you, Dr. Yonghong Wan. It may have been a rocky road, but I am glad that you were patient with me and allowed me the autonomy to develop my own scientific interests and skills. No one loves science more than you, and it has really shaped my research intentions both during my time here and moving forward. Although I still do not know where my path may lead, I will retain that spirit of genuine curiosity and passion for research for as long as I am able. After I defend, let us go for that walk and talk about where our journeys will take us next.

Thank you, Dr. Jonathan Bramson and Dr. Matt Miller. I appreciate both of you taking the time to act as members of my supervisory committee. Your guidance has strengthened my development as a researcher and my confidence in discussing my scientific insights. I hope to one day match the caliber of mentorship that you both have displayed during my study period.

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Thank you, Dr. Lan Chen, Omar, and all the members of Wan Lab. Thank you for enriching my academic experience. I know our paths will cross again someday.

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Abbreviations:

ACT	adoptive cell therapy
ANOVA	analysis of variance
APC	antigen presenting cell
BRCA	breast invasive carcinoma
CAR	chimeric antigen receptor
CFSE	carboxyfluorescein succinimidyl ester
CTL	cytotoxic lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DCT	dopachrome tautomerase
DE	differentially expressed
DEREG	depletion of regulatory T cell
DISC	death-induced signaling complex
DNA	deoxyribonucleic acid
DOX	doxorubicin
DT	diphtheria toxin
DTR	diphtheria toxin receptor
FADD	fas-associated protein with death domain
FC	fold change
FDA	Food and Drug Administration
FDR	false discovery rate
FI	fluorescence intensity
FISH	fluorescence *in situ* hybridization
GDAC	Genome Data Analysis Center
GEO	Gene Expression Omnibus
GFP	green fluorescence protein
GNAT	Gcn5-related *N*-acetyl transferase
GO	gene ontology
GOBP	gene ontology biological processes
GSEA	gene set enrichment analysis
HDAC	histone deacetylase
HIF	hypoxia-inducible factor
HLA	human leukocyte antigen
HNSC	head-neck squamous cell carcinoma
HR	hazard ratio

ICOS	inducible T cell costimulator
ICOSL	inducible T cell costimulator ligand
IDO	indoleamine 2,3-dioxygenase
IF	immunofluorescence
IFN	interferon
IFNG	interferon gamma
IFNGR	interferon gamma receptor
IHC	immunohistochemistry
IMC	immature myeloid cell
IV	intravenous
IVIS	*in vivo* imaging system
JAK	janus kinase
KRAS	kirsten rat sarcoma virus
LCMV	lymphocytic choriomeningitis virus
LMP	latent membrane protein
LPS	lipopolysaccharide
MAGE	melanoma antigen gene
MAPK	mitogen-activated protein kinase
MART-1	melanoma antigen recognized by T cells 1
MDSC	myeloid derived suppressor cell
MEM	memory
MHC	major histocompatibility complex
MICA	MHC class I chain-related protein A
MICB	MHC class I chain-related protein B
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCOR	nuclear receptor co-repressor 1
NK	natural killer cell
NKT	natural killer T cell
NO	nitric oxide
NS	not significant
OV	oncolytic virus
PCA	principal component analysis
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PMN	polymorphonuclear
PPI	protein-protein interaction
RAG2	recombination activating gene 2

RIN	RNA integrity number
RMA	robust multichip average
RNA	ribonucleic acid
ROC	receiver operating characteristic
ROS	reactive oxygen species
SAHA	suberanilohydroxamic acid
SKCM	skin cutaneous melanoma
SOD	superoxide dismutase
SST	signal-space transformation
STAT	signal transducer and activator of transcription
TAA	tumor associated antigen
TAC	Transcriptome Analysis Console
TADC	tumor-associated dendritic cell
TAM	tumor-associated macrophage
TAN	tumor-asssociated neutrophil
TCGA	The Cancer Genome Atlas
TCM	central memory T cells
TCR	T-cell receptor
TEFF	effector T cells
TEMRA	effector memory T cells re-expresses CD45RA
TEX	exhausted T cells
TGFβ	tumor growth factor β
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIL	tumor infiltrating lymphocyte
TIM	T-cell immunoglobulin and mucin-domain
TLR	toll-like receptor
TME	tumor microenvironment
TMEM	memory T cell
TMPEC	memory precursor effector T cell
TNFα	tumor necrosis factor α
TOX	thymocyte selection associated high mobility group box
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TRP-2	tyrosinase-related protein 2
TSA	trichostatin A
TSLEC	short-lived effector T cell
t-SNE	t-distributed stochastic neighbor embedding
TTEC	Tim-3hi effector-like cells
TTMC	Tim-3hi memory-like cells

VEGF	vascular endothelial growth factor
VSV	vesicular stomatitis virus
WHO	World Health Organization
WT	wild-type

Declaration of Academic Achievement:

I, Andrew Nguyen, declare that I have independently authored and assembled the contents of this thesis, with editorial assistance from Dr. Yonghong Wan. With respect to the scholarly works presented in Chapter Two – Four, a detailed breakdown of my academic contributions, as well as my co-authors', is provided in the Preface.

Preface:

This thesis contains five scholarly works (Chapter Two – Four, Appendix). My academic contributions (**bold text**) as well as my co-authors' are detailed as follows:

Chapter Two: **Nguyen, A,** Ho, L, Workenhe, ST, Chen, L, Samson, J, Walsh, SR, Pol, J, Bramson, JL, Wan, Y (2018). "HDACi Delivery Reprograms Tumor-Infiltrating Myeloid Cells to Eliminate Antigen-Loss Variants." *Cell Reports* 24(3): 642-654.

- **A.N. designed and conducted the majority of the experiments described and wrote the manuscript.** L.H. prepared the samples for microarray analysis. L.C. monitored the therapeutic efficacy of ACT + OV + MS-275 in DCT and mERK models. J.S. conducted the FISH staining on treated tumor sections. J.P. constructed the VSV-gp33 viral vector and S.R.W. constructed the VSV-mERK viral vector. S.T.W. and J.L.B. participated in the conception of experimental designs and manuscript writing. Y.W. supervised the study and participated in the conception of experimental designs and manuscript writing.
- This work was conducted from 2014-2016.

Chapter Three: **Nguyen, A,** Ho, L, Walsh, S, Hogg, R, Chen, L, Wan, Y (2021). "HDACi promotes inflammatory remodeling of the tumor microenvironment to enhance epitope spreading and antitumor immunity." *Science Advances*. In submission.

- **A.N. designed and conducted the majority of the experiments described and wrote the manuscript**. L.H. prepared the samples for microarray analysis and qRT-PCR. S.W. constructed the VSV-gp33 viral vector. R.H. constructed the MCA102-p15E cell line. L.C. helped monitor the therapeutic efficacy of ACT+MS-275 in the gp33 model. Y.W. supervised the study, and participated in the conception of experimental designs
- This work was conducted from 2017-2019.

Chapter Four: **Nguyen, A,** Brown, D, Krishnan, R, Walsh, SR, Bastin, D, Deng, L, Chen, L, Bramson, JL, Wan, Y (2021). "T cell transcriptional reprogramming overcomes tumor burden-induced cellular exhaustion." *Immunity*. In submission.

- **A.N. designed and performed the majority of the experiments and wrote the manuscript.** D.B. and R.K. performed animal experiments. S.W. constructed the viral vectors used in the study. D.B. transduced cultured TCM to express luciferase. D.L. performed the cytotoxicity assay. L.C. assisted with in vitro TCM culture and tissue processing. J.L.B. provided guidance during manuscript preparation. Y.W. supervised the study and participated in the conception of experimental designs and manuscript writing.
- This work was conducted from 2019-2021.

Appendix:

Nguyen, A, Salem, O, Wan, Y (2021). "Functional Analysis of Tumor-Infiltrating Myeloid Cells by Flow Cytometry and Adoptive Transfer." *Journal of Visualized Experiments* (169).

- **A.N. wrote the manuscript.** O.S. contributed to the design of key figures. Y.W. supervised the methodology and manuscript writing.
- This work was conducted from 2020.

Workenhe, ST, **Nguyen, A**, Bakhshinyan, D, Wei, J, Hare, DN, MacNeill, KL, et. al. (2020). "De novo necroptosis creates an inflammatory environment mediating tumor susceptibility to immune checkpoint inhibitors." *Communications Biology* **3**(1): 645.

- S.T.W., **A.N.**, Y.W., J.L.B, and K.L.M. **conceived the study and designed experiments.** S.T.W, **A.N.**, A.V, N.E, A.N., D.N.H., J.W., A.O., Kelly L.M., D.N.H. **developed the methodologies, conducted the experiments.** S.T.W, K.L.M, **A.N.**, A.O., J.A.N, A.G.M. S.K.S, D.B. **analyzed and interpreted the experimental results.** S.T.W, A.O., Y.W, J.L.B, and K.L.M. wrote and revised the manuscript.
- This work was conducted from 2020.

1.0   Chapter One – Introduction

This work can be considered a continuation of the Master's thesis (Nguyen, 2013). Parts of the background information in this section were adapted from the Master's thesis.

1.1   Background

1.1.1   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 (*1*). 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 (*2*). Approximately 1% of patients diagnosed with cancer will die on an annual basis (*2*). 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 (*2*). Consequently, the relative ineffectiveness of current therapeutic methods underscores an ever-increasing need for clinically promising treatments against cancer.

1.1.2   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 (*3*): 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 (*4*).

1.1.3 Cancer Immunosurveillance

Paul Ehrlich was the first to suggest that the immune system could repress the majority of carcinomas (*5*), 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 (*6*). 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 (*7, 8*). While caveated studies with immunocompromised athymic nude mice by several investigators dampened enthusiasm to the idea (*9-11*), key observations by Schreiber *et al* renewed interest in the concept of immunosurveillance (*12-14*). He further suggested that host immunity could modulate the immunogenicity of developing tumors by a process in which he termed, "immunoediting" (*15*).

1.1.4 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 (*15*). 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 (*16-18*). 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) (*19-21*), which may facilitate tumor death by anti-proliferative (*22*) and apoptotic (*23*) 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 (*24, 25*).

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.

1.2   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 (*26, 27*). 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 (*28-30*). 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 (*31, 32*).

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) (*33, 34*). 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 (*35*), gp100 (*36*), TRP-2 (*37*)). 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 (*38, 39*). 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 (*40*). Cancer immunotherapy can be implemented in many different ways, whereby each strategy offers a unique method of propagating immune attack on the tumor; however, it is apparent that side-effects and limited clinical efficacy emphasize an ever urgent need to improve current strategies for safe and reliable therapeutic outcomes.

1.2.1 Adoptive Cell 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* (*41-43*). 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 (*44-48*). 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 (*49*), enhance co-stimulation (*50*), prevent apoptosis (*51*), induce inflammation or homeostatic proliferation (*52, 53*), and/or promote T cell migration to the tumor site (*54*).

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 (*55-57*). 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 (*58-62*). Also, toxicities have been associated with the use of nonspecific preconditioning regimens based on chemotherapy and radiation (*43*). On a practical side, ACT is a highly personalized treatment, is labor-intensive, is expensive, and requires laboratory expertise (*41*).

1.2.2 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) (*63*). 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* (*64*).

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) (*65*).

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) (*66, 67*). 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.

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 (*68*). Furthermore, many types of recombinant viruses have been shown to infect professional APCs and express various transgenes (*69-74*), 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 (*75*). 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 (*76*). The existence of pre-existing immunity towards many commonly used viral vectors poses a similar challenge due to the production of neutralizing antibodies (*76*). 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) (*76, 77*). Unfortunately, monotherapies with recombinant viral vectors have not been conducive to significant objective response rates during clinical investigation (*78*).

1.2.3 The Marriage between ACT and Cancer Vaccination

In order to improve clinical responses to immunotherapy, antigen restimulation in the context of ACT may facilitate maximal tumor regression (*79*). Indeed, cancer vaccination has been shown to enhance the *in vivo* antitumor efficacy of adoptively transferred T cells in both preclinical (*80-84*) and clinical studies (*85, 86*). However, a consistent benefit to combination therapy with mono-specific vaccines and ACT was not commonly observed. This could be attributed to the use of relatively weak immunogens such as peptide vaccines with oil-in-water emulsion adjuvants (*87*). By contrast, recombinant poxviruses, fowlpox viruses, and vaccinia viruses encoding human gp100 are strong immunogens that could be rationally combined with ACT to provide an enhanced antitumor immune response (*88*). Indeed, it was found that the viral vaccine titer associated with these vectors correlated with the strength of *in vivo* antigen restimulation during ACT as well as the slope of tumor regression (*88*). Therefore, adoptive cell therapy delivered in tandem with viral vaccination may represent a more optimized immunotherapeutic platform.

1.3   The Challenges Facing Cancer Immunotherapy

However, the design of clinically effective anticancer immune therapies still faces significant challenges. Despite increasing the magnitude and potency of antitumor immune effector cells, generating a higher number of tumor-specific immune effector cells may not necessarily correlate with tumor regression in pre-clinical studies or objective responses in clinical trials. Tumors have evolved mechanisms to inherently evade and suppress antitumor immune responses. It is apparent that current paradigms of immunotherapeutic design should now also consider the elimination of inhibitory factors and prevention of immunoresistant phenotypes.

1.3.1   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 (*89, 90*), adenosine (*91*), prostaglandin E2 (PGE2) (*92, 93*), IL-10 (*94, 95*), and TGFβ (*96-98*) 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 (*99, 100*). 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 (*101-103*).

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 (*104-110*). 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 (*111-118*).

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 (*101*), Treg cells and myeloid lineage cells have received the most consideration in the design of effective cancer immunotherapeutics.

1.3.2 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 (*119*). 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+ (*119*), T_R1 cells are characterized as CD4+ IL-10+ FOXP3- (*120*).

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) (*121-123*). 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 (*124, 125*). Lastly, the secretion of TGFβ may be responsible for the conversion of conventional CD4+ CD25+ T cells into CD4+ CD25+ FOXP3+ Tregs (*126-128*). 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 (*124, 129, 130*).

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 (*131-134*), competitive consumption of IL-2 (*135-137*), direct killing via perforin and granzyme pathways (*138, 139*), and direct subversion of antigen-presenting cell (APC) function through down-regulation of co-stimulatory

molecules (*121, 140-142*). T_R1 cells primarily suppress immune activity through the production of IL-10 (*119*).

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 (*143-145*). 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 (*119*). The expression of IL-10 from these cells has been hypothesized to play a profound role in suppressing APC and T cell function (*146*).

1.3.3   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 (*147*). 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 (*148-152*). 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 (*151, 153*), secretion of immune suppressive factors (*102*), induction of T cell anergy (*154, 155*), and promotion of Treg development (*155*). 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)) (*156*). 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. (*157-159*)

MDSCs migrate to the tumor site via CCL2, CXCL12 and CXCL5 (*160*) and orchestrate a variety of immunosuppressive processes to inhibit tumor-specific immune attack (*161*). 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 (*162*). For instance, a deficit of L-arginine and cysteine can inhibit T cell proliferation and activation, respectively (*163*). MDSCs also increase intratumoral levels of NO and ROS (*164*). NO inhibits E-selectin expression on endothelial cells and thus obstructs T cell recruitment to the tumor (*165*). In addition, NO and ROS are associated with peroxynitrite production, which in turn causes nitration of TCRs and suppression of CD8+ T cell responses (*166, 167*). PGE2 is the main receptor found in MDSCs and up-regulates ARG1 production as well as MDSC recruitment to the tumor (*168-171*). 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) (*172, 173*).

1.3.4 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 (*174*). Several of these outcomes will be described below.

Descriptions of MHC loss have had a great deal of intuitive appeal (*175*), 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) (*176*) and humans (with partial responses to immunotherapies) (*177*) 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 (*178-181*). 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 (*182*).

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 (*183*). 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.

1.3.5   T cell exhaustion

T cell exhaustion is a broad term that is often seen as a dysfunctional response of T cells to chronic antigen stimulation or inadequate activating signals in the context of chronic viral infection or tumor growth (*184*). Exhausted T cells are heterogeneous and express distinct epigenetic and metabolic states (*185*). Upon encountering antigen, effector T cells display a reduced capacity for cytokine secretion and proliferation, and increased expression of multiple inhibitor receptors, including PD1, TIM3, LAG3, CTLA4 and TIGIT (*185*). Recently, it has been demonstrated that the unique expression of transcription factor TOX in exhausted cells facilitates an altered transcriptional programme (*186*).

Developmentally, exhausted T cells share features with effector and memory T cells. Indeed, heterogeneous exhausted T cells are often dichotomized into relevant subsets: terminally differentiated exhausted cells (PD1^{hi} TIM3^{hi} TCF1^{-}) and progenitor exhausted cells (PD1^{hi} TIM3^{lo} TCF1^{+}) that have the ability to self-renew and maintain the former (*187*). Functional rescue of exhausted T cells is dependent on tumor antigen chronicity,

whereby increased exposure results in further chromatin remodeling and initial plastic dysfunctional states give way to fixed dysfunctional states. Indeed, PD-1 blockade-dependent reinvigoration of exhausted T cells can fail with increased epigenetic stability (*188*).

Currently, it is not clearly understood how the chromatin and transcriptional changes of exhaustion can be reversed in a deterministic manner in dysfunctional T cells to restore therapeutic efficacy during cancer immunotherapy.

1.4   The Prospect of Combination Therapy

Most cancer immunotherapeutic strategies fail to fully account 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 target 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 (*189-192*). 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 (*193-196*). Lastly, chemotherapeutics such as paclitaxel (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 (*197*) and even resulted in bystander killing of non-targeted tumor cells (*198*). Consequently, there is therapeutic value in a combinatorial approach. Currently, there is an increasing interest in utilizing epigenetic modifying agents in combination with cancer immunotherapeutic modalities to synergize antitumor attack.

1.4.1   Histone Acetylation as a Target 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 (*199, 200*). Two classes of enzymes can affect histone acetylation status: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (*201*).

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. (*202*)

HATs can be divided into several families on the basis of highly conserved structural motifs (ex. Gcn5-related *N*-acetyl transferase (GNAT) family (*203-207*)). 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 (*202*). 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 (*208*). HATs may also target non-histone protein substrates, including transcription factors and are termed, factor acetyltransferases (FATs) (*209*).

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 (*201*). 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 (*202*). 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) (*210, 211*). 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 (*210*).

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 (*212*). 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) (*202, 212*). Consequently, the original application of HDACi was in transcription-based anticancer therapy.

1.4.2   The Therapeutic Potential of HDAC Inhibitors

HDAC inhibitors (HDACi) have shown much promise as direct anticancer agents and many have progressed to clinical development (*213-223*). 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 (*219, 224, 225*). Direct treatment with HDACi can directly induce tumor cell death through the activation of death-receptor and intrinsic (ex. mitochondrial death pathway (*226*)) 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 (*227-232*). Finally, HDACi can induce apoptosis through indirect regulation of gene expression by modulating the activity of transcriptional factors (E2F1, p53, STAT1/3, and NFκB) (*233-237*) 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 (*238*). 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 (*239, 240*). The common mechanism of these drugs is to bind a critical Zn^{2+} ion required for the catalytic function of HDACs (*241, 242*). 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 (*238*). In particular, synthetic benzamides have shown significant promise for anticancer therapy and have been explored further due to its antiviral and anti-inflammatory properties.

1.4.3 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 (*243*). 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 (*244*). 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 (*245-248*).

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 (*217, 249*). It has been clinically evaluated in phase I clinical trials as treatment against refractory solid tumors, leukemias, and lymphomas (*246, 250-253*). 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) (*254*). It has been found to inhibit tumor proliferation in several cancer lines including breast, colorectal leukemia, lung, ovary, and pancreas (*254*), and is associated with an extensive gene induction (p21$_{WAF1}$, gelsolin, metallothionein, histone H2B) and repression (thymidylate synthase, importin-b, c-myc) profile (*225*). 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 (*255, 256*). 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 (*257, 258*).

1.4.4 HDACi for Cancer Immunotherapy

Aside from direct tumor control, HDACi have been documented to have extensive immunomodulatory properties. 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 (*259, 260*). 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 (*261, 262*). Furthermore, direct histone hyperacetylation may alter the activity of STAT1 (*263*), STAT3 (*236*), and NFκB (*237*), which are considered "master immune regulatory transcription factors". Finally, HDACi are capable of potentiating antigen processing and presentation machinery via upregulation of TAP, LMP, and Tapasin (*264*).

These immunomodulatory properties suggest that HDACi may be complementary to current cancer immunotherapies as a means to further enhance antitumor immune responses. We have previously demonstrated that MS-275 delivery during heterologous prime-boost viral vaccination improved recall responses against the tumor and can abrogate vaccine-induced autoimmunity (*265*). This was in part achieved through selective lymphopenia leaving the boosted antitumor lymphocytes intact while depleting both conventional lymphocytes and Tregs. This provided early evidence that MS-275-dependent immunomodulation may subvert tumor resistance through the removal of immunosuppressive cell subsets. This thesis further characterizes how MS-275 can be utilized to overcome tumor resistance during cancer immunotherapy.

1.5   Thesis Scope and Content

Herein I describe my doctoral studies which utilize pre-clinical models to describe how resistance to cancer immunotherapy can manifest, the tumor resistance mechanisms at play, and how HDAC inhibition via MS-275 can enhance antitumor immunity to provide lasting tumor regression.

2.0 **Chapter Two – HDACi Delivery Reprograms Tumor-Infiltrating Myeloid Cells to Eliminate Antigen-Loss Variants**

2.1 **Introduction**

ACT in solid melanoma tumors promotes acute tumor regression but facilitates the therapeutic selection of immune escape variants, resulting in eventual tumor relapse. This manuscript describes the therapeutic benefit of using of MS-275 in conjunction with ACT.

We observed that combination therapy promotes sustained tumor regression, which is mediated by the reprogramming and functional polarization of tumor-infiltrating myeloid cells from immunosuppressive cells to antitumor effector cells with the ability to reject immune escape variants.

2.2 **Manuscript Status, Copyright, and Citation**

Status: Published manuscript

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2.3 **Published Journal Article**

Starting on the next page.

HDACi Delivery Reprograms Tumor-infiltrating Myeloid Cells to Eliminate Antigen-loss Variants

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Summary

Immune recognition of tumor-expressed antigens by cytotoxic CD8+ T cells is the foundation of adoptive T-cell therapy (ACT) and is shown to elicit significant tumor regression. However, therapy-induced selective pressure can sculpt the antigenicity of tumors, resulting in outgrowth of variants that lose the target antigen. We demonstrate that tumor relapse from ACT and subsequent oncolytic viral vaccination can be prevented using Class I HDACi, MS-275. Drug delivery subverted the phenotype of tumor-infiltrating CD11b+ Ly6Chi Ly6G- myeloid cells, favoring NOS2/ROS secretion and pro-inflammatory genes characteristic of M1 polarization. Simultaneously, MS-275 abrogated the immunosuppressive function of tumor-infiltrating myeloid cells and reprogrammed them to eliminate antigen-negative tumor cells in a caspase-dependent manner. Elevated IFNγ within the tumor microenvironment suggests that MS-275 modulates the local cytokine landscape to favor antitumor myeloid polarization through the IFNγR/STAT1 signaling axis. Exploiting tumor-infiltrating myeloid cell plasticity thus complements T-cell therapy in targeting tumor heterogeneity and immune escape.

Introduction

Cancer immunotherapy has seen major advancement with the success of adoptive cell therapy (ACT), wherein autologous tumor antigen-specific CD8+ T cells expanded ex vivo and subsequently reinfused into the cancer patient. While shown to be capable of promoting tumor regression and enhanced clinical outcomes (Fesnak et al., 2016), a loss of responsiveness to ACT has been observed across multiple tumor types or even within the same tumor type due to genetic and epigenetic instability in growing tumors predisposing them to antigenic heterogeneity (Landsberg et al., 2012; Restifo et al., 2016). Since ACT monotherapies often target a single defined tumor antigen, therapy-induced selection of resistant clones induces proliferation of tumor variants with decreased or absent antigen expression. As a result, tumor recurrence is often associated with a loss of recognition by tumor-specific T cells (Junttila and de Sauvage, 2013). To prevent the outgrowth of antigen-loss variants, therapies incorporating non-parallel tumoricidal pathways must be used in combination to supplement T cell-mediated killing.

In addition to CD8+ T cells, various myeloid cell subsets have been demonstrated to have antitumor effects (Mantovani and Allavena, 2015; Pommier et al., 2013; Powell and Huttenlocher, 2016). It is possible to mobilize these cells in conjunction with ACT to elicit complete tumor destruction and prevent relapse; however, within the immunosuppressive tumor microenvironment, these cells typically exhibit tolerogenic pro-tumor characteristics (Engblom et al., 2016). In particular, the intratumoral myeloid cell compartment comprises a large heterogeneous population of immunosuppressive cells including tumor-associated macrophages, tumor-associated neutrophils, tolerogenic DCs, and myeloid-derived suppressor cells (MDSCs). Interestingly, modulation of the local cytokine milieu can polarize their activation status and functional programming according to classical (M1) or alternatively (M2) activated phenotypes. M1 polarization prototypically involves IFNγ/IFNγR ligation leading to the production of pro-inflammatory mediators (NOS2, TNFα, IL-12) through STAT1 signaling, while M2 polarization is dependent on IL4/IL4R or IL13/IL13R ligation and production of anti-inflammatory mediators (Arg1, Fizz1, Ym1) through STAT3/6 signaling (Yang et al., 2013). Since the opposing polarization programs mutually inhibit one another on an epigenetic and transcriptional level (Piccolo et al., 2017), the relative abundance of M1/M2 polarizing cytokines may dictate if tumor-infiltrating myeloid cells will have a pro- or antitumor function.

We observed that the concomitant delivery of HDAC inhibitor, MS-275, in the context of ACT followed by oncolytic viral vaccination could prevent the outgrowth of antigen-loss variants and promote sustained tumor regression. This coincided with a polarization of tumor-infiltrating myeloid cells from an immunosuppressive M2 phenotype to an inflammatory M1 phenotype as a result of increased local production of IFNγ. This not

only reduced their suppressive function but allowed the cells to directly eliminate antigen-loss variants, thus demonstrating a multi-mechanistic role for myeloid cell polarization in the context of antitumor immunity.

Results

ACT + OV mediates potent antitumor immunity and tumor regression

Oncolytic viruses (OVs) can selectively replicate in and kill tumor cells with minimal impact on normal tissue (Russell et al., 2012). By engineering rhabdoviruses to express tumor antigens (termed oncolytic viral vaccines), we demonstrated that OVs could effectively engage and expand pre-existing tumor-specific central memory T cells (TCM) while retaining their beneficial oncolytic properties (Bridle et al., 2016; Bridle et al., 2010; Pol et al., 2013). Since OV vaccines proved to be robust boosting agents, we wanted to see if they could also enhance the proliferation of ACT responses. To test this, C57BL/6 mice were intradermally (i.d.) injected with 10^5 B16-F10 murine melanoma cells that express an immunodominant epitope from the lymphocytic choriomeningitis virus glycoprotein (LCMV GP33-41) (denoted as B16-gp33). GP33-41-specific CD8+ TCM were generated from donor mice that were infected with LCMV-Armstrong for more than one month. After 5 days of tumor growth, mice were treated with an intravenous (i.v.) injection of 10^4 gp33-specific TCM followed by 2×10^8 pfu of VSV-gp33 24 hours later; control mice received either gp33-specific TCM or VSV-gp33 alone. Figure 1A shows that neither TCM nor VSV-gp33 alone had a significant impact on tumor growth, whereas combining TCM and VSV-gp33 boosting induced complete tumor regression. Moreover, gp33-specific TCM + VSV without the gp33 transgene (VSV-MT) was insufficient to control tumor growth, confirming the synergy and complementarity between ACT and antigen-specific OV vaccination. The potency of this combination therapy was further demonstrated in the regression of much larger tumors where treatment did not start until 10 days after B16-gp33 inoculation (Fig. 1B).

Consistent with the efficacy data, significant T cell responses were only evident in mice treated with TCM + VSV-gp33 (Fig. 1C). To confirm that in vivo CD8+ TCM expansion was responsible for tumor regression, we ablated select lymphocyte populations using monoclonal antibodies specific for CD8, CD4, or NK1.1. As expected, depletion of CD8+ T cells completely abrogated tumor control (Fig. 1D).

To more comprehensively evaluate this combination therapy, we included two additional mouse models targeting dopachrome tautomerase (DCT), a melanocyte-differentiation antigen expressed by murine B16-F10 melanoma cells (Bloom et al., 1997; McGray et al., 2014), and a mutated ERK2-kinase antigen (mERK) that is expressed in CMS-5 fibrosarcoma (Hanson et al., 2000; Ikeda et al., 1997). T cell receptor transgenic mice were used to generate CD8+ TCM that recognize the immunodominant epitope of DCT180-188 and mERK136-144, respectively. Adoptive transfer of TCM followed by boost with VSV vaccines encoding the relevant epitope elicited robust T cell expansion and complete tumor

regression (Fig. S1), indicating that the potency of this combination therapy is independent of the nature of antigens and the strain of mice.

Interestingly, while tumor regression was durable in both DCT and mERK models, it was short-lived in the gp33 model, with tumor relapse evident after 10-20 days (Fig. 1E). This tumor regrowth could not be prevented by increasing the absolute number of transferred TCM before boosting with VSV-gp33, despite gp33-specific T cell responses as high as ~60% circulating CD8+ T cells (Fig. 1F and 1G). This suggests that the relapsing tumors developed resistance to targeted T cell therapy. Indeed, prophylactic immunization of C57BL/6 mice with an adenovirus expressing gp33 (Ad-gp33) led to complete protection against challenge with parental B16-gp33 cells but failed to reject relapsed B16-gp33 explants (B16-gp33R) (Fig. 1H), confirming that B16-gp33R cannot be recognized by gp33-specific CD8+ T cells.

Concomitant MS-275 delivery prevents tumor relapse through complete elimination of antigen-loss variants

We previously demonstrated that MS-275, a Class I HDAC inhibitor, can manipulate secondary CD8+ T cell responses to potentiate antitumor immunity and improve therapeutic outcomes (Bridle et al., 2013). In the context of ACT + OV, we wondered if incorporating MS-275 could similarly enhance the efficacy of our treatment platform. To test this, we monitored tumor growth in B16-gp33-bearing mice after treatment with ACT + OV + MS-275. MS-275 was administered intraperitoneally after OV-boosting and was given daily for 5 days. Treatment of mice with MS-275 alone or in combination with VSV-gp33 was unable to control the initial tumor, whereas gp33-specific TCM + VSV-gp33 + MS-275 treatment completely regressed the initial tumor and prevented tumor relapse (Fig. 2A), leading to durable cure (Fig. 2B).

By contrast, ACT + OV + MS-275 treatment failed to control the growth of B16-gp33R tumors (Fig. 2C), suggesting that immune escape may be due to irreversible antigen loss. Indeed, PCR analysis of genomic DNA from B16-gp33R cells showed no detectable PCR product compared to B16-gp33 cells (Fig. 2D), confirming the loss of gp33 expression on a genomic level. Our findings thus suggest that concomitant MS-275 delivery may prevent the emergence of antigen-loss variants from selective T-cell pressure and/or inhibit immune escape through direct eradication of pre-existing variants. To investigate these possibilities, we mixed 100 luciferase-expressing B16-F10 (B16-F10-luc) cells with 105 B16-gp33 cells to generate chimeric tumors. As shown in Figure 2F, luciferase signal was clearly detectable 10 days post chimeric tumor implantation confirming B16-F10-luc cell survival and expansion. Consistent with previous observations, gp33-specific TCM + VSV-gp33 only induced transient tumor regression (Fig. 2E) and B16-F10-luc cells continued to

expand in relapsed tumors (increased luciferase signal on day 30; Fig. 2F). Comparatively, treatment with gp33-specific TCM + VSV-gp33 + MS-275 led to durable regression of chimeric tumors (Fig. 2E), suggesting that the addition of MS-275 can directly promote the eradication of antigen-loss variants.

ACT + OV + MS-275 subverts the inflammatory status of tumor-infiltrating myeloid cells and reprograms them into killer cells

One possible mechanism for the therapeutic benefit conferred by MS-275 is the functional improvement of antigen-specific CD8+ T cells, which could mediate more complete eradication of the tumor to prevent relapse. However, the frequency and functionality of antigen-specific CD8+ T cells derived from peripheral blood or tumors did not differ between mice receiving ACT + OV and ACT + OV +MS-275 treatment (Fig. S2). Furthermore, qualitative marker expression (TNFα, KLRG1, PD-1) (Fig. S2) and in vitro/in vivo killing (Fig. S3) were relatively unchanged, suggesting that MS-275 may instead leverage alternative cytotoxic cell types or killing mechanisms to eliminate antigen-loss variants.

To gain perspective of the global changes occurring within the tumor microenvironment, we conducted a microarray using tumor RNA from differentially treated mice. Gene ontology term enrichment analyses of differentially expressed genes 5 days post OV-boost indicate a variety of biological processes that were altered as a result of concomitant MS-275 delivery (Table 1); in particular, myeloid leukocyte activation involved in immune response (GO:0002283) and myeloid leukocyte chemotaxis (GO:0030593) along with its associated biological processes seem to show a high degree of enrichment (>100 and 23.07 fold, respectively), indicating that the intratumoral myeloid cell compartment was affected by combination therapy.

Tumor-infiltrating myeloid cells represent a heterogeneous population of CD11b-expressing immune cells with largely immunosuppressive function and can support tumor growth. Cellular subsets can include F4/80+ tumor-associated macrophages, CD11chi tolerogenic DCs, Gr1+ tumor-associated neutrophils, Ly6Chi Ly6G- monocytic MDSCs, and Ly6Clo Ly6G+ granulocytic MDSCs (Engblom et al., 2016). While we were unable to observe significant changes to the total number of tumor-infiltrating myeloid cells (Fig. 3A), there was an increased infiltration of CD11chi cells with ACT + OV treatment, though their frequency was unchanged in the presence of MS-275 (Fig. 3B, S8). Indeed, MS-275 did not seem to alter the frequency of any myeloid subset, including CD11c-/lo F4/80+ cells. Ly6C and Ly6G gating on CD11c-/lo F4/80+ cells showed that, as was consistent with literature (Haverkamp et al., 2011; Mairhofer et al., 2015), untreated tumor masses were more heavily infiltrated with Ly6Chi Ly6G- cells compared to Ly6Clo Ly6G+ cells

and their relative proportion was unaltered by treatment (Fig. 3B, S8). Interestingly, Ly6Chi Ly6G- cells displayed increased expression of Ly6G and fluctuating expression of CD11c upon ACT + OV and, to a larger extent, ACT + OV + MS-275 treatment (Fig. 3C), suggesting that MS-275 may induce a progressive phenotypic shift in tumor-infiltrating myeloid cells. Indeed, morphological analysis of cytospin preparations derived from purified tumor-infiltrating myeloid cells showed a distinct transition from cells with immature/young monocytic cytological features (fine azurophilic granules, greyish blue cytoplasm, no visible nucleolus) to ones with classically activated macrophage-like characteristics (large distended cytoplasm, rounded rather than elongated shape, phagocytosed cellular debris, highly vacuolated) (Fig. 3D) (Goasguen et al., 2009; McWhorter et al., 2013). Based on these therapy-driven morphological changes, we postulated that tumor-infiltrating myeloid cells were undergoing a pro-inflammatory response. Using established markers of pro-inflammatory (NOS2, ROS) and pro-healing (Arg1) phenotypes typically characterized in macrophage polarization studies (Lopez-Castejon et al., 2011), we observed increased NOS2 (Fig. 3E, F) and ROS (Fig. 3G) and decreased Arg1 (Fig. 3H) expression in tumor-derived Ly6Chi Ly6G- gated myeloid cell populations upon ACT + OV treatment. Moreover, in the presence of MS-275, these changes were further pronounced, indicating that the pro-inflammatory status of tumor-infiltrating myeloid cells may be further enhanced. NOS2-related changes (Fig. S4) were also observed in the aforementioned mERK model (Fig. S1), suggesting that these myeloid cell changes were not tumor model or mouse strain specific. Altogether, the data suggest that concomitant MS-275 delivery may potentiate therapy-induced changes to the inflammatory status of tumor-infiltrating myeloid cells.

We next sought to determine if tumor-infiltrating myeloid cells derived from ACT + OV + MS-275-treated mice were functionally dissimilar from those of untreated or ACT + OV-treated mice. We first set up a proliferation assay using CFSE-labeled P14 T cells as responders that were stimulated with gp33 peptide-pulsed dendritic cells. Figure 4A-C shows that tumor-derived myeloid cells from ACT+ OV + MS-275-treated mice possessed a reduced capacity to suppress P14 T cell proliferation compared to those from untreated or ACT + OV-treated mice. We next investigated if tumor-infiltrating myeloid cells, in addition to losing their immunosuppressive function, were directly involved in the killing of antigen-loss variants. Indeed, when co-cultured with B16-F10 cells that do not express gp33, tumor-infiltrating myeloid cells derived from ACT + OV + MS-275-treated mice demonstrated significant killing capacity (Fig. 4E) in a cell contact-dependent manner (Fig. S5). Interestingly, histological staining of tumor tissues showed localized expression of cleaved caspase 3, an apoptosis marker, with CD11b+ cells following ACT + OV + MS-275 therapy (Fig. 4D), also supporting the notion that MS-275-influenced tumor-infiltrating myeloid cells may provide an antitumor effector role by effectively eradicating antigen-

negative tumor cells. To directly address this hypothesis, we isolated tumor-infiltrating myeloid cells from ACT + OV + MS-275-treated mice (CD11bTVM) and injected them into tumors one day before treatment with ACT + OV. Figure 4F shows that this intratumoral injection was able to recapitulate the therapeutic effects of MS-275 delivery resulting in prolonged survival (Fig. 4G). It is therefore apparent that enhanced inflammatory reprogramming of tumor-infiltrating myeloid cells by MS-275 subverts their immunosuppressive function and confers cytotoxic killing capacity, ultimately preventing tumor relapse.

Altered inflammatory status in tumor-infiltrating myeloid cells is representative of M1 polarization

To better understand the mechanisms by which MS-275 influences the function of tumor-infiltrating myeloid cells in the context of ACT + OV therapy, we sorted CD11b+ Ly6Chi Ly6G- cells from tumor tissues and used an nCounter codeset to quantify the gene expression of 179 inflammation-related mouse genes that are commonly used to define the polarization status of macrophages, namely M1 and M2, a paradigm that has also been observed in other cells within the myeloid lineage (Fridlender et al., 2009; Yang et al., 2013). Gene set enrichment analysis showed enrichment of M2 genes in untreated mice (Table S1) while M1 genes were enriched in both ACT + OV and ACT + OV + MS-275-treated mice (Fig. 5), suggesting that combination treatment in the absence of MS-275 is sufficient to reprogram CD11b+ Ly6Chi Ly6G- cells to express genes congruent with an M1 profile. However, as we previously demonstrated, the extent of NOS2 upregulation and Arg1 downregulation in CD11b+ Ly6Chi Ly6G- cells was more pronounced during concomitant MS-275 delivery. We postulate that the drug enables stronger M1-signaling within the tumor microenvironment, leading to a hyper-polarized state.

M1 hyper-polarization is dependent on local IFNγ and enhanced Type II-interferon-mediated signaling

Having delineated the therapeutic role of M1 polarization in T-cell therapy, we set out to answer how concomitant MS-275 delivery alters the tumor microenvironment to promote M1 polarization in tumor-infiltrating myeloid cells. We hypothesized that the functional phenotypes seen in M1 or M2 activation are a dynamic response to combinations of environmental stimuli. Using tumor homogenate from differentially treated mice, the cytokine landscape was assessed with a 31-plex cytokine array and visualized by heat map (Fig. 6A). Principal component analysis indicated that treatment clusters (ACT + OV, ACT + OV + MS-275) were highly removed from untreated clusters (No Tx), confirming that the differential enrichment of M1/M2 genes in CD11b+ Ly6Chi Ly6G- cells corresponded to unique intratumoral cytokine signatures (Fig. 6B). While tightly grouped, ACT + OV

and ACT + OV + MS-275 clusters were also distinct from one another. Indeed, while the relative concentrations of most inflammatory cytokines (including M2-stimulating cytokines IL4 and IL13) did not change between ACT + OV and ACT + OV + MS-275 (Fig. 6A), several inflammatory cytokines were significantly upregulated by MS-275 including M2-related cytokines M-CSF (6.39-fold) and IL10 (2.95-fold) and M1-stimulating cytokine IFNγ (6.3-fold) (Fig. 6C). It should be noted that the absolute concentration of M-CSF/IL10 was near the lower limit of detection (Fig. S6), while IFNγ was present at high concentrations (~1.5-2.0 ng/mL) upon ACT + OV + MS-275 treatment (Fig. 6D). Coupled with qRT-PCR analysis of IFNG from whole tumor RNA (Fig. 6E), the data suggest that enhanced local production of IFNγ from tumor infiltrating lymphocytes such as CD8+ T cells (Fig. S7) may contribute to the hyper-polarization of tumor-infiltrating myeloid cells towards an enhanced M1 phenotype. This possibility was supported by subsequent analyses where enhanced STAT1 phosphorylation was observed in tumor-derived CD11b+ Ly6Chi Ly6G- gated cells when MS-275 was present (Fig. 6F). To firmly establish a causal relationship between IFNγ signalling and myeloid cell polarization, we included a group of tumor bearing IFNγ receptor-deficient mice that were treated with ACT + OV + MS-275. The data in Figure 6G indicate that the effect of MS-275-mediated M1 hyper-polarization was abrogated in IFNγ receptor-deficient mice, resulting in a reduced frequency of NOS2-expressing CD11b+ Ly6Chi Ly6G- cells. Conversely, repeating the study in IFNα receptor-deficient mice did not alter NOS2 expression, suggesting that the role of Type I IFN was not required for myeloid cell polarization (data not shown).

Discussion

In this study, we describe a treatment platform which incorporates the transfer of tumor-specific central memory T cells (TCM) with a recombinant oncolytic virus (OV) expressing the tumor antigen of interest. This combination induced robust T cell expansion and complete tumor regression in three different models, irrespective of the nature of tumor antigens and strain of mice, confirming the synergy and complementarity between ACT and OV vaccination. While this tumor regression was durable in two models (DCT and mERK), it was transient in the gp33 model, with tumors relapsing after 10-20 days. Genetic analyses revealed that relapsed tumor cells no longer expressed the gp33 gene and failed to be recognized by gp33-specific T cells. Strikingly, concomitant MS-275 delivery with ACT + OV treatment induced complete and durable tumor regression. Though HDAC inhibitors modulate transcription on an epigenetic level and can thus instigate broad biological effects, we observed that MS-275 could help reprogram tumor-infiltrating myeloid cells to directly eliminate antigen-loss tumor cell variants and, by transfusing these cells into ACT+OV-treated mice, the enhanced therapeutic benefits that we observed with MS-275 could be recapitulated. The transcriptional changes mediated by MS-275 on other cell types which may potentiate the efficacy of ACT + OV vaccination remain to be determined.

Tumor-infiltrating myeloid cells broadly represent immunosuppressive cells with pro-tumoral activity. During cancer, they accumulate in secondary lymphoid tissue and tumor sites wherein they suppress antigen-specific T cell activity through direct cell-contact, secretion of soluble factors, or competitive uptake of essential amino acids/cytokines (Gordon et al., 2017; Marvel and Gabrilovich, 2015). As a result, the depletion of tumor-infiltrating myeloid cells to enhance antitumor immune responses has been pursued intensely as a therapeutic strategy (De Henau et al., 2016; Lu et al., 2017). However, identifying selective inhibitors of tumor-infiltrating myeloid cells can be difficult due to their phenotypic heterogeneity and readiness to differentiate into one another (Corzo et al., 2010; Kumar et al., 2016; Strauss et al., 2015).

By contrast, it is known that the local cytokine environment can dictate the functional programming of tumor-infiltrating myeloid cells by influencing M1/M2 activation. While tumor-infiltrating myeloid cells predominantly display M2-like phenotypes (Ochando and Chen, 2012), they have been demonstrated to express arginase and nitric oxide as well as both pro-inflammatory M1 and anti-inflammatory M2 genes (Umemura et al., 2008). This may suggest that polarizing myeloid cell function in favor of a pro-inflammmatory phenotype can offer an alternative approach to subverting tumor-infiltrating myeloid cell suppressiveness. Indeed, others have shown that myeloid cell polarizing agents including cationic polymers (He et al., 2016), and inhibitors of CSF-1R (Pyonteck et al., 2013), SHP1

(Ma et al., 2011), TGFβR kinase (Fridlender et al., 2009) can reduce local immunosuppression in order to potentiate antitumor immune responses. In our model, we observed that the addition of MS-275 could also enhance M1 polarization, reducing the inhibitory effects of tumor-infiltrating myeloid cells on T cell proliferation. However, it is apparent that prevention of tumor relapse was unlikely mediated through tumor-specific T cell improvement since the magnitude, quality, and killing capacity of gp33-specific CD8+ T cells were unchanged. In fact, ACT + OV treatment alone was sufficient to completely control the initial tumor, suggesting that local immunosuppression was likely overcome by the rapid and potent therapy-driven antitumor immune response as was suggested by others (McGray et al., 2014). Instead, we observed that concomitant MS-275 delivery with ACT + OV treatment conferred tumoricidal properties to M1-polarized tumor-infiltrating myeloid cells.

Myeloid-derived killer cells have been postulated to suppress tumor growth through the enhanced expression of free radicals and death ligands (Marigo et al., 2016; Pilon-Thomas and Ruffell, 2016); however, until now their clinical relevance has not been clearly defined. In this study, we demonstrate that myeloid-derived killer cells can enhance therapeutic outcomes by directly clearing antigen-loss variants. Interestingly, while ACT + OV + MS-275 can durably regress chimeric tumors that have antigen-negative cells mixed in, B16-gp33R tumors, of which the tumor bulk is composed of antigen-negative cells, cannot be controlled. This suggests that while tumor-infiltrating myeloid cells can decrease the efficacy of ACT through immunosuppression, co-ordinated antitumor responses from both tumor-infiltrating myeloid cells and antigen-specific CD8+ T cells can mediate complete tumor destruction and prevent relapse. Consequently, the polarization of tumor-infiltrating myeloid cells into myeloid-derived killer cells can multi-mechanistically complement ACT by subverting myeloid suppressiveness and enable the eradication of heterogeneous or therapy-resistant tumor cells.

MS-275 possesses pleiotropic effects and has been investigated as an anticancer agent, either alone or in combination with other targeted therapies (Nguyen et al., 2014). While MS-275 is also under investigation as an anti-inflammatory and immunosuppressive drug in the context of autoimmune diseases and allograft survival (Haberland et al., 2009; Shakespear et al., 2011), it has paradoxically been shown to enhance T-cell therapy (Bridle et al., 2013). Using microarray analysis, we confirmed in this study that OV administration induced acute inflammatory responses within the tumor, which were attenuated by MS-275 (Table 1). However, several local inflammatory signals remained at a high level and even increased in the presence of MS-275, potentially creating a more favourable environment for M1 polarization. This may explain why tumor-derived myeloid cells from both ACT + OV- and ACT + OV + MS-275-treated mice showed enrichment for M1-related genes, yet

only concomitant MS-275 delivery was able to confer tumoricidal capability. In particular, the level of local IFNγ was markedly increased and the effect of MS-275-mediated M1 hyper-polarization was abrogated in IFNγ receptor-deficient mice, establishing a critical role of IFNγ in enhancing the polarization of tumor-infiltrating myeloid cells into myeloid-derived killer cells. Consequently, we suggest that modifying the intratumoral cytokine landscape in favor of selective key pro-inflammatory cytokines (such as IFNγ) is necessary to invoke antigen-independent antitumor mechanisms to help promote durable tumor regression.

IFNγ has been well-characterized as an essential component of an effective antitumor immune response. Direct IFNγ has been demonstrated to induce tumor senescence or killing (Chin et al., 1997; Ikeda et al., 2002), and defects in IFNγ pathway genes have been shown to adversely affect patient responsiveness to anti-CTLA-4 therapy (Gao et al., 2016). IFNγ can also have tumor stromal targeting effects leading to regression of tumor vasculature and non-haemorrhagic necrosis (Kammertoens et al., 2017). However, tumor sensing of IFNγ can mediate adaptive alterations in gene expression leading to increased resistance to antitumor immune responses. It has been previously reported that immune checkpoints such as PD-L1 are up-regulated in response to IFNγ resulting in subsequent deactivation of cytotoxic T cells (McGray et al., 2014). Furthermore, there are even reports suggesting that IFNγ facilitates genome immunoediting and the emergence of resistant cancer clones (Takeda et al., 2017). As a result, there is ongoing discussion whether IFNγ is necessary for successful antitumor immunity. Interestingly, while ACT + OV + MS-275 therapy is a significant driver of intratumoral IFNγ, we did not observe reduced functionality in tumor-infiltrating lymphocytes. We postulate that, relative to the slow activation of antitumor immune responses during traditional cancer vaccination, the rapid expansion and tumor infiltration of transferred T cells during ACT + OV treatment mediates significant de-bulking of the tumor before tumor adaptation can occur. Likewise, due to the enhanced therapeutic benefit we observed during concomitant MS-275 delivery, IFNγ's direct impact on antigen loss may be negligible compared to the potent antitumor response elicited by IFNγ-polarized myeloid derived killer cells. Altogether, the combination treatment we described may bypass negative regulation to facilitate an additional antitumor role for IFNγ in the elimination of antigen-loss variants.

Acknowledgements

Author Contributions

A.N. designed and conducted the majority of the experiments described and wrote the manuscript. L.H. prepared the samples for microarray analysis. L.C. monitored the therapeutic efficacy of ACT+OV+MS-275 in DCT and mERK models. J.S. conducted the FISH staining on treated tumor sections. J.P. constructed the VSV-gp33 viral vector and S.R.W. constructed the VSV-mERK viral vector. S.T.W. and J.L.B participated in the conception of experimental designs and manuscript writing. Y.W. supervised the study, and participated in the conception of experimental designs and manuscript writing.

Declaration of Interests

The authors report no conflicts of interest.

Data Availability

The accession numbers for the expression data reported in the paper are GEO: GSE101985.

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Main Figure Titles and Legends

Figure 1: ACT+OV-mediated CD8+ T cell expansion regressed tumors, but subsequent loss of antigen recognition promotes tumor relapse

In C57BL/6 mice (n=5 per group), (A) five to (B) ten day old intradermal B16-gp33 tumors were given adoptive cell therapy (ACT, 104 gp33-specific TCM delivered i.v.) followed by oncolytic viral (OV) vaccination with 2x108 pfu VSV-gp33 i.v. (See also Figure S1). Tumor volumes were calculated based on height, width, and length. (C) Five days post-vaccination, the frequency of gp33-specific CD8+ T cells, as determined by IFNγ expression after ex vivo peptide stimulation, was measured from peripheral blood. (D) Monoclonal antibodies targeting CD8, CD4, and NK1.1 were administered i.p. one day before and after VSV-boosting and every two weeks afterwards. (E) Tumor volumes of ACT+OV-treated mice (n=5) were continuously measured up to 40 days post-tumor challenge. After varying the dose of TCM, (F) the frequency of gp33-specific CD8+ T cells was measured from peripheral blood and (G) tumor volumes were monitored (n=3-4 per group). (H) C57BL/6 mice (n=3 per group) were pre-treated with Ad-gp33 and challenged with parental or relapsed B16-gp33 after two weeks. Results are mean ☐ SEM. To determine significance one way ANOVA was used (**** $p<0.0001$); VSV-MT, VSV without transgene

Figure 2: Concomitant MS-275 delivery prevents tumor relapse by mediating the elimination of gene-loss variants

B16-gp33 tumor-bearing mice (n=5 per group) were treated with ACT+OV. Concomitantly, MS-275 was injected i.p. daily for 5 days. (A) Tumor volumes were used to generate (B) Kaplan-Meier survival curves. (C) B16-gp33R (n=3 per group) or (E) chimeric B16-gp33/B16-F10luc tumor-bearing mice (n=5 per group) were treated with ACT+OV+MS-275. (D) Genomic DNA was used as a template for PCR analysis of gp33 expression. (F) In vivo imaging of B16-F10luc cells in treated mice. Fluorescence signal was quantified by photon flux after i.p. injection of 75 mg/kg D-Luciferin. Results are mean ☐ SEM. B16-gp33R, relapsed B16-gp33 tumor explant; B16-F10luc, luciferase-expressing B16-gp33 cells

Figure 3: Concomitant MS-275 delivery induces a pro-inflammatory phenotype within tumor-infiltrating CD11b+ Ly6Chi Ly6G- myeloid cells

Five days post-treatment, digested tumors (n=3-5 per group) were enriched for CD11b+ cells. (A) Absolute counts of CD45.2+ CD11b+ cells were quantified and (B) frequencies of myeloid cell subsets were depicted (See also Figure S8). (C) Representative histogram overlays of Ly6G and CD11c are shown for CD11b+ CD11c-/lo F4/80+ Ly6Chi Ly6G- myeloid cells and mean fluorescence intensities were enumerated. (D) CD11b+ cells were Giemsa-Wright stained and imaged at 40x magnification (Scale bar: 5 μm). (E) Representative histogram overlays of NOS2 are shown for CD11b+ Ly6Chi Ly6G- and CD11b+ Ly6Clo Ly6G+ cells (See also Figure S4). The frequency of CD11b+ Ly6Chi Ly6G- cells expressing (F) NOS2 and (H) Arg1 was measured. (G) Carboxy-H2DCFDA was used to fluorescently detect reactive oxygen species in tumor-derived CD11b+ cells. Results are mean □ SEM. To determine significance one way ANOVA was used (*** p=0.0004, *** p=0.0003, * p=0.0479)

Figure 4: Pro-inflammatory tumor-infiltrating myeloid cells display decreased suppressiveness and demonstrate tumor killing capacity

(A-C) Enriched CD11b+ cells (n=3 per group) were co-cultured for 3 days with stimulated CFSE-labeled P14 T cells. CFSE dilution is shown as (A) a representative histogram, (B) cellular division index and (C) percentage of divided cells. (D) B16-F10 tumors (n=3 per group) were co-stained with cleaved caspase 3 and CD11b and imaged at 20x magnification (Scale bar: 25 μm). (E) Enriched CD11b+ cells derived from ACT+OV+MS-275-treated mice (CD11bTVM) were co-cultured with B16-F10 target cells at a 10:1 ratio. Killing was measured after 12 hours by MTT reduction and done in triplicate (See also Figure S5). (F, G) CD11bTVM cells were intratumorally injected into B16-gp33 tumor-bearing mice (n=5 per group), treated with ACT+OV, and monitored for (F) tumor growth and (G) survival. Results are mean □ SEM. To determine significance one way ANOVA was used (NS=not significant, **** p<0.0001, *** p=0.0002)

Figure 5: ACT+OV and ACT+OV+MS-275 reprogram tumor-infiltrating myeloid cells from an M2 phenotype to an M1 phenotype

Enriched CD11b+ cells (n=3 per group) were pooled and sorted into Ly6Chi Ly6G- and Ly6Clo Ly6G+ fractions. RNA from sorted Ly6Chi Ly6G- cells was analyzed using an nCounter inflammatory gene codeset. Gene set enrichment analysis was conducted using manually curated lists representing M1 and M2 inflammatory profiles (See also Table S1). Enrichment score displays the extent of 'enrichment' in a specific list when comparing ACT+OV+/-MS-275 to no treatment. Members of each profile are placed in the rank list

('bar code') which displays the total number of genes in the codeset. A signal-to-noise metric is represented by a red/blue gradient (red represents treated and blue represents untreated). Each comparison demonstrates significant enrichment (false discovery rate, <10-2) and data are representative of two independent experiments.

Figure 6: Concomitant MS-275 delivery enhances M1 polarization further through heightened local IFNγ/IFNγR signaling

Five days post-treatment, B16-gp33 tumors (n=4 per group) were homogenized and sent for multiplex cytokine analysis. (A) Heat map displaying the relative changes in RMA-normalized, log2 transformed tumor cytokine concentrations. (B) Principal component analysis of tumor cytokine concentrations showing clustering of treatment groups. (C) Revised heat map showing fold concentration changes for cytokines displaying significant differential expression when comparing ACT+OV+MS-275 to ACT+OV treatment (See also Figure S6). (D) Concentration of intratumoral IFNγ. (E) qRT-PCR of IFNG expression from whole tumor RNA (n=3 per group) (See also Figure S7). (F) Enrichment of CD11b+ cells followed by pSTAT1 staining of Ly6Chi Ly6G- gated cells (n=5 per group). (G) Enrichment of CD11b+ cells from IFNγR -/- mice followed by NOS2 staining of Ly6Chi Ly6G- gated cells (n=5 per group). Results are mean □ SEM. To determine significance one way ANOVA was used (* p=0.0349, ** p<0.01, ** p=0.0033, **** p<0.0001)

Main Table Titles and Legends

Table 1: GO enrichment analysis for differentially expressed genes in ACT+OV+MS-275 vs ACT+OV-treated tumors

Experimental Procedures:

Mice

Female age-matched (6-8 weeks old) C57BL/6 and Balb/C mice were purchased from Charles River Laboratory, B6.129S7-Ifngr1tm1Agt/J (IFNGR KO) mice were purchased from The Jackson Laboratory, and B6.Cg-Tcratm1Mom Tg(TcrLCMV)327Sdz (P14) mice were purchased from Taconic. DUC18 mice were kindly provided by Dr. Lyse Norian (University of Iowa) and 24H9R mice were kindly provided by Dr. Andy Hurwitz (Frederick National Laboratory for Cancer Research, National Cancer Institute). Mice were housed in the Central Animal Facility at McMaster University and all animal studies complied with the Canadian Council on Animal Care guidelines and were approved by McMaster University's Animal Research Ethics Board.

Viral Vectors

LCMV-Armstrong, VSV-gp33, and Ad-gp33 were described previously (Ahmed et al., 1984; Bassett et al., 2011; Zhang et al., 2013). VSV-MT lacks the gp33 transgene and was utilized as a control vector.

Peptides

The H-2Db-restricted peptide of LCMV-GP33-41 (KAVYNFATM) was purchased from the Dalton Chemical Laboratory and dissolved in ddH2O.

Cells and Culture Conditions

B16-gp33 cells were generated as described previously (Prevost-Blondel et al., 1998). B16-gp33, B16-F10, B16-F10luc, B16-gp33R, and CMS-5 cells were grown as previously described (Bridle et al., 2013; Bridle et al., 2016).

Tumor Challenge

Mice were challenged intradermally with 105 B16-gp33 cells in 30 μl PBS. Tumor growth was monitored as previously described (Bridle et al., 2016). Chimeric tumors were

generated by mixing B16-gp33 with B16-F10luc cells at a 1000:1 ratio before intradermal injection. In vivo monitoring of these tumors was conducted using an IVIS Spectrum In Vivo Imaging System (Caliper Life Sciences) following intraperitoneal injection of 75 mg/kg D-luciferin (Caliper Life Sciences).

Challenge with Relapsed Tumor

Relapsing tumors (B16-gp33R) were excised and suspended in EDTA 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 RPMI (7 mL/g of tumor) at 37$\Box$C for 1 hr. B16-gp33R cells were subsequently filtered through a 40 µM strainer, washed, and re-suspended in culture media or PBS.

Adoptive T cell Transfer

Spleens were collected from LCMV-Armstrong-infected mice (>1 month) and a single cell suspension was prepared. Gp33-specific central memory T cells (TCM) were enumerated by staining with H-2Db-GP33 tetramer (Baylor College of Medicine), CD127, and CD62L (BD Biosciences). 104 gp33-specific TCM in 200 µL PBS were adoptively transferred into tumor-challenged mice by intravenous injection.

Oncolytic Viral Vaccination

Twenty-four hours post-adoptive T cell transfer, mice were injected intravenously with 2x108 pfu of VSV-gp33 in 200 µL PBS. Concomitantly, MS-275 (Sigma-Aldrich) was intraperitoneally injected (100 µg/mouse in 50 µL PBS) daily for five days. Selective lymphocyte depletion was conducted using monoclonal antibodies (produced in-house at McMaster University) specific for CD8+ T cells, CD4+ T cells, or NK cells. Mice were injected with 250 µg of mAb in 500 µL PBS on Day -1 and 1 post-vaccination and every two weeks afterwards (150 µg). In some experiments, CD11b-enriched cells (described below) derived from gp33-specific TCM + VSV-gp33 + MS-275-treated mice were injected intratumorally (~3x105 cells in 30 µL PBS) at day 0 post-vaccination instead of MS-275.

Detection of Antigen-specific Responses

Five days post-vaccination, PBMCs were stimulated at for 5 hours with gp33 peptide in the presence of brefeldin A (GolgiPlug; BD Biosciences). Following surface staining for CD8α (BD Biosciences), cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained for intracellular IFNγ (BD Biosciences). Data were acquired using an LSRFortessa flow cytometer with FACSDiva software (BD Biosciences) and analyzed with FlowJo X, version 10.0.7 (Treestar).

Genomic DNA Extraction and PCR

Genomic DNA was extracted from B16-gp33, B16-F10, and B16-gp33R cells using Purelink Genomic DNA Extraction Kits (Life Technologies). PCR was carried out using Taq DNA polymerase (New England Biolabs) using the gp33 primer sequence FWD – GTCCTTTGGGCGCTAACTG, REV – GTGGCGAAATTGTACACAGC.

Tumor RNA Extraction and qRT-PCR

B16-gp33 tumors were excised 5 days post-vaccination and snap-frozen in liquid nitrogen and samples were then homogenized in Trizol (Invitrogen). RNA was extracted and purified using an RNeasy mini kit (Qiagen) and treated with Ambion's DNA-free kit. Reverse transcription was performed with Superscript II First-Strand reverse transcriptase (Thermo Fisher Scientific). qRT-PCR was done as previously described (Workenhe et al., 2016). The primer sequences are as follows: IFNG FWD – CTTGAAAGACAATCAGGCCATC, IFNG REV – CAGCAGCGACTCCTTTTCC; HPRT FWD – ACACCTGCTAATTTTACTGGCAACA, HPRT REV - TGGAAAAGCCAAATACAAAGCCTA

Gene Expression Analysis by Microarray

RNA from B16-gp33 tumors was isolated five days post-vaccination and prepared for profiling on MouseRef8_V2_0_R3_11278551_A beadchips (Illumina) according to the manufacturer's protocol. Global expression profiles were preprocessed with the Illumina package in R (Du et al., 2008), and only annotated and present genes were used for further analyses. During the preprocessing, the obtained values were VST transformed (difference variance stabilizing transformation (Lin et al., 2008)) and background correction was

performed; then the obtained data were normalized with quantile normalization. Genes were considered differentially expressed if the fold change was >2.0 and q value was <0.1. Gene ontology term enrichment analysis was conducted by PANTHER overrepresentation test (release 20160715) using the GO database (released 2016-12-29) with GO biological process complete annotation data set. The reference gene list was the Mus musculus genome and expression data analyses include Bonferroni correction for multiple testing. Overrepresented GO terms were sorted hierarchically and displayed results are statistically significant (P<0.05).

Purification of Tumor-infiltrating Myeloid Cells

B16-gp33 tumors were excised 5 days post-vaccination and digested in 0.5 mg/mL collagenase Type IV (Gibco), and 0.2 mg/mL DNase (Roche) prepared in complete RPMI (Gibco, 10 mL/250 mg tumor) and incubated at 37□C for 30 min. The digested material was CD11b+ enriched through magnetic selection with an EasySep Mouse CD11b Positive Selection Kit II (Stemcell Technologies). CD45.2, CD11b, CD11c, F4/80, Ly6C, and Ly6G (BD Biosciences) staining antibodies were used to differentiate myeloid cell subsets, while Nos2 (Thermo Fisher Scientific), Arg1 (R&D Systems), pStat1 (BD Biosciences) were used to characterize their inflammatory status.

Cytospin of Tumor-infiltrating Myeloid Cells

Tumor-infiltrating myeloid cells were isolated, re-suspended in PBS, and cytospun (200 μl, 300 rpm, 2 min) in a cytocentrifuge (Cytospin 4; Thermo Fisher Scientific) and differentially stained with Giemsa (Sigma-Aldrich) for morphological analyses. Images were captured at 40X magnification using a Micropublisher 5.0 RTV camera (QImaging) on a Leica DMRA microscope (Leica).

Immunofluorescence

B16-gp33 tumors excised 5 days post-vaccination were snap frozen before cryostat sections were obtained and mounted on gelatin-coated histological slides. The slides were fixed in 4% paraformaldehyde for 20 min at room temperature and blocked with PBS containing 5% goat serum and 0.3% Triton X-100 for 30 min. Anti-Cleaved Caspase-3 (1:400, Cell Signaling Technology) and anti-CD11b (1:400, Thermo Fisher Scientific) were added and incubated overnight at 4□C. Slides were incubated for 1h with biotinylated goat anti-rabbit

IgG (Vector Laboratories) and Texas Red-conjugated goat anti-rat IgG (Cedarlane Labs) followed by Alexa Fluor 488-conjugated streptavidin (Thermo Fisher Scientific). Images were captured at 20X magnification using a Micropublisher 5.0 RTV camera (QImaging) on a Leica DMRA microscope (Leica).

ROS assay

Tumor-infiltrating myeloid cells were isolated and re-suspended in PBS before loading with Carboxy-H2DCFDA as reported by others (Wu and Yotnda, 2011) and measured for fluorescence (435 nm excitation, 535 nm emission) using a Synergy microplate reader (Biotek Instruments). Loaded cells were treated with 1 mM H2O2 for 20 minutes as a positive control.

Proliferation assay

Tumor-infiltrating myeloid cells were isolated and co-cultured for 3 days with gp33-peptide pulsed dendritic cells and CFSE-labeled P14 T cells as previously reported (Quah and Parish, 2010). Tumor-infiltrating myeloid cells were added at a 1:1 or 0.5:1 CD11b to T cell ratio. Proliferation was evaluated using several metrics including the division index and the percentage of cells divided (Roederer, 2011).

In vitro Cytotoxicity Assay

B16-F10 cells (1x104 /well) were co-cultured with tumor-infiltrating myeloid cells (1x105/well) in 96-well flat bottom microtiter plates (Corning Inc.) for 12 hours before killing was assessed. Non-adherent cells were washed using warm PBS and 500 μg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thermo Fisher Scientific) solution was added before incubating the plates for 4 hours. Solubilization of the formazan by-product was done by aspirating the MTT solution and adding DMSO. The absorbance was measured at 540 nm using a Synergy microplate reader (Biotek).

Gene Expression by Nanostring

Tumor-infiltrating myeloid cells were sorted using a BD FACSAria Flow Cytometer into CD11b+ Ly6Chi Ly6G- and CD11b+ Ly6Clo Ly6G- fractions. Extracted RNA was

analysed using the nCounter Mouse Inflammation Gene Expression CodeSet v2 (Nanostring Technologies). Data was background-subtracted and normalized using nSolver Analysis Software (Nanostring Technologies). Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) was performed using GenePattern Version X (Reich et al., 2006). We examined the enrichment of two manually curated lists: (a) M1 Profile, reflective of the M1 phenotype (34 genes) and (b) M2 Profile, reflective of the M2 phenotype (25 genes) (Table S1) (Benoit et al., 2008; Butovsky et al., 2012; Lechner et al., 2010; Martinez and Gordon, 2014).Two pair-wise comparisons were performed: gp33-specific TCM + VSV-gp33 vs No Tx and gp33-specific TCM + VSV-gp33 + MS-275 vs No Tx.

Multiplex Cytokine Analysis

B16-gp33 tumors were excised 5 days post-vaccination and homogenized in lysing solution (Bioplex Cell Lysing Kit, Bio-Rad Laboratories). The multiplex assay was performed by Eve Technologies using the Bio-Plex 200 system and the Milliplex Mouse Cytokine/Chemokine Magnetic Bead Panel Kit according to their protocol. Multiplex data were preprocessed with the Affymetrix package in R (Gautier et al., 2004) involving RMA background adjustment and quantile normalization (Irizarry et al., 2003). Resulting expression values were transformed to the log2 scale for further analysis. After preprocessing we performed principal component analysis (princomp function from "stats" package in R) to confirm that samples were clustering into homogeneous groups matching experimental groups. Heat maps were created using the HeatMapImage (version 6) module available on Gene Pattern (http://genepattern.broadinstitute.org/gp/pages/index.jsf). To test for differential expression for pre-specified contrasts, linear models were fit for each cytokine using the "limma" package (Ritchie et al., 2015). Obtained p-values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure.

Fluorescent in situ Hybridization

FISH was done at the MIRC Core Histology Facility with the Automated Leica Bond RX and using probes (IFNγ, CD3, CD11b) and assay kits obtained from Advanced Cell Diagnostics. Staining and analyses were conducted according to manufacturer instructions.

Statistics

GraphPad Prism version 6.00 for Windows (GraphPad Software) was used for graphing and statistical analyses. One-way analysis of variance (ANOVA) was used to query immune response data. All data were presented as means ± SEM and differences between means were considered significant at $p < 0.05$. Error bars indicate 95% confidence intervals throughout.

Supplementary Figure Titles and Legends

Figure S1, related to Figure 1A: Efficacy of ACT+OV in additional tumor models

Balb/c mice or C57BL/6 mice were challenged with CMS-5 or B16-F10 tumors respectively for five days before treatment with ACT+OV (n=5 per group). TCM were derived from TCR-transgenic mice recognizing mERK or DCT (DUC18 and 24H9R mice, respectively).

Figure S2: Concomitant MS-275 does not alter T cell magnitude or phenotype

Five days post-vaccination, the frequency of gp33-specific CD8+ T cells was measured (n=5 per group). Functional (CD107, TNFα) and exhaustion (KLRG1, PD-1) markers were also quantified.

Figure S3: Concomitant MS-275 does not improve T cell killing

(A) Gp33 peptide-pulsed bulk splenocyte targets were labeled with 5μM CFSE and i.v. injected with an equal number of 0.5μM CFSE unlabeled targets before treatment (n=3 per group). Killing was assessed by CFSE five days post-treatment from individual spleens or pooled tumors. (B) Tumor-enriched CD8+ T cells were co-cultured with B16-gp33 target cells at a 1:1 ratio (n=3 per group). Killing was measured after 12 hours by MTT reduction and done in triplicate.

Figure S4, related to Figure 3D, E: Reprogrammed pro-inflammatory tumor-infiltrating myeloid cell phenotype in the CMS-5 tumor model

Five days post-treatment, digested CMS-5 tumors were enriched for CD11b+ cells. Representative histogram and quantification of NOS2 for Ly6Chi Ly6G- gated cells.

Figure S5, related to Figure 4E: Pro-inflammatory tumor-infiltrating myeloid cells kill through cell-contact dependent mechanisms

Enriched CD11b+ cells derived from ACT+OV+MS-275-treated mice (CD11bTVM, n=3) were co-cultured with B16-F10 target cells in the presence of iNOS and ROS inhibitors (L-

NIL and SOD/Catalase, respectively) or transwell permeable supports at a 10:1 ratio. Killing was measured after 12 hours by MTT reduction and done in triplicate.

Figure S6, related to Figure 6C: Concomitant MS-275 delivery alters the intratumoral concentrations of select inflammatory-related cytokines

Concentrations of cytokines showing significant differential expression when comparing ACT+OV+MS-275 to ACT+OV treatment where n=4 per group.

Figure S7, related to Figure 6E: Elevated IFNγ in the context of MS-275 is attributed to tumor-infiltrating CD8+ T cells

B16-gp33 tumor-bearing mice were given gp33-specific TCM one day before vaccination with VSV-gp33 and MS-275. Upon depleting Ab administration, tumor volumes were measured based on height, width, and length (n=5 per group) (A) and the frequency of NOS2-expressing Ly6Chi Ly6G- cells was measured by flow cytometry in digested tumors that were purified for CD11b+ cells (n=3 per group) (B, C). (D) FISH staining of frozen tumor sections derived from ACT+OV+MS-275-treated mice (n=3 per group). (E) In vivo Golgi Plug administration for 6h on day 5 post-ACT+OV+/-MS-275 treatment (n=3 per group) before IFNγ staining of tumor-infiltrating CD8+ T cells and CD11b+ myeloid cells.

Figure S8, related to Figure 3B: Enumeration of myeloid cell subsets during ACT + OV + MS-275 treatment

Supplementary Table Titles and Legends

Table S1: M1/M2 Gene Profile, related to Figure 5

Table S2: Differentially expressed genes from the Nanostring nCounter inflammatory gene codeset, related to Figure 5

Figure 1
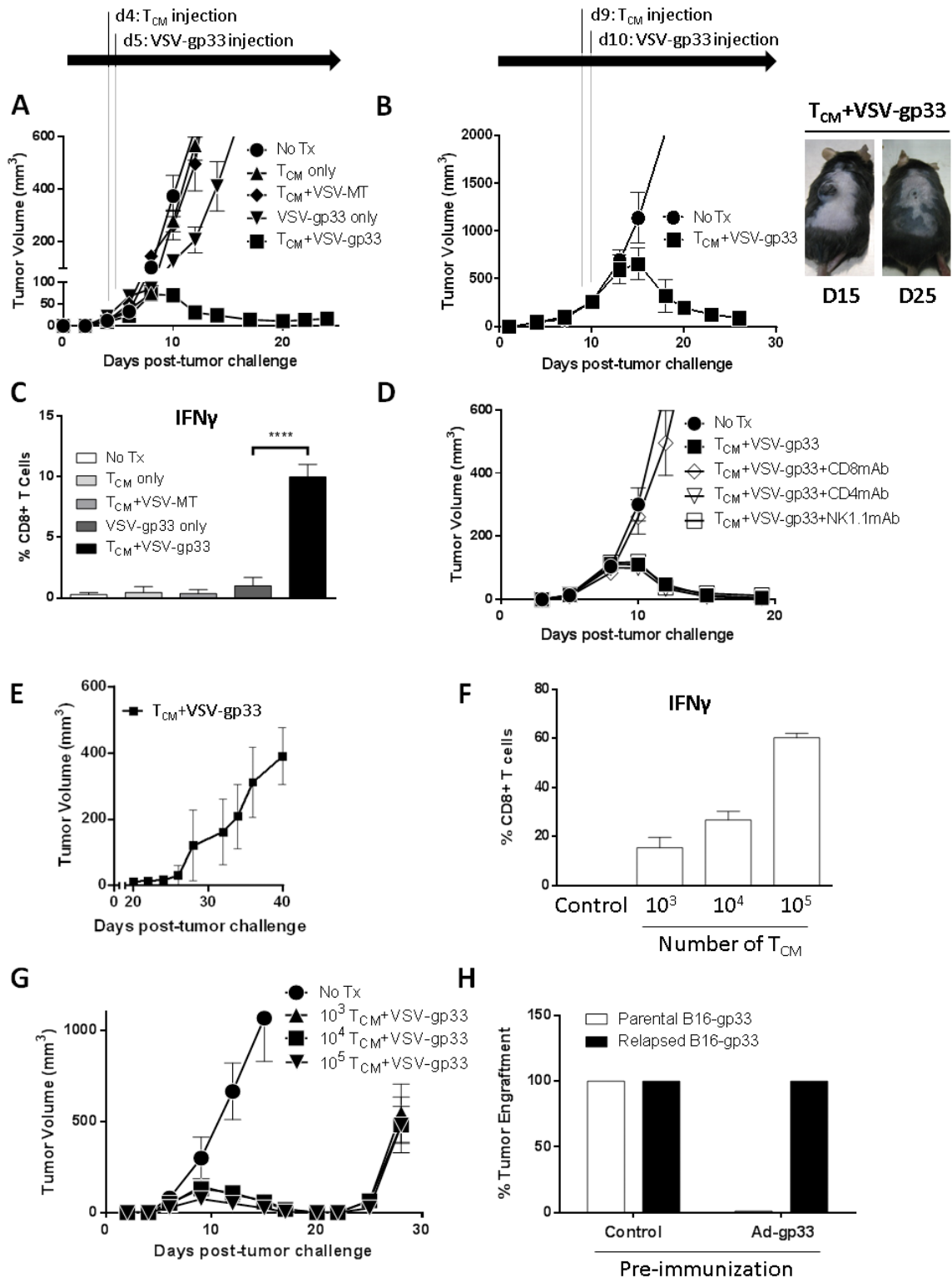
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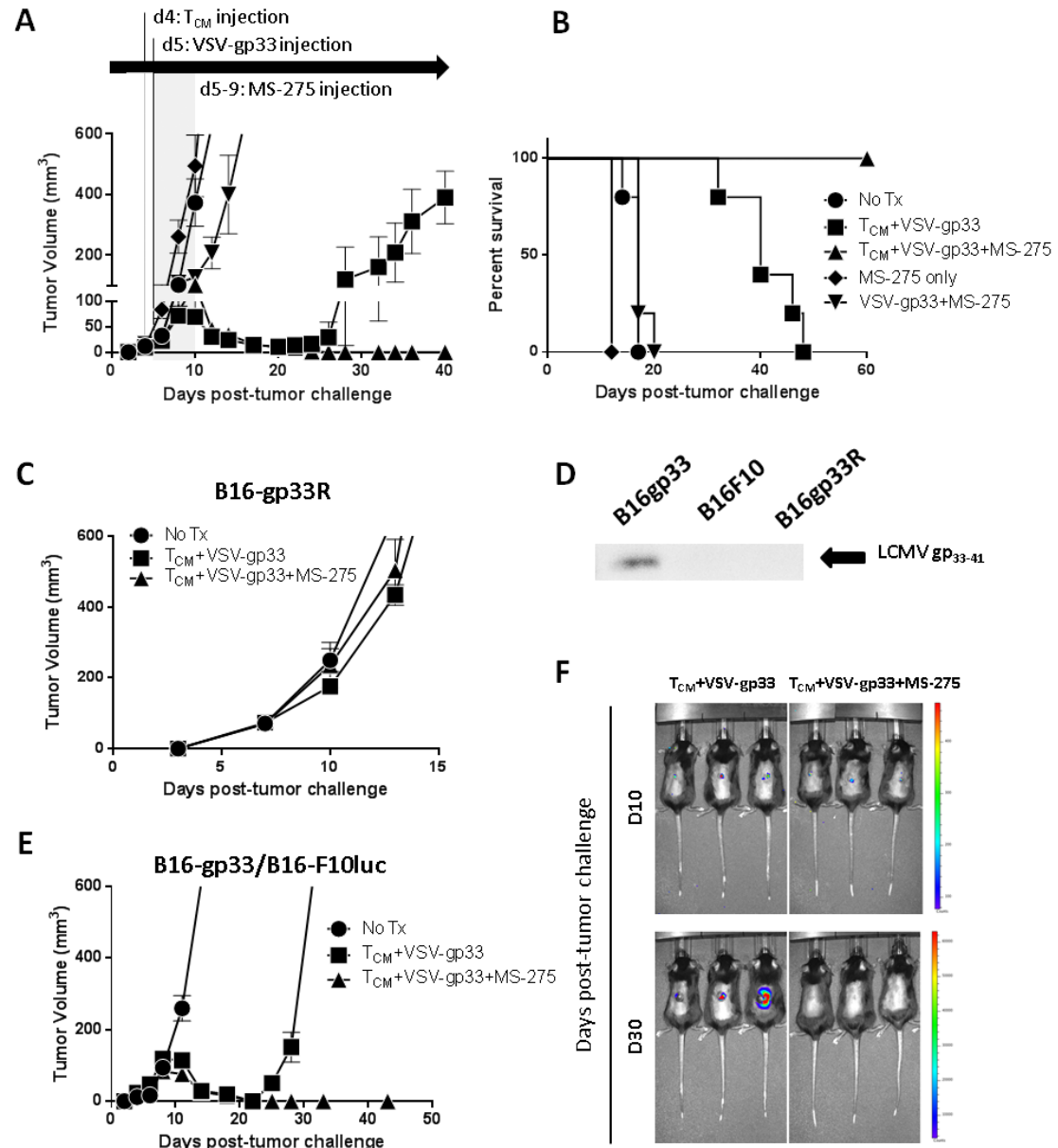
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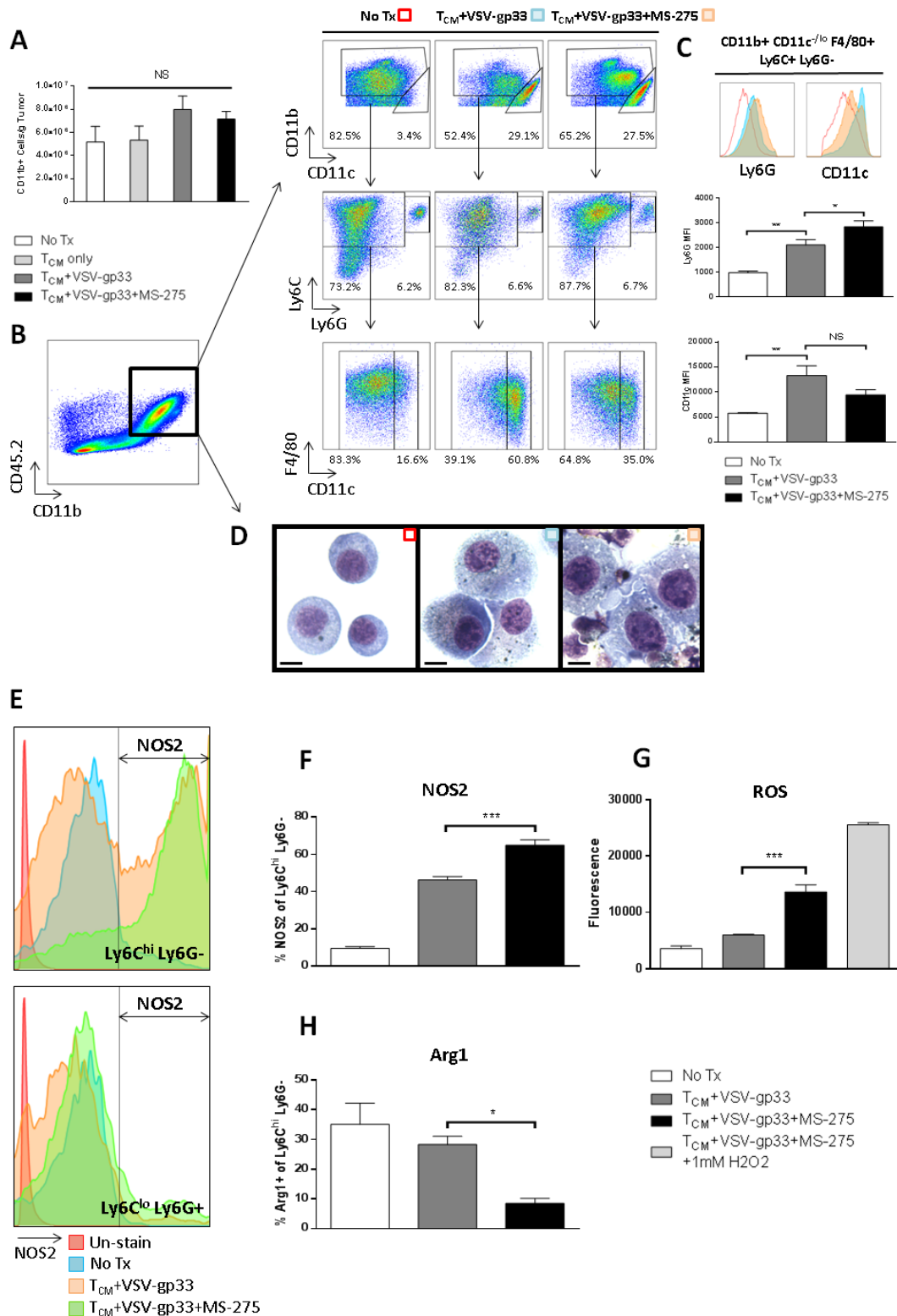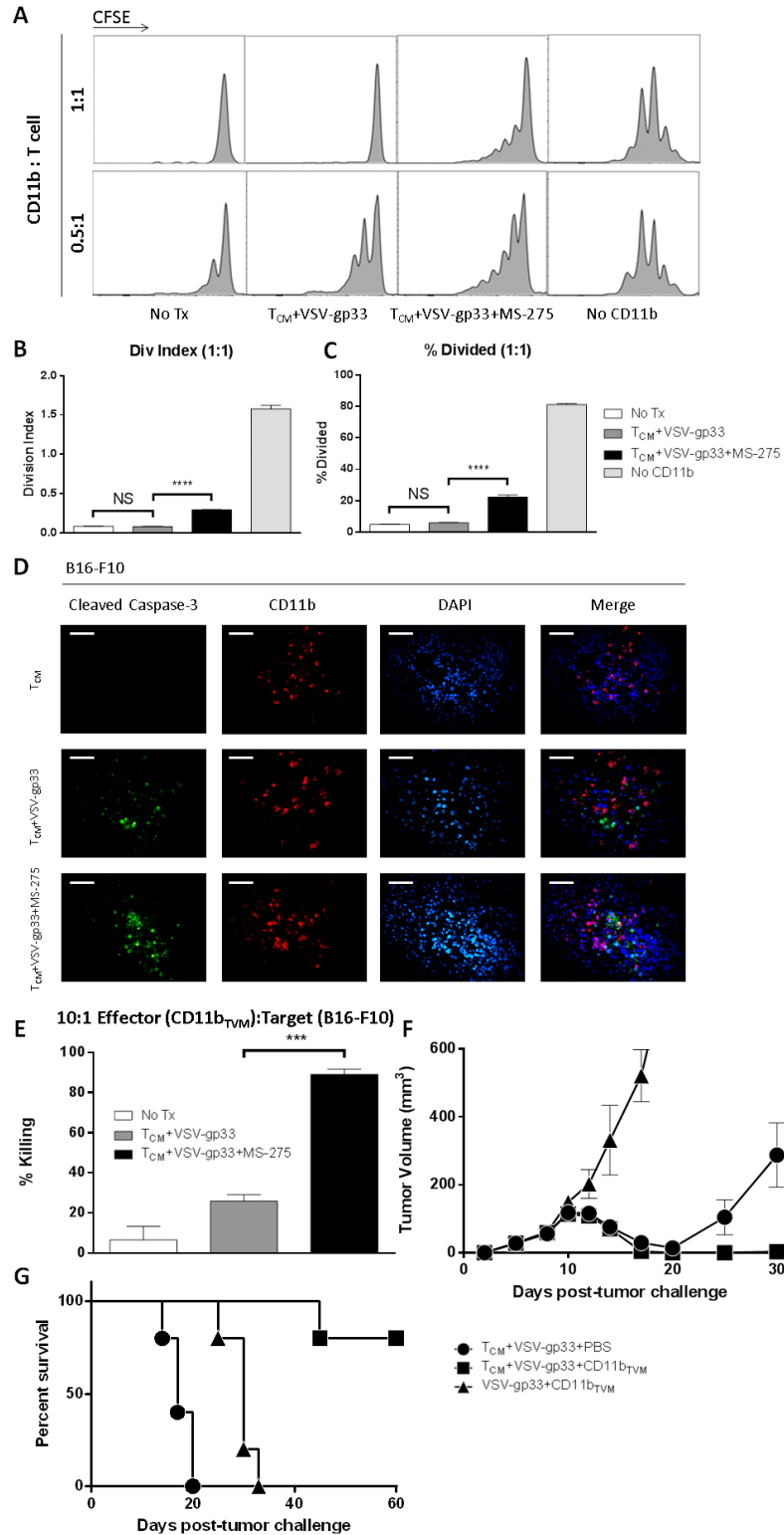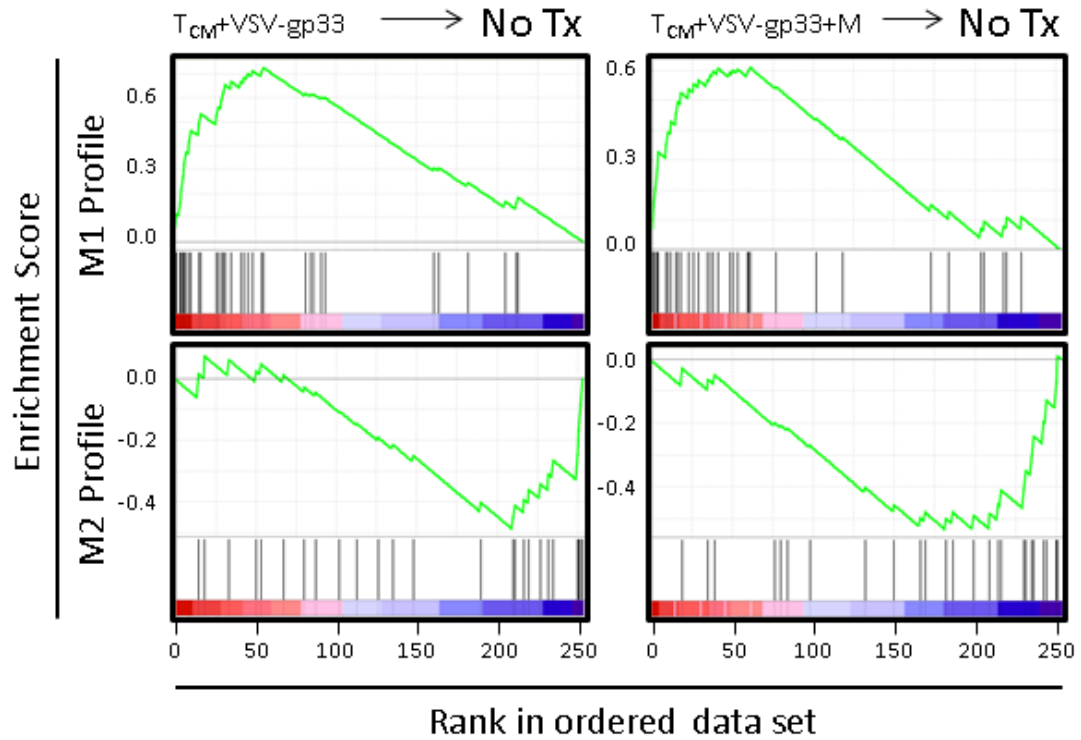
Figure 4

Figure 5

Figure 6
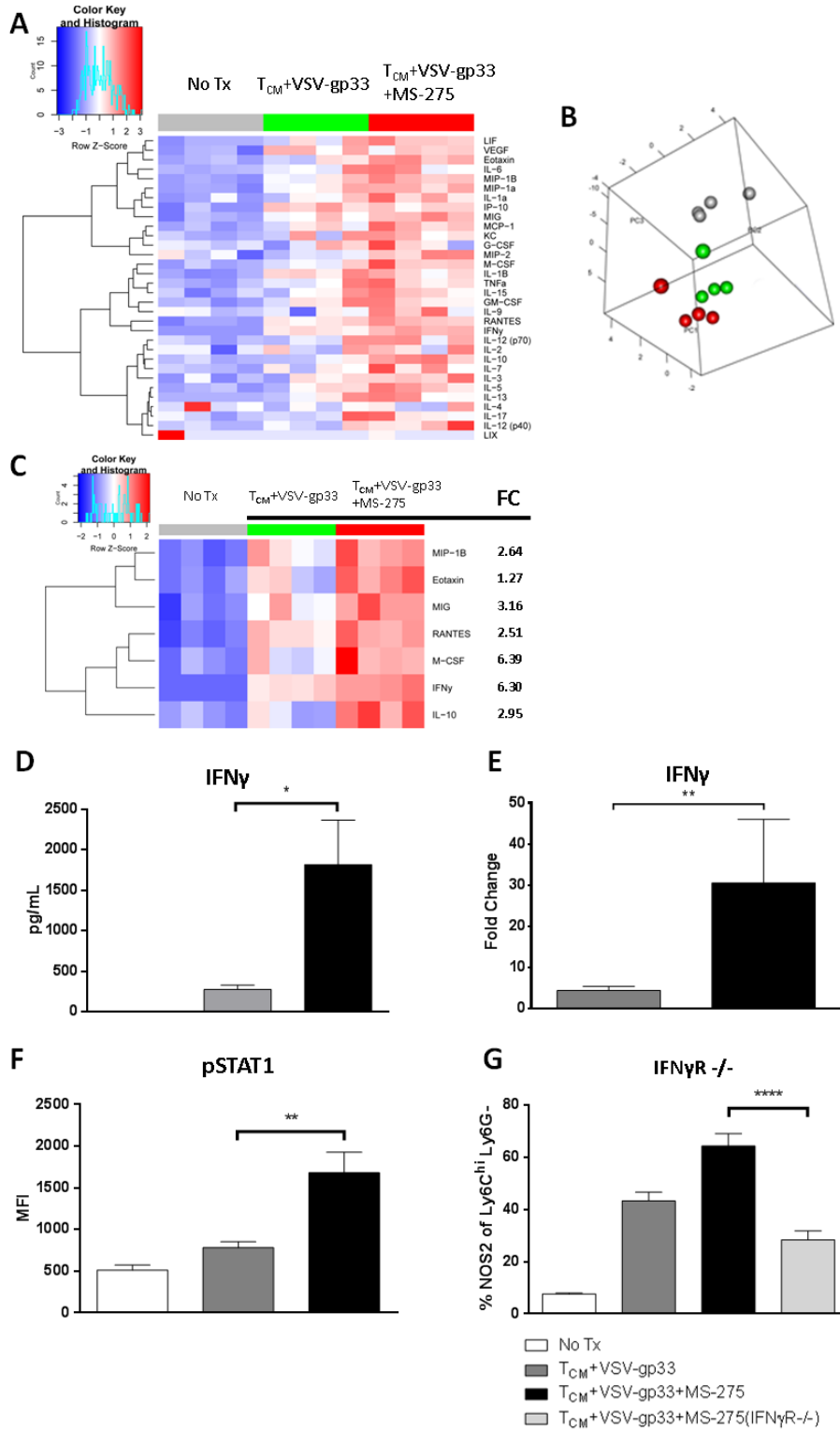
Table 1

GO Biological Process (Downregulated)	Fold Enrichment	P value
myeloid leukocyte activation involved in immune response (GO:0002283)	> 100	3.15E-02
leukocyte activation involved in immune response (GO:0002366)	12.96	1.05E-02
leukocyte activation (GO:0045321)	9.01	8.90E-07
cell activation (GO:0001775)	7.51	1.07E-05
immune system process (GO:0002376)	6.45	1.84E-19
cell activation involved in immune response (GO:0002263)	12.79	1.15E-02
immune effector process (GO:0002252)	8.91	2.00E-05
immune response (GO:0006955)	8.51	2.49E-14
myeloid leukocyte activation (GO:0002274)	27.5	3.72E-08
neutrophil activation (GO:0042119)	77.78	2.06E-03
granulocyte activation (GO:0036230)	68.05	3.49E-03
positive regulation of acute inflammatory response (GO:0002675)	43.91	1.02E-03
positive regulation of inflammatory response (GO:0050729)	14.98	2.73E-02
regulation of response to stimulus (GO:0048583)	2.56	6.17E-03
positive regulation of response to stimulus (GO:0048584)	3.51	1.19E-03
positive regulation of response to external stimulus (GO:0032103)	9.61	3.62E-03
regulation of acute inflammatory response (GO:0002673)	23.88	1.99E-02
mast cell activation (GO:0045576)	40.33	2.74E-02
myeloid leukocyte chemotaxis (GO:0030593)	23.07	2.35E-02
granulocyte chemotaxis (GO:0071621)	20.94	3.75E-02
leukocyte migration (GO:0050900)	17.01	4.85E-07
leukocyte chemotaxis (GO:0030595)	17.17	1.63E-03
cell chemotaxis (GO:0060326)	16.6	4.79E-06
chemotaxis (GO:0006935)	7.15	3.31E-03
taxis (GO:0042330)	7.11	3.47E-03
response to external stimulus (GO:0009605)	3.88	4.57E-04
neutrophil migration (GO:1990266)	21.27	3.48E-02
lymphocyte mediated immunity (GO:0002449)	15.67	4.23E-04
leukocyte mediated immunity (GO:0002443)	15.74	7.95E-06
immune response-activating cell surface receptor signaling pathway (GO:0002429)	14.08	3.89E-02
immune response-regulating cell surface receptor signaling pathway (GO:0002768)	15.12	3.79E-03
immune response-regulating signaling pathway (GO:0002764)	10.65	3.79E-02
regulation of immune response (GO:0050776)	7.5	2.86E-06
regulation of immune system process (GO:0002682)	5.32	1.77E-06
activation of immune response (GO:0002253)	12.96	4.96E-05
positive regulation of immune response (GO:0050778)	8.73	1.08E-04
positive regulation of immune system process (GO:0002684)	6.92	6.97E-07
adaptive immune response (GO:0002250)	10.01	5.49E-04
innate immune response (GO:0045087)	9.72	1.33E-08
defense response (GO:0006952)	6.95	1.45E-11

response to stress (GO:0006950)	2.9	7.63E-04
inflammatory response (GO:0006954)	8.57	3.19E-05
positive regulation of cytokine production (GO:0001819)	7.8	5.29E-03
response to other organism (GO:0051707)	5.01	3.77E-02
response to external biotic stimulus (GO:0043207)	5.01	3.77E-02
GO Biological Process (Upregulated)	Fold Enrichment	P value
DNA replication	33.62	2.54E-03
cell cycle	9.51	9.77E-04

Supplementary Figure 1
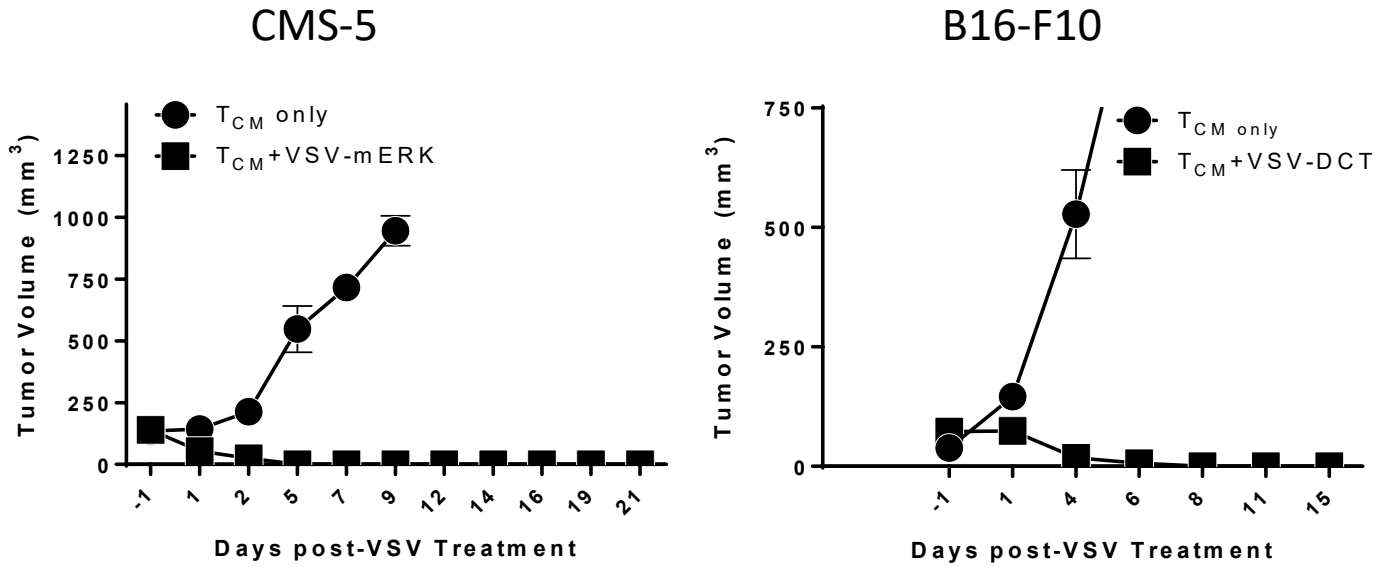
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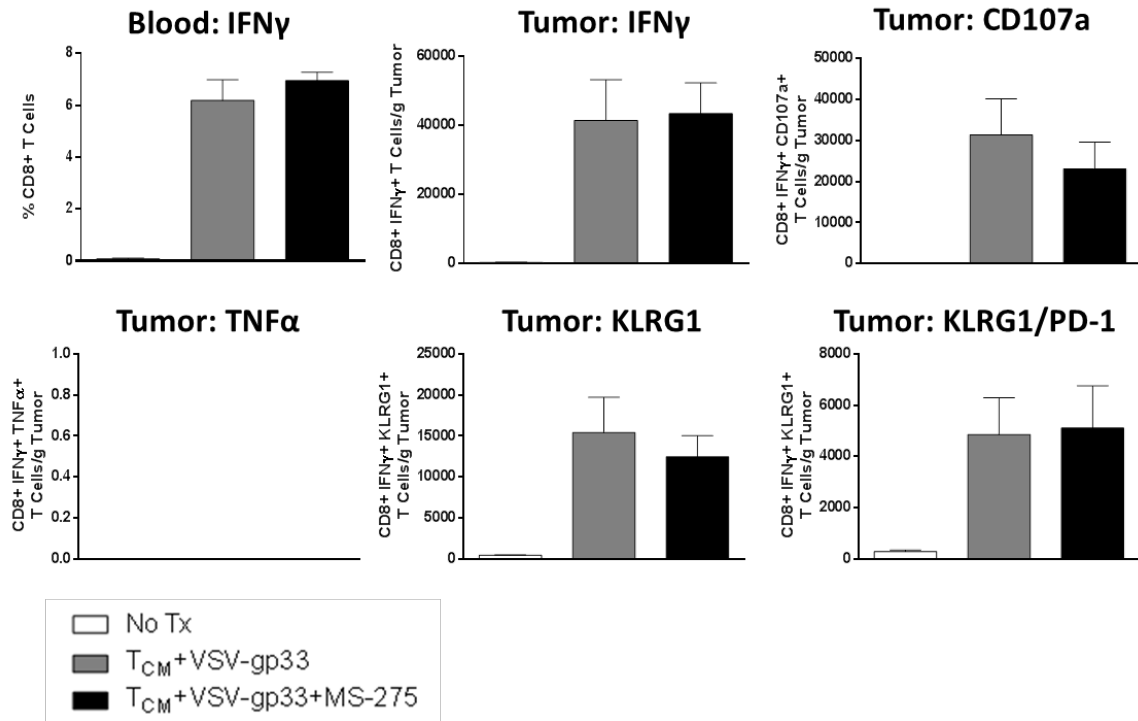
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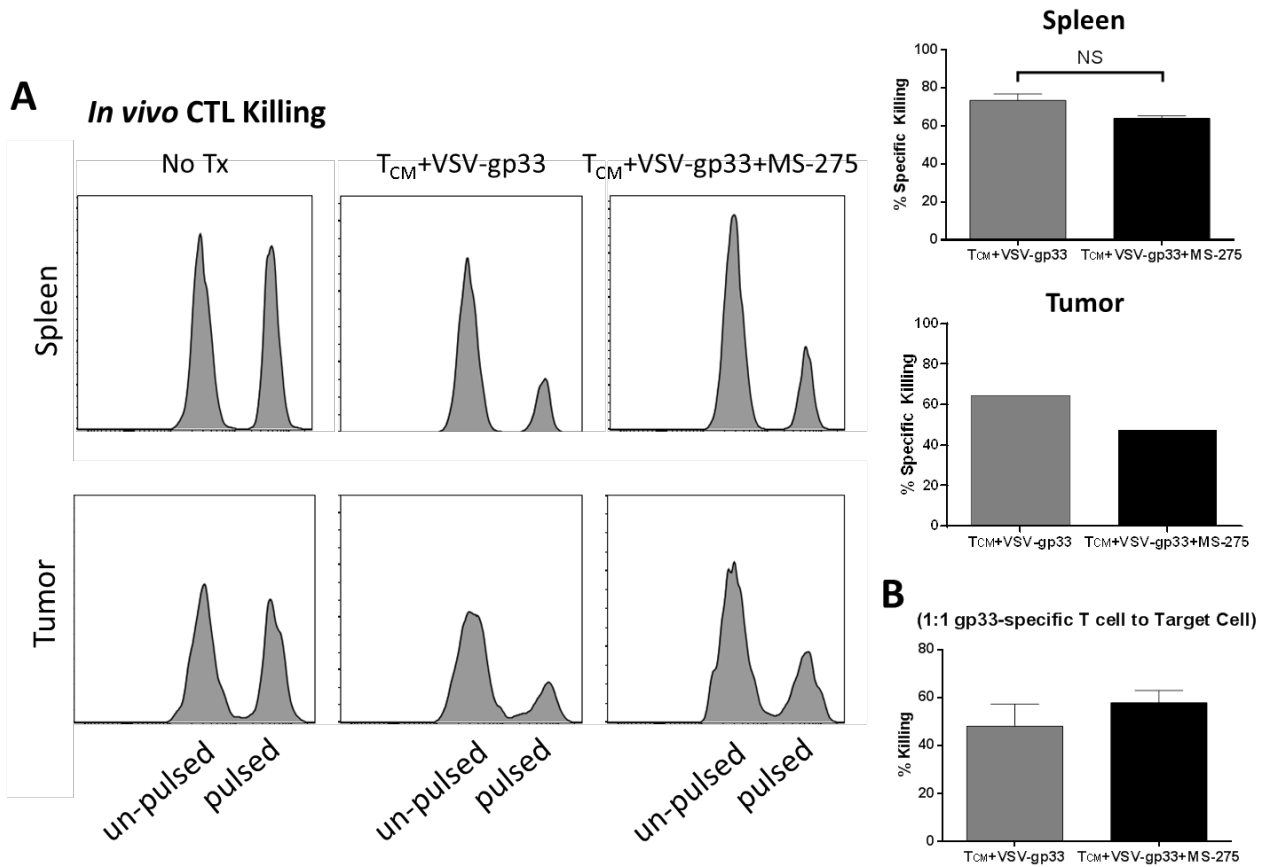
Supplementary Figure 4

Supplementary Figure 5

10:1 Effector (CD11b$_{TVM}$):Target (B16-F10)
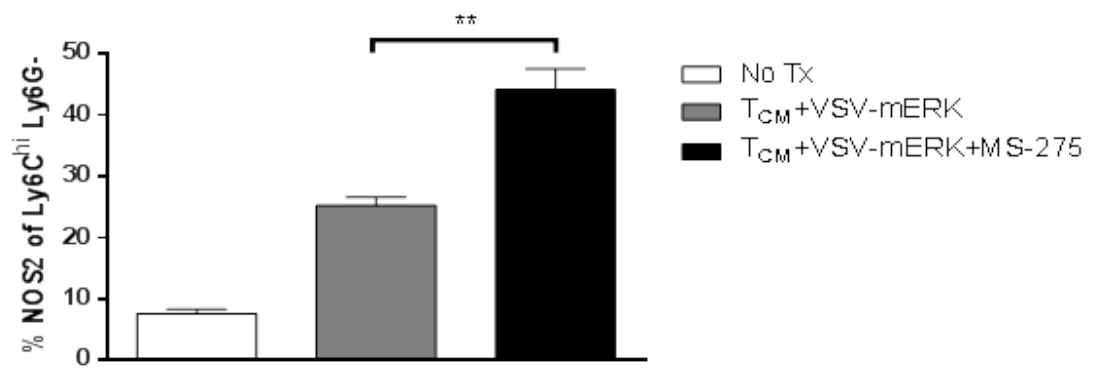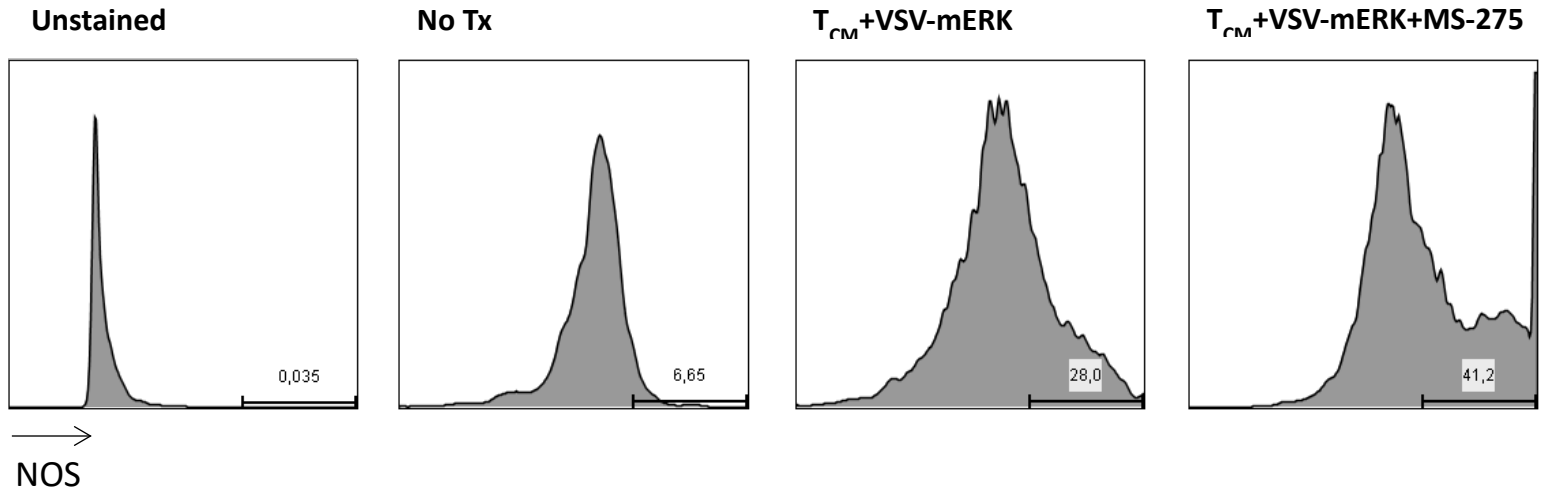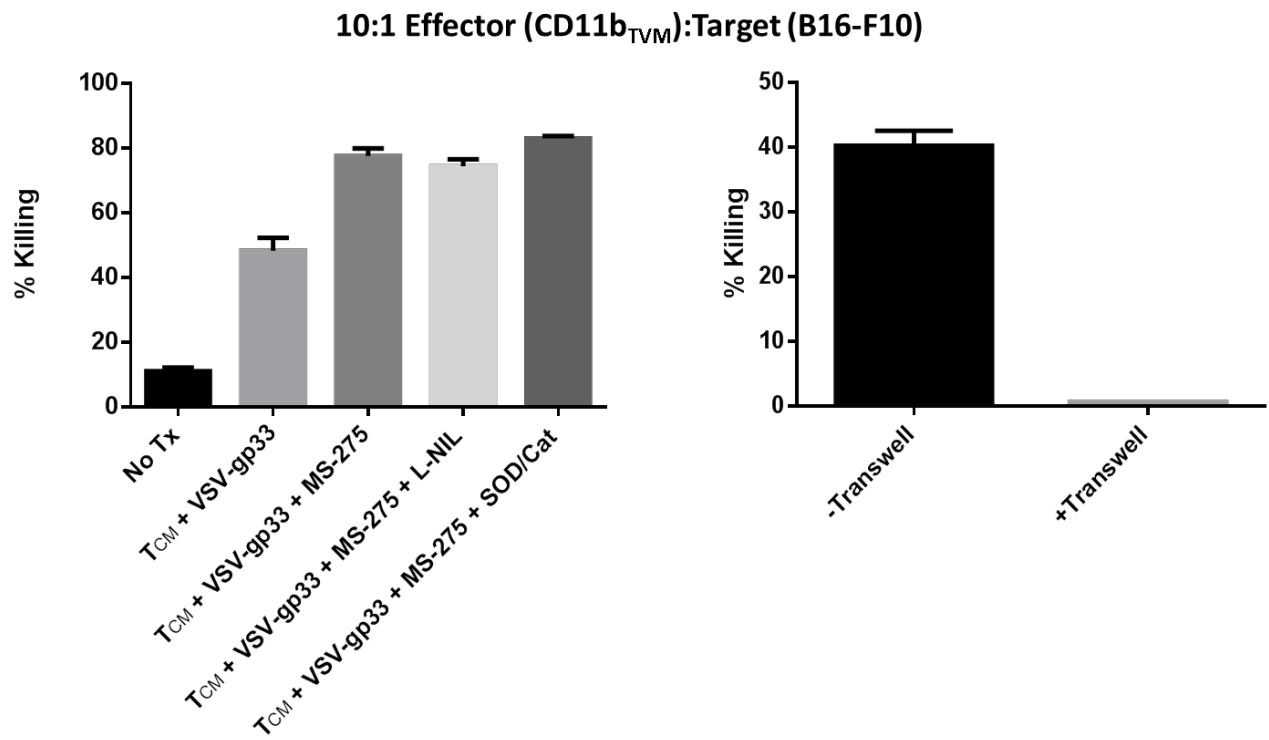
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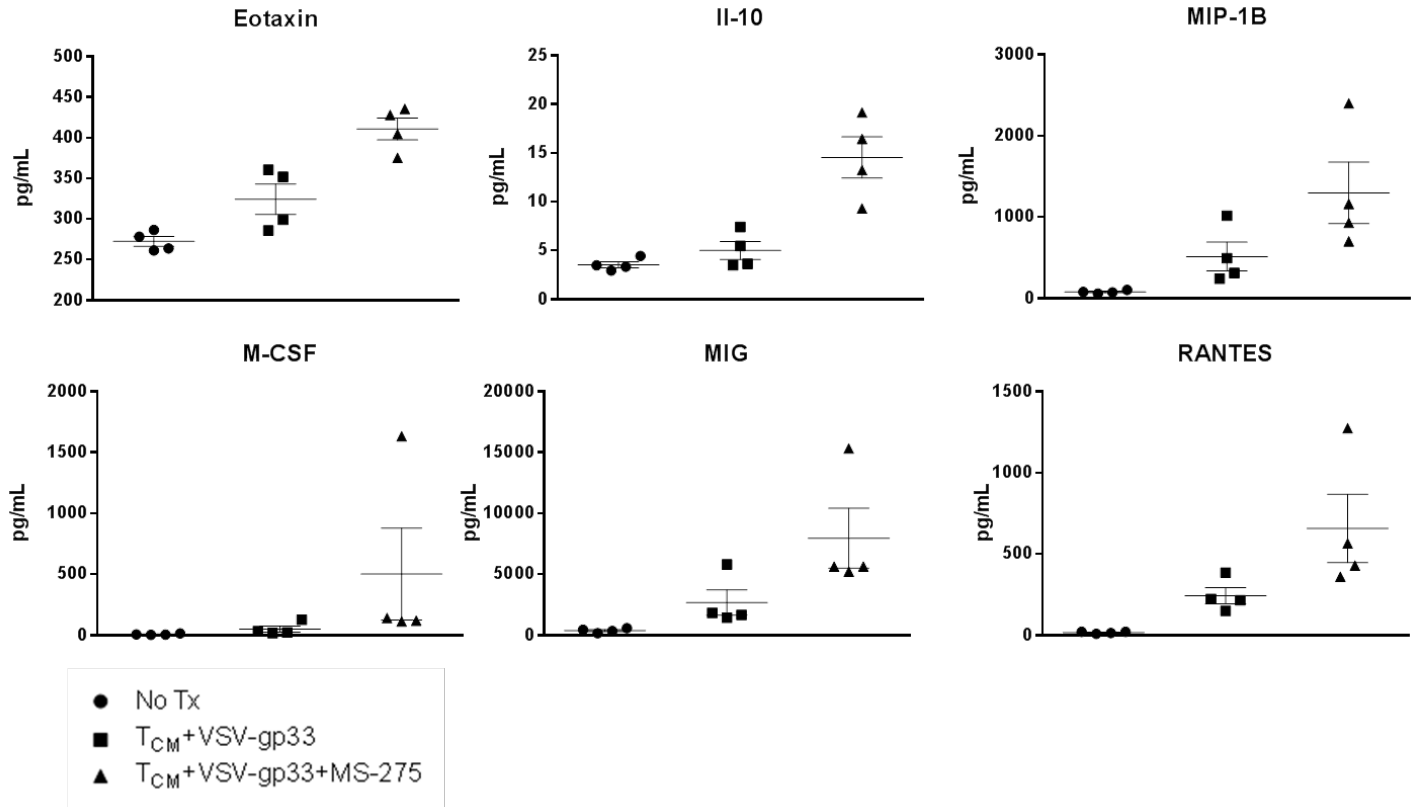
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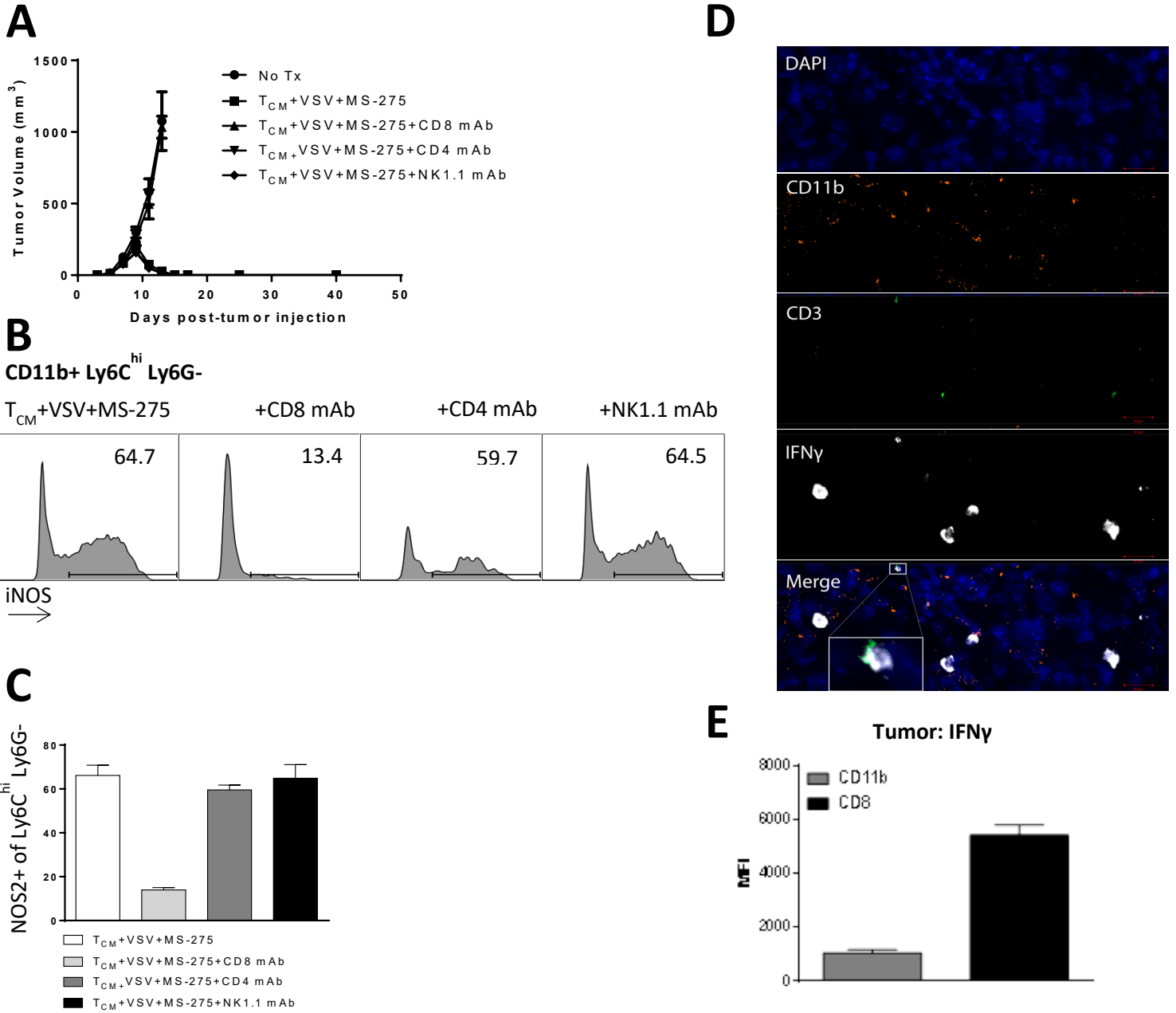
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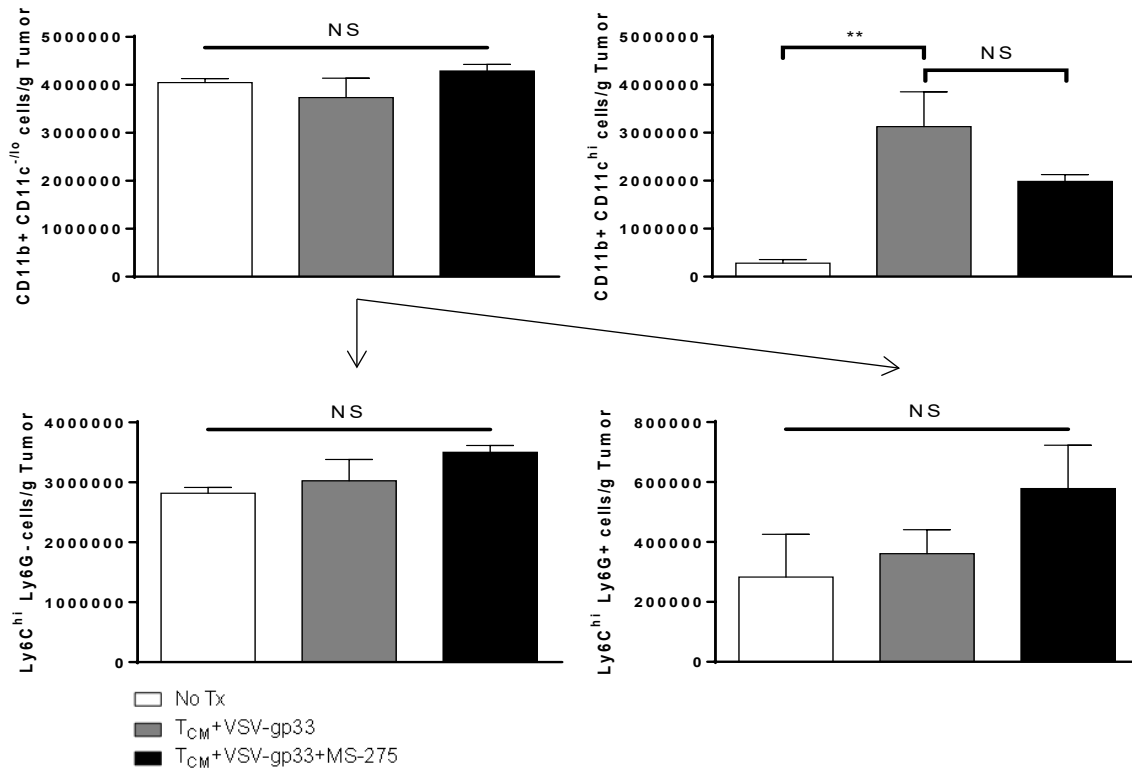
Supplementary Table 1

	M1 Profile				M2 Profile	
Ccl11	Cxcl10	Iigp1	Nlrp3	Arg1	Cxcr2	Ptgfr
Ccl2	Cxcl2	Il12a	Nos2	Ccl17	Flt1	Ptgir
Ccl3	Cxcl3	Il12b	Tnf	Ccl22	Il10	Retnla
Ccl4	Cxcl5	Il15	Tnfaip3	Ccl24	Il13	Tgfb1
Ccl5	Cxcl9	Il18		Ccr2	Il1rn	Tgfb2
Ccl8	Fasl	Il1a		Cd163	Il4	
Cd40	Hif1a	Il1b		Cebpb	Il6ra	
Cd86	Hmgb1	Il1r1		Chi3l3	Mmp9	
Csf2	Hmgb2	Il23a		Csf1	Mrc1	
Cxcl1	Ifng	Il6		Cxcr1	Ptger3	

Supplementary Table 2

Gene Name	TCM+VSV+MS-275 vs. TCM+VSV
Ifng	4.19
Ltb4r1	3.51
Il6ra	3.1
Tnfsf14	2.99
Ccr2	2.88
Oas2	2.82
Chi3l3	2.81
Tlr9	2.76
Cxcr2	2.71
Il18	2.63
Tlr4	2.42
Ifi27l2a	2.4
Tlr1	2.35
Cebpb	2.34
Ccl2	2.32
Il18rap	2.17
Plcb1	2.16
Myd88	2.1
Ccl24	2.07
Mknk1	2.04
Nod2	1.98
Ccr1	1.97
Stat2	1.94
Daxx	1.82
Bcl6	1.76
Ccl5	1.75
Ptger2	1.75
Irf5	1.72
Ltb	1.68
Irf7	1.67
Gngt1	1.65
Ptgs1	1.62
Oas1a	1.61
Prkcb	1.61
Tlr5	1.59
Cd86	1.55

Tlr6	1.51
Iigp1	1.5
Hif1a	1.49
Pla2g4a	1.45
Hsh2d	1.44
Mafk	1.41
Cd40lg	1.38
Flt1	1.38
Fos	1.38
Ccl7	1.37
Stat1	1.36
Tyrobp	1.36
C3ar1	1.34
Nod1	1.34
Irf1	1.33
Twist2	1.33
Map2k4	1.32
Grb2	1.31
Ager	1.29
Ccr4	1.29
Map2k6	1.29
Nfe2l2	1.29
Fasl	1.27
Ripk1	1.26
Cfl1	1.25
Jun	1.25
Rps6ka5	1.25
Ccl20	1.23
Map3k1	1.23
Mafg	1.2
Mef2a	1.19
Rac1	1.19
Fxyd2	1.18
Birc2	1.16
Cxcl9	1.16
Hmgb2	1.15
Limk1	1.15
Cysltr1	1.12
Stat3	1.12

Tradd		1.12
Itgb2		1.11
Mapk14		1.11
Max		1.11
Cysltr2		1.1
Ifit3		1.1
Shc1		1.1
Map3k9		1.09
Csf1		1.08
Prkca		1.08
C1qb		1.06
Mapk1		1.06
C3		1.05
Rhoa		1.05
Cfb		1.04
Il11		1.03
Il7		1.03
Mef2d		1.02
Tlr8		1.02
Cdc42		1.01
Ly96		1.01
Nfatc3		1.01
Lta		-1.01
Alox5		-1.02
Ccr3		-1.02
Cd163		-1.02
Tlr2		-1.02
Bcl2l1		-1.03
Mapkapk2		-1.03
Nos2		-1.03
Masp2		-1.04
C1qa		-1.05
Keap1		-1.05
Mapk3		-1.07
Oasl1		-1.07
Ifit2		-1.08
Raf1		-1.08
Cd4		-1.09
Gnb1		-1.1

Map3k7	-1.1
Mx1	-1.1
Ifi44	-1.11
Tcf4	-1.11
Il13	-1.12
Ppp1r12b	-1.12
Pik3c2g	-1.13
Traf2	-1.13
Hdac4	-1.14
Ccl19	-1.15
Gnas	-1.15
H2-Ea-ps	-1.16
Gnaq	-1.17
Nr3c1	-1.18
Tgfb1	-1.18
C4a	-1.19
H2-Eb1	-1.19
Il10rb	-1.19
Il21	-1.19
C7	-1.21
Hmgb1	-1.21
Il9	-1.21
Tollip	-1.22
Tnfaip3	-1.23
Myc	-1.24
Hspb1	-1.26
Mx2	-1.26
Rapgef2	-1.26
Smad7	-1.27
Ptk2	-1.28
Cfd	-1.29
Irf3	-1.29
C1ra	-1.3
Tgfb3	-1.3
Il1rap	-1.31
Mrc1	-1.32
Nfkb1	-1.32
Il1r1	-1.33
Map2k1	-1.34

Kng1	-1.35
Alox15	-1.37
C9	-1.37
Map3k5	-1.37
Elk1	-1.39
Il15	-1.39
Ptger3	-1.39
Atf2	-1.4
Cxcr4	-1.4
Ddit3	-1.41
Il2	-1.41
Tlr7	-1.41
Il4	-1.42
Relb	-1.43
Rela	-1.45
Nox1	-1.48
Ccl8	-1.49
Ccl11	-1.5
Crp	-1.5
Cxcl3	-1.5
Mmp9	-1.51
Hspb2	-1.52
Ptgir	-1.52
Ifna1	-1.53
Csf2	-1.55
Hmgn1	-1.55
Tlr3	-1.55
Trem2	-1.56
Mapk8	-1.57
Ptger1	-1.58
Il22	-1.61
Il5	-1.61
Cxcl10	-1.63
C8b	-1.73
Il23r	-1.74
Cxcl2	-1.76
C1s	-1.77
Masp1	-1.77
Ccl4	-1.78

Ptger4	-1.79
Cd55	-1.85
Myl2	-1.86
Ccl3	-1.87
Hras1	-1.87
Rock2	-1.89
Mef2c_Mm	-1.91
Tgfbr1	-1.92
Cxcr1	-1.93
Ripk2	-1.95
Ccl17	-2.02
Tbxa2r	-2.02
Hc	-2.1
Il17a	-2.1
Retnla	-2.15
Mbl2	-2.18
Il10	-2.19
Tslp	-2.2
Cxcl5	-2.21
Nlrp3	-2.22
Il1b	-2.24
Defa-rs1	-2.3
Maff	-2.35
Mmp3	-2.36
Ptgfr	-2.36
Creb1	-2.37
Il22ra2	-2.42
Il3	-2.45
Gpr44	-2.46
C6	-2.54
Mapkapk5	-2.58
Mef2b	-2.66
Ccl21a	-2.67
Cd40	-2.68
Ltb4r2	-2.83
Ifit1	-2.85
Il1rn	-2.91
C2	-3.18
Tgfb2	-3.22

C8a	-3.39
Il12a	-3.55
Tnf	-4.13
Areg	-4.31
Il23a	-4.53
Cxcl1	-4.67
Ccr7	-4.78
Ptgs2	-4.81
Arg1	-5.15
Alox12	-5.8
Il6	-5.8
Pdgfa	-5.91
Ifnb1	-6.29
Il1a	-6.45
Il12b	-8.81
Ccl22	-11.02
Csf3	-14.59

3.0   Chapter Three – HDACi promotes inflammatory remodeling of the tumor microenvironment to enhance epitope spreading and antitumor immunity

3.1   Introduction

Given that HDACi have been documented to induce co-stimulatory molecule expression and up-regulate antigen processing and presentation machinery, we questioned whether MS-275 was capable of altering the inflammatory tumor landscape to promote local immunoactivation and if this was clinically beneficial in our model of tumor relapse.

Utilizing the same tumor model and treatment platform as Chapter Two, we observed that MS-275 changed the inflammatory microenvironment to support the maturation of cross-presenting dendritic cells while discouraging the accumulation of regulatory T cells. This corresponded with an activation of endogenous antitumor T cell responses targeting non-target tumor antigens.

3.2   Manuscript Status, Copyright, and Citation

Status: Submitted to Science Advances

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3.3   Preprint of the Journal Article

Starting on the next page.

HDACi promotes inflammatory remodeling of the tumor microenvironment to enhance epitope spreading and antitumor immunity

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Abstract:

Adoptive cell therapy (ACT) with tumor-specific memory T cells has shown increasing efficacy in regressing solid tumors. However, tumor antigen heterogeneity represents a longitudinal challenge for durable clinical responses due to the therapeutic selective pressure for immune escape variants. Here, we demonstrate that delivery of class I histone deacetylase inhibitor, MS-275, promotes sustained tumor regression by synergizing with ACT in a coordinated manner to enhance cellular apoptosis. We find that MS-275 alters the tumor inflammatory landscape to support antitumor immunoactivation through the recruitment and maturation of cross-presenting CD103+ and CD8+ dendritic cells and depletion of regulatory T cells. Activated endogenous CD8+ T cell responses against non-target tumor antigens was critically required for the prevention of tumor recurrence. Importantly, MS-275 alters the immunodominance hierarchy by directing epitope spreading towards endogenous retroviral tumor-associated antigen, p15E. Our data suggest that MS-275 multi-mechanistically improves epitope spreading to promote long-term clearance of solid tumors.

Introduction

Adoptive cell therapy (ACT) involves the ex vivo expansion and infusion of antigen-specific T cells, tumor-infiltrating lymphocytes (TIL) or engineered T cells expressing TCRs or chimeric antigen receptors (CAR) into tumor-bearing patients (1). These personalized cellular products potentiate tumor recognition and killing and has demonstrated clinical efficacy in the treatment of malignant disease (2). However, incomplete responses and tumor recurrence have been reported following adoptive transfer (3). Since patient tumors can harbor extensive variability in antigen expression, targeted therapies such as ACT may create selective pressure for antigen-negative or antigen-low immune escape variants (4). As a result, tumor antigen heterogeneity may reduce the probability of durable responses. It has been demonstrated that the detection of epitope spreading during ACT is highly correlated with improved clinical outcomes (5-7). 'Epitope' or 'determinant' spreading during cancer immunotherapy is characterized by early tumor lysis and release of immunogenic tumor-derived antigens (8). Cross-presentation of these antigens by tumor-infiltrating dendritic cells (DC) can engage endogenous T cells to facilitate recognition and killing of a wider tumor antigen repertoire (9). Therefore, deliberate induction of broad-spectrum antitumor immunity during ACT may promote comprehensive eradication of heterogeneous solid tumors.

In opposition, the tumor microenvironment (TME) employs an abundant array of immunoregulatory mechanisms that can suppress the activation of endogenous T cell responses. The secretion of immunosuppressive factors by tumors and tumor-infiltrating leukocytes including myeloid-derived suppressor cells, regulatory T cells, and tumor associated macrophages/neutrophils has been shown to curb the development/survival (eg. VEGF, IL6, PGE2) and function (eg. TGFβ, IL10) of cross-presenting DCs and render them tolerogenic (10, 11). Additionally, the TME impairs the production of pro-inflammatory DC-activating cytokines (eg. IFNα/β, Hmgb1) which further inhibits the de novo activation of endogenous T cell responses (12-14). As such, targeting TME immunosuppressiveness may improve epitope spreading and improve the therapeutic impact of ACT. For instance, it has been demonstrated that selective MDSC/Treg depletion (eg. doxorubicin, paclitaxel (15-17)) and targeted molecular inhibition (eg. IDO, STAT3 (18, 19)) can foster DC maturation and activity. Alternatively, several methods have been employed to directly potentiate DC function including local up-regulation of DC-recruiting cytokines (eg. GM-CSF, FLT3L (20-22)) and administration of immunostimulatory adjuvants (eg. TLR/CD40/STING agonists (10)). Overall, while immunosuppressive pressures within the TME present significant barriers to epitope spreading, strategies to enhance DC activation and cross-presentation may propel the advancement of combinatorial ACT approaches.

In this study, we determined that MS-275, a class I histone deacetylase inhibitor, delivered in conjunction with ACT could promote sustained tumor regression and prevent relapse. This was associated with inflammatory remodeling of the TME in favor of recruitment and activation of cross-presenting DCs. Decreased immunosuppressive signaling also corresponded with a depletion of tumor-infiltrating regulatory T cells. These changes collectively promoted epitope spreading to non-target tumor antigens which were critical for long-term tumor control. Overall, MS-275 multi-mechanistically improves epitope spreading to enhance the efficacy of ACT.

Results

MS-275 delivery enhances ACT leading to sustained tumor regression

During adoptive cell therapy (ACT), the in vivo magnitude and persistence of infused tumor-specific T cells are considered determinants of successful clinical responses (23). It has been increasingly accepted that less differentiated subsets of memory T cells contribute to durable antitumor immune responses with exceptional proliferative capacity upon antigenic stimulation (24, 25).

To generate LCMV GP33–41-specific memory T cells (TMEM) for adoptive transfer, we infected C57BL/6 mice with lymphocytic choriomeningitis virus (LCMV, Armstrong strain) and harvested bulk splenocytes after one month. In mice intradermally challenged with B16-F10 murine melanoma cells expressing the immunodominant LCMV GP33-41 epitope (B16-gp33), ACT treatment utilizing TMEM was sufficient to completely regress five-day old tumors; however, the tumors quickly relapsed within one month after initial tumor challenge. Class I histone deacetylase inhibitor (HDACi) MS-275 has been previously shown to potentiate immunotherapeutic outcomes (26, 27), so we speculated whether incorporating the drug could prolong tumor regression. Daily injections of MS-275 were delivered concomitantly with ACT or two days prior/later and continued for four additional days. While MS-275 alone did not provide tumor protection, ACT+MS-275 completely sustained tumor regression relative to ACT alone (Figure 1A). Interestingly, early or late delivery of MS-275 abrogated any therapeutic benefit, signifying that timing-dependent interactions between ACT and MS-275 were necessary to prevent tumor relapse.

Since MS-275 has anticancer properties (28, 29), we considered whether the drug was additively contributing to tumor regression by direct elimination of resistant tumor variants despite being unable to control tumor growth on its own. In frozen tumor sections derived five days post-treatment, immunofluorescence staining for cleaved caspase-3 revealed that MS-275 alone did not seem to directly promote tumor apoptosis (Figure 1B). By contrast, ACT+MS-275 treatment demonstrated drastically higher levels of cleaved caspase-3 relative to ACT alone, suggesting that MS-275 synergizes with ACT to drive tumor apoptosis.

Hematoxylin and eosin staining of tumor tissue revealed that that ACT-treated tumors were heavily infiltrated with leukocytes both in the presence or absence of MS-275 (Figure 1C). To determine whether MS-275 enhances ACT by selectively increasing the total number of tumor-infiltrating CD8+ T cells, we stained tumor sections with anti-CD8 antibody for immunohistochemistry five days post-treatment. There was no significant difference in total CD8 staining with the addition of MS-275 (Figure 1D), which was further validated by flow cytometric analysis (Figure 1E). Furthermore, the total number of LCMV GP33–

41-specific CD8+ T cells was not affected by MS-275 (Figure 1F). While MS-275 could mobilize other lymphocytes with antitumor potential, selective depletion of CD4 or NK1.1-expressing cells during ACT+MS-275 treatment provided no loss of sustained tumor regression compared to CD8 depletion (Figure 1G). Taken together, despite tumor control being primarily CD8+ T cell dependent, the additional therapeutic benefit afforded by concomitant MS-275 delivery may not rely on the additional recruitment of target antigen-specific CD8+ T cells or other antitumor lymphocyte populations.

Inflammatory remodeling of the tumor microenvironment favors immunoactivation

Since the data suggest that ACT-MS-275 synergy occurs within a restricted time frame, we wanted to investigate the dynamic changes within the tumor microenvironment that could facilitate enhanced tumor killing in the presence of the drug. To accomplish this, we conducted microarray analyses (GSE179337) of bulk tumor RNA from ACT-treated mice with or without MS-275 at one, three, and five-days post-treatment.

Using connectivity mapping (CMap), we first confirmed if the gene changes observed were due to the direct influence of MS-275. Time course analysis was performed by measuring differential gene expression between ACT +/- MS-275 from Day 1 to Day 5 post-treatment. Lists of differentially expressed genes (DEGs) that showed >1.5 or >2.0 absolute fold change (FC) were used to query the CMap library and compared to expression data from chemical perturbation studies. Of the 32 chemicals that showed significant overlap in differential gene expression, chemical studies utilizing MS-275 displayed the highest similarity (Figure S1). As a result, we had confidence that during ACT therapy, MS-275 was altering the local genetic landscape through direct chemical perturbation.

To assess the tumor inflammatory state over the course of treatment, custom gene sets (Table S1) representing specific inflammatory pathways were analyzed for statistical enrichment within the expression data of each treatment group (Figure 2A, Table S2). Relative to ACT alone, ACT+MS-275 up-regulated Type I IFN signaling, inflammatory cytokine signaling, and inflammatory responses at an early timepoint (Day 1) while down-regulating those processes at a later timepoint (Day 5). Despite having a progressive anti-inflammatory effect, qRT-PCR validation showed that ACT+MS-275 also enhanced the expression of select pro-inflammatory cytokines at various time points. In particular, MS-275 up-regulated ISG56 and IL-12 expression at Day 1 and IFNG expression at Day 5 (Figure 2B, S2). Taken together, MS-275-driven modulation of tumor inflammation may activate biological pathways that can facilitate sustained tumor regression.

Gene set enrichment analysis (GSEA) was then used to interrogate the effect of MS-275 on biological pathways derived from curated gene sets (C2) in the Molecular Signatures Database (MSigDB) (Figure 2C). An enrichment map was constructed to group significantly enriched gene sets containing similar genes. Based on the differential enrichment pattern of clustered gene sets, we noted that ACT+MS-275 treatment promoted early up-regulation (Day 1) of TCR signaling, JAK-STAT signaling, and innate immunity followed by late up-regulation (Day 5) of antigen processing, cross-presentation and lymphocyte-dependent antigen-dependent responses. Since ACT-only treatment showed an inverse enrichment pattern with the aforementioned biological processes, the data confirmed that MS-275-induced inflammatory changes correlated with enhanced immunoactivation.

Gene ontology (GO) analysis allowed us to determine if terms representing annotated biological processes were statistically overrepresented in our DEGs and comparable to our GSEA-enriched MSigDB pathways. Using DEGs obtained from comparing ACT+MS-275 vs ACT alone over a time-course (D3 vs D1 and D5 vs D1), a protein-protein interaction (PPI) network was constructed and gene modules were identified from the network (Table S3). GO analysis allowed us to functionally classify the modules according to annotated biological processes. After ordering the modules by the percentage of constituent genes up-regulated by MS-275, we observed that antigen processing and cross-presentation was highly upregulated throughout the time-course (Figure 2D, S3).

Since immunoactivation may represent a crucial mechanism for promoting sustained tumor regression, we hypothesized that MS-275's effect on tumor inflammation may alter the composition and/or activation of tumor-infiltrating antigen-presenting myeloid cells. qRT-PCR analysis of various myeloid chemotactic genes such as CCR5, CCR2, and CXCL12 suggested that MS-275 may influence myeloid cell recruitment to the tumor (Figure 2E). Furthermore, GSEA of immunologic signatures (C7) from the MSigDB revealed that gene sets related to activated myeloid cells from LPS treatment, viral infection, or vaccination were enriched by ACT+MS-275 treatment from Day 1 to Day 5 (Figure 2F, S4). Taken together, further examination of tumor-infiltrating myeloid cells was necessary to determine how enhanced antitumor immunity occurred within the context of MS-275.

MS-275 drives myeloid cell recomposition and maturation within the tumor and dLN

Myeloid cells are a heterogenous group of innate immune cells that exist within varying activation and differentiation states (30, 31). Tumors exploit myeloid cell plasticity through the secretion of soluble factors that can divert myelopoiesis and skew myeloid cell function to support tumor growth (32). In the context of ACT, we questioned whether MS-275 could

reprogram tumor-infiltrating myeloid cells into effective antigen-presenting cells with CD8+ T cell activating capacity.

Five days post-treatment with ACT+/-MS-275, myeloid populations were characterized in the tumor and draining lymph nodes (dLN) by flow cytometry. After excluding lymphocytes, we identified several cell subsets including monocytic (CD11c- CD11b+ Ly6Chi Ly6G-), granulocytic (CD11c- CD11b+ Ly6Clo Ly6G-), as well as dendritic cell (DC, CD11c+ CD11b+ Ly6C- CD8-/CD11c+ CD11b- Ly6C+ CD8+/CD11c+ CD11b- CD8- CD103+) populations (Figure 3A). While we had previously reported MS-275's influence on tumor-infiltrating monocytic/granulocytic myeloid cells (33), we expanded our analysis to include DCs and observed cellular subset recomposition within the tumor and dLN (Figure 3B, C). In the tumor, ACT+MS-275 treatment reduced the frequency of CD11b+ DCs while increasing the frequency of CD8+ and CD103+ DCs (Figure 3D); in draining lymph nodes however, there was a reduced frequency of CD103+ DCs (Figure 3E). In addition, DCs in the tumor and dLN demonstrated a significant increase in maturation marker expression including MHC Class II (I-Ab) and co-stimulatory ligand CD86 (B7-2) (Figure 3F, G). This coincided with higher immunoactivation potential when we pulsed them with LCMV GP33–41 peptide and co-cultured them with CFSE-labeled LCMV-P14 TCR-transgenic naïve T cells ex vivo (Figure 3H, I). Overall, MS-275-dependent tumor remodeling may mobilize immunoactivating DCs to promote endogenous CD8+ T cell responses.

Activation of endogenous CD8+ T cell responses promote sustained tumor regression

To confirm that endogenous CD8+ T cells enhanced the therapeutic efficacy of ACT treatment, lymphocyte-deficient RAG2/Il2rg knockout mice were treated with ACT+MS-275. We observed a failure to recapitulate the therapeutic effects of MS-275, resulting in tumor control similar to that of ACT alone (Figure 4A). The data therefore suggest that endogenous CD8+ T cells were necessary for tumor clearance.

As demonstrated previously, the magnitude of LCMV GP33–41-specific CD8+ T cell responses remained unchanged during MS-275 delivery. If cross-presentation of tumor peptides during ACT enhanced endogenous CD8+ T cell responses against targets other than LCMV GP33–41, then broadening the spectrum of antitumor killing may represent an important mechanism to prolong tumor regression. To confirm that MS-275-potentiated tumor killing transcended LCMV GP33–41 epitope recognition, we investigated whether tumor-infiltrating endogenous CD8+ T cells derived from ACT+MS-275-treated mice could recognize and kill parental B16F10 cells, which do not express LCMV GP33–41. Using a 10:1 effector to target co-culture approach, we did not observe improvements to

B16-gp33 lysis, but there was a significant increase in B16F10 cell death (Figure 4B). Moreover, the inclusion of MCA102 fibrosarcoma cells as an irrelevant line suggested that enhanced tumor killing was antigen-dependent.

Using a limited epitope screening process, we established the antigen specificity of endogenous CD8+ T cell responses during ACT+MS-275 treatment. In B16-gp33 tumors, there was an increased frequency of CD8+ T cells specific for p15E, an endogenous retroviral tumor-associated antigen (Figure 4C). Interestingly, T cell responses against defined melanoma antigens (gp100, svy) and other retroviral gene products (gp70) were unchanged. To confirm if epitope spreading altered the immunodominance hierarchy to disproportionally favor p15E-specific responses, we repeated the experiment with a known p15e-expressing tumor model such as MC38 colon adenocarcinoma. In MC38 tumors expressing LCMV GP33-41¬ (MC38-gp33), MS-275 raised the frequency of T cell responses to p15E as well as to a neo-epitope corresponding to a mutation in ADP-dependent glucokinase (Adpgk) (Figure 4D). Although the extent of epitope spreading may be model dependent, we demonstrated that MS-275 could promote endogenous immune responses against non-target tumor antigens.

However, it was yet unclear whether these responses translated to in vivo antigen-specific killing. We pulsed CFSE-labeled bulk splenocytes with p15E peptide and infused them into B16-gp33 tumor-bearing mice five days post-ACT+MS-275 treatment. Interestingly, we observed that enhanced p15E responses correlated with improved killing of p15E-pulsed target cells in vivo (Figure 4E, F). Since MC38 tumor cells share an endogenous antigen with B16F10 in the expression of p15E (34, 35), we wondered whether ACT+MS-275-driven p15E responses alone could facilitate tumor rejection. In mice that were cured of B16-gp33 tumors during ACT+MS-275 treatment, re-challenge with parental MC38 tumors resulted in delayed tumor growth and longer survival compared to naïve mice (Figure 4G, H). This suggested that persisting immunity from ACT+MS-275 treatment could have mild protective benefits against tumors expressing p15E. To eliminate cell line differences, we repeated the experiment but re-challenged cured mice with parental MCA102 cells (inherently p15E-negative) or MCA102 cells engineered to express p15E (MCA102-p15E). Again, we demonstrated that MCA102-p15E cells showed delayed tumor growth in cured mice relative to parental MCA102 cells (Figure 4J, I). Ultimately, MS-275-dependent immunoactivation of endogenous CD8+ T cells allow for tumor rejection beyond target antigen recognition.

Intratumoral down-regulation of immunosuppressive signals coincide with Treg depletion

Regulatory T cells (Tregs) exert indispensable functions in inducing and maintaining self-tolerance and immune homeostasis and their presence is often associated with poor clinical prognosis during cancer (36). Many studies support a mutualistic relationship between immunosuppressive tumor-infiltrating myeloid cells and Tregs. Myeloid cells can secrete IL10 and TGFβ while Tregs secrete IL4, IL13, and IL10, which result in reciprocal activation (37). Furthermore, nitric oxide synthase and arginase production by myeloid cells have been found to be potent inducers of Treg activity (38-40). Since MS-275 remodels the tumor microenvironment and facilitates myeloid cell reprogramming, we questioned whether that would create a subversive effect on tumor-infiltrating Tregs.

qRT-PCR analysis of bulk tumor RNA indicated that ACT+MS-275 treatment down-regulated ARG1, NOS2, and TGFB1 expression, which may prohibit Treg expansion (Figure 5A). To examine its corresponding impact on Treg abundance, we stained frozen tumor sections with anti-Foxp3 antibody for immunohistochemistry and immunofluorescence imaging. While ACT alone increased Treg numbers relative to untreated mice, ACT+MS-275 increased Tregs initially (Day 1), but decreased their number significantly afterwards (Day 3-5) (Figure 5B, C). These trends were validated by qRT-PCR analysis of FOXP3 gene expression in bulk tumor RNA (Figure 5D). Flow cytometric staining of CD45.2-enriched tumor-derived cells further revealed that depletion of tumor-infiltrating CD4+ CD25+ Foxp3+ Tregs was accompanied by a more general and severe depletion of tumor-infiltrating CD4+ T cells (Figure 5E, F).

Tregs inhibit endogenous CD8+ T cell responses and prevent long-term tumor control

Tregs can induce immunosuppression through a variety of mechanisms including: secretion of immunosuppressive cytokines, competitive consumption of IL-2, direct killing via perforin and granzyme pathways, and direct subversion of antigen-presenting cell function through down-regulation of co-stimulatory molecules (41). To evaluate the therapeutic contribution of Treg ablation in the context of ACT, we depleted CD4+ T cells using monoclonal antibodies or utilized "depletion of regulatory T cell" (DEREG) BAC transgenic mice, which express a simian diphtheria toxin receptor-enhanced green fluorescent protein (DTR-eGFP) fusion protein under control of the endogenous forkhead box P3 promoter/enhancer regions on the BAC transgene (42), to ablate Treg via intraperitoneal diphtheria toxin (DT) administration.

Flow cytometric analysis of peripheral blood five days post-treatment revealed that, while MS-275 induced partial depletion of bulk CD4+ T cells and CD4+ CD25+ Foxp3+ Tregs, CD4 mAb delivery completely depleted both subsets (Figure 6A, B). Comparatively, DT administration preserved the bulk CD4+ T cell compartment while completely ablating

CD4+ CD25+ Foxp3+ Tregs. In either scenario, the therapeutic benefit of MS-275 was recapitulated and sustained tumor regression was achieved (Figure 6C, D). Therefore, MS-275's influence on Treg numbers may play a critical role in its therapeutic efficacy. Furthermore, DT administration also increased the frequency of p15E-specific CD8+ T cells while having negligible impact on LCMV GP33–41-specific CD8+ T cells (Figure 6E, F), suggesting that Tregs selectively inhibit the magnitude of endogenous CD8+ T cell responses to facilitate tumor relapse. Indeed, CD11c+ cells isolated from ACT+DT-treated tumors were able to elicit better proliferation during co-culture with P14 naïve T cells compared to ACT alone. Taken together, MS-275 may be instigating concordant mechanisms of enhanced immunoactivation to promote sustained tumor regression via myeloid reprogramming and Treg depletion.

Discussion

The density and composition of tumor infiltrating immune cells often predict the efficacy of immunotherapy. Broadly speaking, "cold tumors" are characterized by low pro-inflammatory cytokine production, T cell infiltrate, and molecular signatures of immune activation (43). Furthermore, cold tumors orchestrate the poor cellular fate of tumor-infiltrating lymphocytes by cultivating an immunosuppressive microenvironment. Reciprocal host-tumor interactions lead to the propagation of local anti-inflammatory signals and influx of immunosuppressive cells. In particular, myeloid-derived suppressor cells (MDSC) regulatory T cells (Treg), tumor-associated dendritic cells (TADC), and type 2-polarized macrophages (M2) are intrinsically associated with the developing TME and coordinate anti-inflammatory mechanisms to obstruct the function of cytotoxic CD8+ T cells and antigen-presenting cells (44). As a result, strategies that promote tumor inflammation ("hot tumors") may circumvent TME immunosuppressive phenotypes and enhance the efficacy of T cell immunotherapy.

With our ACT platform, we were able to observe significant T-cell infiltration in solid tumors and complete acute regression. ACT alone also induced higher local inflammatory signaling relative to ACT+MS-275, suggesting hot tumor induction; however, this corresponded with worse immunologic/clinical outcomes including reduced antigen-presentation/antigen-dependent responses and tumor relapse. Indeed, tumor inflammation does not always predispose the TME towards immunostimulation and can instead promote immune escape (45). It has been demonstrated that tumor exposure to IFN-γ up-regulates PD-L1 expression such that subsequent engagement to PD-1 expressing T-cells attenuates their antitumor response (46). Similarly, PD-L1 up-regulation on CD103+ DCs from tumor dLNs impaired cross-presentation while PD-L1 and PD-1 blockade mitigated DC dysfunction (47). Finally, inflammation has been shown to drive the accumulation of MDSCs and Tregs (48), enhancing tumor immunosuppressive effects on infiltrating immune cells. Attenuating excessive inflammation in hot tumors may therefore allow immunotherapeutic strategies to have a durable clinical outcome.

Histone acetylation can alter chromatin structure and gene transcription to affect various aspects of the TME including tumor immunogenicity, T-cell infiltration, and immunosuppression (49). In the context of ACT, we demonstrated that class I HDAC inhibitor MS-275 promotes sustained tumor regression. Despite having a broadly anti-inflammatory effect over time relative to ACT-only treatment, ACT+MS-275 enhanced gene signatures related to immunoactivation. We speculate that MS-275 achieves this by altering the composition and dynamics of intratumoral inflammatory signaling with emphasis on early up-regulation of Type I IFN signaling and late up-regulation of Type II IFN signaling. IFN-α has been shown to enhance DC maturation and cross-presentation

through antigen survival, endocytic routing, and processing (50). In support of this, we detected increased ISG56 and IL-12 expression (mature DC-derived cytokines) within 24h of treatment that coincided with an accumulation of inflammatory myeloid gene signatures over time. As previously stated, IFN-γ may have deleterious effects on antigen presentation and can potentiate immune escape. During ACT+MS-275 treatment, IFNG expression within the tumor peaked 5 days post-treatment coinciding with peak systemic T cell responses. ACT-only treatment produced peak IFNG expression at Day 3, suggesting that earlier exposure to IFN-γ may predispose mice to eventual therapeutic failure. Ultimately, if coordinated expression of select inflammatory signals can curtail tumor immunosuppression and maximize antitumor immunity, MS-275 may promote features of what we term a "warm tumor".

In the TME, the acquisition, processing and cross-presentation of extracellular tumor antigen released from dying tumor cells by tumor-infiltrating dendritic cells (DC) is critical for antitumor immunity. Mouse conventional DCs (cDCs) comprise two main subsets, CD8+ or CD103+ cDC1 subsets and CD11b+ cDC2 subsets. cDC1s are often associated with superior antigen cross-presentation, stronger CD8+ T cell immunity, and improved clinical prognosis (10). However, tumors often subvert the maturation and function of infiltrating cDCs such that they become protumorigenic and suppress immune activation. These tolerogenic DCs present tumor antigen without proper co-stimulation (CD80/CD86), express inhibitory molecules (PDL1/CTLA4), and secrete immunosuppressive factors (TGFβ, IL10, IL27, NO, Arg and IDO) (51). Depletion of tolerogenic DCs has been associated with reduced tumor growth and angiogenesis (52, 53) and as such, targeting these cells may improve the recruitment, infiltration and effector activity of T cells in the TME. During ACT+MS-275 treatment, we observed that intratumoral changes to the chemokine milieu were associated with cDC subset recomposition in the tumor/dLN favoring CD8+ and CD103+ cDC1 accumulation within the TME. Additionally, MS-275-induced tumor inflammation was accompanied by an increase in co-stimulatory molecule expression (CD86/I-Ab) and antigen-presenting capability. Overall, MS-275 simultaneously subverts tolerogenic DC activity and promotes immunoactivation by recomposing the TME and providing maturation signals to support tumor-infiltrating cDC1s.

Cross-presentation of tumor antigens by cDCs are a pre-requisite to epitope spreading. In cancer immunotherapy, this process leads to the enhancement and diversification of endogenous T-cell responses against different epitopes from the original target. In the context of ACT and other immunotherapies, epitope spreading was observed in patients achieving remission of metastatic lesions and may thus contribute to treatment responsiveness (54). However, the association between epitope spreading and clinical

benefit has been mostly correlative. It is still unclear if and how therapeutic success is mechanistically dependent on epitope spreading. It has been suggested that epitope spreading can eliminate emergent immune escape variants as a result of therapeutic selective pressure (55). Alternatively, it may prolong antitumor immunity by stimulating endogenous T cell responses that can persist after the contraction of the initial therapy (56). During ACT+MS-275 treatment, we demonstrate that endogenous CD8+ T cells are critical for preventing tumor relapse and selective enhancement of p15E-specific responses provide long-lived recognition and killing of p15E-expressing tumors. Epitope spreading is therefore critical for the prevention of tumor relapse during adoptive cell therapy.

Regulatory T cells (Treg) exert indispensable functions in inducing and maintaining self-tolerance and immune homeostasis. During cancer, Tregs infiltrate into tumor tissue and their presence is associated with poor clinical prognosis (36, 57). Correspondingly, the systemic removal of Treg cells can invoke effective anti-tumor immunity (58, 59). In this study, we observed that MS-275 directly affected the TME and/or recomposed the myeloid department to reduce intratumoral production of TGFβ, NO, and Arg leading to partial depletion of Treg. This provided direct therapeutic value since complete ablation of Treg during ACT was able to prevent tumor relapse in lieu of MS-275 treatment. Moreover, Treg ablation did not impact LCMV GP33-41-specific T cell responses but significantly up-regulated p15E-specific responses, suggesting that Treg accumulation in tumors selectively inhibits epitope spreading responses.

Author contributions:

A.N. designed and conducted the majority of the experiments described and wrote the manuscript. L.H. prepared the samples for microarray analysis and qRT-PCR. S.W. constructed the VSV-gp33 viral vector. R.H. constructed the MCA102-p15E cell line. L.C. helped monitor the therapeutic efficacy of ACT+MS-275 in the gp33 model. Y.W. supervised the study, and participated in the conception of experimental designs.

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Main Figure Titles and Legends

Figure 1: Concomitant MS-275 delivery prevents tumor relapse during adoptive cell therapy

In C57BL/6 mice (n=5 per group), (A) five-day old intradermal B16-gp33 tumors were treated with adoptive cell therapy (ACT, 104 LCMV GP33–41-specific TMEM i.v. followed by viral vaccination). MS-275 was injected i.p. daily for 5 days starting at various timepoints. Tumor volumes were calculated based on height, width, and length. (B) Immunofluorescence-staining with cleaved caspase-3 antibody and TO-PRO-3 nuclear stain, (C) hematoxylin and eosin staining, or (D) immunohistochemical CD8 antibody staining of frozen tumor sections harvested five days post-treatment. Digested tumors (n=5 per group) were enriched for CD45.2+ cells and the (E) frequency of CD45.2+ CD8+ T cells and (F) absolute count of LCMV GP33–41-specific CD8+ T cells as determined by IFNγ expression after ex vivo peptide stimulation was measured. (G) Tumor volume measurements following selective lymphocyte depletion prior and during ACT+MS-275 treatment using monoclonal antibodies specific for CD8, CD4, and NK1.1. Data are represented as mean ± SEM. NS=not significant, **** p<0.0001; ACT, adoptive cell therapy

Figure 2: MS-275 remodels the inflammatory landscape of the tumor microenvironment to promote antigen processing and presentation

Bulk tumor RNA was derived from ACT-treated mice +/- MS-275 for microarray analysis (n=4 per group). (A) heatmap and gene set enrichment analysis (GSEA) of custom gene sets representing specific inflammatory pathways at D1 and D5 (see also Table S2). (B) qRT-PCR of pro-inflammatory cytokines at specific time points post-treatment (n=3 per group). (C) GSEA of curated gene sets (C2) derived from the Molecular Signatures Database (MSigDB) and displayed as an enrichment map (see also Figure S2). (D) Gene ontology (GO) analysis of modules (see also Table S3) derived from DE genes (D3 vs D1 and D5 vs D1, FDR p<0.05) within a protein-protein interaction (PPI) network. Modules were sorted by ratio of up-regulated to down-regulated genes when comparing ACT+MS-275 to ACT alone (see also Figure S3). (E) qRT-PCR of myeloid-related chemokines at specific time points post-treatment (n=3 per group). (F) GSEA of immunologic signatures (C7) derived from the Molecular Signatures Database (MSigDB) where highlighted groups represent gene sets related to activated myeloid cells (see also Figure S4). Data are represented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; ACT, adoptive cell therapy, DE, differentially expressed

Figure 3: MS-275 alters myeloid cell composition within the tumor/dLN and enhances co-stimulation

Five days post-treatment, digested tumors (n=3 per group) were positively enriched for CD45.2 cells. (A) Representative scatter plots outlining the gating strategy for characterizing myeloid populations in the tumor and draining lymph nodes (dLN). Myeloid cell composition changes in the tumor and dLN during ACT +/- MS-275 treatment were depicted by (B, C) representative contour plots and by (D, E) frequency as a percentage of total CD11c+ cells. Maturation marker expression in total CD11c+ cells in the (F) tumor and (G) dLN were determined by MHC Class II (I-Ab) and co-stimulatory ligand CD86 (B7-2)-specific flow staining. (H, I) Enriched CD11c+ cells were pulsed with LCMV GP33–41 peptide and co-cultured with CFSE-labeled LCMV-P14 TCR-transgenic naïve T cells. Representative histograms show CFSE dilution after 3 days and changes in proliferation due to treatment were quantified by division index. Data are represented as mean ± SEM. NS=not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; ACT, adoptive cell therapy

Figure 4: Epitope spreading mobilizes tumor-rejecting p15E-specific endogenous CD8+ T cell responses

(A) In lymphocyte-deficient RAG2/Il2rg knockout mice (n=7-8 per group), five-day old intradermal B16-gp33 tumors were treated with ACT +/- MS-275. Tumor volumes were calculated based on height, width, and length. Using C57BL/6 tumor-bearing mice treated with ACT+MS-275, (B) CD8+ T cells were positively enriched from digested tumors and co-cultured with target cell lines at a 10:1 effector to target ratio. Killing was measured by MTT reduction and done in triplicate. (C, D) Five days post-vaccination, the frequency of tumor antigen-specific CD8+ T cells in peripheral blood was determined by IFNγ expression after ex vivo stimulation with peptides (n=3 per group). (E, F) CFSEhi-labeled bulk splenocytes were pulsed with p15E peptide, mixed with CFSElo-labeled, unpulsed splenocytes at a 1:1 ratio, and infused into tumor-bearing mice five days post-ACT+MS-275 treatment. P15E-specific killing was measured by the recovery of labeled, pulsed targets relative to unpulsed targets (n=4 per group). Mice that were cured of B16-gp33 tumors during ACT+MS-275 were re-challenged with (G, H) natural, p15E-expressing MC38 tumors or (I, J) engineered, p15E-overexpressing MC102 tumors and monitored for tumor growth and survival (n=4 per group). The dotted line represents endpoint tumor volume. Data are represented as mean ± SEM. NS=not significant, * $p<0.05$, ** $p<0.01$; ACT, adoptive cell therapy

Figure 5: Reduced local immunosuppressive signals coincide with reduced regulatory T cell infiltration

(A, D) Bulk tumor RNA was derived from ACT-treated mice +/- MS-275 and qRT-PCR analysis was performed at specific time points post-treatment (n=3 per group). (B, C) Five days post-treatment, frozen tumor sections were stained with anti-Foxp3 antibody for immunohistochemistry and immunofluorescence imaging. (E, F) Positive enrichment of CD45.2+ cells from digested tumors in treated mice was followed by flow cytometric staining and absolute regulatory T cell counts were measured (n=5 per group). Data are represented as mean ± SEM. * p<0.05, ** p<0.01, **** p<0.0001; ACT, adoptive cell therapy

Figure 6: Regulatory T cells selectively obstruct the magnitude of endogenous responses from epitope spreading and their depletion promotes sustained tumor regression

In "depletion of regulatory T cell" (DEREG) BAC transgenic mice (n=5 per group), five-day old intradermal B16-gp33 tumors were treated with adoptive cell therapy followed by depletion of regulatory T cells using anti-CD4 monoclonal antibodies or diphtheria toxin. (A, B) Five days post-treatment, regulatory T cells were quantified within peripheral blood by flow cytometry. (C, D) Tumor volume measurements following regulatory T cell depletion in the context of ACT. (E, F) Five days post-vaccination, the frequency of tumor antigen-specific CD8+ T cells in peripheral blood was determined by IFNγ expression after ex vivo stimulation with peptides. (G) Five days post-treatment, digested tumors (n=3 per group) were positively enriched for CD11c+ cells, pulsed with LCMV GP33–41 peptide and co-cultured with CFSE-labeled LCMV-P14 TCR-transgenic naïve T cells. Representative histograms show CFSE dilution after 3 days and changes in proliferation due to treatment were quantified by division index. Data are represented as mean ± SEM. NS=not significant, * p<0.05, ** p<0.01, *** p<0.001; ACT, adoptive cell therapy, DT, diphtheria toxin

Materials and Methods

Study Design

The overall objective of the study was to determine how epigenetic modification during adoptive T-cell therapy can prevent tumor recurrence. The in vivo experiments were conducted to examine the differential therapeutic effect of histone deacetylase inhibitor delivery, to characterize local genetic, inflammatory, and immunological changes, and to determine the therapeutic impact of epitope spreading in the context of tumor recurrence prevention. In vivo studies were performed with n = 5 female age-matched (6-8 weeks old) mice. Experiments were repeated at least three times. Tumor-challenged mice were randomized prior to blinded treatments. Mice were monitored for signs of distress and humane endpoints were determined by decreased body condition. Veterinary staff monitored mice daily and alerted researchers when a humane endpoint had been reached. All animal studies complied with the Canadian Council on Animal Care guidelines and were approved by McMaster University's Animal Research Ethics Board.

Animals

C57BL/6 mice were purchased from Charles River Laboratory and B6.Cg-Tcratm1Mom Tg(TcrLCMV)327Sdz (P14) and Rag2/Il2rg Double Knockout (RAG KO) mice were purchased from Taconic. Mice were housed in the Central Animal Facility at McMaster University.

Viral Vectors

LCMV-Armstrong and VSV-gp33 were described previously (60-62).

Tumor Challenge

Naïve C57BL/6 mice were challenged intradermally with 105 B16-gp33 or MC38-gp33 cells in 30 µl PBS. Cured mice were re-challenged with 105 MC38, MCA102, or MCA102-p15E cells in 30 µl PBS. Tumor growth was monitored as previously described (63).

Adoptive T cell Transfer

Spleens were collected from LCMV-Armstrong-infected mice (>1 month) and a single cell suspension was prepared. LCMV GP33–41-specific memory T cells (TMEM) were enumerated by staining with H-2Db-GP33 tetramer (Baylor College of Medicine), CD127, and CD62L (BD Biosciences). 104 LCMV GP33–41-specific TMEM in 200 μL PBS were adoptively transferred into tumor-challenged mice by intravenous injection. Twenty-four hours post-adoptive T cell transfer, mice were injected intravenously with 2x108 pfu of VSV-gp33 in 200 μL PBS. Concomitantly, MS-275 (Sigma-Aldrich) was intraperitoneally injected (100 μg/mouse in 50 μL PBS) daily for five days. Selective lymphocyte depletion was conducted using monoclonal antibodies (Bio X Cell) specific for CD8, CD4, or NK1.1. Mice were injected with 250 μg of mAb in 500 μL PBS on Day -1 and 1 post-vaccination and every two weeks afterwards (150 μg).

Detection of Antigen-specific Responses

Five days post-vaccination, PBMCs were stimulated for 5 hours with LCMV GP33-41 peptide in the presence of brefeldin A (GolgiPlug; BD Biosciences). Following surface staining for CD8α (BD Biosciences), cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained for intracellular IFNγ (BD Biosciences). Data were acquired using an LSRFortessa flow cytometer with FACSDiva software (BD Biosciences) and analyzed with FlowJo X, version 10.0.7 (Treestar).

Tumor RNA Extraction

B16-gp33 tumors were excised 5 days post-vaccination and snap-frozen in liquid nitrogen and samples were then homogenized in Trizol (Invitrogen). RNA was extracted and purified using a RNeasy mini kit (Qiagen) and treated with Ambion's DNA-free kit.

Gene Expression Microarray

Samples were profiled using Illumina MouseRef8v2 arrays (GSE179337). The obtained data were processed with VST transformation and quantile normalization (lumi package (64)). Only annotated and detected genes were selected yielding a list of 13,088 genes for further analyses. Time course analysis using limma package (65) was performed with the following contrasts: (ACT+MS-275(D5)-ACT+MS-275(D1)) - (ACT only(D5)-ACT only(D1)) and (ACT+MS-275(D3)-ACT+MS-275(D1)) - (ACT only(D3)-ACT only(D1)). Obtained p-values were corrected with Benjamini-Hochberg correction for multiple testing (66); corrected values <0.05 were considered to be significant. All significant genes from

the comparisons were used for Protein-Protein Interaction Network construction, using Reactome FI plugin (67) in Cytoscape environment (68). Next, the modules of nodes in the network were defubed and analyzed for Pathway enrichment and GO Biological Process component. The obtained lists of significantly enriched and over-represented pathways and biological processes were used to categorize and label the modules in the network.

Gene set enrichment analysis (GSEA) (69) was performed using the whole gene expression profiles to examine the following comparisons: (a) ACT+MS-275(D1)-ACT only(D1), (b) ACT+MS-275(D3)-ACT only(D3), and (c) ACT+MS-275(D5)-ACT only(D5). We have performed the analysis using 3 sets of gene sets: C2v4 and C7v4 from MSigDB [https://www.gsea-msigdb.org/gsea/msigdb/index.jsp], and a custom gene set (Table S1). FDR corrected P-values < 0.05 were considered to be significant. Next, the results obtained for C2v4 and C7v4 were visualized using Enrichment Map plugin (70) in Cytoscape environment.

Purification of Tumor-infiltrating Dendritic Cells

B16-gp33 tumors were excised 5 days post-vaccination and digested in 0.5 mg/mL collagenase Type IV (Gibco), and 0.2 mg/mL DNase (Roche) prepared in complete RPMI (Gibco, 10 mL/250 mg tumor) and incubated at 37□C for 30 min as previously described (71). The digested material was CD45.2 or CD11c-enriched through magnetic selection with an EasySep Mouse Biotin or CD11c Positive Selection Kit (Stemcell Technologies).

Histology

B16-gp33 tumors excised 5 days post-vaccination were snap frozen before cryostat sections were obtained and mounted on gelatin-coated histological slides. The slides were fixed in 4% paraformaldehyde for 20 min at room temperature and blocked with PBS containing 5% goat serum and 0.3% Triton X-100 for 30 min. Slides were pretreated with Leica Bond Epitope Retrieval buffer #2 (Leica Biosystems) for 20 minutes before staining using hematoxylin and eosin (H&E), immunohistochemistry (IHC), or immunofluorescence (IF). IHC: Anti-CD8 (1:1000, Thermo Fisher Scientific) was added and incubated overnight at at 4□C. Color was developed using the Leica Bond Polymer Refine Detection Kit (Leica Biosystems) and with rabbit anti-rat antibody (1:100, Vector Laboratories). IF: Anti-Cleaved Caspase-3 (1:400, Cell Signaling Technology) or anti-FoxP3 (1:400, Thermo Fisher Scientific) were added and incubated overnight at 4□C. Slides were incubated for 1h with biotinylated goat anti-rabbit IgG (Vector Laboratories) followed by Alexa Fluor 488-conjugated streptavidin (Thermo Fisher Scientific).

Proliferation assay

Tumor-infiltrating dendritic cells were isolated, pulsed with LCMV GP33-41 peptide and co-cultured for 3 days with CFSE-labeled P14 T cells as previously reported (72). Proliferation was evaluated using several metrics including division index (73).

Cytotoxicity Assay

In vitro: B16-F10 cells (1x104 /well) were co-cultured with tumor-infiltrating myeloid cells (1x105/well) in 96-well flat bottom microtiter plates (Corning Inc.) for 12 hours before killing was assessed. Non-adherent cells were washed using warm PBS and 500 µg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thermo Fisher Scientific) solution was added before incubating the plates for 4 hours. Solubilization of the formazan by-product was done by aspirating the MTT solution and adding DMSO. The absorbance was measured at 540 nm using a Synergy microplate reader (Biotek). In vivo: Target bulk splenocytes were isolated and pulsed with 1 µg/mL LCMV GP33-41 peptide for 1 hour and labeled with CFSE at a final concentration of 5 µM/mL (CFSEhi) in RPMI media 1640 with 2% FBS for 15 min as previously described (74). The cells were mixed at a 1:1 ratio with unpulsed bulk splenocytes labeled with 0.5 µM/mL CFSE (CFSElo) and infused i.v. into treated mice 4 days post-treatment. After 24 hours, spleens were harvested and processed for flow cytometric detection of CFSE-expressing cells.

Statistics

GraphPad Prism (GraphPad Software) was used for graphing and statistical analyses. Student's t-test and one-way/two-way analysis of variance (ANOVA) were 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.

Supplementary Figure Titles and Legends

Figure S1, related to Figure 2: Connectivity mapping of differentially expressed (DE) genes

(A) DE genes expressed in ACT+MS-275 relative to ACT only treatment over the period of the 5 days (Day 5 vs. Day 1) were used to create two signatures: genes with fold change of at least 1.5, and genes with fold change of at least 2.0 (FDR p<0.05). Both signatures were used to query the Connectivity Map database and overlapped with chemical perturbation gene signatures. (B) Venn diagram of compounds that show significant overlap (C) Ranking of 32 compounds found to overlap both signatures based on similarity to the FC >= 2.0 signature

Figure S2, related to Figure 2C: Fully-annotated curated gene sets (C2) enrichment map

Enrichment map with full annotations for every gene set cluster.

Figure S3, related to Figure 2D: Gene module ranking based on MS-275 up-regulation

(A) Differential gene expression comparison of ACT+MS-275 treatment vs ACT alone from Day 1 to Day 5 (FDR p<0.05). (B) Protein-protein interaction network where clusters of differentially expressed genes (DE) were assembled into gene modules based on D3 vs D1 and D5 vs D1. (C) Expression heatmap and sorting of modules based on percentage of genes up-regulated by MS-275 for D3 vs D1 and D5 vs D1 comparisons (D) GO term assignment to each module and color assignment based on relative up-regulation (red) or down-regulation (green) by MS-275.

Figure S4, related to Figure 2F: Fully-annotated immunologic signatures (C7) enrichment map

Enrichment map with full annotations for every gene set cluster.

Supplementary Table Titles and Legends

Table S1: Custom inflammatory gene sets, related to Figure 2A

Table S2: Enriched custom gene sets after gene set enrichment analysis (GSEA), related to Figure 2A

Table S3: Modules derived from the network of differentially expressed genes, related to Figure 2D

Figure 1
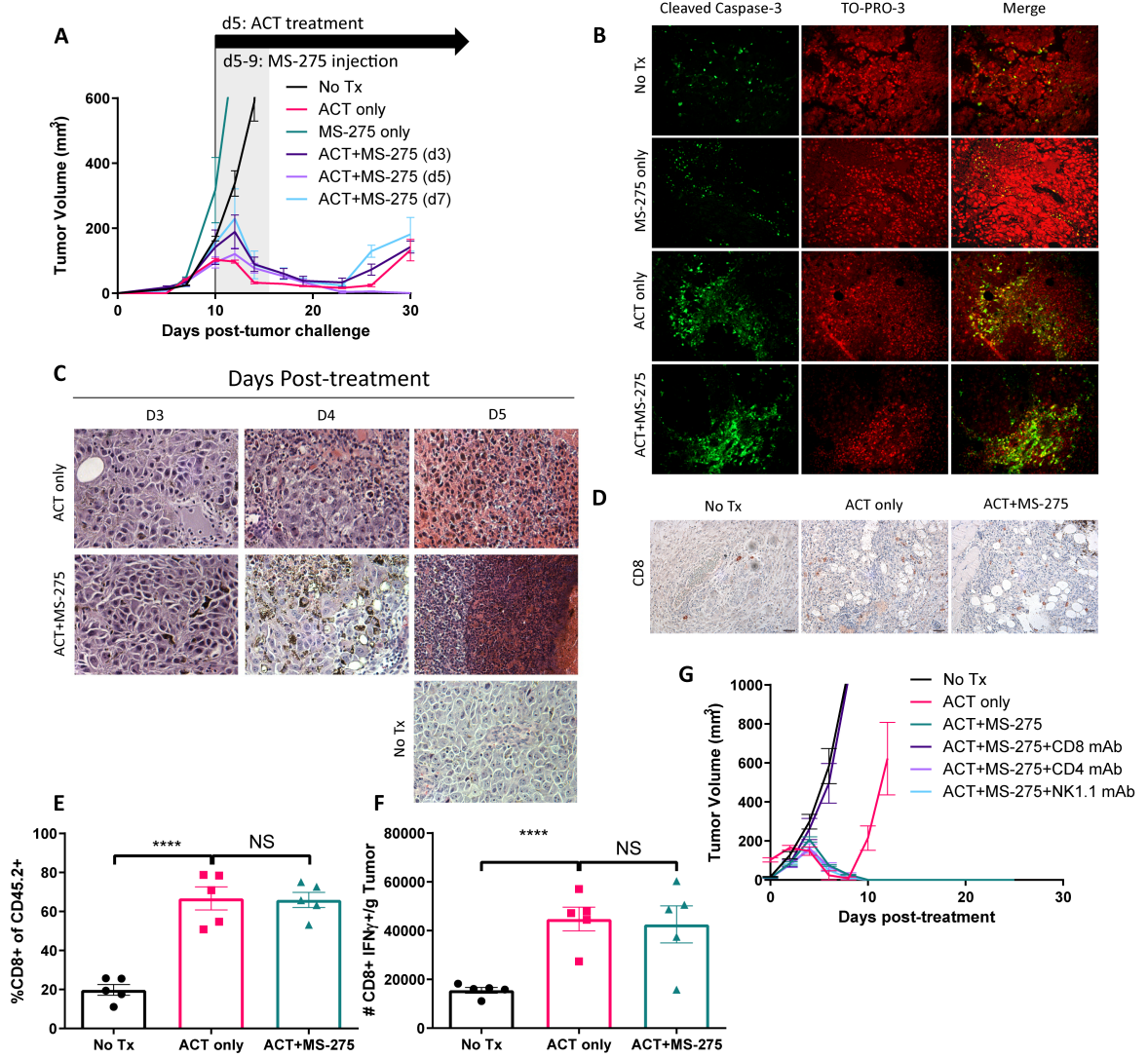
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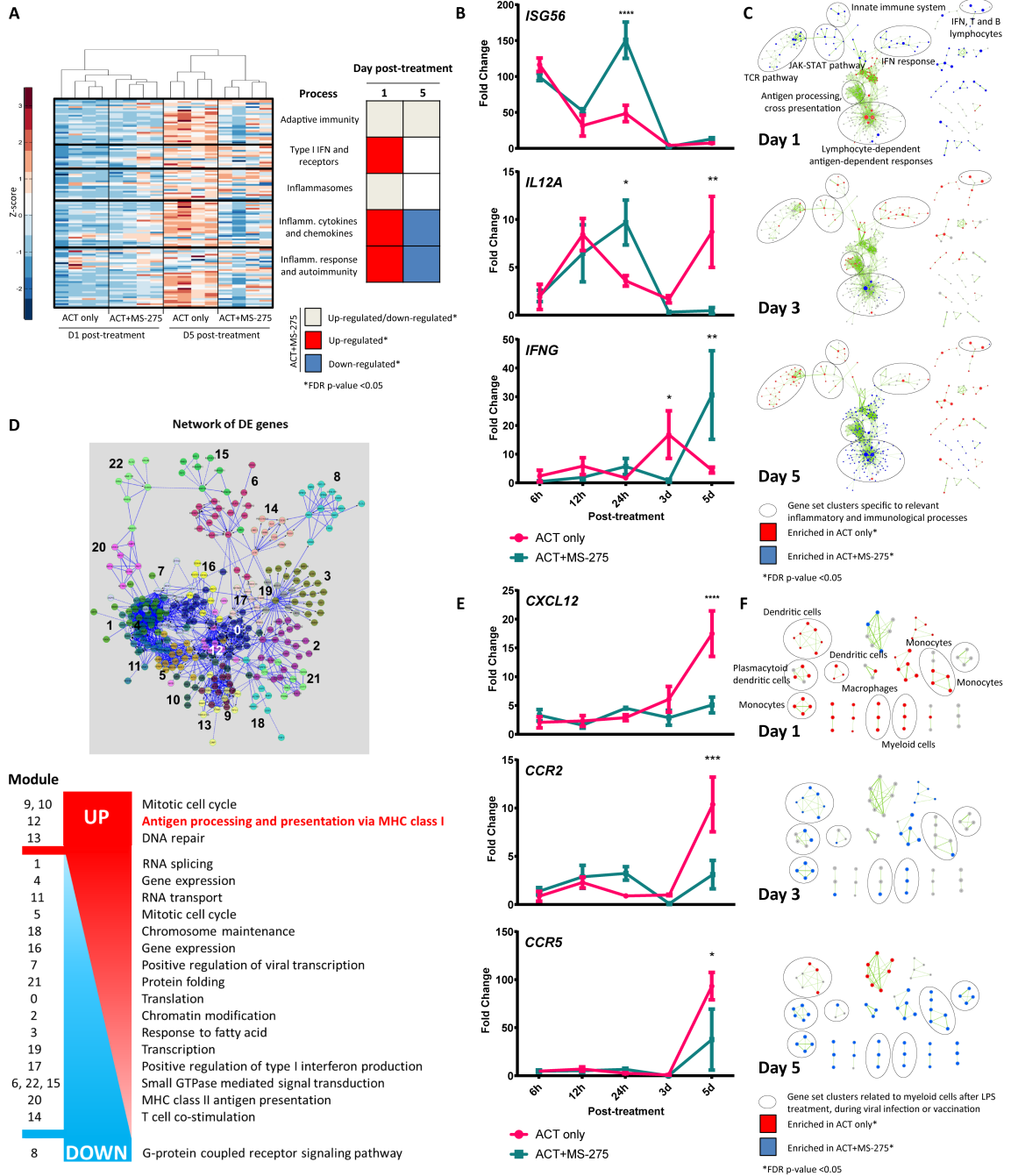
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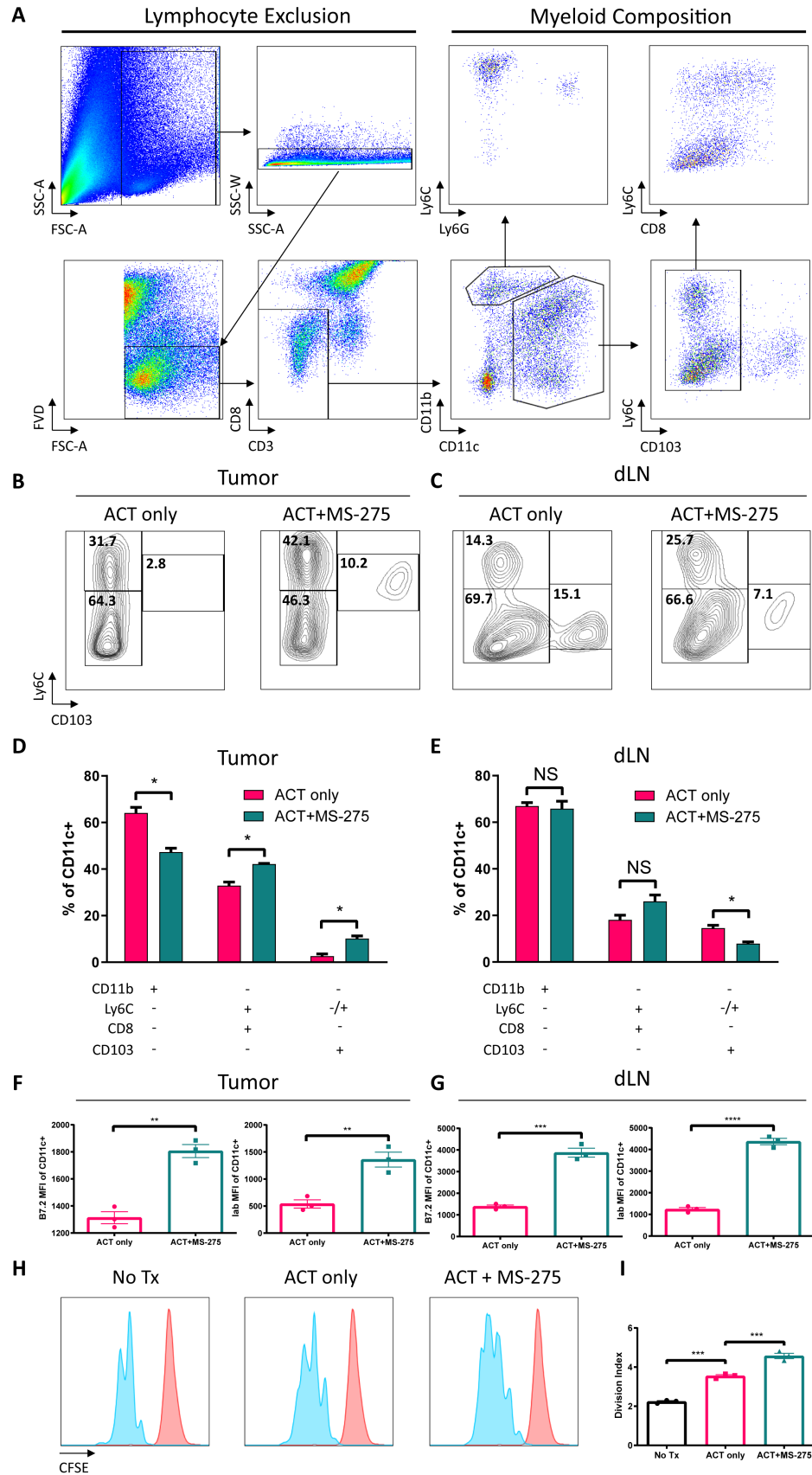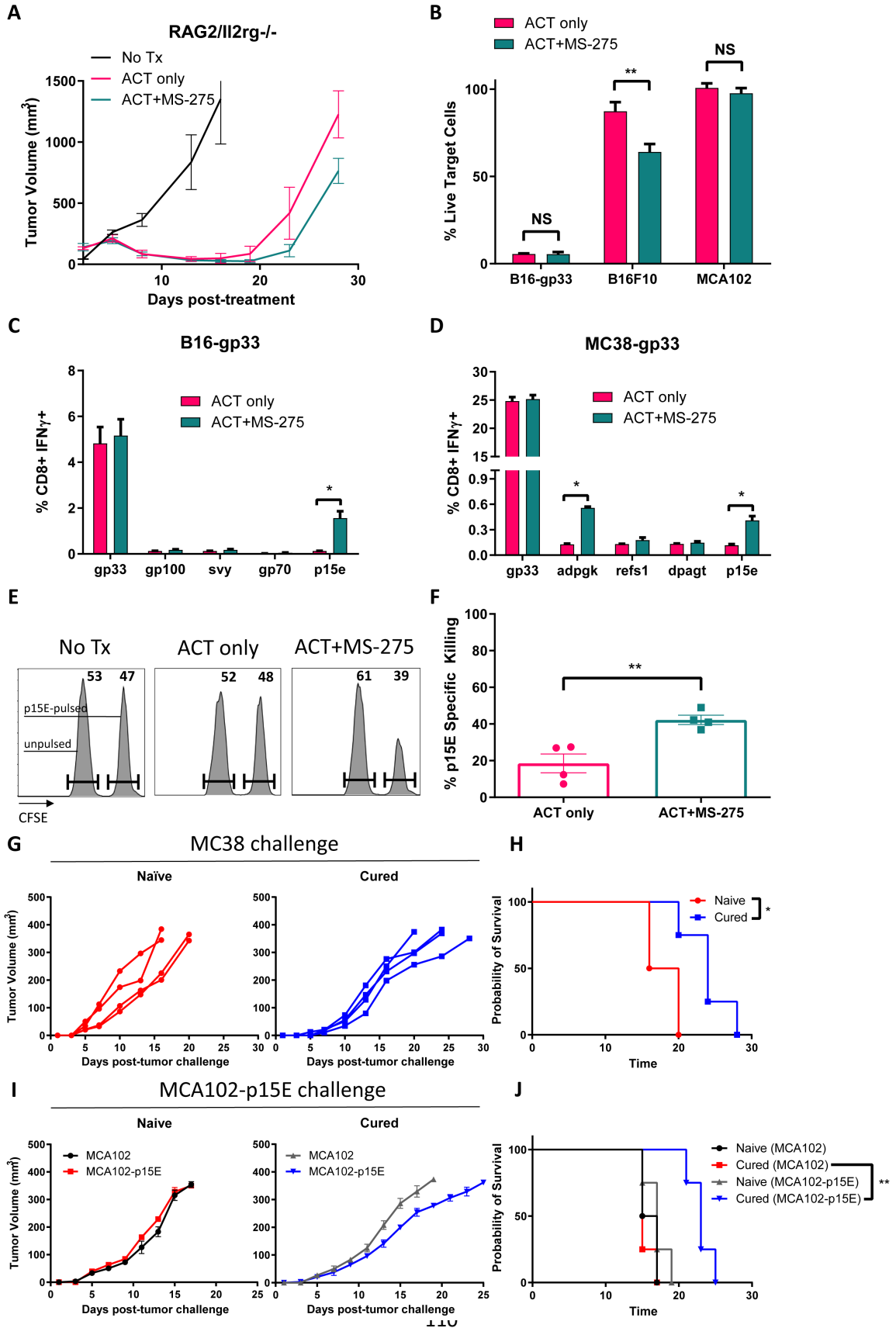
Figure 4

Figure 5

Figure 6

Supplementary Figure 1

A

Differentially expressed genes (limma, decideTests, BH correction)

Regulation	↑			↓		
Absolute Fold Change	All	>1.5	>2.0	All	>1.5	>2.0
3d vs. 1d	61	24	3	19	7	2
5d vs. 3d	327	99	9	321	110	34
5d vs. 1d	1262	299	24	1147	411	160

→ cMap1 ←

→ cMap2 ←

B

FC >= 2.0   22 | 32 | 33   FC >= 1.5
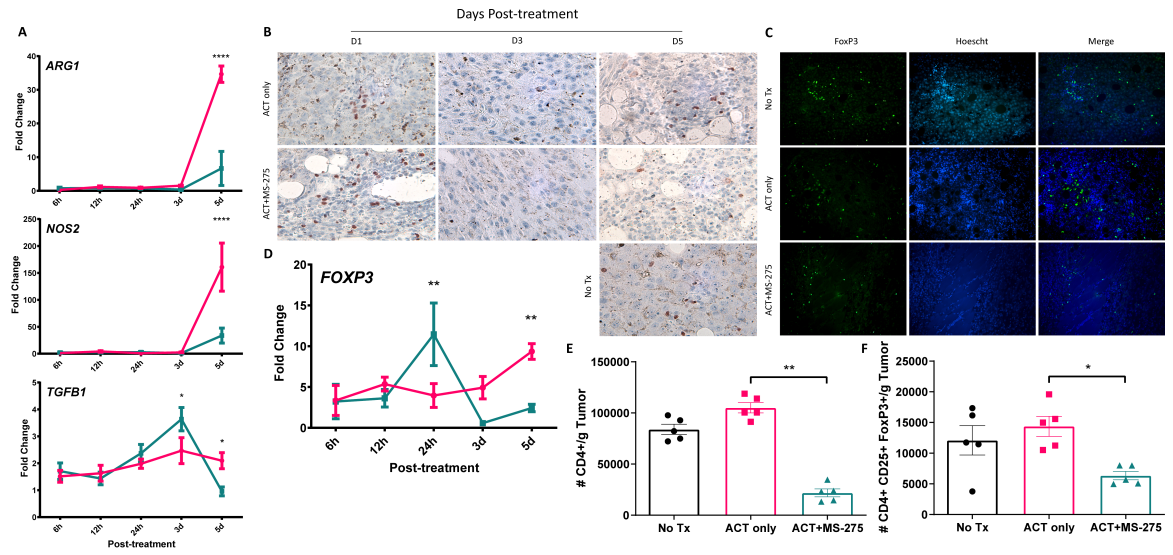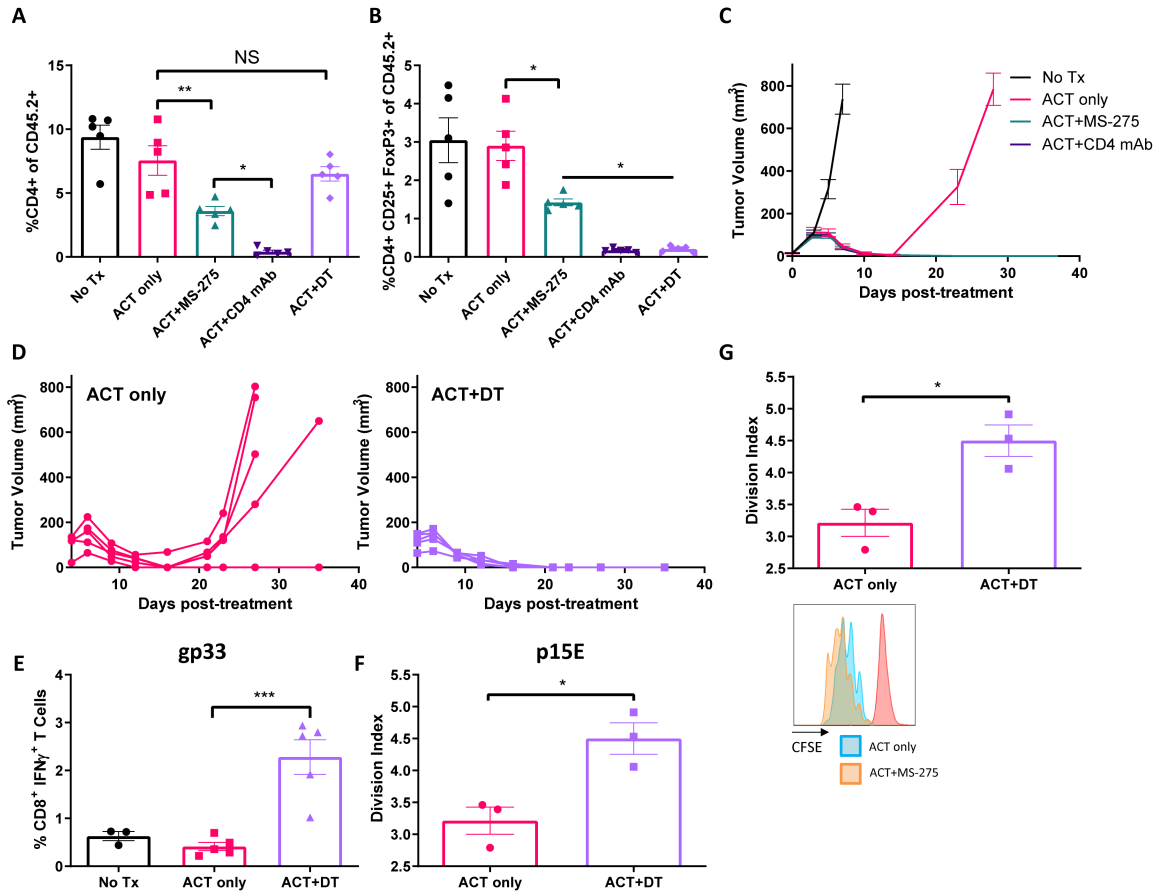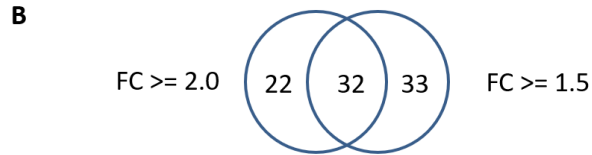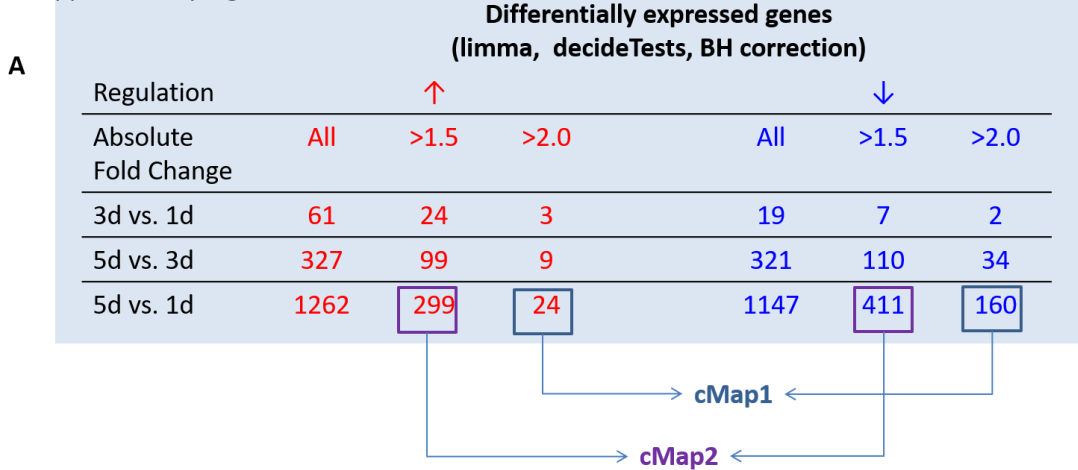
C

Compound		Similarity	
		FC2.0	FC1.5
MS-275	HDAC inhibitor	-0.929	-0.782
DL-thiorphan	membrane metalloendopeptidase inhibitor	-0.89	-0.722
quinostatin	PI3K inhibitor	-0.807	-0.87
rottlerin	Enzyme inhibitor	-0.793	-0.729
0297417-0002B		-0.75	-0.829
alexidine		-0.717	-0.573
puromycin	antibiotics	-0.709	-0.65
pyrvinium	antihelmintic	-0.672	-0.717
propafenone	Sodium-channel blocker	-0.665	-0.509
etacrynic acid	Enzyme inhibitor	-0.66	-0.709
ivermectin	Activates chloride channels	-0.658	-0.662
phthalylsulfathiazole		-0.624	-0.593
medrysone	corticosteroid	-0.616	-0.597
methylergometrine	oxytocics	-0.58	-0.644
dequalinium chloride	Disinfectant	-0.567	-0.533
thioridazine	Dopamine antagonist	-0.563	-0.649
levonorgestrel	Synthetic progesterone	-0.558	-0.56
withaferin A		-0.541	-0.61
dipyridamole	phosphodiesterase inhibitor	-0.535	-0.549
trichostatin A	HDAC inhibitor	-0.535	-0.653
flunisolide	corticosteroid	-0.514	-0.487
vorinostat	HDAC inhibitor	-0.512	-0.556
pimozide	Dopamine blocker	-0.51	-0.58
LY-294002	Enzyme inhibitor	-0.507	-0.613
0175029-0000		-0.498	-0.634
chlorpromazine	Dopamine antagonist	-0.458	-0.441
tretinoin	ATRA, retinoic acid	-0.45	-0.427
trifluoperazine	Dopamine antagonist	-0.439	-0.653
sirolimus	Immunosupressive agent	-0.323	-0.615
wortmannin	Immunosupressive agent; PI3K inhibitor	-0.3	-0.576
15-delta prostaglandin J2		-0.296	-0.387
valproic acid	HDAC inhibitor	-0.206	-0.307

Supplementary Figure 2

Supplementary Figure 3

A

**Differentially expressed genes
(limma, decideTests, BH correction)**

Regulation	↑			↓		
Absolute Fold Change	All	>1.5	>2.0	All	>1.5	>2.0
3d vs. 1d	61	24	3	19	7	2
5d vs. 3d	327	99	9	321	110	34
5d vs. 1d	1262	299	24	1147	411	160

Network (no linkers) – all DE genes (3vs1 & 5vs1)

B

Regulated in 3vs1
(all 3 cases – in the same direction as 5vs1)

C

Color Key and Histogram

D

Supplementary Figure 4

Genes down-regulated in dendritic cells after Newcastle disease virus infection

Genes up-regulated in CD8 T cells after stimulation with antigen-B7-1, IL12, TSA,

Genes up-regulated in peripheral blood mononuclear cells after YF17D vaccination

Genes up-regulated in effector and memory CD8 T cells vs naïve CD8 T cells

Genes down-regulated in monocytes treated with LPS (TLR4 agonist) vs monocytes treated with anti-TREM1

Up-regulated in pDC vs B cells and vs. myeloid DC (without and after influenza vaccination) and vs. monocytes

Genes up-regulated in dendritic cells stimulated with R848, LPS and both

Genes down-regulated in CD8 T cells stimulated antigen B7-1, IL12

Genes down-regulated in monocytes treated with anti-TREM1 and LPS (TLR4 agonist)

Genes up-regulated in naïve monocytes vs. B cells, T cells, CD4 and CD8 T cells

Genes up-regulated in memory, effector memory, central memory T cells vs. naïve T cells

Genes up-regulated in macrophage cells stimulated with LPS (TLR4 agonist) vs unstimulated macrophage cells

Genes up-regulated in systemic lupus erythematosus myeloid cells vs. systemic lupus erythematosus CD4 T and B cells and in healthy myeloid cells vs healthy B cells

Genes down-regulated in naïve CD4 T cells treated with IL4 and anti-IL12

Genes up-regulated in naïve CD4 and CD8 T cells vs naïve B cells

Genes up-regulated in CD4 T treated with with IL4 and anti-IL12

Supplementary Table 2

Day 1 GSEA - positively enriched in ACT+MS-275 vs ACT only

NAME	SIZE	FDR q-val	Leading Edge
ADAPTIVEIMMUNITY_DOWN	25	0.001	TLR4, CTLA4, RORC, STAT3, IFNG, TGFB1, SLC11A1, IL18, CXCL10, CCR5, C3
ADAPTIVEIMMUNITY_UP	18	0.001	ICAM1, IFNG, CD83, CD40, PRF1, CD86, CD68, SLC11A1, IL18
ANTIVIRALRESPONSE_UP	74	0.000	MX1, IRAK4, TLR13, AZI2, RELA, IFIH1, TYK2, IFNAR2, CASP8, IFITM2, TLR4, DDX3X, TBK1, RIPK1, DDX58, MAPK3, MAP3K1, MAP2K1, IL15, TLR2, CD40, TLR3, MYD88, TLR7, PSTPIP1, CD86, SPP1, TLR13, CTSB, IRF5, STAT1, CTSS, IL18, CXCL10, CASP1, IFIT2
IFNS_AND_RECEPTORS_DOWN	48	0.000	IL15, IL7R, IFNG, EBI3, IL12RB1, IL28RA, IL13RA1, IRF2, IL10RB, IFIT3, IRF8, IRF5, IRF1, CXCL10, IFIT2
INFLAMMASOMES_DOWN	46	0.001	MAPK3, IL33, IFNG, BIRC2, MAPK13, NLRX1, MYD88, CCL7, TXNIP, IL18, IRF1, CIITA, NOD1, CASP1
INFLAMMASOMES_UP	9	0.048	BCL2L1, HSP90B1, PSTPIP1, CTSB
INFLAMMATORYCYT_CHE_DOWN	43	0.000	IL33, IL15, CSF1, IFNG, CCL2, IL10RA, CCL3, CXCL13, CXCL16, CXCL9, IL10RB, CCL6, CCL7, TNFRSF11B, CCL9, CXCL10, CCR5
INFLAMMATORYRES	49	0.000	IL1RN, CCL25, FASL, RIPK2, BCL6, TLR4, CCL11, LY96, CSF1, IFNG, TLR2, CCL2, CD40, TLR3, MYD88, CCL3, TLR7, CXCL9, IL10RB, CCL7, C4B, CEBPB, IL18, CXCL10, C3
MELANOMA_UP	18	0.018	ENC1, TRP53INP1, RB1, SOCS2, SOCS3, IRF8

Day 5 GSEA - negatively enriched in ACT+MS-275 vs ACT only

NAME	SIZE	FDR q-val	Leading Edge
INFLAMMATORYRESPONSE_AUTOIMMUNITY_DOWN	49	0.000	CCL5, IL1B, CD14, IL1RN, CD40, CCL7, C4B, IL10RB, TLR2, C3, TNF, IL18, NOS2, TLR7, CCL2, BCL6, CEBPB, IL1A, LTB, FASL, ITGB2, CXCR4, NFKB1, CXCL9
INFLAMMATORYCYT_CHE_DOWN	43	0.002	CXCL16, CCR5, CCL6, TNFRSF11B, CCL5, IL1B, IL1RN, CCL7, IL10RB, IL15, IL10RA, TNF, IL2RG, CCL9, CCL2, IL1A, LTB, FASL, CXCL9, IL33, CXCL12, OSM, IL16
ANTIVIRALRESPONSE_UP	74	0.008	CASP1, CCL5, CTSS, TLR13, IL1B, CD14, CD86, CD40, TLR2, IL15, IRF5, TNF, IL18, TLR7, NFKBIA, IL1A, ATG12, NFKB1, AZI2, CTSB
ADAPTIVEIMMUNITY_UP	18	0.008	CD68, SLC11A1, CD86, CD40, CD83, IL18, ICAM1
ADAPTIVEIMMUNITY_DOWN	25	0.016	CCR5, SLC11A1, CCL5, C3, TNF, IL18, NOS2, PDCD1LG2, TGFB1, TLR4, STAT4, TLR6

Supplementary Table 3

Module	Nodes in Module	Genes
0	28	ANAPC4, ANAPC5, FZR1, GSPT2, HECTD3, MGRN1, PARD6A, PJA1, PSMB4, RBCK1, RNF4, RPL21, RPL23, RPL30, RPL38, RPS10, RPS13, RPS19, RPS27A, RPS6, RSL1D1, SKP2, SPSB1, SRP14, TRIP12, UBA2, UBE2L3, UBE2S
1	22	ALYREF, CBFB, CCAR1, CD164, EFTUD2, HNRNPA2B1, HNRNPM, NCBP1, PABPN1, PHF5A, PTBP1, RALY, RBM4, RBM8A, RBMX, SF3B2, SRSF1, SRSF2, SRSF3, SRSF6, THOC5, TRA2A
2	22	ASF1B, BCL11B, CHD4, CTSH, DNMT1, DNMT3B, H2AFJ, H2AFY, HDAC11, HDAC2, HIRA, HIST1H2AH, HIST2H2AB, IKZF1, KLF7, NAP1L1, SALL4, SMARCA2, SMARCE1, SUV39H1, TCF19, UHRF1
3	20	ALPL, ASAH2, BZW2, CHMP1B, CRABP2, CTF1, DBP, DRAM2, EP300, FABP3, HCN2, ILF2, INSIG2, MFGE8, NDRG2, PSTPIP1, PTGDS, VAMP5, WFDC1, ZHX2
4	19	CSTF1, DDX20, GEMIN5, GEMIN6, HNRNPK, LSM10, LSM2, LSM4, LSM6, PRPF19, SF3A3, SF3B3, SMN1, SNRNP70, SNRPA, SNRPB, SNRPD2, SNRPD3, TXNL4A
5	17	APITD1, AURKA, AZI1, BUB1B, CENPM, CENPN, CLIP1, KIF2C, MAD1L1, NDC80, NDE1, NUDC, PLK1, PMF1, SGOL1, SPC24, ZW10
6	17	ABR, ARFIP2, ARHGAP17, ARHGAP25, ARHGDIG, ARHGEF3, CDC42EP2, CTTN, DEPDC1B, HAP1, MTSS1, NCKAP1L, PLEK, RAC2, RHOBTB2, RHOF, SELPLG
7	15	DCPS, ERCC1, ERCC2, GTF2E2, GTF2H3, NFIB, NFIX, POLR2D, POLR2E, POLR2F, POLR2H, POLR2J, RNMT, WHSC2, ZFP30
8	14	CXCL16, CYSLTR1, FPR2, GNA15, GNAS, GNG2, GNGT2, GPR18, GPR65, GPR68, GRK6, P2RY14, P2RY6, PLCL2
9	12	CDC45, CDC7, CDT1, GINS4, MCM10, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, ORC6
10	12	CDC25A, CDK1, FOXM1, LMNB1, MELK, NCAPH2, PBK, PKMYT1, PRC1, SPAG5, TUBG1, UCK2
11	11	AAAS, BANF1, MCFD2, NUP133, NUP210, NUP43, NUP50, NUP85, NUP93, RANGAP1, SNUPN
12	11	EARS2, MVD, NVL, PSMA7, PSMB1, PSMC2, PSMC5, PSMD13, PSMD4, PSMD7, RAD23A
13	10	ATRIP, CINP, CLSPN, MSH6, POLE, RAD1, RFC1, RFC5, RPA1, TIMELESS
14	9	CD28, CD3E, DBNL, LCK, LIME1, PDCD1LG2, PTPN22, RGS1, SLA
15	9	APBB1IP, ELMO1, RAP1B, RAPGEF1, RAPGEF5, RASGRP1, RGL1, RIT1, RND3
16	8	CNOT2, DDX49, EIF2S2, EIF3G, EIF4A1, EIF4E3, EIF4G1, RQCD1
17	8	NFKB2, NFRKB, NR1H3, RELA, RELB, S100B, TSC22D3, ZDHHC13
18	8	ACTR5, LEO1, MORF4L1, NHP2, PAF1, RUVBL2, TFPT, WRAP53
19	7	ACOX3, FADS2, LMO2, MED15, MED18, MED8, RXRA
20	7	ACTR1B, DCTN4, KIF22, KIF3C, LAMP2, RILP, STX12
21	7	CCT3, CCT6A, CCT8, FBXL5, FBXW4, PFDN2, PFDN4
22	7	ALS2, ARL8B, RAB11B, RAB5A, RAB5B, RABEP1, RIN2

4.0 Chapter Four – T cell transcriptional reprogramming overcomes tumor burden-induced cellular exhaustion

4.1 Introduction

Tumor size or burden is an intrinsic property of solid tumors that has negative clinical implications for cancer immunotherapy. Indeed, systemic immunosuppression associated with increased tumor burden can lead to rapid deletion of transferred cells during ACT. This manuscript characterizes the immunosuppressive pathways associated with tumor burden and illustrates how MS-275 can restore therapeutic efficacy in large tumors.

In this manuscript, large fibrosarcoma tumors are defined by a unique immunosuppressive pathway signature that is conducive to the systemic shutdown of transferred T cell responses through induction of cellular exhaustion. MS-275 promotes tumor tissue normalization by reversing large tumor-intrinsic pathways. This was accompanied by a reprogramming of transferred T cells from an exhausted phenotype to activated effector phenotype.

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T cell transcriptional reprogramming overcomes tumor burden-induced cellular exhaustion

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Summary

Adoptive cell therapy (ACT) for solid tumors is a clinical challenge that is progressively being addressed. However, tumor burden is still an inherent barrier to therapeutic responsiveness. Here we identified that in contrast to small tumors, large syngeneic tumors systemically ablated T cell expansion and mitigated tumor control. This was accompanied by clinically pervasive immunosuppressive pathway changes in the tumor microenvironment. In our study, we demonstrated that Class I histone deacetylase inhibitor MS-275 promoted tumor normalization and in conjunction with ACT restored T cell responses and tumor regression. Transferred CD8+ T cells displayed higher proliferation and cytotoxicity which we associated with T cell activation-favored transcriptome remodeling. Additionally, Tim-3 was selectively up-regulated, which correlated with reduced signatures of T cell exhaustion and differentiation into a predominantly terminal effector-like Tim3hi TCF1- CD127- KLRG1+ population. Taken together, concomitant MS-275 delivery may mediate conversion between exhaustion and effector lineage differentiation states.

Introduction

Immunotherapy for the treatment of cancer has gained much attention in recent years for its successes in enabling immune responses that can elicit durable tumor control. One of the major cancer immunotherapy strategies, adoptive cell therapy (ACT), involves the autologous/allogeneic transplant of personalized cellular products that demonstrate potent tumor recognition and killing. These are usually tumor antigen-specific CD8+ T cells that were derived from tumor-infiltrating lymphocytes or were genetically modified to express specific T-cell receptors or chimeric antigen receptors (Mardiana et al., 2019). ACT and immune checkpoint blockade currently represent the overwhelming number of active trials for immunotherapy and have shown consistent efficacy in the treatment of hematological malignancies (Xin Yu et al., 2019). However, the clinical success of cancer immunotherapy is contingent on a CD8+ T cell-inflamed tumor microenvironment (TME), and unlike non-solid cancers, solid cancers have often produced compromised T cell responses (Morotti et al., 2021). As a result. the number of clinical trials that have shown success in the treatment of non-melanoma solid cancers is disproportionately low. Currently, the field is looking to address the unique challenges imposed by solid tumors to improve the curative potential of ACT.

The TME in solid tumors presents significant hurdles for T cell inflammation. Tumor secretion of inhibitory chemokines and stromal/vascular changes can impede immune infiltration, while tumor-intrinsic/tumor-extrinsic immunosuppressive factors may reduce their viability and function (Wagner et al., 2020). Furthermore, variability in tumor antigen expression can facilitate therapeutic selective pressure for immune escape variants (Landsberg et al., 2012; Restifo et al., 2016). Fortunately, increasing comprehension of optimal antitumor T cell parameters have fostered improvements to existing ACT protocols. In addition to T cell dose (Hanson et al., 2000), several variables were shown to be determinants of therapeutic efficacy: 1) the differentiation status of adoptively transferred T cells, which is inversely correlated with in vivo proliferation capacity (Klebanoff et al., 2011), and 2) the inclusion of post-transfer viral vaccination for in vivo antigenic re-stimulation, which enhances cytotoxic effector differentiation and homing of adoptively transferred T cells (Smith et al., 2009). Accordingly, these conditions have been shown to promote the quick accumulation of tumor-infiltrating T cells to bypass TME resistance and regress solid tumors (Nguyen et al., 2018). ACT may also facilitate the induction of secondary endogenous T cell responses against other tumor antigens, resulting in broad spectrum antitumor immunity (Walsh et al., 2019). Taken together, ACT protocols are becoming increasingly efficacious in the control and resolution of solid tumors.

Despite this, the extent of tumor burden presents an additional layer of difficulty for solid tumor eradication. It has been previously demonstrated that successful ACT treatment in small tumors may not be recapturable in large tumors (Kim et al., 2020). This has been attributed to the functional Impairment and rapid deletion of transferred cells (Prato et al., 2013). Indeed, increased tumor burden may promote immunosuppressive cytokine/immunoregulatory cell accumulation and dysregulation of antigen presenting cells, which stifles the antigenic re-stimulation of transferred T cells (Hegde et al., 2020; Lin et al., 2020). Deficiencies in cellular activation, along with antigen chronicity, are primary determinants of T cell exhaustion, a broad categorical term that encompasses hyporesponsive effector T cells with unique transcriptional programmes and differentiation states (Blank et al., 2019; Jiang et al., 2015; Thommen and Schumacher, 2018). Therefore, cellular exhaustion may be viewed as a prolific functional consequence of increased tumor burden. While the use of checkpoint blockade and similar strategies can restore the antitumor capacity of exhausted T cells, its therapeutic efficacy in large tumors has been shown to be limited by the degree of reinvigoration relative to the extent of tumor burden (Huang et al., 2017). An alternative strategy is to target the local changes inherent to increased tumor burden that could create systemic immunosuppressive outcomes. However, few studies have examined these upstream biological pathway changes despite large tumors being more clinically representative in terms of size/duration of growth (Wen et al., 2012). Increased comprehension of large tumors may lead to purposeful re-design of the ACT platform to reliably induce positive clinical outcomes.

Here we determined that successful adoptive cell therapy in solid tumors is abrogated by increased tumor burden. Large tumors expressed a unique pathway signature that coincided with the functional exhaustion of transferred T cells. Interestingly, concomitant delivery of Class I histone deacetylase inhibitor, MS-275, restored therapeutic efficacy by promoting tumor tissue homeostasis in large tumors and subverting T cell exhaustion programming. We found that transferred CD8+ T cells displayed features of heightened activation-dependent signaling and effector differentiation. Overall, our findings suggest that epigenetic remodeling can reliably sensitize large tumors to immunotherapy.

Results

Tumor burden correlates with reduced responsiveness to immunotherapy and negative clinical outcomes

In a murine CMS5 fibrosarcoma tumor model wherein the immunodominant rejection antigen is mutated ERK2136–144 (mERK2136–144), ex vivo culture of DUC18 T cell receptor (TCR) transgenic T cells into a central memory phenotype (TCM) followed by adoptive transfer and viral vaccination (VSVΔM51-mERK2136–144) sustainably regressed 7-day old (<150 mm3) established tumors. However, when we increased the tumor burden to 14 days (>600 mm3), our ACT protocol was incapable of controlling tumor growth and prolonging survival (Figure 1A, B). To determine if there was an adverse effect on transferred T cell responses, we harvested peripheral mononuclear blood cells (PBMCs) 5 days post vaccination. In contrast to 7-day old tumors wherein we detected a ~26% circulating mERK2136–144-specific CD8+ T cell frequency, ACT treatment of 14-day old tumors resulted in complete ablation of the T cell response (Figure 1C).

In addition, 14-day old tumors were surgically removed 24 hours prior to ACT treatment. Unlike intact tumors, surgical resection restored the peripheral T cell response (Figure 1D). As such, we reasoned that increased tumor burden did not induce permanent host biological changes and that the systemic shutdown of antitumor T cell expansion, while potent, was inherently reversible.

To preserve transferred T cell responses during ACT, we first sought to understand the local changes that predisposed large tumors to therapeutic resistance. We harvested RNA from 7- and 14-day old tumors and analyzed them by bulk-cell RNA-seq. 14-day old tumors acquired a distinct transcriptomic signature compared to 7-day tumors by principal component analysis (PCA) (Figure 1E). Correspondingly, gene set enrichment analysis (GSEA) of 14- vs 7-day old tumors using Hallmark, Curated (C2), and Ontology (C5) gene set collections from the Molecular Signatures Database (MSigDB) allowed us to identify biological processes affected by increased tumor burden. Enrichment redundancy across collections affirmed that 14-day old tumors positively enriched pathways related to transcription/translation, cellular metabolism, and hypoxia, and negatively enriched pathways related to inflammatory cytokine signaling and lymphocyte activation (Figure 1F-H). Altogether, unrestrained gene expression and increased bioenergetic demand within large tumors may stimulate pathophysiological signaling processes associated with anti-inflammation and immunosuppression.

If these processes are clinically represented in patients with high tumor burden, it could signify a translational challenge for ACT. We investigated this possibility using human skin cutaneous melanoma (SKCM) cohort data from The Cancer Gene Atlas (TCGA)

project. A training set composed of stratified patient samples based on primary tumor size (T4 vs T1) was used to elucidate overlaps in enriched Hallmark gene sets from 14- vs 7-day old tumors. We found that T4 tumors enriched several pathways previously observed in 14-day old tumors (positive: 'cholesterol homeostasis', 'glycolysis'; negative: 'KRAS signaling up', 'complement', 'Il2 STAT5 signaling', 'inflammatory response', 'Il6 JAK STAT3 signaling', 'interferon alpha response', 'interferon gamma response', 'allograft rejection') (Figure 1I).

To determine if these enriched pathways could serve as a prognostic signature, single-sample GSEA (ssGSEA) was used to calculate enrichment scores for each sample within the dataset (Figure 1J). Based on the enrichment score and its relative contribution to overall survival per multivariate Cox regression analysis, we developed a prognostic model with the risk score method for survival prediction. The training set was stratified into high- and low-risk groups using the median risk score as a cutoff threshold (Figure 1K). Since the high-risk group had a significantly shorter survival duration compared to the low-risk group (Figure 1L), we confirmed that risk scores derived from our pathway signature had predictive value. While univariate Cox regression indicated that clinical variables 'pathologic stage', 'T stage', 'N stage', and 'risk score' were significantly associated with patient survival, multivariate analysis confirmed that only 'T stage', or tumor stage, was an independent prognostic factor (Table 1). Interestingly, 'risk score' demonstrated the highest hazard ratio (HR = 2.7007) amongst the four prognostic variables. This confirmed our hypothesis that expression of the pathway signature in the context of increased tumor burden contributed to poor clinical outcomes.

Finally, we examined the prevalence of the pathway signature in other tumor cohorts. Using receiver operating characteristic (ROC) curves, we found that risk scores were sensitive and specific in the prediction of tumor burden (T1/T4) in SKCM (AUC = 0.688), head-neck squamous cell carcinoma (HNSC, AUC = 0.709), and breast invasive carcinoma (BRCA, AUC = 0.653) (Figure 1M-O). In each of these studies, T4 patients had a lower survival duration relative to T1 patients. Consequently, large tumor-related pathway changes represent a pervasive clinical issue that should be strategically targeted to improve the efficacy of ACT and other T cell therapies.

MS-275 restores tissue homeostasis and promotes tumor clearance during immunotherapy

Pathway changes observed during tumor progression are often associated with dysregulation of the chromatin landscape which leads to disruption of cellular homeostasis and reconfiguration of the TME (Cheng et al., 2019). This drives local reprogramming of

stromal and immune cell populations to support immunosuppression and resistance to immunotherapy (Pan and Zheng, 2020). By contrast, histone deacetylase inhibitors (HDACi) have been shown to reprogram aberrant regulation of chromatin (Anastas et al., 2019), leading to tumor sensitization and augmentation of antitumor immune responses (Li et al., 2021; McCaw et al., 2019). In this study, we wondered if epigenetic rewriting of the TME using HDACi could promote tissue normalization and prevent shutdown of transferred T cell responses.

We treated 14-day old CMS5 tumors with Class I HDACi MS-275 and harvested tumor RNA for bulk-cell RNA-seq. Interestingly, GSEA of treated vs un-treated 14-day old tumors using MSigDB Hallmark gene sets revealed that the direction of enriched pathways was in complete opposition to that of 14- vs 7-day old tumors (Figure 2A). We validated this observation by analyzing both comparisons ((a) 14- vs 7-day old tumors, (b) treated vs un-treated 14-day old tumors) against the entire database, which comprised the following gene set collections: Positional (C1), Curated (C2), Regulatory target (C3), Computational (C4), Ontology (C5), Oncogenic signature (C6), Immunologic signature (C7), Cell type signature (C8) (Figure 2B). While the number of enriched gene sets was substantially higher in (a) than (b), 77.1% of gene sets in (b) overlapped with (a) with 99.8% opposing enrichment direction (Figure 2C). As a result, MS-275 treatment induced key biological changes that may restore tissue homeostasis within large tumors and improve the outcome of cancer immunotherapy.

Although the treatment of 7-day old CMS5 tumors with ACT+MS-275 did not show additional benefit to tumor regression, survival, and peripheral antitumor immune response (Figure 2D-F), combination therapy completely regressed 14-day old tumors and improved overall survival (Figure 2D, E). Correspondingly, mERK2136–144-specific CD8+ T cell expansion was restored and comparable to ACT in tumor-free and 7-day old tumors (Figure 2F). Altogether, MS-275-dependent rescue of ablated T cell responses improved the resolution of large, highly immunosuppressive, solid tumors.

Using a different tumor model and treatment modality, we were able to recapitulate the effects of both tumor burden and MS-275 on T cell-dependent therapeutic outcomes. 7-day old MC38 murine colon adenocarcinoma tumors that were engineered to overexpress surrogate tumor antigen LCMV GP33-41 (MC38-gp33) were treated with viral vaccine VSVΔM51-gp33 and found to elicit a sufficient endogenous LCMV GP33-41-specific CD8+ T cell response to completely regress tumors and prolong survival (Figure 2G-I). By contrast, 14-day old tumors could not be controlled by VSVΔM51-gp33 treatment, which coincided with a significant reduction in antitumor immune response. Concomitant MS-275 delivery was able to restore both tumor regression and immune response resulting in improved overall survival. These findings suggest that MS-275's effects on large tumors

may be widely applicable across tumor types and antigen-specific T cells, both adoptively transferred and endogenously activated.

T cells demonstrate increased persistence and cytotoxicity in immunosuppressive large tumors

Comprehensive analysis of the events which led to the peripheral disappearance of antitumor T cells gave us insight into the consequences of increased tumor burden on T cell fate. We transduced cultured TCM to express luciferase and monitored their accumulation in 14-day old tumor-bearing mice during ACT treatment by in vivo imaging system (IVIS) (Figure 3A). Over the course of 5 days post vaccination, we clearly observed T cell migration to the tumor, axillary lymph nodes, and inguinal lymph nodes (dorsal view) as well as the spleen and cervical lymph nodes (vental view). Interestingly, ACT treatment was able to elicit early expansion of adoptively transferred T cells which peaked at around day 3-4 (day 4 by tumor flux, Figure 3B); however, by day 5 we were unable to detect a bioluminescent signal, indicating total loss of transferred T cells. Conversely, ACT+MS-275 treatment promoted continuous expansion of adoptively transferred T cells and peak flux at day 5 (Figure 3A, B). Time course analysis of peripheral T cell responses further indicated that tumor-specific CD8+ T cells persisted beyond day 5 and remained detectable for the length of the study (Figure 3C). Therefore, the disappearance of transferred T cells was likely from large tumors truncating the overall T cell response rather than preventing initial T cell expansion.

To investigate the functional changes incurred by adoptively transferred T cells, we conducted multi-tissue analysis at a timepoint prior to their disappearance (day 4.5). Ki67 and annexin V staining of transferred DUC18-Thy1.1 congenic CD8+ T cells allowed us to quantify proliferation and early apoptosis during treatment (Figure 3D, E). Thy1.1+ T cells from 14-day old tumor-bearing mice showed reduced Ki67 expression (blood, tumor) and elevated annexin V expression (tumor) relative to 7-day old tumor-bearing mice. This may suggest that increased tumor burden precipitated T cell dysfunction most severely within the tumor itself while having a disseminated systemic effect. Interestingly, ACT+MS-275 treatment in 14-day old tumors increased Ki67 expression (spleen, blood) compared to ACT alone while having minimal impact on annexin V expression (Figure 3D, E). Since the Ki67 expression level was proportionate to T cell accumulation over time (Figure S1), MS-275 delivery may support the persistence of adoptively transferred T cells by enhancing cellular proliferation.

Although its immune-potentiating capacity was reflected most obviously by increased T cell accumulation, we were curious if MS-275 provided further T cell benefits that could

promote tumor control. We repeated the multi-tissue analysis with P14-Thy1.1 CD8+ T cells/MC38-gp33 tumors and additionally monitored polyfunctional marker expression to assess degranulation and secretion of multiple inflammatory cytokines. While the frequency of degranulation (CD107a+) remained largely unchanged (slightly increased in the tumor), we observed a significant up-regulation of granzyme B (GzB) production (Figure 3F, G). This was accompanied by a slight decrease in interferon γ (IFNγ) and significant down-regulation of tumor necrosis factor α (TNFα) production (Figure 3H, I). To determine the net cytotoxic outcome of these polyfunctional changes, we positively enriched Thy1.1+ cells from bulk splenocytes harvested from treated mice. We then co-cultured them with dye-labeled target cells and measured cell lysis by flow cytometry. At a 0.5:1 effector to target ratio, we observed clear improvement to tumor killing using T cells derived from ACT+MS-275-treated mice (Figure 3J). Overall, MS-275 broadly altered the functional programming of adoptively transferred T cells, which allowed them to persist and demonstrate elevated antitumor activity.

T cell transcriptional changes potentiate activation-dependent signaling

Since MS-275 is an epigenetic agent, we wanted to examine the transcriptional landscape of adoptively transferred CD8+ T cells during combination treatment. Despite its pleiotropic nature, our aim was to deconvolute the global gene changes associated with MS-275 in order to: 1) discover perturbed biological pathways, 2) correlate them with improved T cell function, and 3) identify prognostic markers of MS-275-induced functional reprogramming.

Bulk splenocytes were harvested from ACT±MS-275-treated, 14-day old MC38-gp33 tumor-bearing mice and flow sorted for Thy1.1+ P14-Thy1.1 cells. T cell RNA was extracted for microarray-based whole transcriptome profiling, whereby PCA revealed that T cells acquired a distinct transcriptomic signature after MS-275 delivery (Figure 4A). GSEA with MSigDB Hallmark gene sets indicated that ACT+MS-275 samples positively enriched pathways related to cell cycle progression, cellular metabolism, and immune response signaling and negatively enriched pathways related to inflammatory cytokine production (Figure 4B). This reinforced our earlier observations that ACT+MS-275 treatment enhanced cellular proliferation and down-regulated IFNγ and TNFα production.

Cellular processes are often controlled by interacting proteins that are frequently co-regulated or co-expressed across conditions. Detecting groups of co-expressed genes allow for the identification of novel prognostic markers. To identify markers corresponding to MS-275's effects, we attempted to reproduce our pathway enrichment results using gene co-expression modules. We utilized Co-Expression Module identification Tool

(CEMiTool) analysis for unsupervised dimensionality reduction of the gene expression data to create six modules (M1-M6) of highly correlated genes. Despite the subtle change in median gene activity within each module (Figure 4C), GSEA with M1-M6 gene lists showed positive enrichment of M2 and M3 and negative enrichment of M4 and M6 during ACT+MS-275 treatment (Figure 4D). Of the four gene modules, only M2 showed an overlap in enriched Hallmark pathways (Figure S2). Therefore, it was the module of interest for additional functional characterization and prognostic marker identification.

The co-expressed genes in M2 were submitted as a list for GO-based network construction (Figure 4E). Functional clustering revealed that the majority of overrepresented GO terms associated with M2 were primarily associated with 'lymphocyte activation' (Cluster 12, 30.09%) and 'regulation of cell activation' (Cluster 13, 31.86%) (Figure 4F) and included relevant biological processes such as 'positive regulation of lymphocyte activation' (GO:0051251), 'positive regulation of lymphocyte proliferation' (GO:0050671), 'positive regulation of immune system process' (GO:0002684), 'positive regulation of immune response' (GO:0050778), and 'positive regulation of reactive oxygen species metabolic process' (GO:2000379) (Table S1). Since M2 was positively enriched in ACT+MS-275-treated samples, Hallmark GSEA and GO collectively suggested that improvements to T cell functionality were largely driven by enhanced cellular activation.

Hub genes are defined by high module membership and intermodular connectivity, and are often investigated as important prognostic markers (Zhou et al., 2018). We utilized CEMiTool to identify key hub genes within M2 (HAVCR2, PDE8A, MR1, SKAP2, DUSP22), and visualized them in a protein-protein interaction (PPI) network (Figure 4G). Additionally, interactome data integration through GeneMania allowed us to identify several proteins (TNF, CDK1, BRCA1, ASF1B, RRM2, IFI30, CCNB2, PTTG1, BIRC5, CCNA2) with high annotated physical interactivity within M2. All hub genes were highly up-regulated during ACT+MS-275 treatment; in particular, HAVCR2 was one of the top differentially expressed genes in the study (6.754207-fold change) (Figure 4H, S3). Therefore, we reasoned that HAVCR2 and other hub genes comprised a unique gene signature that was conducive to T cell activation.

Gene correlation provides a means by which the roles of poorly characterized proteins can be inferred from better-annotated counterparts. We were curious if individual hub genes participated in any of the aforementioned pathways that potentiated T cell activation during MS-275 delivery. To address this question, we used ssGSEA to calculate enrichment scores for each sample from the Hallmark GSEA analysis (Figure 4I). Using ACT+MS-275 samples, we then constructed a hierarchically-clustered pairwise correlation matrix by combining hub gene expression values with ssGSEA enrichment scores (Figure 4J). After filtering for immunologically relevant pathways, hub genes showed an association with

'IL2 STAT5 signaling', 'MYC targets V1', 'oxidative phosphorylation', and 'MTORC1 signaling'. In particular, DUSP22 showed weak association with 'MYC targets V1' (r=0.4053, p=0.4984) and 'oxidative phosphorylation' (r=0.3011, p=0.6225) (Figure S4), while HAVCR2 showed moderate association with 'MYC targets V1' (r=0.5464, p=0.3407), 'oxidative phosphorylation' (r=0.5202, p=0.3688), and 'MTORC1 signaling' (r=0.4926, p=0.3992). More importantly, HAVCR2 demonstrated very strong and statistically significant association with 'IL2 STAT5 signaling' (r=0.9524, p=0.0124) (Figure 4K). Thus, HAVCR2 expression may have a role within the IL2/STAT5 signaling axis that contributed to improved T cell activation.

Uncoupling Tim-3 expression from cellular exhaustion is associated with terminal effector lineage differentiation

HAVCR2/Tim-3 is a member of the T-cell immunoglobulin and mucin-domain (TIM) family and has been widely investigated for its involvement in regulating immune responses within autoimmunity and cancer (Ferris et al., 2014). Primarily viewed as checkpoint or co-inhibitory receptor, Tim-3 is often reported in the context of T cell dysfunction or exhaustion (Wolf et al., 2020). However, accumulating evidence suggests it may also have a co-stimulatory role (Avery et al., 2018; Kataoka et al., 2021; Lee et al., 2011). Since our data associated Tim-3 with T cell activation, we wanted to affirm its expression level in the context of increased tumor burden/MS-275 treatment and reconcile its function.

Using DUC18-Thy1.1 CD8+ T cells for adoptive transfer, we treated 7- or 14-day old CMS5 tumors with ACT±MS-275 and conducted multi-tissue analysis 4.5 days post vaccination. We stained for the expression of Tim-3 as well as PD-1, another exhaustion marker/checkpoint receptor. Thy1.1+ T cells from ACT-treated 14-day old tumor-bearing mice showed significantly elevated Tim-3 expression (blood, tumor) and PD-1 expression (blood, tumor) relative to 7-day old tumor-bearing mice (Figure 5A, B). This was congruent with the typical T cell exhaustion phenotype, and was reinforced by aforementioned observations of reduced proliferation (blood, tumor). Surprisingly, ACT+MS-275 treatment of 14-day old tumors increased Tim-3 expression further (spleen, blood) compared to ACT alone despite a reduction of PD-1 expression in the spleen. Since MS-275 delivery coincided with enhanced proliferation, Tim-3 expression may have been uncoupled from cellular exhaustion. For further validation, we repeated the experiment and additionally stained for TOX, a potent transcriptional inducer of T cell exhaustion (Khan et al., 2019). In 14-day old tumor-bearing mice, we found no significant change to TOX expression in the spleen, blood, or tumor (Figure 5C). Finally, ssGSEA using a compiled

list of exhausted T cell (TEX) markers allowed us to correlate HAVCR2 expression with TEX gene signature enrichment. There was a strong association in ACT-treated T cells (r=0.9123, p=0.0308) but none whatsoever in ACT+MS-275-treated T cells (r=-0.09578, p=0.8782) (Figure 5D). Instead, MS-275 may correspond with activation-dependent changes that influence T cell state.

To better define enhanced cellular activation in the context of T cell state, we utilized GSEA to compare ACT+MS-275- vs ACT-treated T cells using gene lists of known T cell lineages including exhausted (TEX), effector (TEFF), memory (TMEM), memory precursor effector cell (TMPEC), short lived effector cell (TSLEC), and effector memory cells re-expressing CD45A (TEMRA) (Table S2). ACT+MS-275 showed negative enrichment of TEX and TMPEC signatures and positive enrichment of TSLEC and TEMRA signatures (Figure 5E). Heightened activation may therefore have skewed T cell programming away from precursory memory and exhaustion and drove key features of terminal effector differentiation.

In 14-day old MC38-gp33 tumor-bearing mice, ACT±MS-275 treatment with P14-Thy1.1 CD8+ T cells allowed us to stain splenic Thy1.1+ cells with a panel of T cell lineage differentiation markers. Following viability, singlet, and exclusion gating as well as artifact removal, a single concatenated sample was created by combining down-sampled events from both ACT and ACT+MS-275 groups. Unsupervised cell clustering was performed with an integrated analysis method using t-stochastic neighbor embedding (t-SNE) and FlowSOM algorithms. Metaclustering revealed extensive heterogeneity in post vaccinated T cells consisting of six major populations (Figure 5F) that underwent significant recomposition during MS-275 co-delivery (Figure 5G). Surface marker staining intensity on t-SNE dimension-reduced space showed differential expression patterns that aligned with the territories inhabited by metaclusters (Figure 5H). Unsurprisingly, T cells from ACT+MS-275 treatment were primarily characterized by very high Tim-3 staining intensity as indicated by heatmap hierarchical clustering (Figure 5I). Indeed, T cell phenotype assignment revealed that MS-275 replaced effector-like (Tim-3+ CD62L-TCF1- CD127-) and memory-like (Tim-3+ CD62L- TCF1lo CD127+) cells with Tim-3hi effector-like (Tim-3hi CD62L- TCF1- CD127-) and Tim-3hi, TCF1int memory-like (Tim-3hi CD62L- TCF1int CD127+) cells (Figure 5J). Importantly, 68.42% of adoptively transferred T cells were Tim-3hi effector-like cells (TTEC), suggesting that combination treatment facilitated a much more polarized antitumor response.

TSLEC signature enrichment observed during ACT+MS-275 treatment prompted us to explore KLRG1 expression (a defining marker of TSLEC (Herndler-Brandstetter et al., 2018)) in effector-like T cells harvested from multiple tissues post vaccination. Since KLRG1 expression has been shown to be negatively correlated with cellular exhaustion

(Herndler-Brandstetter et al., 2018; Khan et al., 2019; Wherry and Kurachi, 2015), we were interested whether TTEC displayed elevated KLRG1 during ACT+MS-275 treatment compared to ACT alone. Indeed, after gating on CD8+ Thy1.1+ CD44+ Tim-3+ TCF1- CD62L- CD127- cells (Figure 5K), we observed a systemic increase in KLRG1 expression (spleen, blood, tumor) (Figure 5L). As a result, this phenotypic overlap further suggested that TTEC-restricted Tim-3 expression was uninvolved with exhaustion.

Discussion

Despite the unprecedented success of adoptive cell therapy for treating hematological malignances, there are substantial challenges in developing the platform for solid tumors. Improved understanding of the parameters influencing T cell inflammation allowed us to implement a treatment protocol that could durably regress established tumors. However, when we increased the initial tumor burden, we observed a systemic loss of antitumor immunity, enhanced cellular exhaustion, and resistance to immunotherapy. This was associated with tumor microenvironmental (TME) changes that were clinically pervasive in patients with large tumors and were predictive for worse survival outcomes. In this study, we found that histone deacetylase inhibition in conjunction with T cell therapy normalized large tumors to restore immune responses and therapeutic efficacy. This was consistent across multiple tumors and therapeutic modalities. Antitumor T cells underwent transcriptome reprogramming to enhance biological processes associated with cellular activation, which corresponded with improved proliferation and cytotoxicity. Surprisingly, we found that Tim-3 expression was markedly up-regulated but was not functionally correlated with exhaustion; rather, Tim-3hi cells shared genomic and phenotypic features of non-exhausted, terminally differentiated effector lineages. Overall, our findings reveal a novel sensitization approach for effective resolution of highly immunosuppressive large tumors.

Solid tumors are uniquely difficult to treat due to a complexity of multifactorial resistance mechanisms that are further compounded by increased tumor burden. In this study, we determined that large tumors are often driven by an accumulation of key biological pathway changes that predispose them to worse therapeutic responsiveness. In our evaluation of tumor cohort studies, patients with increased primary tumor burden were prone to these changes, which were negative prognostic factors for overall survival. Due to the prevalence of this pathway signature in multiple tumor types, the translational potential of ACT in the treatment of solid tumors may therefore hinge on the subversion of these biological processes. Fortunately, these pathways are well-studied; for instance, cholesterol accumulation, glucose and amino acid deprivation, and hypoxia are known inducers of local T cell hyporesponsiveness (Chang et al., 2015; Franco et al., 2020; Hung et al., 2021; Ma et al., 2019; Scharping et al., 2021). However, concurrent up-regulation of these processes may also facilitate systemic immunosuppressive outcomes. The systemic immune landscape during tumor development has been shown to be quite plastic (Allen et al., 2020), and thus, the prolific dissemination of immunosuppressive factors arising from pathway signature expression may impair the quality of peripheral T cell activation. In our models, we demonstrated that therapy-driven T cell expansion was rapidly ablated in large tumors, resulting in minimal T cell persistence and loss of tumor control. Ultimately, our

data suggest that large tumors worsen the dysfunctional outcomes of cellular exhaustion to enhance the severity of therapeutic resistance.

Despite this, combinatorial approaches may be capable of improving clinical outcomes in large, established tumors. One notable precedent showed that maximal antitumor efficacy could be achieved using a four-component treatment platform consisting of a tumor-targeting antibody, recombinant IL-2, PD-1-blocking antibody, and adjuvanted peptide vaccine (Moynihan et al., 2016). Therapeutic resistance can thus be overwhelmed by effective mobilization of innate and adaptive immune cells to promote broad spectrum antitumor immunity. The authors also demonstrated that concurrent targeting of tumor immunosuppression may be a necessary prerequisite for eradicating large tumors. However, our findings suggested that increased tumor burden was associated with numerous TME pathway changes that can independently promote immunosuppression. As a result, incorporating therapeutic components to target each pathway may not be logistically feasible. By contrast, epigenetic agents have emerged as an increasingly viable combinatorial option for their ability to reprogram the tumor transcriptional landscape to simultaneously affect multiple pathways of therapeutic resistance. In particular, histone deacetylase (HDAC) inhibitors are FDA approved as anti-cancer drugs but also have broad immunopotentiating effects. This includes reducing regulatory T cells, tumor-associated macrophages, and myeloid derived suppressor cells, remodeling the tumor inflammatory landscape, and promoting cross-presentation of tumor antigens (Briere et al., 2018; Li et al., 2021; Ritter et al., 2017; Shen and Pili, 2012; Truong et al., 2021). In our study, we investigated Class I HDAC inhibitor, MS-275 (Entinostat), and found that treatment of large tumors reversed most pathway changes caused by increased burden. In particular, we observed that correction of metabolic, MYC, and MTORC1 signaling was accompanied by restored inflammatory responses. Since MYC and MTORC1 are known regulators of cellular metabolism and have been linked to tumor-driven exhaustion programming (Kim et al., 2017; Topper et al., 2017), MS-275-dependent tumor normalization may prevent T cell exhaustion. Indeed, combination immunotherapy with MS-275 restored T cell fitness and therapeutic efficacy in large tumors.

In addition to tumor normalization, we provide indirect evidence to suggest that MS-275 overwrites the distinct epigenetic features of exhausted T cells to promote effector function. This may address a known issue with exhausted T cells in that intrinsic epigenetic fate inflexibility can limit the durability of reinvigoration strategies such as PD-1 blockade (Ghoneim et al., 2017; Pauken et al., 2016). T cell exhaustion is commonly defined by multiple inhibitory receptor expression (PD1, Tim-3, Lag3, CTLA4, TIGIT) (Blackburn et al., 2009), and in our study, we demonstrated that PD-1 and Tim-3 was up-regulated in transferred T cells in 14-day old tumors compared to 7-day old tumors. However, MS-275

delivery did not reduce Tim-3 expression in 14-day old tumors; rather, Tim-3 was further up-regulated and became correlated with cellular activation rather than exhaustion. Therefore, MS-275's role may not just be corrective, but potentiating as well.

Interestingly, the uncoupling of Tim-3 expression from genomic and functional signatures of cellular exhaustion may suggest that, in certain contexts, Tim-3 may not be a reliable exhaustion marker. Since Tim-3 does not inherently express an inhibitory signaling motif (Wolf et al., 2020), its regulatory role is often conditional. Binding to specific ligands like galectin 9 (Zhu et al., 2005) or co-expression with other regulatory molecules (PD1, CEACAM1) (Huang et al., 2015; Jin et al., 2010; Sakuishi et al., 2010) has been shown to confer inhibitory function. However, the ectopic expression of Tim-3 has been associated with recruitment to the immune synapse and enhanced TCR signaling in a phosphotyrosine-dependent manner (Kataoka et al., 2021; Lee et al., 2011). In our study, MS-275 selectively up-regulated Tim-3 and decorrelated its expression from PD1. Thus, it is possible that combination treatment allowed Tim-3 to exercise its co-stimulatory role to improve the quality of T cell activation and promote a divergent cellular fate. Indeed, Tim-3 expression was strongly associated with the IL-2/STAT5 signaling axis, suggesting its potential involvement in post-activation metabolic reprogramming, proliferation induction, and lineage commitment.

Exhausted T cells exist on a phenotypic continuum like many other differentiation states (Philip et al., 2017). In our study, post-vaccinated antitumor T cells derived from large tumors displayed surface marker heterogeneity while collectively enriching for gene signatures consistent with TEX and TMPEC. These include effector-like Tim3+ TCF1- CD127- cells and memory-like Tim3+ TCF1lo CD127+ cells, which may represent different stages of cellular exhaustion such as terminally exhausted and progenitor exhausted T cells, respectively (Kallies et al., 2020; Siddiqui et al., 2019; Wu et al., 2016). Interestingly, both populations deteriorated over time, which affirmed their dysfunction within large tumors. During MS-275 treatment, T cell heterogeneity was reconciled into a polarized effector response whereby TTEC (Tim3hi TCF1- CD127- KLRG1+) was the predominant T cell population and displayed superior post vaccination proliferation and killing. Interestingly, TTEC showed gene signature enrichment for TSLEC and TEMRA, the former of which is a differentiation outcome that has been previously associated with Tim-3 co-stimulation (Avery et al., 2018). We also observed a minor T cell population with memory-like characteristics (TTMC, Tim-3hi TCF1int CD127+). Unlike memory-like Tim3+ TCF1lo CD127+ cells, TTMC displayed increased proliferation in association with higher Tim-3 and TCF1 expression. Similar to how others have associated the success of immune checkpoint blockade with the proliferation of stem-like tumor-infiltrating lymphocyte subsets (Kallies et al., 2020; Siddiqui et al., 2019), TTMC may represent a

stem-like, self-renewing population that can persist and repopulate TTEC to prolong antitumor immune responses. Ultimately, modulation of the epigenetic landscape may mediate conversion between exhaustion and effector/memory T cell states.

Acknowledgements

Author Contributions

A.N. designed and performed the majority of the experiments and wrote the manuscript. D.B. and R.K. performed animal experiments. S.R.W. constructed the viral vectors used in the study. D.B. transduced cultured TCM to express luciferase. D.L. performed the cytotoxicity assay. L.C. assisted with in vitro TCM culture and tissue processing. J.L.B. provided guidance during manuscript preparation. Y.W. supervised the study and participated in the conception of experimental designs and manuscript writing.

Declaration of Interests

The authors declare no competing interests.

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Main Figure Titles and Legends

Figure 1: Tumor burden potentiates immunotherapeutic resistance and decreased clinical outcomes through the expression of a unique pathway signature

BALB/c mice were implanted i.d. with CMS5 tumor cells and allowed to grow for 7 or 14 days. Mice were treated with adoptive cell therapy (ACT) consisting of transferred TCM¬-cultured DUC18 T cells and vaccination with VSVΔM51-mERK2136–144.

(A) Tumor volume in 7-day or 14-day tumors left un-treated or treated with ACT.

(B) Fraction of mice that did not reach endpoint (decreased body condition). No palpable tumors were observed in surviving mice.

(C, D) Frequency of mERK2136–144-specific CD8+ T cells as determined by ex vivo peptide stimulation and IFNγ staining 5 days after vaccination. (D) 14-day tumors were surgically removed 24 hours prior to ACT.

(E) PCA was performed on TMM-normalized counts for bulk tumor RNA-seq samples derived from un-treated tumors.

(F-H) GSEA showing enriched pathways in 7-day (negative enrichment score) or 14-day tumors (positive enrichment score). Analyzed using the MSigDB (F) Hallmark, (G) Curated (C2), and (H) Ontology (C5) gene set collections. (G, H) Enrichment Map and AutoAnnotate were used to identify clusters of enriched gene sets.

(I) Using the MSigDB Hallmark gene set collection, GSEA was used to enrich pathways in SKCM patients stratified by tumor size/extent (T1, negative enrichment score; T4, positive enrichment score). Emphasized pathways were also enriched in the 14-day vs 7-day tumor comparison.

(J) Hierarchically-clustered heatmap of single-sample GSEA (ssGSEA)-determined enrichment scores for each patient in the T1/T4 training set using the commonly enriched pathways, forming a pathway signature.

(K) Multivariate Cox regression analysis was used to create risk scores for survival prediction. The distribution of risk scores within the T1/T4 training set allowed for patient stratification into low- and high-risk groups based on the median value.

(L) Kaplan-Meier curves of overall survival of the low- and high-risk groups.

(M-O) ROC curve for tumor burden prediction by pathway signature and corresponding Kaplan-Meier curve of overall survival based on tumor burden (T1/T4). (M) SKCM as well

as (N, O) two additional representative TCGA tumor types HNSC and BRCA were analyzed.

Data in (A-C) are representative of at least 3 experiments with n = 5 mice per group. Data in (D) was obtained with n = 5 mice per group. Data in (E-H) utilized n = 3 mice per group and statistical significance was defined at (F, I) FDR < 0.05 or (G, H) FDR < 0.001. Error bars are SEM and statistics were based on log rank test, unpaired T-test, or one-way ANOVA test.

Figure 2: MS275 promotes tissue homeostasis and in conjunction with ACT restores therapeutic efficacy in large, immunosuppressive tumors

(A-C) Bulk tumor RNA-seq samples were derived from MS-275-treated 14-day tumors. (A) Using the MSigDB Hallmark gene set collection, GSEA was used to enrich pathways in un-treated (negative enrichment score) or MS-275-treated tumors (positive enrichment score). Corresponding pathways in the 14-day vs 7-day tumor comparison were enriched in the opposing direction.

(B) Pie charts illustrating total enriched gene sets from the complete MSigDB collection.

(C) Venn diagram showing the number of overlapping enriched gene sets. Pie charts were used to show the direction of enrichment for overlapped gene sets.

(D-F) BALB/c mice were implanted i.d. with CMS5 tumor cells and allowed to grow for 7 or 14 days. Mice were treated with ACT with or without concomitant MS-275 delivery. (D) Tumor volume and (E) overall survival curves were plotted. (F) Frequency of mERK2136–144-specific CD8+ T cells with tumor-free controls.

(G-I) C57BL/6 mice were implanted i.d. with MC38-gp33 tumor cells and treated with VSVΔM51-gp33 with or without MS-275 delivery. VSVΔM51-GFP was used as a vector control. (D) Tumor volume, (E) overall survival, and (F) frequency of LCMV GP33-41-specific CD8+ T cells.

Data in (A-C) utilized n = 3 mice per group and statistical significance was defined at (A) FDR < 0.05 or (B, C) FDR < 0.001. Data in (D-I) are representative of at least 2 experiments with n = 5 mice per group. Error bars are SEM and statistics were based on log rank test or one-way ANOVA test.

Figure 3: T cell reinvigoration is associated with increased proliferation and cytotoxicity

(A-B) TCM¬-cultured DUC18 T cells were transduced to express luciferase prior to ACT and monitored post-vaccination by in vivo imaging. (A) Representative images were selected to illustrate T cell accumulation and persistence for each treatment group. (B) Selective gating of the tumor region for quantitation of local T cell inflammation.

(C) Frequency of mERK2136–144-specific CD8+ T cells over time.

(D-J) Transferred Thy1.1+ congenic TCM were analyzed 4.5 days post-vaccination and stained for markers of (D, E) proliferation, cell death, and (J-I) polyfunctionality. Fluorescence intensity was determined by geometric mean. (J) Thy1.1+ cells were positively enriched from bulk splenocytes and co-cultured with dye-labeled target cells. Percent lysis was determined relative to 'target-only' controls.

Data in (A, B) are representative of at least 2 experiments with n = 3 mice per group. Data in (C) was obtained with n = 5 mice per group. Data in (D-J) are representative of at least 2 experiments with n = 4-5 per group. Error bars are SEM and statistics were based on unpaired T-test or one-way ANOVA test.

Figure 4: T cell whole transcriptome analysis reveals enhanced activation-dependent signaling

Transferred T cells were flow sorted from bulk splenocytes in 14-day MC38-gp33 tumor-bearing mice 4.5 days post-vaccination. RNA samples were analyzed by Clariom D microarray.

(A) PCA was performed on SST-RMA-normalized signal values.

(B) Using the MSigDB Hallmark gene set collection, GSEA was used to enrich pathways in ACT-treated (negative enrichment score) or ACT+MS-275-treated tumors (positive enrichment score).

(C) CEMiTool was used to perform weighted gene-centric network analysis and identify modules of co-expressed genes. Graphs demonstrate median gene expression per module across treatment groups.

(D) Using the module gene sets, GSEA was used to enrich pathways in ACT-treated (negative enrichment score) or ACT+MS-275-treated tumors (positive enrichment score).

(E, F) ClueGO was used to perform Gene Ontology Biological Processes (GOBP) pathway analysis for the M2 module. (E) Overrepresented pathways were functionally clustered. (F) Pie chart detailing the percentage of overrepresented pathways per cluster.

(G) CEMiTool-defined hub genes visualized with other M2 genes in a protein-protein interaction (PPI) network. Additional genes were identified based on the degree of physical interactivity within the module.

(H) Fold change in hub gene expression when comparing ACT+MS-275- to ACT-treated T cells.

(I) Hierarchially-clustered heatmap of ssGSEA-determined enrichment scores using the MSigDB Hallmark gene set collection.

(J) Hierarchical clustering matrix of pairwise correlated pathway enrichment scores and hub gene expression values derived from the ACT+MS-275 sample group. A black square was used to represent a correlated cluster of hub genes and pathways. A white square was used to a indicate high gene-pathway correlation involving HAVCR2.

(K) Scatter plots showing correlation between Hallmark pathways and HAVCR2 gene expression.

Data in Figure 4 was obtained with n = 5 mice per group. Statistical significance in (B, D) was defined at FDR < 0.05. All pathways in (E, F) are at least $p < 0.05$.

Figure 5: Tim-3 up-regulation is associated with reduced exhaustion and polarization towards terminal effector lineage differentiation

(A-C) Transferred Thy1.1+ congenic TCM were analyzed 4.5 days post-vaccination and stained for typical markers of exhaustion. Fluorescence intensity was determined by (A, B) geometric mean or (C) median.

(D) Scatter plot showing correlation between T cell exhaustion signature and HAVCR2 gene expression for ACT or ACT+MS-275 treatment.

(E) GSEA plots of enriched T cell signatures in ACT+MS-275- vs ACT-treated T cells.

(F-J) tSNE visualization and flowSOM clustering of transferred T cells was created by subsampling and concatenating events from all ACT±MS-275-treated samples. (F) FlowSOM clusters were identified and colored. (G) Visualization separated by treatment group. Protein expression levels of lineage differentiation markers were (G) overlaid onto the tSNE map and (H) used by flowSOM to create hierarchically clustered populations with unique phenotypes. (J) Frequencies of flowSOM populations in each treatment group.

(G) Flow cytometry gating strategy to identify effector-like T cells in ACT±MS-275-treated samples.

(H) Frequency of KLRG1+ cells within effector-like T cell populations.

Data in (A-C) are representative of at least 2 experiments with n = 4-5 mice per group. Data in (D-L) was obtained with n = 5 mice per group and statistical significance in (E) was defined at FDR < 0.05. Error bars are SEM and statistics were based on unpaired T-test or one-way ANOVA test.

Main Table Titles and Legends

Table 1: Cox regression analysis of prognostic factors for SKCM T1/T4 patients

STAR Methods

Study design

The overall objective of the study was to determine how epigenetic modification during adoptive T-cell therapy can reinvigorate exhausted T cell responses in large, immunosuppressive tumors. The in vivo experiments were used to examine the immunosuppressive effect of increased tumor burden, the therapeutic benefit of histone deacetylase inhibitor delivery, and the functional improvements to antitumor T cells. In vivo studies were performed with female, age-matched (6-8 weeks old) mice. Tumor-challenged mice were randomized prior to blinded treatments. Tumor growth was monitored and measured with calipers. Tumor volume was calculated as the width times the length times the depth. Mice were monitored for signs of distress and humane endpoints were determined by decreased body condition. Veterinary staff monitored mice daily and alerted researchers when a humane endpoint had been reached. All animal studies complied with the Canadian Council on Animal Care guidelines and were approved by McMaster University's Animal Research Ethics Board.

Animals

C57BL/6 and BALB/C mice were purchased from Charles River Laboratories and B6.Cg-Tcratm1Mom Tg(TcrLCMV)327Sdz (P14) mice were purchased from Taconic. DUC18 mice were provided by Lyse Norian (University of Iowa, Iowa City, Iowa, USA) (Hanson et al., 2000). Mice were housed in a pathogen-free room in the Central Animal Facility at McMaster University.

Viral vectors

Recombinant VSVΔM51 was engineered to express an H-2Kd–restricted epitope corresponding to amino acids 136–144 of a mutated ERK2 protein (mERK2136–144), denoted as VSVΔM51-mERK2 (Walsh et al., 2019). VSVΔM51-gp33 expresses the dominant CD8+ and CD4+ T cell epitopes of the lymphocytic choriomeningitis virus glycoprotein (LCMV GP33–41 and LCMV GP61–80, respectively) in a minigene cassette (Zhang et al., 2013).

Peptides

Peptides for mERK2136–144 (QYIHSANVL) and LCMV GP33–41 (KAVYNFATM) were purchased from Biomer Technologies and dissolved in PBS supplemented with 0.5% BSA.

Cell lines and tumor challenge

All cells were maintained at 37°C in a humidified atmosphere with 5% CO2. CMS5 (a gift from Lyse Norian) (Ikeda et al., 1997), were cultured in DMEM supplemented with 10% FBS, penicillin/streptomycin (100 U/mL and 100 ng/mL, respectively), and 2 mM l-glutamine (Thermo Fisher Scientific). MC38-gp33 cells (MC38 cells stably transfected with a minigene corresponding to the GP33–41 peptide) (Prevost-Blondel et al., 1998) were maintained in MEM/F11 containing 10% FBS, 2 mM l-glutamine, 5 ml sodium pyruvate, 5 mL nonessential amino acids, 5 mL vitamin solution (Thermo Fisher Scientific), 55 μM 2-mercaptoethanol (Sigma-Aldrich), 100 U/mL penicillin, and 100 ng/ml streptomycin.

Tumor cells were washed twice with PBS and re-suspended in PBS at a concentration of 106 cells/30 μL for CMS5 cells or 105 cells/30 μL for MC38-gp33 cells. Mice were challenged via i.d. injection, and tumors were allowed to grow to a mean volume of ≤150 mm3 (7-day old) or ≥600 mm3 (14-day old) prior to treatment.

Surgical resection of tumors

Primary tumors were surgically resected as described (Piranlioglu et al., 2019). Using a scalpel, an incision was made around the tumor mass, which was then carefully removed. Arteries supplying the tumor were cauterized and skin flaps were sutured by metal clips.

In vitro TCM differentiation

Bulk splenocytes from TCR transgenic mice were isolated and cultured for 7 days in RPMI 1640 (GIBCO) supplemented with 10% FBS, penicillin-streptomycin (100 U/ml and 100 ng/ml, respectively), l-glutamine (2 mM), and 2-mercaptoethanol (55 μM). Splenocytes were stimulated with 100 ng/ml mERK2136–144 or LCMV GP33–41 peptide (Biomer Technologies) in the presence of 10 ng/ml IL-15, 10 ng/ml IL-21 (BioLegend), and 20 ng/ml rapamycin (Sigma-Aldrich).

Luciferase transduction

Retrovirus encoding Luciferase (RV2011oFL) (Rabinovich et al., 2008) was prepared and concentrated as previously described (Hammill et al., 2015) and supplemented with 20ug/mL lipofectamine 2000 (Invitrogen) and 16ug/mL polybrene (ABM) for transduction. DUC18 splenocytes were resuspended at 2 x 10⁶/mL in T cell culture media lacking cytokine and inhibitor additives and transduced with RV2011oFL via spinfection before incubation at 37°C for 2 hours. Cells were then utilized for TCM culture. In vivo monitoring of transferred cells was conducted using an IVIS Spectrum In Vivo Imaging System (Caliper Life Sciences) following intraperitoneal injection of 75 mg/kg D-luciferin (Caliper Life Sciences).

Adoptive cell therapy

In vitro-differentiated TCR transgenic CD8+ TCM were injected i.v. into mice at a dose of 10^6 cells/200 μL of PBS. After 24 hours, mice were treated i.v. with VSVΔM51-mERK2 or VSVΔM51-gp33 at 2×10^8 pfu. Concomitantly, MS-275 (Sigma-Aldrich) was injected i.p. (100 μg/mouse in 50 μL PBS) daily for 5 days.

Tumor digestion

For RNA extraction: Tumors were excised, snap-frozen in liquid nitrogen, and homogenized in Trizol (Invitrogen). RNA was extracted and purified using a RNeasy mini kit (QIAGEN).

For T cell studies: Tumors were excised and digested in 0.5 mg/mL collagenase type IV (GIBCO), 0.2 mg/mL DNase (Roche), and 5 mM calcium chloride prepared in RPMI-1640 (GIBCO) supplemented with 10% FBS, penicillin-streptomycin (100 U/ml and 100 ng/ml) and incubated at 37°C with 200 rpm agitation for 30 min on a temperature-controlled orbital shaker. Digestion was neutralized with two volumes of cold RPMI-1640 with 10% FBS and 2 mM ethylenediamine tetraacetic acid (EDTA), and refrigerated for 10 min at 4 °C. Samples were filtered with a 40 μm strainer, centrifuged ($500 \times g$, 4°C), and re-suspended in PBS with 2% FBS and 1 mM EDTA.

Spleen and blood processing

Harvested spleens were mechanically dissociated with a syringe plunger and treated with ACK lysis buffer (GIBCO) to remove red blood cells. Blood samples were similarly treated

with ACK lysis buffer to extract PBMCs. Cells were re-suspended in PBS with 2% FBS and 1 mM EDTA.

T cell enrichment

For RNA extraction: Re-suspended cells were stained for CD8, Thy1.1, and viability (see below). Stained cells were centrifuged (500 × g, 4°C) and re-suspended in cold sorting buffer (PBS with 1% w/v BSA, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1 mM EDTA). Cell sorting was conducted as previously described (Nguyen et al., 2021). RNA was extracted and purified using a RNeasy mini kit (QIAGEN).

For T cell studies: Re-suspended cells were positively enriched for Thy1.1+ cells using an EasySep Mouse Thy1.1+ T Cell Isolation Kit (STEMCELL Technologies).

Surface and intracellular staining of T cells

For surface staining, cells were treated with Fc Block solution (purified rat anti-mouse CD16/CD32, BD Biosciences) and stained for surface markers followed by viability staining. For detection of cytokine production, cells were stimulated with 1 μg/ml mERK2136–144 or LCMV GP33–41 peptide (Biomer Technologies) in culture at 37°C for 4 hours. Brefeldin A (GolgiPlug, BD Biosciences; 1 μg/mL) was added for the last 3 hours of incubation. Cells were Fc blocked, surface stained, and viability stained before fixation and permeablization (Cytofix/Cytoperm, BD Biosciences), followed by intracellular staining.

Staining fluorescence was detected using a BD LSRFortessa or LSR II flow cytometer (BD Biosciences). Data were analyzed using FlowJo (version 10) flow cytometry analysis software (Tree Star). Sample data was pre-gated, downsampled (7500 events per sample), and concatenated in FlowJo in preparation for tSNE/flowSOM visualization. tSNE dimensionality reduction (Van der Maaten and Hinton, 2008) was calculated with default parameters: perplexity = 30, max iterations = 1000, learning rate (eta) = 4378, theta = 0.5 using channels corresponding to CD62L, TCFF1, CD127, and Tim3. Resulting data was clustered using the integrated FlowSOM plugin (Van Gassen et al., 2015) with the aforementioned channels and set for automatically generated number of metaclusters.

Cytotoxicity assay

MC38-gp33 cells were labeled with 5 µM CFSE (MilliporeSigma), seeded in a 96-well plate at 105 cells per well, and co-cultured for 6 hours with splenocyte-derived Thy1.1-enriched T cells from treated mice at a 0.5:1 effector to target ratio. Cells were stained with eBioscience Fixable Viability Dye eFluor 780 (Thermo Fisher), and staining was evaluated by flow cytometry (see above). The percentage of specific lysis was calculated using the following equation: % specific lysis = 100 × ((% specific cell death - % basal cell death)/(100 - % basal cell death)), where specific cell death is determined from viability dye staining of CFSE-positive cells in T cell co-culture wells and basal cell death from wells lacking co-cultured T cells.

RNA-seq analysis

Bulk tumor RNA was extracted (see above) and mRNA was enriched using the NEBNext Poly(A) mRNA Isolation Module (New England Biolabs) where each sample had an RNA integrity number (RIN) score of >7. A NEBNext Ultra II Directional RNA Library kit (New England Biolabs) was used for RNA-seq library construction. Samples were sequenced on the Illumina HiSeq 2500 with the Illumina HiSeq Rapid v2 kit using a single-read 1x50 bp configuration. Gene counts were normalized using trimmed means of M-values (TMM) and converted to log2 counts per million with the Limma-Voom package (Law et al., 2014). The normalized counts were used for subsequent analyses.

Transcriptome microarray analysis

RNA was extracted from flow sorted CD8+ Thy1.1+ T cells (see above) and utilized for gene expression analysis by Clariom D array (Applied Biosystems). Sample preparation for microarray hybridization was conducted according to the GeneChip WT Pico Reagent Kit User Manual (Applied Biosystems). Samples were run on the GeneChip Scanner 3000 instrument system (Applied Biosystems). CEL files were analyzed on the Transcriptome Analysis Console (TAC 4.0) and probe set signals were SST-RMA-normalized prior to further analysis.

Gene set enrichment analysis

Utilizing GSEA4.1 (Mootha et al., 2003; Subramanian et al., 2005), enrichment analysis was conducted using gene sets from the Molecular Signatures Database (MSigDB, version 7.4) in the Hallmark gene category (Liberzon et al., 2015), Curated (C2) gene category, and Ontology (C5) gene category. Enriched gene sets from C2 and C5 were organized into

networks using the EnrichmentMap plugin (Merico et al., 2010) for Cytoscape 3.8.2 (Shannon et al., 2003), and automatic identification and labeling of network clusters was facilitated by the AutoAnnotate plugin (Kucera et al., 2016). The gene lists that were chosen to represent defined T cell signatures were adapted from previously published characterizations of exhausted T cells (TEX), effector T cells (TEFF), memory T cells (TMEM), memory precursor effector cells (TMPEC), short lived effector cells (TSLEC), and effector memory cells re-expressing CD45A (TEMRA) (Joshi et al., 2007; Knaus et al., 2018; Wherry et al., 2007). GSEA was performed with the following parameters: number of permutations: 2000, permutation type: gene set, metric for ranking genes: T-test.

The ssGSEA (v10.0.x) projection module (Barbie et al., 2009) within GenePattern (Reich et al., 2006) was used to calculate separate enrichment scores for individual sample-gene set pairings, independent of phenotype labeling. ssGSEA was performed on untransformed gene expression signal values with the following parameters: sample normalization method: rank, weighting component: 0.75, minimum gene set size: 10. From ACT+MS-275 treated samples, Pearson's correlation coefficient was used to calculate the correlation between enriched Hallmark pathways (ssGSEA enrichment scores) and differentially expressed hub genes (signal values) and visualized as a correlation matrix or individual scatter plots.

Unsupervised weighted gene-centric network analysis

Module identification: Co-expressed modules were identified with the CEMiTool method (Russo et al., 2018) using the web application, webCEMiTool (Cardozo et al., 2019). Analysis was performed with log2-transformed gene expression signal values using default parameters: variance filter (p-value): 0.05, correlation method: Pearson, beta: 18, dissimilarity threshold: 0.8. Identified modules were denoted as M1-M6. CEMiTool was then used to generate a protein-protein interaction (PPI) network from the module 2 (M2) gene list based on interactome data provided by Genemania (Mostafavi et al., 2008).

Pathway analysis: A functional network was constructed from the module 2 (M2) gene list utilizing the ClueGO plugin (Bindea et al., 2009) for Cytoscape 3.8.2. The Gene Ontology Biological Processes (GOBP) database was used and overrepresented terms were displayed according to the following parameters: GO Term Fusion: yes, display only pathways with p: <0.05.

Survival analysis of TCGA data

Normalized RNA-seq data from The Cancer Genome Atlas (TCGA) were downloaded from the Broad Genome Data Analysis Center (GDAC) Firehose repository (doi: 10.7908/C11G0KM9) (Broad_Institute_TCGA_Genome_Data_Analysis_Center, 2016) through the Firebrowse portal (Deng et al., 2017). Patients were filtered to select for primary tumor samples and tumor stage I or IV (extent of primary tumor burden), denoted as T1 or T4. ssGSEA analysis was used to determine Hallmark pathway enrichment scores for each patient (see above) and pathways that overlapped with preclinical data were chosen to represent a pathway signature. Pathways were fitted in a multivariate Cox regression model to assess relative contribution to survival prediction. Using the regression coefficients in the model, a prognostic risk score for predicting overall survival was calculated,

$$Risk\ score = \sum_{i=1}^{n} exp_i * \beta_i$$

where n is the number of prognostic pathways, exp_i is the enrichment score of pathway i, and β_i is the regression coefficient of pathway i. The median risk score was used to stratify patients into high- and low-risk groups.

Statistical analysis

Statistical analyses were performed using Prism 8.4.3 (Graphpad Software) and MedCalc. Bar graphs depict means ± SEM. Un-paired T-tests (two-tailed) and one-way ANOVA was used to query immune response or characterization data. p values < 0.05 were considered significant (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$); p-values > 0.05; non-significant (NS). For survival studies, Kaplan-Meier analysis was used and log-rank test was used to assess the statistical significance ($p < 0.05$).

Univariate and multivariate Cox regression and patient stratification were used to determine whether a pathway signature unique to increased tumor burden was predictive of decreased overall survival and was independent of other clinical features, where $p < 0.05$. Receiver operating characteristic (ROC) curves were used to determine sensitivity and specificity of the pathway signature in predicting increased tumor burden in patients. The area under the curve (AUC) is a ROC performance measurement where a higher value corresponds to an increased ability to distinguish between classes.

Supplementary Figure Titles and Legends

Figure S1: Transferred T cell proliferation and cell death over time, related to Figure 3D, E

Transferred Thy1.1+ congenic TCM were analyzed at several timepoints post-vaccination and stained for markers of proliferation and cell death. Fluorescence intensity was determined by geometric mean. Data was obtained with n = 5 mice per group. Statistics were based on one-way ANOVA test.

Figure S2: Enriched Hallmark pathways using the Module (M2) list, related to Figure 4D

Using the MSigDB Hallmark gene set collection, GSEA was used to enrich pathways in ACT-treated (negative enrichment score) or ACT+MS-275-treated tumors (positive enrichment score). Gene expression data was filtered from the Module 2 (M2) gene list.

Figure S3: Differentially expressed genes when comparing ACT+MS-275- to ACT-treated T cells, related to Figure 4D

(A) Scatter plot outlining average log2 signal values for expressed genes and volcano plot outlining differentially expressed genes (FDR < 0.05) during ACT±MS-275 treatment. (B) Pie charts showing locus type breakdown for differentially expressed genes. (C) Top differentially expressed genes by absolute fold change.

Figure S4: DUSP22-pathway correlation, related to Figure 4I, J

Scatter plots showing correlation between Hallmark pathways and DUSP22 gene expression.

Supplementary Table Titles and Legends

Table S1: Overrepresented GO terms associated with Module 2 (M2), related to Figure 4E, F

Table S2: Gene lists of T cell signatures, related to Figure 5E

Figure 1
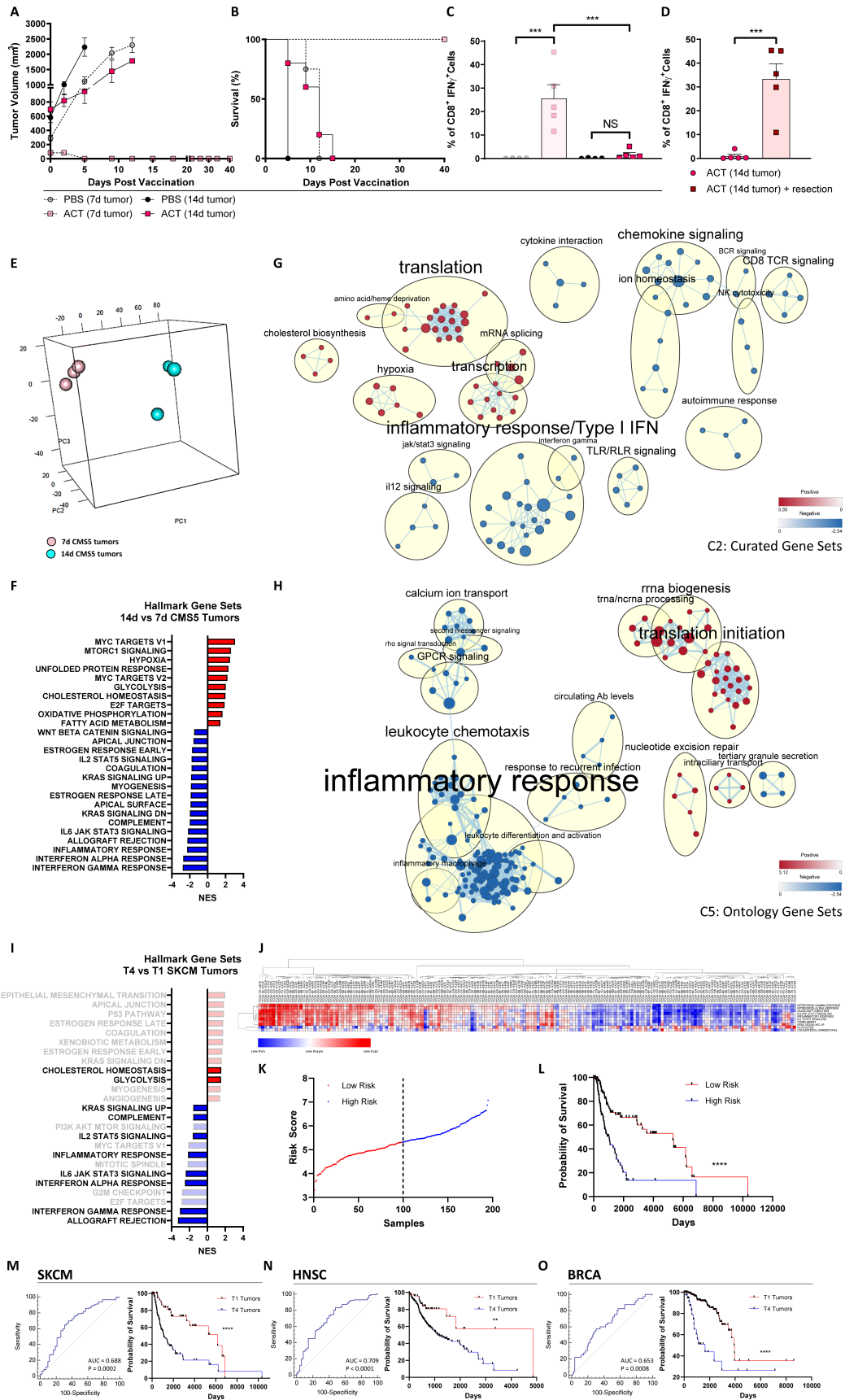
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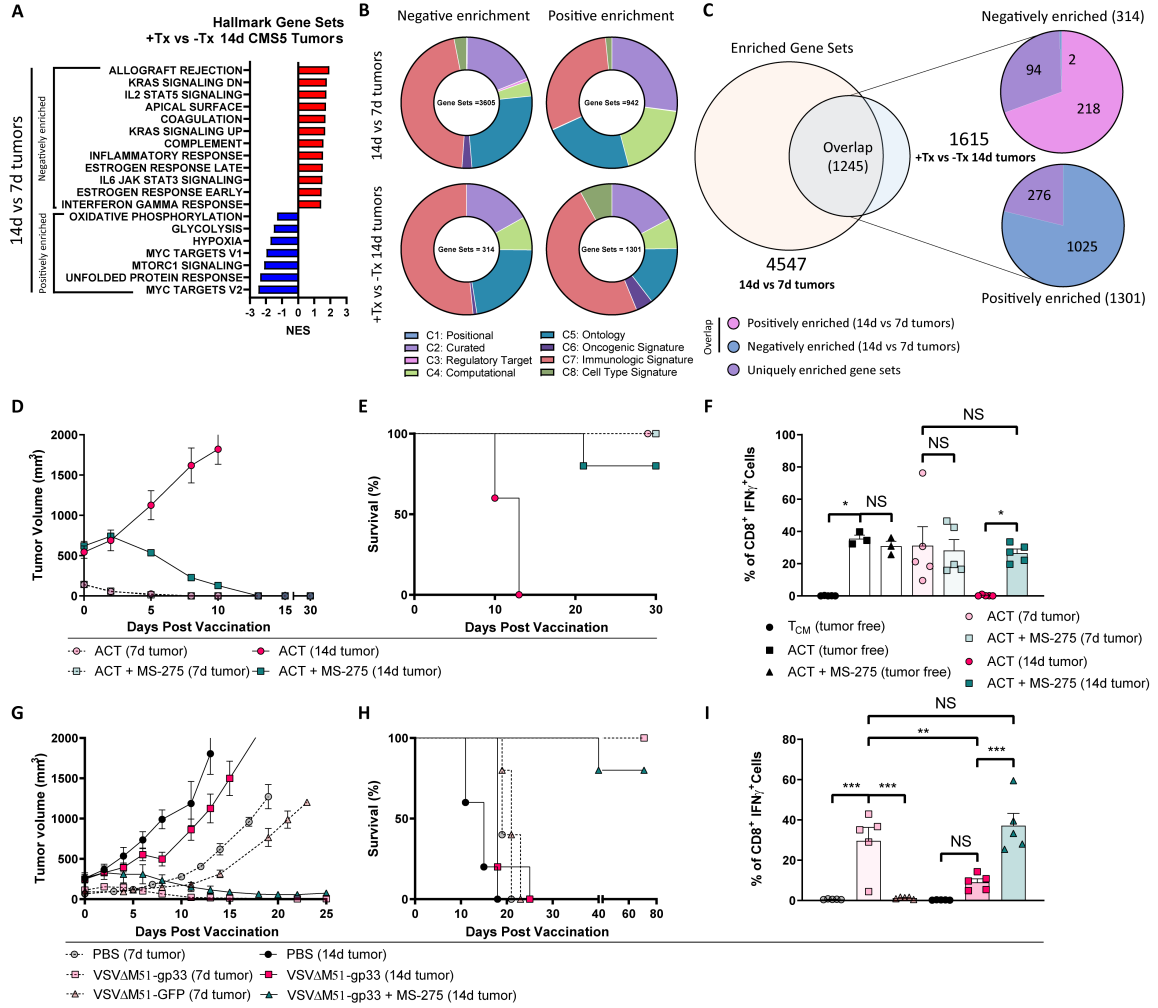
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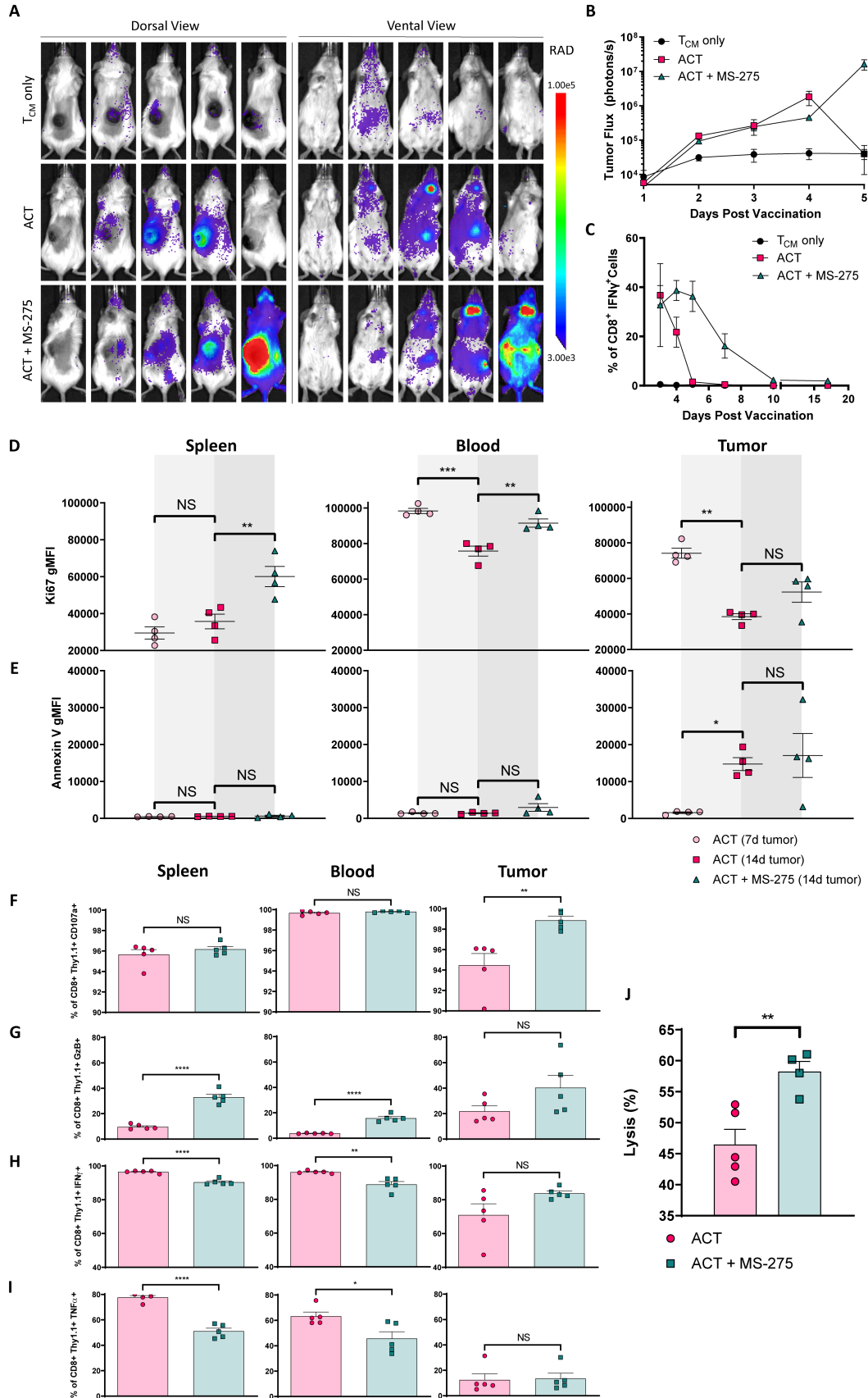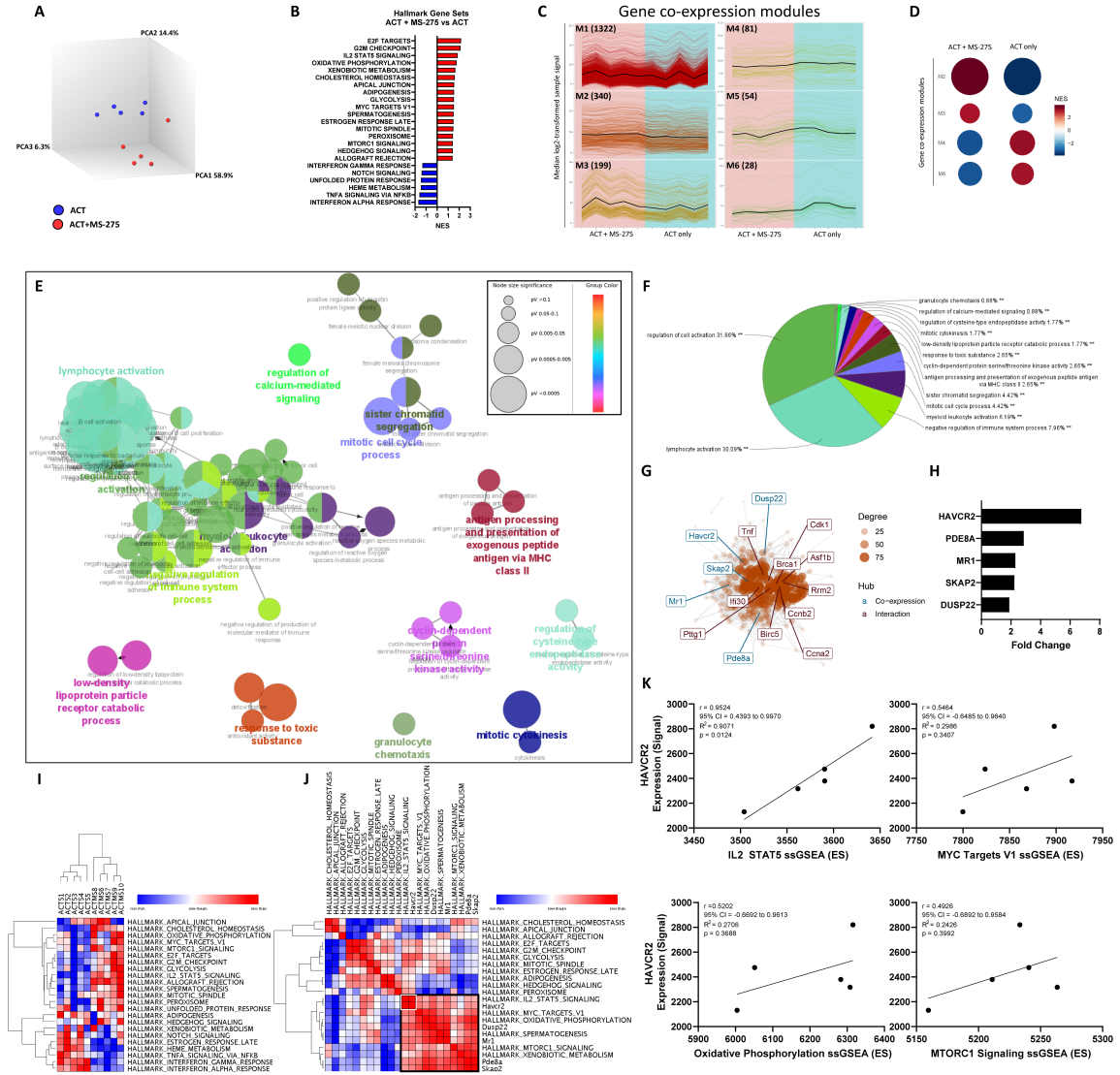
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Figure 5
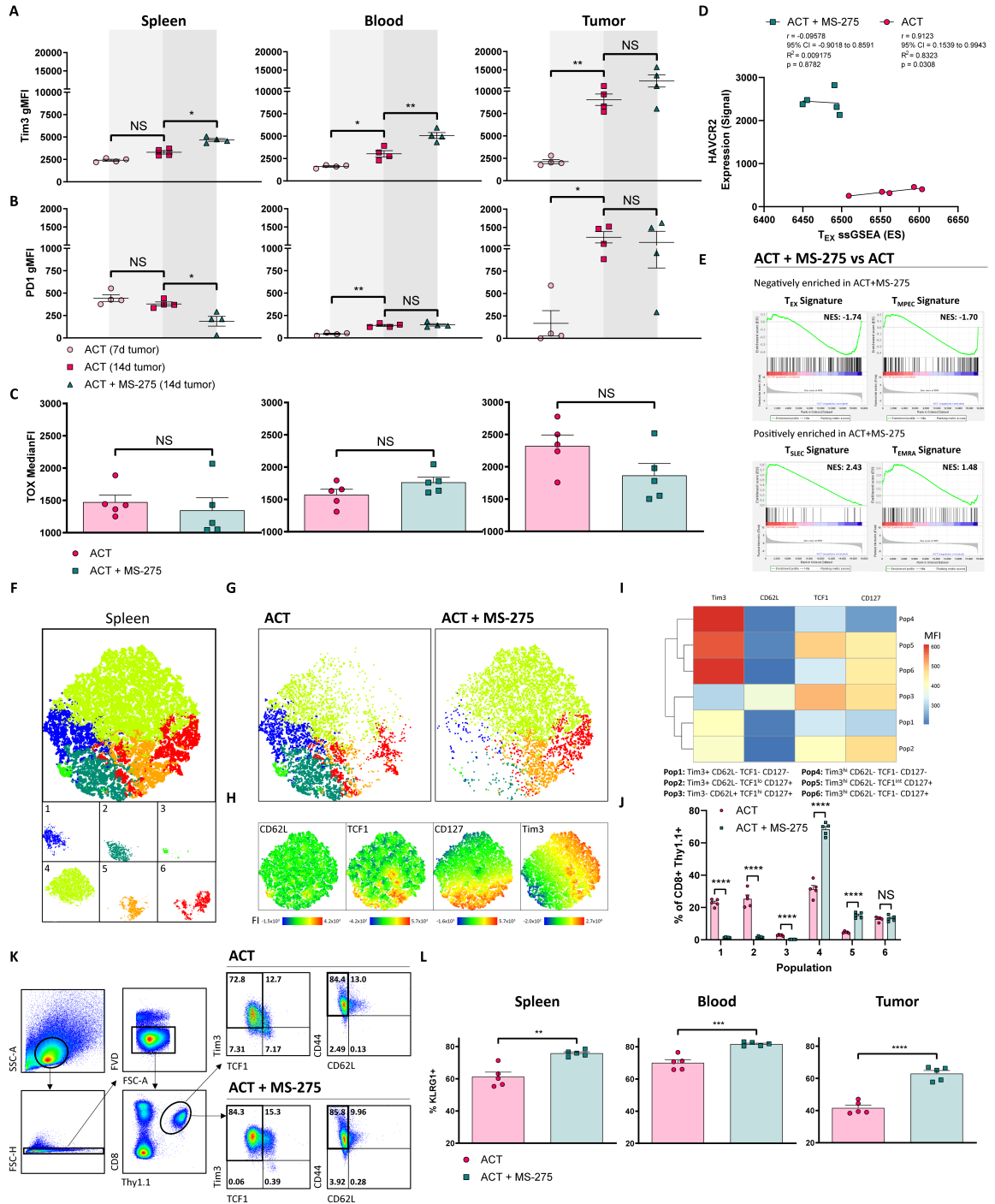
Table 1

Variables		Patients (N)	Univariate Survival		Multivariate Survival	
			HR (95% CI)	P-value	HR (95% CI)	P-value
Age	<=61 / >61	99/96	1.4703 (0.9296 to 2.3254)	0.0994		
Gender	Female / Male	82/113	1.3077 (0.8350 to 2.0479)	0.2411		
Pathologic Stage	**I-II / III-IV**	**117/74**	**2.6947 (1.7179 to 4.2269)**	**<0.0001**	**1.6867 (0.4996 to 5.6942)**	**0.3997**
T Stage	**T1 / T4**	**42/153**	**3.1487 (1.7690 to 5.6046)**	**0.0001**	**2.4563 (1.3357 to 4.5172)**	**0.0038**
N Stage	**N0 / N1-3**	**108/67**	**2.4027 (1.4844 to 3.8891)**	**0.0004**	**0.4566 (0.1142 to 1.8257)**	**0.2676**
M Stage	M0 / M1	183/7	1.2430 (0.3905 to 3.9566)	0.7127		
Radiation Therapy	No / Yes	184/11	0.6911 (0.2790 to 1.7118)	0.4247		
Risk Score	**Low / High**	**98/97**	**2.8692 (1.8019 to 4.5688)**	**<0.0001**	**2.7007 (0.7039 to 10.3625)**	**0.1476**

HR, Hazard Ratio; CI, Confidence Interval; T, Tumor; N, Node; M, Metastasis

Supplementary Figure 1
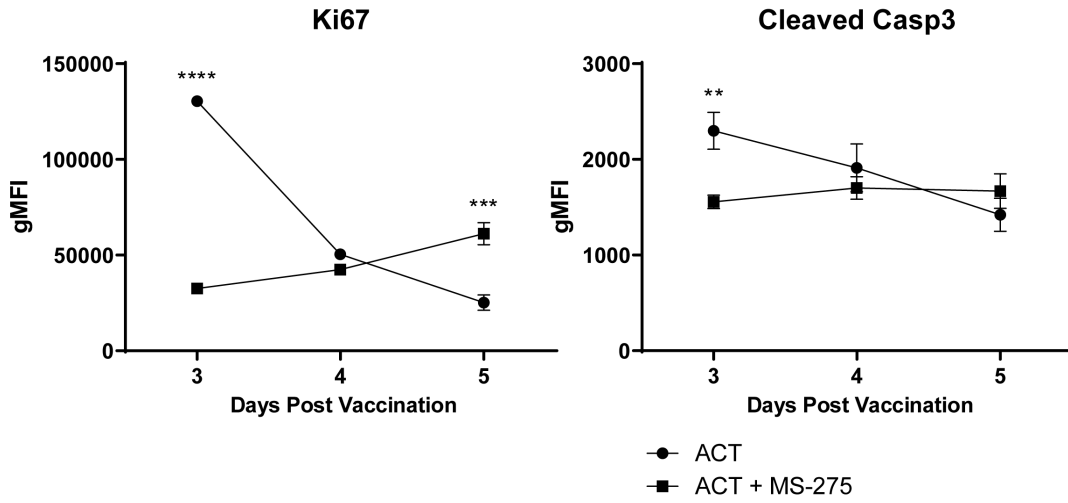
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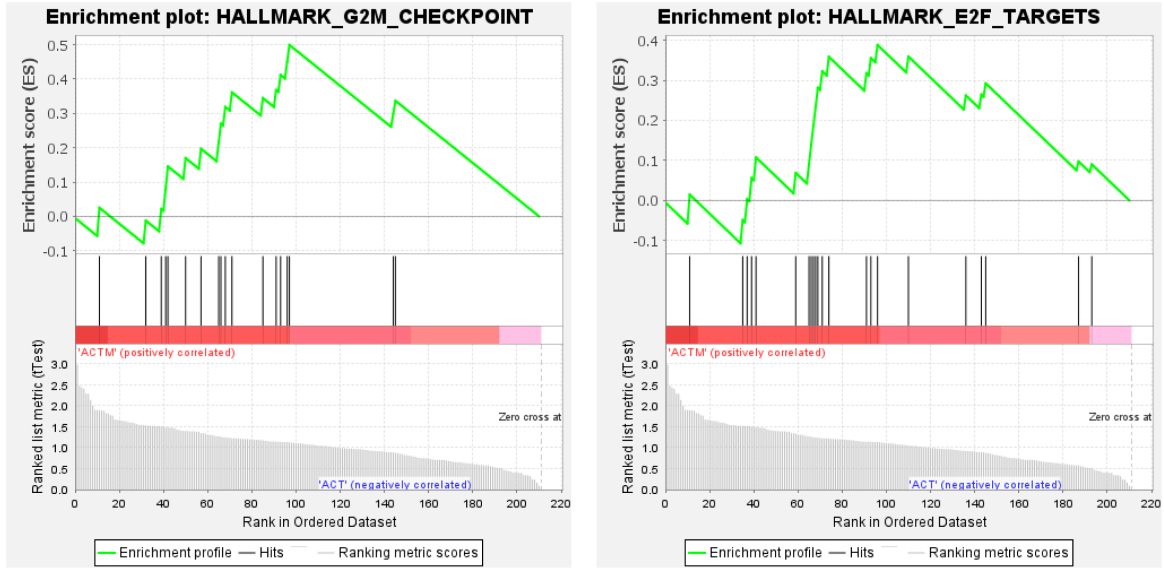
GS	GS DETAILS	ES	NES	NOM p-val	FDR q-val	FWER p-val	RANK AT MAX	LEADING EDGE
1	HALLMARK_G2M_CHECKPOINT	18	0.5	1.7	0.011	0.021	0.019	97
2	HALLMARK_E2F_TARGETS	22	0.39	1.4	0.093	0.092	0.162	96

Supplementary Figure 3
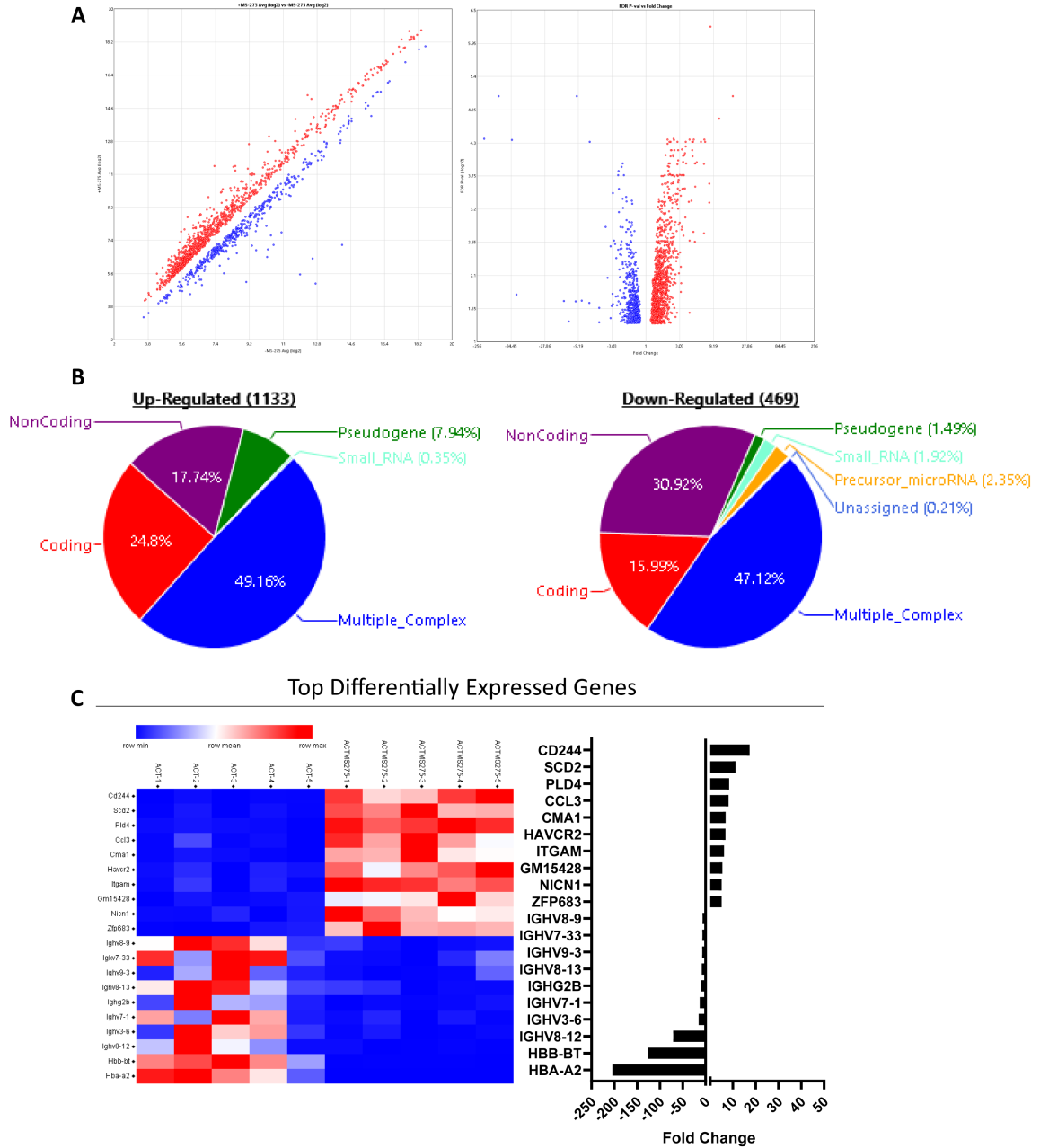
Supplementary Figure 4
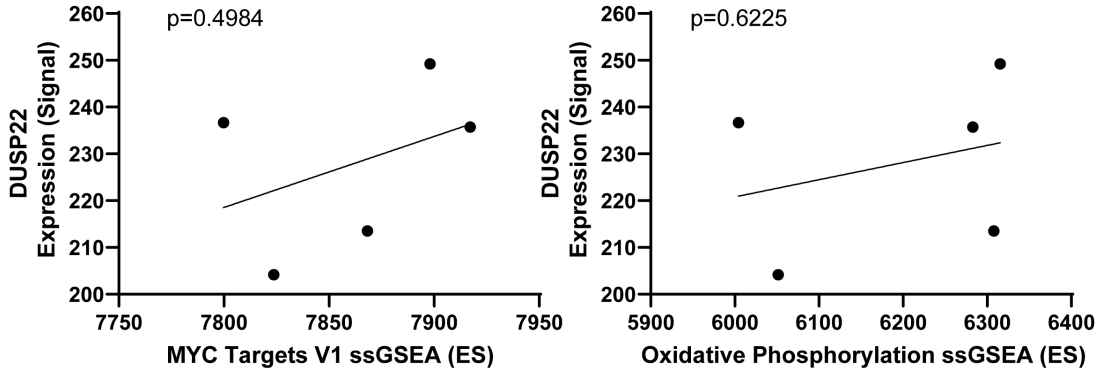
	ACT + MS-275		ACT + MS-275
r	0.4053	r	0.3011
95% confidence interval	-0.7425 to 0.9484	95% confidence interval	-0.7914 to 0.9350
R squared	0.1643	R squared	0.09066

Supplementary Table 1

GO ID	Term				Group		
GO:0071621	granulocyte chemotaxis	3.79E-04	3.67E-02	3.79E-04	1.14E-03 [4, 5, 6, 7, 8]	Group00	5.80	8.00 [Ccl3, Ccl4, Ccl9, Itgam, S100a11, S100a8, S100a9, Slamf1]
GO:0050848	regulation of calcium-mediated signaling	5.31E-04	4.99E-02	5.31E-04	1.06E-03 [5, 6, 7, 8]	Group01	7.50	6.00 [Ccl3, Ccl4, Cd22, L1cam, Tmbim4, Tnf]
GO:2000116	regulation of cysteine-type endopeptidase activity	1.35E-04	1.52E-02	1.35E-04	6.74E-04 [5, 8, 9, 11, 12]	Group02	4.58	12.00 [Aim2, Birc5, Ccna2, Dap, Dhcr24, Fas, Nr4a1, Pdcd5os, S100a8, S100a9, Tnf, Xdh]
GO:0001056	positive regulation of cysteine-type endopeptidase activity	1.71E-04	1.88E-02	1.35E-04	6.74E-04 [6, 8, 9, 10, 11, 12, 13]	Group02	5.77	9.00 [Aim2, Ccna2, Dap, Fas, Pdcd5os, S100a8, S100a9, Tnf, Xdh]
GO:0000910	cytokinesis	2.60E-04	2.68E-02	2.60E-04	1.04E-03 [3, 4]	Group03	5.45	9.00 [Arl3, Birc5, E2f8, Kif20a, Nusap1, Plk1, Srx9, Sptbn1, Stmn1]
GO:0000281	mitotic cytokinesis	2.52E-06	3.56E-04	2.60E-04	1.04E-03 [4, 5, 6]	Group03	11.59	8.00 [Arl3, Birc5, Kif20a, Nusap1, Plk1, Srx9, Sptbn1, Stmn1]
GO:0032802	low-density lipoprotein particle receptor catabolic process	3.89E-05	4.90E-03	3.89E-05	2.72E-04 [5, 6, 7]	Group04	50.00	3.00 [Abca2, Anxa2, Myltp]
GO:0032803	regulation of low-density lipoprotein particle receptor catabolic process	3.89E-05	4.90E-03	3.89E-05	2.72E-04 [5, 6, 7, 8]	Group04	50.00	3.00 [Abca2, Anxa2, Myltp]
GO:0098754	detoxification	1.12E-05	1.50E-03	3.57E-07	2.85E-06 [1, 4]	Group05	7.19	10.00 [Gpx8, Gstk1, Hbb-bs, Mt1, Mt2, Prdx1, Prdx4, S100a8, S100a9, Tnf]
GO:0009636	response to toxic substance	3.57E-07	5.17E-05	3.57E-07	2.85E-06 [3, 4]	Group05	5.95	16.00 [Ass1, Ccl3, Ccl4, Cdk1, Cdkn1a, Fas, Gpx8, Gstk1, Hbb-bs, Mt1, Mt2, Prdx1, Prdx4, S100a8, S100a9, Tnf]
GO:0016209	antioxidant activity	4.31E-05	5.38E-03	3.57E-07	2.85E-06 [4, 7]	Group05	7.92	8.00 [Gpx8, Gstk1, Hbb-bs, Prdx1, Prdx4, S100a8, S100a9, Tnf]
GO:0004693	cyclin-dependent protein serine/threonine kinase activity	9.57E-05	1.17E-02	9.57E-05	5.74E-04 [5, 6, 9, 10, 11]	Group06	7.08	8.00 [Ccna2, Ccnb2, Cdk1, Cdkn1a, Cks1b, Mnat1, Plk1, Psmd10]
GO:0000079	regulation of cyclin-dependent protein serine/threonine kinase activity	1.18E-04	1.34E-02	9.57E-05	5.74E-04 [5, 6, 7, 9, 10, 11, 12]	Group06	8.05	7.00 [Ccna2, Ccnb2, Cdkn1a, Cks1b, Mnat1, Plk1, Psmd10]
GO:0016538	cyclin-dependent protein serine/threonine kinase regulator activity	3.09E-04	3.06E-02	9.57E-05	5.74E-04 [5, 6, 7, 8, 9, 10, 11, 12, 13]	Group06	10.64	5.00 [Ccna2, Ccnb2, Cdkn1a, Cks1b, Mnat1]
GO:0019884	antigen processing and presentation of exogenous antigen	1.81E-04	1.97E-02	5.67E-04	5.67E-04 [3]	Group07	11.90	5.00 [Ctse, H2-DMa, H2-Oa, If30, Mr1]
GO:0048002	antigen processing and presentation of peptide antigen	3.75E-04	3.67E-02	5.67E-04	5.67E-04 [3]	Group07	8.00	6.00 [Ctse, Ctsl, H2-DMa, If30, Mr1]
GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	5.22E-05	6.42E-03	5.67E-04	5.67E-04 [5]	Group07	23.53	4.00 [Ctse, H2-DMa, H2-Oa, If30]
GO:0000819	sister chromatid segregation	3.84E-05	4.88E-03	1.64E-07	1.48E-06 [4, 5, 6]	Group08	5.67	11.00 [Birc5, Esco2, Kif18b, Knstrn, Ncaph, Nusap1, Plk1, Pttg1, Sgo1, Top2a, Ube2c]
GO:0007143	female meiotic nuclear division	2.26E-04	2.39E-02	1.64E-07	1.48E-06 [3, 4, 5, 6, 7, 8]	Group08	11.36	5.00 [Ccnb2, Mastl, Ncaph, Plk1, Top2a]
GO:0016321	female meiosis chromosome segregation	1.07E-04	1.25E-02	1.64E-07	1.48E-06 [3, 4, 5, 6, 7, 8, 9]	Group08	37.50	3.00 [Ncaph, Plk1, Top2a]
GO:0030261	chromosome condensation	2.76E-04	2.82E-02	1.64E-07	1.48E-06 [8]	Group08	10.87	5.00 [Cdk1, H1f5, Ncaph, Nusap1, Top2a]
GO:1904668	positive regulation of ubiquitin protein ligase activity	2.25E-04	2.40E-02	1.64E-07	1.48E-06 [5, 7, 9, 10, 11, 12, 13]	Group08	30.00	3.00 [Cdc20b, Mastl, Plk1]
GO:1903047	mitotic cell cycle process	9.09E-09	1.37E-06	4.61E-09	5.07E-08 [3, 4]	Group09	4.20	30.00 [Arl3, Birc5, Ccna2, Ccnb2, Cdk1, Cdkn1a, Cks1b, Kif11, Kif18b, Kif20a, Knstrn, Mastl, Mki67, Mnat1, Nabp1, Ncaph, Nusap1, Plk1, Pttg1, ...]
GO:0000819	sister chromatid segregation	3.84E-05	4.88E-03	4.61E-09	5.07E-08 [4, 5, 6]	Group09	5.67	11.00 [Birc5, Esco2, Kif18b, Knstrn, Ncaph, Nusap1, Plk1, Pttg1, Sgo1, Top2a, Ube2c]
GO:0140014	mitotic nuclear division	9.81E-05	1.18E-02	4.61E-09	5.07E-08 [4, 5, 7]	Group09	4.44	13.00 [Birc5, Kif11, Kif18b, Knstrn, Mki67, Ncaph, Nusap1, Plk1, Pttg1, Sgo1, Tnf, Top2a, Ube2c]
GO:0000070	mitotic sister chromatid segregation	2.37E-04	2.47E-02	4.61E-09	5.07E-08 [4, 5, 6, 7, 8]	Group09	5.52	9.00 [Birc5, Kif18b, Knstrn, Ncaph, Nusap1, Plk1, Pttg1, Sgo1, Ube2c]
GO:0016321	female meiosis chromosome segregation	1.07E-04	1.25E-02	4.61E-09	5.07E-08 [3, 4, 5, 6, 7, 8, 9]	Group09	37.50	3.00 [Ncaph, Plk1, Top2a]
GO:0072593	reactive oxygen species metabolic process	1.31E-05	1.74E-03	5.98E-09	5.98E-08 [3]	Group10	4.75	15.00 [Ass1, COX2, Ccna2, Cdkn1a, Hbb-bs, If18, Itgam, Ndufc2, Prcp, Prdx1, Prdx4, Tnf, Tyrobp, Ulbp1, Xdh]
GO:0001909	leukocyte mediated cytotoxicity	3.48E-05	4.46E-03	5.98E-09	5.98E-08 [3, 4]	Group10	7.09	9.00 [Gzmb, Havcr2, If18, Itgam, Klre1, Mr1, Prdx1, Tyrobp, Ulbp1]
GO:0002274	myeloid leukocyte activation	6.74E-07	9.64E-05	5.98E-09	5.98E-08 [3, 4]	Group10	6.05	15.00 [Ccl3, Cd244a, Fgr, Havcr2, If18, If4, Itgam, Rbpj, Slamf1, Syt11, Tnf, Tnfsf9, Tspan32, Tyrobp, Ulbp1]
GO:0002703	regulation of leukocyte mediated immunity	9.70E-05	1.17E-02	5.98E-09	5.98E-08 [4, 5]	Group10	4.74	12.00 [Bst2, Dusp22, Fgr, Havcr2, If18, Itgam, Klre1, Mr1, Slamf1, Tnf, Tyrobp, Ulbp1]
GO:0036230	granulocyte activation	1.81E-04	1.97E-02	5.98E-09	5.98E-08 [4, 5]	Group10	11.90	5.00 [Ccl3, If18, Itgam, Tnf, Tyrobp]
GO:2000377	regulation of reactive oxygen species metabolic process	1.05E-04	1.24E-02	5.98E-09	5.98E-08 [4, 5]	Group10	5.07	11.00 [Ass1, COX2, Cdkn1a, If18, Itgam, Ndufc2, Prcp, Tnf, Tyrobp, Ulbp1, Xdh]
GO:2000379	positive regulation of reactive oxygen species metabolic process	1.81E-05	2.36E-03	5.98E-09	5.98E-08 [4, 5, 6]	Group10	7.69	9.00 [Ass1, COX2, Cdkn1a, If18, Itgam, Tnf, Tyrobp, Ulbp1, Xdh]
GO:0002683	negative regulation of immune system process	9.74E-09	1.46E-06	3.06E-09	3.67E-08 [3, 4]	Group11	5.25	23.00 [Aim2, Bst2, Cd22, Cd244a, Cuedc2, Dusp3, Fas, Fgr, Gpr55, H2-Oa, Havcr2, Il2ra, Klre1, Pdcd1, Slamf1, Syt11, Tigit, T...
GO:0002697	regulation of immune effector process	3.00E-06	4.17E-04	3.06E-09	3.67E-08 [3, 4]	Group11	4.38	19.00 [Aim2, Bst2, Cd22, Cd244a, Cuedc2, Dusp22, Fgr, Havcr2, Il2ra, Itgam, Klre1, Mr1, Slamf1, Tnf, Tspan32, Tyrobp, Ulbp1]
GO:0002698	negative regulation of immune effector process	1.09E-04	1.26E-02	3.06E-09	3.67E-08 [3, 4, 5]	Group11	6.12	9.00 [Bst2, Cd22, Cuedc2, Dusp22, Havcr2, Il2ra, Klre1, Slamf1, Tnf]
GO:0050866	negative regulation of cell activation	5.12E-06	7.02E-04	3.06E-09	3.67E-08 [3, 4, 5]	Group11	5.88	13.00 [Cd80, Cd9, Ctla4, Dusp22, Dusp3, Fas, Fgr, Havcr2, Il2ra, Syt11, Tnf, Tspan32, Tyrobp]
GO:0002703	regulation of leukocyte mediated immunity	9.70E-05	1.17E-02	3.06E-09	3.67E-08 [4, 5]	Group11	4.74	12.00 [Bst2, Dusp22, Fgr, Havcr2, If18, Itgam, Klre1, Mr1, Slamf1, Tnf, Tyrobp, Ulbp1]
GO:0002701	negative regulation of production of molecular mediator of immune response	1.11E-04	1.28E-02	3.06E-09	3.67E-08 [3, 4, 5, 6, 7]	Group11	13.16	5.00 [Bst2, Cd22, Cuedc2, Slamf1, Tnf]
GO:0051250	negative regulation of lymphocyte activation	4.69E-05	5.81E-03	3.06E-09	3.67E-08 [3, 4, 5, 6, 7]	Group11	6.10	10.00 [Cd80, Ctla4, Dusp22, Dusp3, Fas, Fgr, Havcr2, Il2ra, Tigit, Tyrobp]
GO:0032729	positive regulation of interferon-gamma production	7.06E-06	9.53E-04	3.06E-09	3.67E-08 [4, 5, 6, 7, 8]	Group11	10.13	8.00 [Cd244a, Havcr2, If18, Klre1, Slamf1, Tnf, Tnfsf9, Ulbp1]
GO:0050863	regulation of T cell activation	1.01E-04	1.20E-02	3.68E-03	3.67E-08 [5, 6]	Group11	4.18	14.00 [Cd80, Ctla4, Dusp22, Dusp3, H2-DMa, H2-Oa, Havcr2, If18, Il2ra, If6st, Slamf1, Tigit, Tnfsf9]
GO:0046321	leukocyte activation	3.12E-15	5.33E-13	6.57E-14	8.55E-13 [2, 3]	Group12	4.58	49.00 [Ccl3, Cd22, Cd244a, Cd80, Cdkn1a, Ctla4, Ctsl, Dusp22, Dusp3, Fas, Fgr, Gpr55, H2-Oa, Havcr2, Il2ra, Klre1, Pdcd1, Slamf1, Syt11, Tigit, Tnfsf9]
GO:0002250	adaptive immune response	6.84E-11	1.10E-08	6.57E-14	8.55E-13 [3]	Group12	5.17	30.00 [Cd244a, Ctla4, Dusp22, Fas, Gzmb, H2-DMa, H2-Oa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-...
GO:0002443	leukocyte mediated immunity	1.26E-13	2.11E-11	6.57E-14	8.55E-13 [3]	Group12	6.19	32.00 [Bst2, Ccl3, Dusp22, Fas, Fgr, Gzmb, H2-DMa, H2-Oa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-1, Ighv3-6, Ighv6-6, Ighv7-1, Ighv...
GO:0002684	positive regulation of immune system process	4.19E-11	6.80E-09	6.57E-14	8.55E-13 [2, 3, 4]	Group12	4.04	41.00 [Aim2, Ccl3, Ccl4, Cd22, Cd244a, Cd80, Cdkn1a, Ctla4, Dusp22, Dusp3, Fas, Fgr, ...]
GO:0006959	humoral immune response	9.66E-08	1.41E-05	6.57E-14	8.55E-13 [3]	Group12	5.01	21.00 [H2-DMa, H2bc4, H2bc6, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-6, ...]
GO:0002697	regulation of immune effector process	3.00E-06	4.17E-04	6.57E-14	8.55E-13 [3, 4]	Group12	4.38	19.00 [Aim2, Bst2, Cd22, Cd244a, Cuedc2, Dusp22, Fgr, Havcr2, Il2ra, Itgam, Klre1, Mr1, Slamf1, Tnf, Tspan32, Tyrobp, Ulbp1]
GO:0046649	lymphocyte activation	1.59E-15	2.75E-13	6.57E-14	8.55E-13 [3, 4]	Group12	5.04	45.00 [Cd22, Cd244a, Cd80, Cdkn1a, Ctla4, Ctsl, Dusp22, Dusp3, Fas, Fgr, H2-DMa, H2-Oa, Havcr2, Ighm, Ighv1-37, Ighv1-80, Ighv1-80, ...]
GO:0050865	regulation of cell activation	2.60E-15	4.48E-13	6.57E-14	8.55E-13 [3, 4]	Group12	5.44	41.00 [Cd22, Cd244a, Cd9, Cdkn1a, Ctla4, Dusp22, Dusp3, Fas, Fgr, H2-DMa, H2-Oa, Havcr2, Ighm, Ighv1-37, Ighv1-80, ...]
GO:0002440	lymphocyte mediated immunity	3.48E-12	5.78E-10	6.57E-14	8.55E-13 [4]	Group12	6.52	27.00 [Dusp22, Fas, Gzmb, H2-DMa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-1, Ighv3-6, Ighv6-6, Ighv7-1, ...]
GO:0002460	adaptive immune response based on somatic recombination of immune receptors bu...	2.27E-10	3.61E-08	6.57E-14	8.55E-13 [4]	Group12	5.85	25.00 [Dusp22, Fas, Gzmb, H2-DMa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-1, Ighv3-6, Ighv6-6, Ighv7-1, ...]
GO:0006909	phagocytosis	2.38E-10	3.76E-08	6.57E-14	8.55E-13 [3, 5]	Group12	6.37	23.00 [Fgr, Ighm, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv1-1, Ighv8-12, Ighv8-13, Ighv8-6, Itgam, ...]
GO:0050778	positive regulation of immune response	1.66E-09	2.57E-07	6.57E-14	8.55E-13 [3, 4, 5]	Group12	4.42	30.00 [Aim2, Cd22, Cd244a, Dusp22, Dusp3, Fgr, H2-DMa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-6, Ighv6-6, Ighv7-1, ...]
GO:0002253	activation of immune response	4.74E-08	6.97E-06	6.57E-14	8.55E-13 [2, 4, 5, 6]	Group12	5.01	22.00 [Aim2, Ctla4, Dusp22, Dusp3, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-1, Ighv6-6, Ighv7-1, Ighv8-12, ...]
GO:0006910	phagocytosis, recognition	1.27E-09	1.99E-07	6.57E-14	8.55E-13 [3, 4]	Group12	9.50	15.00 [Ighm, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv3-6, Ighv8-12, Ighv8-13, Ighv8-6, ...]
GO:0002696	positive regulation of leukocyte activation	9.03E-14	1.53E-11	6.57E-14	8.55E-13 [3, 4, 5, 6]	Group12	6.49	31.00 [Cd244a, Cd80, Cdkn1a, Fgr, H2-DMa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-80, Ighv14-3, Ighv2-1, Ighv3-1, Ighv3-6, Ighv6-6, Ighv7-...
GO:0002703	regulation of leukocyte mediated immunity	9.70E-05	1.17E-02	6.57E-14	8.55E-13 [4, 5]	Group12	4.74	12.00 [Bst2, Dusp22, Fgr, Havcr2, If18, Itgam, Klre1, Mr1, Slamf1, Tnf, Tyrobp, Ulbp1]
GO:0042113	B cell activation	3.37E-09	5.18E-07	6.57E-14	8.55E-13 [4, 5]	Group12	5.56	23.00 [Cd244a, Cdkn1a, Ctla4, Fas, Ighm, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv6-6, Ighv7-1, Ighm14-...
GO:0045087	innate immune response	2.94E-11	4.79E-09	6.57E-14	8.55E-13 [3, 4, 5, 6]	Group12	4.17	40.00 [Aim2, Ass1, Bst2, Capg, Ccl3, Ccl4, Ccl9, Cd244a, Fgr, Gzmb, H2bc4, H2bc6, Havcr2, Ighm, Ighv1-37, Ighv1-49, Ighv1-80, ...]
GO:0046651	lymphocyte proliferation	5.78E-06	7.85E-04	6.57E-14	8.55E-13 [4, 5, 6]	Group12	4.80	16.00 [Cd22, Cd80, Cdkn1a, Ctla4, Havcr2, Ighm, If18, Il2ra, If6st, Ins2, Itgam, Slamf1, Tnfsf9, Tyrobp, Zbtb32]
GO:0010324	membrane invagination	2.26E-11	3.73E-09	6.57E-14	8.55E-13 [5]	Group12	9.09	19.00 [Ighm, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv3-6, Ighv6-6, Ighv7-1, Ighv8-12, Ighv8-13, Ighv8-6, Itgam, ...]
GO:0019724	B cell mediated immunity	1.01E-08	1.50E-06	6.57E-14	8.55E-13 [5]	Group12	6.32	18.00 [Fas, H2-DMa, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-1, Ighv3-6, Ighv6-6, ...]
GO:0042742	defense response to bacterium	8.78E-10	1.38E-07	6.57E-14	8.55E-13 [3, 4, 6]	Group12	5.28	26.00 [Fgr, H2bc4, H2bc6, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-6, Ighv3-...
GO:0051249	regulation of lymphocyte activation	5.82E-15	9.89E-13	6.57E-14	8.55E-13 [4, 5, 6]	Group12	6.07	36.00 [Cd22, Cd244a, Cd80, Cdkn1a, Ctla4, Dusp22, Dusp3, Fas, Fgr, H2-DMa, Havcr2, Ighm, Ighv1-37, Ighv1-49, Ighv1-80, ...]
GO:0051251	positive regulation of lymphocyte activation	6.28E-13	1.05E-10	6.57E-14	8.55E-13 [4, 5, 6, 7]	Group12	6.73	28.00 [Cd244a, Cd80, Cdkn1a, H2-DMa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-1, Ighv3-6, Ighv6-6, Ighv7-1, Ighv...
GO:1903037	regulation of leukocyte cell-cell adhesion	1.52E-05	2.00E-03	6.57E-14	8.55E-13 [5, 6]	Group12	4.69	15.00 [Ass1, Cd244a, Cd80, Ctla4, Dusp22, Dusp3, H2-DMa, Havcr2, If18, If2ra, If6st, Slamf1, Tnf, Tnfsf9]
GO:0002429	immune response-activating cell surface receptor signaling pathway	2.33E-09	3.06E-07	6.57E-14	8.55E-13 [4, 5, 6, 7, 8]	Group12	6.21	21.00 [Cd22, Ctla4, Dusp22, Dusp3, Ighm, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-6, Ighv6-6, Ighv7-1, Ighv8-12, ...]
GO:0050670	regulation of lymphocyte proliferation	2.83E-06	3.96E-04	6.57E-14	8.55E-13 [5, 6, 7]	Group12	5.76	14.00 [Cd22, Cd80, Cdkn1a, Ctla4, Havcr2, Ighm, If18, Il2ra, If6st, Ins2, Slamf1, Tnfsf9, Tyrobp]
GO:0050863	regulation of T cell activation	1.01E-04	1.20E-02	6.57E-14	8.55E-13 [5, 6, 7]	Group12	4.18	14.00 [Cd80, Ctla4, Dusp22, Dusp3, H2-DMa, H2-Oa, Havcr2, If18, Il2ra, If6st, Slamf1, Tigit, Tnfsf9]
GO:0050864	regulation of B cell activation	2.67E-11	4.38E-09	6.57E-14	8.55E-13 [5, 6, 7]	Group12	7.92	21.00 [Cd22, Cd244a, Ctla4, Fas, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-1, Ighv3-6, Ighv6-6, Ighv7-1, Ighv8-12, Ighv...
GO:0050671	positive regulation of lymphocyte proliferation	2.28E-06	3.23E-04	6.57E-14	8.55E-13 [5, 6, 7, 8]	Group12	7.64	11.00 [Cd244a, Cd80, Cdkn1a, Havcr2, Ighm, If18, Il2ra, If6st, Ins2, Slamf1, Tnfsf9]
GO:0050871	positive regulation of B cell activation	2.44E-09	3.73E-07	6.57E-14	8.55E-13 [5, 6, 7, 8]	Group12	7.87	17.00 [Cd244a, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-1, Ighv3-6, Ighv6-6, Ighv7-1, Ighv8-12, Ighv8-13, Ighv8-9...
GO:0030888	regulation of B cell proliferation	2.78E-04	3.04E-02	6.57E-14	8.55E-13 [6, 7, 8]	Group12	8.45	6.00 [Cd22, Cdkn1a, Ctla4, Ighm, Ins2, Tyrobp]
GO:0050851	antigen receptor-mediated signaling pathway	1.19E-08	1.76E-06	6.57E-14	8.55E-13 [5, 6, 7, 8, 9]	Group12	6.27	19.00 [Cd22, Ctla4, Dusp22, Dusp3, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-6, Ighv6-6, Ighv7-1, Ighv8-12, Ighv8-13, Ighv8-6, ...]
GO:0050863	B cell receptor signaling pathway	1.32E-10	2.11E-08	6.57E-14	8.55E-13 [6, 7, 8, 9, 10]	Group12	9.50	17.00 [Cd22, Ctla4, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-6, Ighv6-6, Ighv7-1, Ighv8-12, Ighv8-13, Ighv8-6, ...]
GO:0002443	leukocyte mediated immunity	1.26E-13	2.11E-11	2.14E-14	3.00E-13 [3]	Group13	6.19	32.00 [Bst2, Ccl3, Dusp22, Fas, Fgr, Gzmb, H2-DMa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-1, Ighv3-6, Ighv6-6, Ighv7-1, Ighv...
GO:0002683	negative regulation of immune system process	9.74E-09	1.46E-06	2.14E-14	3.00E-13 [3, 4]	Group13	5.25	23.00 [Bst2, Ccl3, Cd22, Cd80, Ctla4, Cuedc2, Dusp22, Fas, Fgr, Gpr55, H2-Oa, Havcr2, Il2ra, Klre1, Pdcd1, Slamf1, Syt11, Tigit, T...
GO:0002684	positive regulation of immune system process	4.19E-11	6.80E-09	2.14E-14	3.00E-13 [2, 3, 4]	Group13	4.04	41.00 [Aim2, Ccl3, Ccl4, Cd22, Cd244a, Cd80, Cdkn1a, Ctla4, Dusp22, Dusp3, Fas, Fgr, H2-DMa, Havcr2, Il2ra, Klre1, Pdcd1, Slamf1, Tnf, Tnfsf9]
GO:0001909	leukocyte mediated cytotoxicity	3.48E-05	4.46E-03	2.14E-14	3.00E-13 [3, 4]	Group13	7.09	9.00 [Gzmb, Havcr2, If18, Itgam, Klre1, Mr1, Prdx1, Tyrobp, Ulbp1]
GO:0002274	myeloid leukocyte activation	6.74E-07	9.64E-05	2.14E-14	3.00E-13 [3, 4]	Group13	6.05	15.00 [Ccl3, Cd244a, Fgr, Havcr2, If18, If4, Itgam, Rbpj, Slamf1, Syt11, Tnf, Tnfsf9, Tspan32, Tyrobp, Ulbp1]
GO:0002419	immune response to tumor cell	2.35E-04	2.56E-02	2.14E-14	3.00E-13 [3, 4]	Group13	15.38	4.00 [Havcr2, Klre1, Mr1, Ulbp1]
GO:0002697	regulation of immune effector process	3.00E-06	4.17E-04	2.14E-14	3.00E-13 [3, 4]	Group13	4.38	19.00 [Aim2, Bst2, Cd22, Cd244a, Cuedc2, Dusp22, Fgr, Havcr2, If18, Il2ra, Itgam, Klre1, Mr1, Slamf1, Tnf, Tspan32, Tyrobp, Ulbp1]
GO:0046649	lymphocyte activation	1.59E-15	2.75E-13	2.14E-14	3.00E-13 [3, 4]	Group13	5.04	45.00 [Cd22, Cd244a, Cd80, Cdkn1a, Ctla4, Ctsl, Dusp22, Dusp3, Fas, Fgr, H2-DMa, H2-Oa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, ...]
GO:0050865	regulation of cell activation	2.60E-15	4.48E-13	2.14E-14	3.00E-13 [3, 4, 5]	Group13	5.88	13.00 [Cd80, Cd9, Ctla4, Dusp22, Dusp3, Fas, Fgr, Havcr2, Il2ra, Syt11, Tnf, Tspan32, Tyrobp]
GO:0002698	negative regulation of immune effector process	1.09E-04	1.26E-02	2.14E-14	3.00E-13 [3, 4, 5]	Group13	6.12	9.00 [Bst2, Cd22, Cuedc2, Dusp22, Havcr2, Il2ra, Klre1, Slamf1, Tnf]
GO:0050778	positive regulation of immune response	1.66E-09	2.57E-07	2.14E-14	3.00E-13 [3, 4, 5]	Group13	4.52	30.00 [Aim2, Cd22, Cd244a, Dusp22, Dusp3, Fgr, H2-DMa, Havcr2, Il2ra, Ighm, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-6, Ighv...
GO:0050866	negative regulation of cell activation	5.12E-06	7.02E-04	2.14E-14	3.00E-13 [3, 4, 5]	Group13	5.88	13.00 [Cd80, Cd9, Ctla4, Dusp22, Dusp3, Fas, Fgr, Havcr2, Il2ra, Syt11, Tnf, Tspan32, Tyrobp]
GO:0001773	myeloid dendritic cell activation	4.39E-04	4.39E-02	2.14E-14	3.00E-13 [3, 4, 5]	Group13	18.42	7.00 [Cd244a, If4, Rbpj, Slamf1, Tnfsf9, Tspan32]
GO:0002696	positive regulation of leukocyte activation	9.03E-14	1.53E-11	2.14E-14	3.00E-13 [4, 5]	Group13	6.49	31.00 [Cd244a, Cd80, Cdkn1a, Fgr, H2-DMa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-1, Ighv3-6, Ighv6-6, Ighv7-...
GO:0002703	regulation of leukocyte mediated immunity	9.70E-05	1.17E-02	2.14E-14	3.00E-13 [4, 5]	Group13	4.74	12.00 [Bst2, Dusp22, Fgr, Havcr2, If18, Itgam, Klre1, Mr1, Slamf1, Tnf, Tyrobp, Ulbp1]
GO:0002407	regulation of cell-cell adhesion	1.56E-05	2.04E-03	2.14E-14	3.00E-13 [4, 5]	Group13	4.05	14.00 [Ass1, Cd244a, Cd9, Ctla4, Dusp22, Dusp3, Havcr2, If18, If2ra, If6st, L1cam, Myadm, Slamf1, Tigit, Tnf, Tnfsf9]
GO:0030101	natural killer cell activation	5.03E-06	6.94E-04	2.14E-14	3.00E-13 [4, 5]	Group13	9.00	9.00 [Cd244a, Fgr, Havcr2, If18, Klre1, Prdx1, Slamf1, Tyrobp, Ulbp1]
GO:0036230	granulocyte activation	1.81E-04	1.97E-02	2.14E-14	3.00E-13 [4, 5]	Group13	11.90	5.00 [Ccl3, If18, Itgam, Tnf, Tyrobp]
GO:0046651	lymphocyte proliferation	5.78E-06	7.85E-04	2.14E-14	3.00E-13 [4, 5]	Group13	4.80	16.00 [Cd22, Cd80, Cdkn1a, Ctla4, Havcr2, Ighm, If18, If2ra, If6st, Ins2, Slamf1, Tnfsf9, Tyrobp, Zbtb32]
GO:0002837	regulation of immune response to tumor cell	2.17E-04	2.35E-02	2.14E-14	3.00E-13 [4, 5]	Group13	16.67	4.00 [Havcr2, Klre1, Mr1, Ulbp1]
GO:0022408	negative regulation of cell-cell adhesion	3.23E-04	2.44E-02	2.14E-14	3.00E-13 [4, 5, 6]	Group13	5.03	10.00 [Cd22, Cd9, Ctla4, Dusp22, Dusp3, Havcr2, If2ra, Myadm, Tigit]
GO:0051249	regulation of lymphocyte activation	5.82E-15	9.89E-13	2.14E-14	3.00E-13 [4, 5, 6]	Group13	6.07	36.00 [Cd22, Cd244a, Cd80, Cdkn1a, Ctla4, Dusp22, Dusp3, Fas, Fgr, H2-DMa, Havcr2, Ighm, Ighv1-37, Ighv1-49, Ighv1-80, ...]
GO:2000379	positive regulation of reactive oxygen species metabolic process	1.81E-05	2.36E-03	2.14E-14	3.00E-13 [4, 5, 6]	Group13	7.69	9.00 [Ass1, COX2, Cdkn1a, If18, Itgam, Tnf, Tyrobp, Ulbp1, Xdh]
GO:0001787	natural killer cell proliferation	3.98E-04	3.98E-02	2.14E-14	3.00E-13 [4, 5, 6]	Group13	25.00	3.00 [Cd244a, If18, Slamf1]
GO:0042098	T cell proliferation	1.39E-04	1.98E-02	2.14E-14	3.00E-13 [4, 5, 6, 7]	Group13	6.22	13.00 [Cd244a, Cd80, Ctla4, Havcr2, If18, If2ra, If6st, Ins2, Slamf1, Tnfsf9, Zbtb32]
GO:0051250	negative regulation of lymphocyte activation	4.69E-05	5.81E-03	2.14E-14	3.00E-13 [4, 5, 6, 7]	Group13	6.10	10.00 [Cd80, Ctla4, Dusp22, Dusp3, Fas, Fgr, Havcr2, Il2ra, Tigit, Tyrobp]
GO:0051251	positive regulation of lymphocyte activation	6.28E-13	1.05E-10	2.14E-14	3.00E-13 [4, 5, 6, 7]	Group13	6.73	28.00 [Cd244a, Cd80, Cdkn1a, H2-DMa, Havcr2, Ighm, Ighv1-22, Ighv1-37, Ighv1-49, Ighv1-80, Ighv14-3, Ighv2-2, Ighv3-1, Ighv3-6, Ighv6-6, Ighv7-1, Ighv...
GO:1903037	regulation of leukocyte cell-cell adhesion	1.52E-05	2.00E-03	2.14E-14	3.00E-13 [4, 5, 6, 7]	Group13	4.69	15.00 [Ass1, Cd244a, Cd80, Ctla4, Dusp22, Dusp3, H2-DMa, Havcr2, If18, If2ra, If6st, Slamf1, Tnf, Tnfsf9]
GO:0032729	positive regulation of interferon-gamma production	7.06E-06	9.53E-04	2.14E-14	3.00E-13 [4, 5, 6, 7, 8]	Group13	10.13	8.00 [Cd244a, Havcr2, If18, Klre1, Slamf1, Tnf, Tnfsf9, Ulbp1]
GO:0032814	regulation of natural killer cell mediated cytotoxicity	2.68E-05	3.46E-03	2.14E-14	3.00E-13 [4, 5, 6, 7, 8]	Group13	12.77	6.00 [Cd244a, Fgr, Havcr2, If18, Klre1, Prdx1, Ulbp1]
GO:0042267	natural killer cell mediated cytotoxicity	1.43E-04	1.96E-02	2.14E-14	3.00E-13 [5, 6, 7]	Group13	9.52	6.00 [Cd244a, Fgr, Havcr2, Prdx1, Ulbp1]
GO:0050670	regulation of lymphocyte proliferation	2.83E-06	3.96E-04	2.14E-14	3.00E-13 [5, 6, 7]	Group13	5.76	14.00 [Cd22, Cd80, Cdkn1a, Ctla4, Havcr2, Ighm, If18, If2ra, If6st, Ins2, Slamf1, Tnfsf9, Tyrobp]
GO:0050863	regulation of T cell activation	1.01E-04	1.20E-02	2.14E-14	3.00E-13 [5, 6, 7]	Group13	4.18	14.00 [Cd80, Ctla4, Dusp22, Dusp3, H2-DMa, H2-Oa, Havcr2, If18, Il2ra, If6st, Slamf1, Tigit, Tnfsf9]
GO:1903038	negative regulation of leukocyte cell-cell adhesion	3.79E-04	3.67E-02	2.14E-14	3.00E-13 [5, 6, 7]	Group13	5.80	8.00 [Ass1, Cd80, Ctla4, Dusp22, Dusp3, H2-DMa, H2-Oa, Havcr2, If18, If2ra, Tigit]
GO:0050671	positive regulation of lymphocyte proliferation	2.28E-06	3.23E-04	2.14E-14	3.00E-13 [5, 6, 7, 8]	Group13	7.64	11.00 [Cd244a, Cd80, Cdkn1a, Havcr2, Ighm, If18, If2ra, If6st, Ins2, Slamf1, Tnfsf9]
GO:0030888	regulation of B cell proliferation	2.78E-04	2.83E-02	2.14E-14	3.00E-13 [6, 7, 8]	Group13	8.45	6.00 [Cd22, Cdkn1a, Ctla4, Ighm, Ins2, Tyrobp]

Supplementary Table 2

tex	teff	tmem	tmpec	tslec	temra
1110061A1	FOLR4	ABCA3	2010300C02RIK	44450	ANKDD1A
1500001L2	8430438L05RIK	ABCC4	ABCA3	2610528A11RIK	ARHGEF3
2010200L2	A330103N21RIK	CAMK4	ABI2	4933413G19RIK	BAG2
ABCF2	ATP8A1	DNMT3A	ACPP	AA414768	CASZ1
ACAS2	BC030940	LFNG	ACTN1	ACSS2	CCDC117
ACTB	BCL2	PIGN	ADK	ANXA4	CD244
ACTB	CD2AP	PPM1B	AFF3	APOBEC2	CEP78
ACTR1B	CD69	SNN	AFP	AS3MT	CTBP2
ADD3	CEPT1	TRIM34	AHR	BORCS7	CUL4A
ADD3	D630014A15RIK	ZFP207	ALS2CL	BZRAP1	CX3CR1
ADD3	D630014A15RIK		APOL7B	CAR5B	DGKD
ADNP	DAF1		AR	CCL27A	DOCK5
AGPS	DIRC2		ARHGAP5	CCL3	DPYSL2
AKT2	DIRC2		ARMCX2	CCL6	EMC2
AP1GBP1	DSCR5		ART2B	CCL9	EXOC4
AP3M2	FOXO1		ARXES2	CD244	FAM122B
APEH	FOXP1		AXL	CHIT1	FAM126A
APEH	HSD17B7		BACH2	CMA1	FAM204A
ARCN1	IKBKB		BCL2	CMKLR1	FAM49A
ARFIP1	IRF2BP2		BTBD11	CX3CR1	FAM63B
ARHGAP12	RASA2		BTG1	CYP17A1	FAM69A
ARHGEF18	REV1L		BTLA	DAPK2	FCGR3A
ASH1L	RPL12		CCR6	DIXDC1	FGL2
ATIC	SATB1		CCR7	ESM1	FNIP2
ATP10D	SLC14A1		CCR9	FKBP10	GBP3
ATP11B	TMLHE		CD200	GAS2L3	GNPTAB
ATP8A1	UBE2I		CD2AP	GPX8	GOLIM4
ATP9B	ZFP318		CD55	GSG2	GSAP
AW536289	ZHX2		CD7	GZMA	HDAC8
BCLAF1			CD86	HAVCR2	HENMT1
BFAR			CLYBL	HIST1H2BC	HMGN3
BFAR			CMPK2	IFITM10	HSPB11
BRMS1L			CTLA4	INSL6	IDH1
BTBD14A			CXCR5	ITGAM	IGF2R
BTBD5			DAPL1	KCNJ8	ITGAM
CCNT2			DDR1	KLRA21	JAZF1
CCNT2			DDX60	KLRA3	KIR2DL1
CDC14B			DHX58	KLRE1	KIR2DL2

CDC14B	DOCK9	KLRG1	KIR2DL3
CDC27	ELOVL6	MASTL	KIR2DL4
CDC40	EMB	MDM1	KIR2DL5A
CDH11	EVL	MEGF9	KIR2DS1
CDK5RAP1	F2RL1	MIAT	KIR2DS2
CDS2	FAAH	NHSL2	KIR2DS3
CEPT1	FAM101B	NRARP	KIR2DS4
CEPT1	FAM102A	OSBPL3	KIR2DS5
CHC1L	FAM134B	P3H4	KIR3DL1
COMMD8	FAM169B	PRDM1	KIR3DL2
CPNE3	FAM213A	PRF1	KIR3DL3
CPSF1	FAM26F	RACGAP1	KIR3DS1
CREBBP	FAM46C	RAP1GAP2	KLRC3
CRNKL1	FARSB	RHOQ	KLRD1
CTSO	FCHSD2	S1PR5	KLRF1
CUL3	FCRL1	SMPDL3B	LILRB1
CUL3	FILIP1L	SNX10	LUC7L3
CUL3	GBP6	SORD	LYN
CUL4B	GBP9	SPAG1	LYPLA1
CUL5	GM10406	STMN1	MAPK1
D1ERTD762	GM10409	TCRG-C1	MCTP2
DCTN3	GM9706	TKTL1	MDM1
DDX46	GPR183	TPPP3	METTL3
DNM1L	H2-OA	TRAF3IP1	MINPP1
DNMT3A	H2-OB	TSGA10	MYL12A
EDEM1	HVCN1	TSPAN32	NBN
EDEM1	ID3	ZEB2	NFYB
EEF1E1	IER3		OSGEPL1
ELK4	IFI205		P2RX7
ENC1	IFI44		PALLD
ENTPD5	IFIT1		PCID2
EPS8L1	IFIT3		PKD2
FARSLB	IFITM1		PROK2
FBXL12	IFITM3		PRSS23
FBXL4	IGHM		PSTPIP2
FOXK1	IIGP1		PTCH1
FTHFSDC1	IL7R		PTGDS
FVT1	INPP4B		PTPN12
FXYD6	IPO4		PTPN4
GALNT6	IRAK3		RAB29

GMFB

GMPS

GNAI3

GOSR1

HARSL

HBP1

HIPK3

IAN4

INPP5F

INSIG1

IQGAP1

IVNS1ABP

JAG2

KATNA1

KBTBD2

KCTD4

KIF1B

KLF3

KLF7

KLHL6

LBR

LIN7C

LTA4H

MADD

MAK3

MAN1B

MAN1B

ME2

MLL3

MMD

MSL2

MTA3

MTAP

MYO1G

NAPG

NDUFS1

NEK9

NFATC1

NGLY1

NR2C2

ISG20

JMJD1C

KBTBD11

KLF4

LILR4B

LTB

LY6E

LYPD6B

LYSMD2

MAP7

MS4A4C

MTMR7

MYB

MYC

NCF1

NRP1

NSG2

NT5E

OAS3

OASL1

OASL2

P2RX7

PAK6

PANK1

PARP12

PARP8

PDCD1LG2

PDK1

PFN2

PGS1

PIK3IP1

PIM2

PLEKHA1

POU2AF1

POU6F1

PRMT2

PRSS30

PTGER2

PTPN3

PYDC4

RHEBL1

RNF135

SAMD3

SEC23IP

SLC15A4

SLFN13

SPIN4

SRPK2

SSBP3

STK39

STX8

TBK1

TEP1

TGFBR1

TMEM14A

TMEM64

TTC38

TTC39B

TYROBP

UBE2F

USP28

VRK2

YME1L1

ZBP1

ZEB2

ZNF600

NRF1	QPCT
NT5C3	QTRTD1
NUP205	RAB37
NUP210	RGS10
OSGEP	RNF144A
PAG	RSAD2
PCCA	RTP4
PCOLN3	SELL
PDE4B	SERPINA3G
PEX13	SESN3
PIGT	SGMS1
PIK3C2A	SH2B3
PITRM1	SH3BP5
PLP2	SIRT5
PPARBP	SLAMF6
PPM1B	SLC14A1
PPP2R5A	SLFN1
PPP3CB	SMAD1
PPP4R2	SOCS3
PRKCN	SOCS5
PRPF4B	SPINT2
PRPS2	SSBP2
PURA	ST6GAL1
RAB14	ST8SIA1
RAB3IP	TACC2
RAD51AP1	TAF4B
RAPGEF2	TBC1D4
RASSF2	TCF12
RASSF3	TCF7
RBM3	TDRP
RBM5	TESPA1
RCOR3	THA1
RNF111	TLR1
RNF111	TNFRSF22
RNF125	TNFRSF25
RNMT	TOX
RPL3L	TPD52
RPP14	TRAF1
RUTBC2	TREML2
SATB1	TRIB2

SATB1

SENP2

7-Sep

SERPINB4

SERTAD3

SFRS6

SFRS7

SFXN2

SGK3

SIRT1

SLC35A5

SLC35B3

SLC38A1

SLC39A10

SLC9A6

SLCO3A1

SMAD5

SP100

SRPK2

STAG1

STAT5B

SYAP1

TARDBP

TAZ

TBC1D17

TBPL1

TGFBR1

TIA1

TMOD3

TNRC15

TRIM23

TRP53BP2

TRPM7

TTC13

TTC14

UBE2D3

UCHL5

USP22

USP28

UTRN

TRIB3

TRMT61A

TSC22D1

UGT1A1

USP18

USP53

VAV3

VWA5A

WDR12

WFIKKN2

ZBTB18

ZC3H12D

ZEB1

VDRIP
WDR11
WDR37
WDR37
WDR37
ZC3HDC7
ZFP148
ZFP191
ZFP369
ZNRF2

5.0   Chapter Five – Conclusions

The findings of this work illustrate the effectiveness of HDAC inhibition in subverting tumor resistance during ACT. MS-275 delivery promoted epigenetic and transcriptional reprogramming within the TME to propagate broad immunomodulatory effects that enhanced the therapeutic efficacy of ACT. Combination therapy can therefore allow for the durable regression of highly immunosuppressive solid tumors.

5.1   Tumor resistance can supersede potent therapy-driven antitumor immune responses

In Chapter Two – Four, our implemented ACT protocol utilized adoptive transfer of memory-cultured tumor-specific T cells followed by viral vaccination. As was previously described, antigen re-stimulation following adoptive transfer potentiates the magnitude of tumor regression; indeed, in B16, MC38, and CMS5 7-day old tumors, we were consistently able to achieve complete acute tumor regression. However, tumor resistance superseded the potency of our vaccination platform in determining clinical outcomes. Resistance to immunotherapy manifested as two separate observations: 1) Acquired resistance, wherein tumor relapse occurred from an outgrowth of immune escape variants (Chapter Two and Three) and 2) primary resistance, wherein increased tumor burden facilitated loss of immediate tumor control due to systemic ablation of antitumor immunity (Chapter Four). In each situation, the rapid expansion of transferred cells failed to prevent loss of tumor recognition or prolific induction of T cell exhaustion. These tumor resistance mechanisms arose independently but are not mutually exclusive; as a result, it is difficult to rationally design immunotherapies to target every manifestation of this multifaceted issue.

5.2   TME reprogramming induces a cascade of pro-inflammatory events that disrupt tumor immunosuppression and potentiate antitumor immunity

MS-275 delivery may provide the conceptual framework to comprehensively address multiple mechanisms of tumor resistance that may not have an overlapping origin. Indeed, the work described suggests that epigenetic reprogramming via HDAC inhibition can reprogram the TME to induce a cascade of immunomodulatory changes that are therapeutically beneficial. In Chapter Four, we showed that MS-275 treatment reversed the expression of unique immunosuppressive pathways in growing tumors to facilitate a pro-inflammatory antitumor response. In Chapter Three, the local inflammatory changes were conducive to the expression of immunoactivating signals and, in Chapter Two, polarization

signals. The net benefits of these microenvironmental changes include subverting tumor immunosuppression and broadening the spectrum of antitumor immunity.

In Chapter Two, tumor-infiltrating myeloid cells were highly immunosuppressive; however, during MS-275 delivery, pro-inflammatory signals including IFNγ polarized these cells into cytotoxic effectors with the ability to reject immune escape variants and prevent tumor relapse. In Chapter Three, changes in the local inflammatory milieu promoted the maturation of CD8+ and CD103+ cross-presenting DCs, leading to the activation of endogenous T cell responses that targeted p15E, another tumor-rejection antigen. Simultaneously, the reduction of local immunosuppressive factors such as nitric oxide, arginase, and TGFβ led to partial ablation of Tregs, which were capable of suppressing the magnitude of p15E-specific endogenous T cell responses. In Chapter Four, MS-275 reversed tumor burden-dependent immunosuppressive pathway signatures in the TME to prevent induction of T cell exhaustion in transferred cells and restore tumor control during ACT. Overall, TME reprogramming via HDAC inhibition has interconnecting consequences that ultimately benefit cancer immunotherapies in overcoming a plethora of tumor resistance mechanisms.

5.3 Selective inhibition of Class I HDACs may ubiquitously promote antitumor programmes

Interestingly, despite having broad immunological significance, HDACi have been found to affect only a relatively small number of genes, depending on cell type and HDACi used (*266*). While different HDACi inhibit different HDAC classes, they share some commonalities in immunotherapeutic potential such as lymphocyte activation and antigen presentation (*266*). However, HDACi have shown conflicting effects on immunosuppressive cell accumulation and pro-/anti-inflammation (*266*). As a result, the HDAC inhibition profile likely contributes to the breadth of its subversive potential against tumor immunosuppressiveness. In Chapter Three, we found that MS-275, which selectively inhibits HDAC1 and HDAC3, had a progressive anti-inflammatory effect over time, but showed early and acute pro-inflammatory up-regulation with particular emphasis on Type I IFN and IL-12 production. Additionally, in Chapter Two and Three, IFNγ production was up-regulated at later timepoints. It is possible that these selective inflammatory changes can only be observed with MS-275's HDAC inhibition profile. By extension, potentiation of antitumor immunity to overcome tumor resistance can only be observed using MS-275 or similar HDACi.

5.4 Reinvigoration of endogenous T cells is critical for cancer immunotherapy in resistant tumors

In cancer immunotherapy, endogenously activated CD8+ T cells are a valuable contributor for effective tumor clearance regardless if they play a primary or secondary therapeutic role. In Chapter Three, ACT targeting a defined tumor antigen can be supplemented by immunoactivating endogenous responses against other tumor antigens to broaden the spectrum of antitumor immune attack and prevent tumor relapse. In Chapter Four, viral vaccination was utilized to mobilize endogenous CD8+ T cell responses as a primary means of tumor control. Finally, the effectiveness of PD-1 checkpoint therapy has in recent times been less attributed to curbing the tolerizing effect of tumor-expressed PD-L1 on effector T cell function, but rather to promoting the non-specific expansion of endogenous tumor-infiltrating lymphocytes (*187*).

However, in Chapter Four, we demonstrated that tumor burden facilitates dysfunction within CD8+ T cells through the systemic induction of cellular exhaustion programmes. Exogenously- and endogenously- derived tumor-specific CD8+ T cells alike displayed curbed proliferative potential in response to antigen restimulation and their expansion was quickly ablated. Indeed, for PD-1 checkpoint therapy, extent of tumor burden was shown to be a negative determinant of successful clinical outcomes (*267*). As a result, tumor burden-induced exhaustion is a severe issue for many forms of cancer immunotherapy, not just ACT. By contrast, in Chapter Four we demonstrated that concomitant MS-275 delivery was able to reinvigorate tumor-specific CD8+ T cells to potentiate their proliferation and persistence in large, solid tumors. This suggests that the effectiveness of MS-275 extends to any cancer immunotherapy that relies on the *in vivo* activation of antitumor CD8+ T cell responses.

5.5 The Future of HDAC Inhibition in Cancer Immunotherapy

In Chapter Four, we provided evidence to suggest that MS-275 was able to restore antitumor immune responses and tumor control during ACT. While its effects on tumor normalization confirmed that HDAC inhibition has corrective value in subverting the immunosuppressive pathways being up-regulated within the TME, we also provided some indirect evidence to suggest that HDACi can have a potentiating effect on T cells directly. Indeed, adoptively transferred CD8+ T cells recovered from tumors treated with MS-275 displayed a unique transcriptional signature, marked by enhanced activation-dependent signaling, up-regulated Tim-3 expression (Tim3^{hi}), and terminal effector-like lineage commitment. These characteristics were not expressed in small, ACT-treated tumors in the absence of MS-275 which would have represented a corrective outcome. More importantly,

Tim3^{hi} cells showed higher expression of Ki-67, suggesting a proliferative advantage upon antigen re-stimulation. It may be possible that MS-275 may predispose tumor-specific T cells to better functional outcomes post-activation which in turn may allow them to be inert to the immunosuppressivenes of large, solid tumors. If true, then there would be a decreased requirement for cancer immunotherapies to address the plethora of tumor resistance mechanisms or seek a "silver bullet" solution for tumor resistance. HDAC inhibition can be used instead for "T cell doping" which would theoretically create a more powerful antitumor immune response that is resistant to tumor immunosuppression.

5.6   Desired Post-activation T Cell Phenotypes for Cancer Immunotherapy

It has been traditionally accepted that the adoptive transfer of less differentiated T lymphocytes during ACT allowed for higher proliferative potential compared to fully or terminally differentiated effector cells. Although memory cells do not have comparable cytotoxic potential, the increased magnitude of the antitumor immune response upon *in vivo* reactivation and differentiation of these transferred cells into effector cells is what contributes to therapeutic improvement. While the *in vivo* reactivation step thus plays a major role in determining the fate of transferred T cells, it is unclear whether this process can be sculpted to achieve a desired post-activation effector phenotype.

In Chapter Four, it was demonstrated that reinvigoration of endogenous and transferred tumor-specific CD8+ cells in highly immunosuppressive large tumors correlated with significant up-regulation of Tim-3. Despite being perceived as a checkpoint or co-inhibitory receptor, Tim-3 itself does not inherently express an inhibitory signaling motif. Indeed, our data may suggest that Tim-3 influences T cell activation and differentiation in favor of the short-lived effector lineage. This was functionally associated with higher proliferative potential, persistence, and killing. Moving forward, it would be interesting to examine how epigenetic modulation of memory T cells prior and during re-stimulation influences the expression of Tim-3 and if/how that potentiates T cell function, regardless of tumor burden or resistance.

6.0   Chapter Six – References

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Functional Analysis of Tumor-Infiltrating Myeloid Cells by Flow Cytometry and Adoptive Transfer

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Abstract

The tumor-infiltrating myeloid cell compartment represents a heterogeneous population of broadly immunosuppressive cells that have been exploited by the tumor to support its growth. Their accumulation in tumor and secondary lymphoid tissue leads to the suppression of antitumor immune responses and is thus a target for therapeutic intervention. As it is known that the local cytokine milieu can dictate the functional programming of tumor-infiltrating myeloid cells, strategies have been devised to manipulate the tumor microenvironment (TME) to express a cytokine landscape more conducive to antitumor myeloid cell activity. To evaluate therapy-induced changes in tumor-infiltrating myeloid cells, this paper will outline the procedure to dissociate intradermal/subcutaneous tumor tissue from solid tumor-bearing mice in preparation for leukocyte recovery. Strategies for flow cytometric analysis will be provided to enable the identification of heterogeneous myeloid populations within isolated leukocytes and the characterization of unique myeloid phenotypes. Lastly, this paper will describe a means of purifying viable myeloid cells for functional assays and determining their therapeutic value in the context of adoptive transfer.

Introduction

The tumor microenvironment (TME) is comprised of rapidly proliferating neoplastic cells and a surrounding heterogeneous stromal cell compartment. As growing tumors are often poorly vascularized, the TME is a peripheral site uniquely characterized by hypoxia, nutrient deprivation, and acidosis[1]. To survive in this landscape, tumor stress responses and metabolic reprogramming result in the secretion of soluble factors that promote tissue remodeling

and angiogenesis as well as the selective recruitment of immune cells[2]. As myeloid cells are one of the most abundant type of hematopoietic cells in the TME, there is increasing interest in examining the role of tumor-infiltrating myeloid cells in the TME.

Myeloid cells are a heterogenous and plastic group of innate immune cells including monocytes, macrophages, dendritic cells, and granulocytes. Although they have critical

roles in tissue homeostasis and adaptive immune response regulation, their function can be polarizing depending on the composition of activation signals within the local microenvironment[3]. Tumors take advantage of myeloid cell characteristics through the secretion of soluble factors within the TME. These alternative signals can divert myelopoiesis towards immature differentiation and skew the function of existing tumor-infiltrating myeloid cells[3]. Indeed, myeloid cells within the TME often promote cancer progression and can suppress antitumor immune responses, leading to adverse effects on cancer therapy.

Although therapeutic strategies promoting the depletion of immunosuppressive myeloid cells have been shown to delay tumor growth[4], the lack of target specificity risks the removal of immunostimulatory myeloid cells, which by contrast, aid in the resolution of cancer. These inflammatory myeloid cells can exert profound antitumor effects including direct tumor cell killing and activation of cytotoxic CD8^{+} T cells[5]. Alternatively, strategies normalizing the composition and function of myeloid cells in the TME have shown therapeutic success[6]; however, the biological mechanisms underlying their re-education towards an antitumor phenotype have still not been fully understood. Ultimately, a comprehensive characterization of tumor myeloid cells is necessary for further improvement of cancer therapy.

Unfortunately, reproducible disaggregation of tumors for myeloid cell isolation is challenging. Tumor-derived myeloid cells are sensitive to *ex vivo* manipulation compared to other leukocyte subsets, and the aggressiveness of tumor processing can lead to enzymatic epitope cleavage and reduced viability of recovered cells[7]. The purpose of this method is to provide a reliable means of tumor dissociation to preserve surface marker integrity for analysis

and cellular vitality for functional study. In comparison to tumor-infiltrating leukocyte (TIL) isolation protocols that favor harsher enzymatic mixes to enhance the reproducible release of various cellular subsets, this method favors more conservative enzymatic digestion to maximize myeloid cell recovery. High-level multi-color flow gating strategies are also provided to identify murine tumor myeloid cell subsets for further characterization and/or sorting.

Protocol

NOTE: All animal studies complied with the Canadian Council on Animal Care guidelines and were approved by McMaster University's Animal Research Ethics Board.

1. Tumor harvest and dissociation

1. Inoculate 6-8-week-old, female, C57BL/6 mice intradermally/subcutaneously with 2×10^5 B16 melanoma cells as described by Nguyen *et al.*[8] Allow tumors to grow for 7 days before harvesting.

2. Euthanize the mouse by cervical dislocation while making sure to not disrupt the tumor when doing so. Spray the mouse down with 70% ethanol before harvesting.

3. Using a scalpel and scissors, surgically remove the intradermal/subcutaneous tumor from surrounding tissue (including attached tumor-draining lymph nodes), and place the tumors into a preweighed microfuge tube. Keep on ice.
   NOTE: Conduct tumor harvest in an animal-use biosafety cabinet. Use a 15 mL conical tube for larger tumors.

4. Weigh the tumors, and add 500 µL of RPMI-1640 medium with 10% fetal bovine serum (FBS) to each tube,

using scissors to cut the tumors into small pieces within the tube or in a 6-well plate.

NOTE: The tumor pieces should be small enough to be mixed by an electric pipettor once the digestion medium has been added.

5. Prepare the dissociation mix by dissolving collagenase type IV at 0.5 mg/mL and DNase at 0.2 mg/mL in RPMI-1640 medium with 10% FBS and 5 mM calcium chloride.

NOTE: Dissociation mix must be prepared fresh to maximize collagenase activity.

6. Transfer the minced tumor suspension to a 15 mL conical tube, and add 10 mL of dissociation mix per 0.25 mg of tumor. Place the tube in a temperature-controlled orbital shaker for 30 min at 37 °C with 200 rpm agitation. Neutralize the collagenase activity by adding two volumes of cold RPMI-1640 medium with 10% FBS and 2 mM ethylenediamine tetraacetic acid (EDTA), and refrigerate for 10 min at 4 °C.

7. Briefly vortex and pipette the suspension into a 40 µm strainer on a 50 mL conical tube. Use a syringe plunger and neutralizing media to disaggregate the residual tumor tissue, and wash it through the strainer. Centrifuge the suspension for 5 min (500 × g, 4 °C), discard the supernatant, and resuspend the pellet in phosphate-buffered saline (PBS) with 2% FBS and 1 mM EDTA.

2. TIL enrichment and flow cytometric staining (FACS)

1. To enrich TILs for myeloid cell characterization, use a magnetic cell separation kit designed for biotin-positive selection with biotinylated CD45.2 antibodies according to the manufacturer's instructions (see the **Table of Materials**).

2. Resuspend the cells in 200 µL of FACS buffer (PBS with 0.5% w/v bovine serum albumin (BSA)), and transfer them to a 96-well U-bottom plate.

NOTE: Do not exceed a staining concentration of 1 × 10^8 cells/mL. Adjust the volume, and split samples into multiple wells to compensate for high cell numbers.

3. Centrifuge the plate for 5 min (500 × g, 4 °C), and discard the supernatant. Add 50 µL of Fc block solution (1:200 dilution of purified rat anti-mouse CD16/CD32 [see the **Table of Materials**), in FACS buffer, final concentration of 2.5 µg/mL), and resuspend the cells by pipetting. Incubate for 10 min at 4 °C.

4. Add 50 µL of FACS buffer containing 2x concentration of surface-staining antibody (1:50 dilution of CD45.2, NK1.1, CD11c, F4/80, CD8a, Ly6C, CD11b, CD4, Ly6G) and fixable viability stain (FVS, 1:500 dilution), and mix the cells by pipetting. Incubate for 20 min at 4 °C.

NOTE: Cover the plate with aluminum foil to minimize light exposure. Antibodies should be titrated prior to the experiment to empirically determine the optimal dilution.

5. Wash the cells twice by adding 200 µL of FACS buffer to each well, centrifuging the suspension (5 min, 500 × g, 4 °C), and discarding the supernatant.

6. Add 100 µL of fixation/permeabilization solution (see the **Table of Materials**) to each well, mix the cells by pipetting, and incubate for 20 min at 4 °C.

7. Add 100 µL of 1x permeabilization buffer (see the **Table of Materials**) to each well, centrifuge the plate for 5 min (500 × g, 4 °C), and discard the supernatant.

8. Wash the cells by adding 200 µL of 1x permeabilization buffer to each well, centrifuging the suspension (5 min, 500 × g, 4 °C), and discarding the supernatant.

NOTE: The experiment can be paused overnight after resuspending the cells in permeabilization buffer. Store the sample at 4 °C and protected from light. Resume after briefly mixing the cells before centrifuging.

9. Add 100 µL of permeabilization buffer containing 1x concentration of intracellular staining antibody (1:100 dilution of nitric oxide synthase 2 (NOS2), arginase 1 (Arg1)), and mix the cells by pipetting. Incubate for 20 min at 4 °C.

    NOTE: Cover the plate with aluminum foil to minimize light exposure. Antibodies should be titrated prior to the experiment to empirically determine the optimal dilution.

10. Add 100 µL of 1x permeabilization buffer to each well, centrifuge the plate for 5 min (500 × g, 4 °C), and discard the supernatant.

11. Wash the cells by adding 200 µL of 1x permeabilization buffer to each well, centrifuging the suspension (5 min, 500 × g, 4 °C), and discarding the supernatant.

12. Resuspend the cells in 300 µL of FACS buffer. Filter the sample through a 5 mL round-bottom polystyrene tube with 40 µm strainer cap before performing flow cytometry analysis.

3. Tumor myeloid cell sorting for functional studies

1. After identifying the desired myeloid cell populations by flow cytometry analysis, pre-enrich bulk myeloid cells for sorting with a magnetic cell separation kit designed for CD11b- or CD11c-positive selection according to the manufacturer's instructions (see the **Table of Materials**).

2. Using surface-staining antibodies specific for the desired myeloid cell subsets (1:100 dilution of CD11b, Ly6C, Ly6G), stain the pre-enriched cells as described in steps 2.2-2.5.

    NOTE: Include fixable viability stain to ensure the sorting of live myeloid cells. Do not exceed a staining concentration of 1×10^8 cells/mL. Adjust the volume, and split the samples into multiple wells to compensate for high cell numbers.

3. Resuspend the cells in cold sorting buffer (PBS with 1% w/v BSA, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1 mM EDTA). Filter the sample through a 5 mL round-bottom polypropylene tube with a 40 µm strainer cap. Keep the cells on ice, and cover the tube with aluminum foil.

    NOTE: Adjust the concentration of cells/volume to the desired instrument specification for sorting.

4. Prepare a sample collection tube with capture medium (5 mL round-bottom polypropylene tube containing PBS with 50% FBS).

    NOTE: Coat tubes with 5 mL capture medium overnight prior to sorting. Discard all but 1-2 mL of the capture medium on the next day before running the sample.

5. Modify the sorter instrument settings to decrease sample pressure and prevent perturbations in droplet formation. Equip the 130 µm nozzle tip, and utilize the 10 psi setting. Run the sample at a low flow rate with periodic sample agitation at 100 rpm, ensuring that the deposition of droplets is in the center of the tube. After the sort, keep the sample on ice.

6. Incubate the sample for 10 min at 4 °C. Centrifuge the tube for 5 min (500 × g, 4 °C), discard the supernatant, and resuspend the pellet in the desired medium for functional assays or adoptive transfer.

4. Adoptive transfer of purified tumor myeloid cells

1. Inoculate 6-8-week-old, female, C57BL/6 mice intradermally with 1×10^6 B16 melanoma tumor cells resuspended in 30 µL of PBS.
   NOTE: Inoculate mice in advance such that tumor growth does not exceed 100 mm^3 by the time of adoptive transfer.

2. Resuspend the sorted tumor myeloid cells in PBS with 25 mM HEPES and 1 mM EDTA at a concentration of 2×10^6 cells/mL. Filter the sample through a 5 mL round-bottom polystyrene tube with a 40 µm strainer cap. Keep on ice.

3. Induce and maintain mice under anesthesia with 3% isoflurane. Apply ophthalmic ointment to prevent ocular dryness/injury.

4. Load a 31 G syringe with 50 µL of cell suspension. Dislodge air bubbles by gently flicking the syringe. Clean the injection site using an alcohol swab.

5. Using sterile forceps, lift the skin at the base of the tumor. Insert the needle into the subcutaneous space at a slight upward angle to enter the tumor from below the skin. Use the forceps to pinch the skin surrounding the needle, and slowly dispense the syringe volume. Continue to pinch the skin with the forceps while removing the needle slowly, and use a cotton swab to clean potential leakage.
   NOTE: Proceed with any additional therapeutic treatments if desired.

6. Allow the mice to recover from anesthesia.

Representative Results

The results demonstrate that this method produces a high yield of myeloid cells from solid murine tumors. The preservation of receptor integrity and cellular viability facilitates reliable functional analysis of the desired myeloid subsets. These improvements to myeloid cell isolation allowed the discernment of the changing function of intratumoral myeloid cells upon normalization of the TME with the class I histone deacetylase inhibitor (HDACi), MS-275, during adoptive T cell therapy. TIL isolation protocols typically do not take the steps to maximize myeloid cell yield[9]. As a result, enzymatic digestion is typically too harsh and leads to a loss of sample viability. When processed B16 melanoma tumors were treated with collagenase type I for 1 h (a commonly used condition) before positively enriching for CD45.2$^+$ cells by magnetic selection, the morphology (forward Scatter-area (FSC-A) vs. side scatter-area (SSC-A)) and myeloid cell subgating (CD11b vs. CD11c) indicated that the yield of myeloid cells (CD11b$^+$ or CD11c$^+$) and non-myeloid cells (CD11b$^-$ CD11c$^-$) was extremely low (**Figure 1**). To reduce the potentially excessive specific activity of collagenase, the duration of tumor digestion was decreased to 30 min. While there was a slight improvement in myeloid cell recovery, the overall yield was still low and there was no improvement in non-myeloid cell recovery.

Because lot variation may introduce proteases with specific activity high enough to cause excessive cell death, a separate lot of collagenase type I was requested from a different commercial supplier for comparison. Interestingly, the overall yield of myeloid and non-myeloid cells was much higher, with a slight enhancement in myeloid cell number upon the addition of FBS. Although FBS was added to stabilize the myeloid cells to collagenase-induced damage, this raised

the question as to whether FBS was also neutralizing the tryptic activity of the collagenase type I preparation, which could be impairing cell recovery. As collagenase type I preparations have collagenase, caseinase, clostripain, and tryptic activities[10], to reduce protease exposure, the digestion was attempted using collagenase type IV, which has higher collagenase-specific activity and lower tryptic activity. This condition resulted in a greater increase in myeloid cell yield, with the addition of FBS resulting in the highest yield. Interestingly, collagenase type I and type IV, with or without FBS, did not markedly change the overall yield of non-myeloid cells.

With these optimized enzymatic digestion conditions, leukocytes were isolated from murine B16 melanoma tumors, and flow cytometry was used to phenotype the different myeloid cell populations within the TME based on their expression of surface markers. Tumors were harvested and processed and the leukocytes isolated using a CD45.2 magnetic selection kit. The cells were then stained using a carefully designed panel of cell surface markers (**Figure 2**). The gating strategy described here starts with a morphological assessment of the cells using FSC-A *vs.* SSC-A. This allows the exclusion of cellular debris based on their small size. FSC-H *vs.* FSC-W was used to select single cells and exclude the doublets. Total live leukocytes were then gated based on CD45.2 and viability staining. Lymphocytes were excluded based on NK1.1, CD4, and CD8 staining; note that in BALB/c mice, Asialo-GM1 and/or DX5 can be used to exclude natural killer (NK) cells as NK1.1 is not expressed on BALB/c-derived NK cells. CD11b was then plotted against CD11c to identify tumor-associated dendritic cells (TADCs)/ conventional dendritic cells (cDCs).

Cells that are negative for CD11c represent the bulk myeloid cells, which can be further separated based on Ly6C and Ly6G staining. Cells that express intermediate levels of Ly6C and high levels of Ly6G represent the neutrophils. This population shares the same phenotype as the granulocytic myeloid-derived suppressor cells (G-MDSCs). CD11b$^+$ cells that stain negative for Ly6G, but positive for Ly6C can be divided into Ly6C^{hi}, Ly6C^{int}, and Ly6C^{lo}. Ly6C$^{int/hi}$ cells express lower levels of F4/80 and represent inflammatory monocytes. However, Ly6C^{hi} cells also share the same phenotype with monocytic myeloid-derived suppressor cells (M-MDSCs). Finally, Ly6C^{int} cells express high levels of F4/80 and are usually associated with tumor-associated macrophages (TAMs). While this characterization may not fully identify the myeloid cell subsets of interest, it provides a useful gating strategy to sort myeloid cell populations within the TME for further functional or genomic analyses. Within the context of immunotherapy, adoptive T-cell therapy incorporating epigenetic modifying drugs, such as the class I histone deacetylase inhibitor (HDACi) MS-275, can affect the TME to promote sustained tumor regression, while its absence results in tumor relapse[8].

Although microarray analysis of bulk tumor RNA suggests a role for tumor-infiltrating myeloid cells, this phenotypic characterization did not indicate major surface marker changes during MS-275 treatment[8]. Interestingly, functional markers present in the flow cytometry staining panel identified a certain myeloid cell subset (CD11b$^+$ Ly6C^{hi} Ly6G$^-$) differentially producing nitric oxide synthase 2 (NOS2) and arginase 1 (Arg1), which are implicit readouts of polarizing or divergent functional programming (**Figure 3A**). By sorting CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells from differentially treated, tumor-bearing mice, more extensive functional studies could be performed to understand their role. Using carboxyfluorescein

succinimidyl ester (CFSE) labelling to monitor lymphocyte proliferation[11], sorted myeloid cells derived from naïve and vaccinated mice were found to suppress T cell proliferation *in vitro,* while cells derived from vaccinated + MS-275-treated mice had reduced immunosuppressive function (**Figure 3B**).

Adoptive transfer of these cells revealed that they instead possessed antitumor capability and promoted sustained regression of tumors during vaccination and prolonged mouse survival (**Figure 3C**).
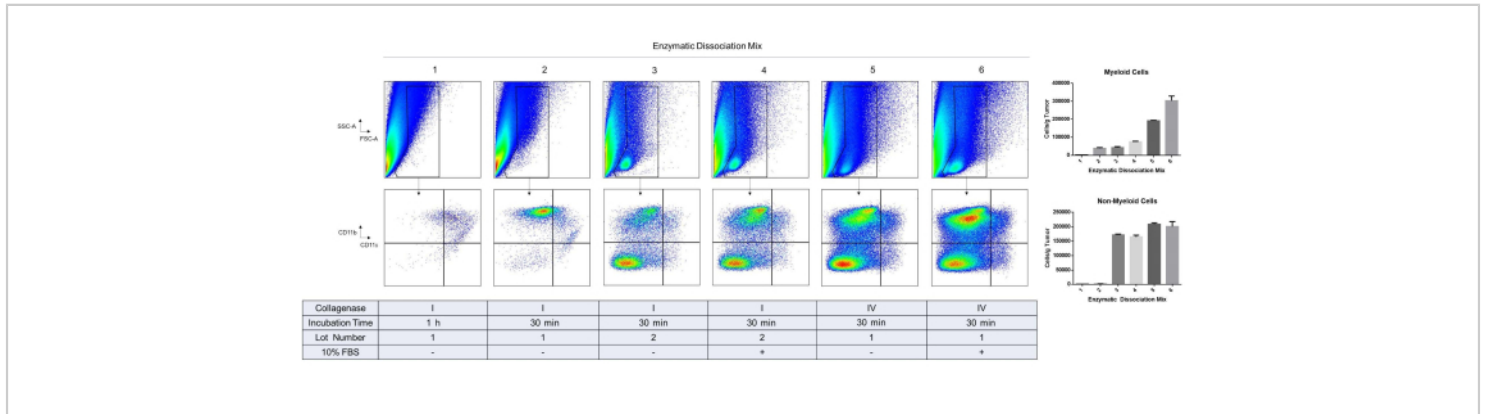

Figure 1: Representative data showing the effectiveness of varying dissociation conditions. In C57BL/6 mice (n=3 per group), untreated intradermal B16F10-gp33 tumors were processed and dissociated under various enzymatic conditions before CD45.2 selection. Shown above are the flow cytometry gating strategies used to demonstrate cellular yield differences (FSC-A *vs*. SSC-A) illustrates tumor-infiltrating leukocyte yield implicitly by cell size/granularity discrimination. (CD11b *vs.* CD11c) allows for the quantification of myeloid (CD11b$^+$ or CD11c$^+$) or non-myeloid (CD11b$^-$ CD11c$^-$) cells. Error is defined by standard error of the mean. Abbreviations: CD = cluster of differentiation; SSC-A = side scatter-area; FSC-A = forward scatter area; FBS =fetal bovine serum. Please click here to view a larger version of this figure.
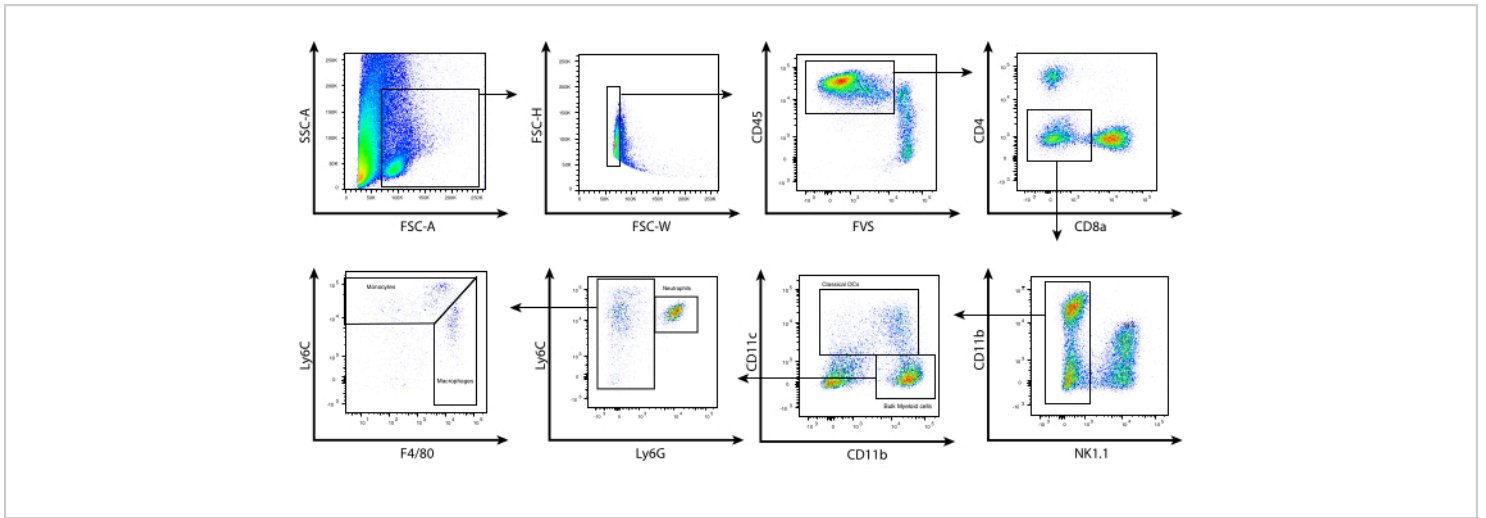
Figure 2: Representative flow cytometry analysis of tumor-infiltrating myeloid cells. Following tumor processing and CD45.2 selection, enriched cells were surface-stained as described in the protocol. Shown above is the gating strategy used to exclude the lymphocytes and identify the individual subsets of myeloid cells within the tumor microenvironment. Debris (SSC-A *vs*. FSC-A) and doublets (FSC-H *vs.* FSC-W) were excluded, and CD45.2$^+$ live cells were determined using the fixable viability stain FVS510 (CD45.2 *vs*. FVS). CD4$^+$, CD8$^+$, NK1.1$^+$ cells were gated out. CD11b$^{hi/lo}$ CD11c$^+$ cells represent cDCs. CD11b$^+$ CD11c$^-$ were then subgated based on Ly6C and Ly6G expression. Three populations were identified: (i) Ly6C^{int} Ly6G$^+$ (neutrophils/G-MDSCs), (ii) Ly6C^{hi} Ly6G$^-$ F4/80$^{lo/int}$ (monocytes/M-MDSCs), (iii) Ly6C$^{lo/int}$ Ly6G$^-$ F4/80^{hi} (macrophages/TAMs). Abbreviations: CD = cluster of differentiation; SSC-A = side scatter-area; FSC-A = forward scatter area; FSC-H = forward scatter-height; FSC-W = forward scatter-width; FVS = fixable viability stain; cDCs = conventional dendritic cells; Ly = lymphocyte antigen; G-MDSCs = granulocytic myeloid-derived suppressor cells; M-MDSCs = monocytic myeloid-derived suppressor cells; TAMs= tumor-associated macrophages. Please click here to view a larger version of this figure.
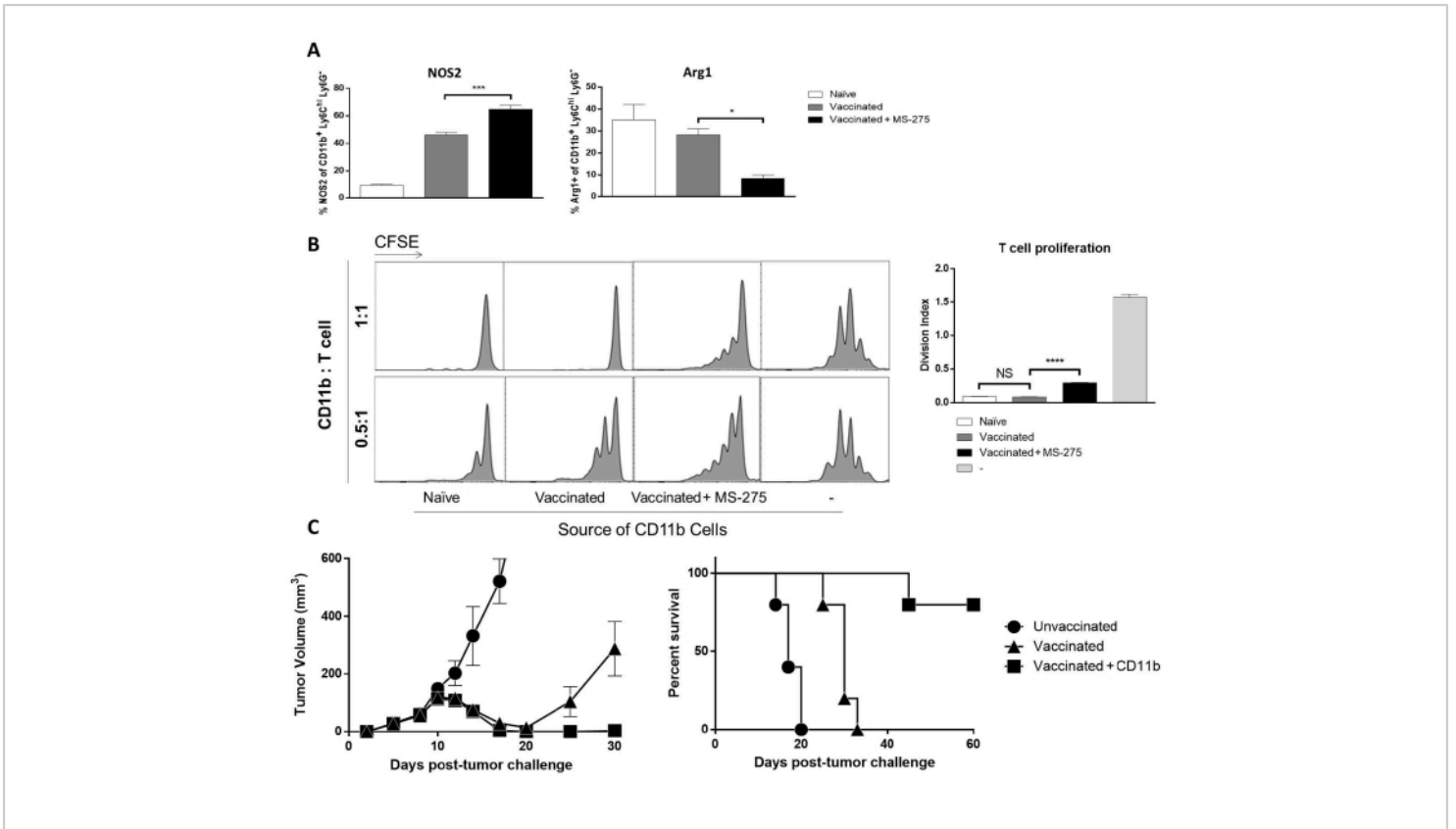
Figure 3: Functional analyses of purified tumor-infiltrating myeloid cells. In C57BL/6 mice (n=3-5 per group), intradermal B16F10-gp33 tumors were either unvaccinated or administered adoptive T cell therapy in the presence or absence of the HDAC inhibitor, MS-275. Five days posttreatment, tumors were processed and positively enriched for CD11b+ cells. The cells were surface-stained to subgate on the desired tumor-infiltrating myeloid cell subset (CD11b+ Ly6Chi Ly6G-). (**A**) These cells were further stained intracellularly for markers that can delineate the polarity of functional activation, and the data are presented as the frequency of CD11b+ Ly6Chi Ly6G- cells that produce NOS2 or Arg1. Alternatively, the surface-stained CD11b+-enriched myeloid cells were sorted to obtain a pure Ly6Chi Ly6G- cell population. (**B**) These purified cells were peptide-pulsed and cocultured with CFSE-labeled, naïve TCR-transgenic T cells in varying ratios. CFSE dilution, as determined by flow cytometry, is shown as a representative histogram and quantified by cellular division index (1:1). (**C**) Separately purified CD11b+ Ly6Chi Ly6G- cells derived from vaccinated + MS-275-treated tumor-bearing mice were adoptively transferred into new tumor-bearing mice (n=5) in conjunction with vaccination and tumor regression, and survival curves were monitored. Error is defined by the standard error of the mean. *** p=0.0004, * p=0.0479, **** p<0.0001. This figure has been modified from Nguyen *et al.*[8]. Abbreviations: CD = cluster of differentiation; HDAC = histone deacetylase inhibitor; NOS2 = nitric oxide synthase 2; Arg1 = arginase 1 ; CFSE = carboxyfluorescein succinimidyl ester; TCR = T-cell receptor; Ly = lymphocyte antigen; NS= not significant. Please click here to view a larger version of this figure.

Discussion

Although tumor-infiltrating myeloid cells exist in varying activation and differentiation states within the tumor, several subsets have been identified including tumor-associated DCs (TADCs), tumor-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs)[12]. Unfortunately, the overlapping expression of cell-surface markers used to identify these myeloid cell subsets makes it currently challenging to phenotypically differentiate tumor myeloid cells from other myeloid cells[13]. Similarly, therapy-induced phenotypic changes in tumor-infiltrating myeloid cells may not be easily observed with existing myeloid antibody staining panels. Taken together, the insufficiency of unique surface markers complicates the understanding of myeloid cell biology. To delineate natural, tumor-driven, and therapy-influenced myeloid cells, the evaluation of myeloid cells subsets must be done according to their function in addition to their phenotypic characteristics.

The methods described herein to harvest and dissociate tumor tissue allow for the isolation of myeloid cells with preserved viability and surface marker integrity. As tumor-infiltrating myeloid cells are sensitive to *ex vivo* manipulation[7], the emphasis of the protocol is on less aggressive mechanical and enzymatic dissociation. However, depending on the tumor tissue type, different samples require variably aggressive treatment to generate a single-cell suspension. For more collagen-rich tumor models (*i.e.,* CMS5 fibrosarcoma), a syringe plunger could be used in addition to scissors to more fully mechanically disaggregate tumor tissue prior to enzymatic treatment. Conversely, less collagen-rich tumor models (*i.e.,* B16 melanoma) do not necessarily require enzymatic treatment. As demonstrated in these results, the type of collagenase preparation and the lot from which it was

derived can significantly influence the variety and potency of proteases that cells are exposed to during enzymatic dissociation.

While non-myeloid leukocytes (*i.e.*, lymphocytes) do not seem to be as sensitive, these data suggest that myeloid cells, in particular, may be very susceptible to excessive protease exposure (**Figure 1**), resulting in damage to membrane proteins and decreased viability. As a result, the use of collagenase type IV over collagenase type I is recommended for its increased collagenase-specific proteolytic activity and the incorporation of FBS into the digestion mix to neutralize residual tryptic activity. Furthermore, utilizing purified collagenase products, as opposed to crude preparations from commercial sources, can increase the reproducibility of cellular recovery. Once the digestion is complete, the addition of EDTA and incubation at 4 °C is mandatory to chelate the Ca^{2+} ions and reduce the temperature needed for collagenase activity[14].

To characterize myeloid cell infiltrate within a heterogeneous TIL population, high-level gating strategies have been provided (**Figure 2**). The polychromatic flow cytometry panel should be designed in such a way that 1) dead and non-myeloid cells are excluded, 2) myeloid cell subsets can be identified, and 3) altered functionality can be implicitly observed across groups of samples (**Figure 3**). Depending on the number of myeloid cell subsets of interest and the depth of functional characterization, these criteria may not be fully accommodated within one staining panel. To free up channels for the panel, markers could be strategically pooled into a single dump channel. Although this method allows for high-dimensional analysis of tumor-infiltrating myeloid cells, it is still constrained by a relatively low parameter limit. More recent technologies using heavy metal reporter ions, such as

mass cytometry, enable up to 40 independent parameters within a single panel, which will allow better study of the cellular and functional diversity of myeloid cells within the TME[15].

However, the cost of instrumentation limits the ubiquity of its use. Flow cytometry is more accessible and produces reliable characterization data, although the mixing and matching of markers across multiple panels necessitates panel validation as an extremely important step prior to the experiment. Upon identification of tumor-infiltrating myeloid subsets of interest, several researchers[15] describe techniques to sort these cells for functional assays and studies. Sorted cells can be reliably analyzed for their T cell-suppressive capacity *in vitro* as well as their antitumor capability *in vivo* after adoptively transferring them into mouse models of tumor relapse (**Figure 3**). Overall, the significance of the described methods is that, in comparison to other TIL isolation protocols, the conditions of tumor harvest, dissociation, cell enrichment, and cell sorting were tailored towards reproducibly acquiring a high yield of the myeloid cell compartment over all other leukocytes and without compromising their function.

Disclosures

No conflicts of interest declared.

Acknowledgments

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COMMUNICATIONS BIOLOGY

De novo necroptosis creates an inflammatory environment mediating tumor susceptibility to immune checkpoint inhibitors

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Cancer immunotherapies using monoclonal antibodies to block inhibitory checkpoints are showing durable remissions in many types of cancer patients, although the majority of breast cancer patients acquire little benefit. Human melanoma and lung cancer patient studies suggest that immune checkpoint inhibitors are often potent in patients that already have intratumoral T cell infiltrate; although it remains unknown what types of interventions can result in an intratumoral T cell infiltrate in breast cancer. Using non-T cell-inflamed mammary tumors, we assessed what biological processes and downstream inflammation can overcome the barriers to spontaneous T cell priming. Here we show a specific type of combination therapy, consisting of oncolytic virus and chemotherapy, activates necroptosis and limits tumor growth in autochthonous tumors. Combination therapy activates proinflammatory cytokines; intratumoral influx of myeloid cells and cytotoxic T cell infiltrate in locally treated and distant autochthonous tumors to render them susceptible to immune checkpoint inhibitors.

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The immune system maintains homeostasis through antigen-specific elimination of tumor cells and preventing tumor growth[1]. Successful generation of T-cell-mediated immunity involves tumor antigen presentation, a process fine-tuned by co-stimulatory/inhibitory signals and cytokines that regulate the activation of tumor-specific naive T cells to become effector T cells[2]. Various types of immunotherapies were developed through the modulation of immune regulatory mechanisms governing antitumor T cells. Of particular interest, the use of monoclonal antibodies to block inhibitory T-cell receptor signaling, collectively called immune checkpoint inhibitors (ICI), are integrated into patient standard of care to treat multiple cancer types[3–6]. However, the overall proportion of patients that respond to ICI is remarkably low owing to numerous adaptive resistance mechanisms orchestrated by tumors to defeat intrinsically developed antitumor immunity[7,8]. There is no definitive biomarker of ICI yet, although correlates such as, neoantigen load[9,10], expression of inhibitory checkpoint ligands, interferon[11] and proinflammatory signatures[12], were showed to predict efficacy of ICI.

Certain types of cell death modalities emit proinflammatory signals to stimulate tumor antigen presentation and intratumoral T-cell infiltration. Cell death stimuli initiated by anthracyclines[13], photodynamic therapy[14], and oncolytic viruses[15–17] elicit premortem tumor cell endoplasmic reticulum stress leading to calreticulin exposure[18] along with release of ATP[19–21] and high mobility group box 1[20,21], resulting in potent antigen presentation[22] and adaptive immunity. Although cell death inducers were extensively studied in immune-responsive transplanted tumors[13–22], it remains largely unknown what types of combination therapies, cell death modalities, and downstream inflammatory signals can heat-up cold and immunosuppressive tumor types such as breast cancer[23,24].

Genetically engineered mouse models of breast cancer have assisted the functional characterization of biological processes implicated in human breast cancers although they have not been extensively used to develop breast cancer immunotherapies[25,26]. Autochthonous tumors driven by overexpression of the rat oncogene Neu (NeuT) in BALB-NeuT mice are highly immunosuppressive and fail to spontaneously prime T cells or after immunogenic chemotherapy[23]. Consistent with previous reports[25,26], here we report that monotherapies of chemotherapy and oncolytic virus had significant anticancer activity. The list of ICD-inducing agents is expanding although their immunomodulatory effects when applied in combination are understudied. A combined administration of oHSV-1 and Mitomycin-C extends survival of autochthonous tumor-bearing mice. This immunogenic combination therapy relies on necroptosis to activate immune-dependent anticancer effect during therapy and prophylactic vaccination. Ablation of necrosome formation in the context of therapy and dying tumor cell vaccination results in loss of efficacy. Further characterization of the tumor immune landscape shows that immunogenic therapy activates desirable inflammation and cytokine/chemokine secretion to elicit intracellular T-cell infiltration thereby rendering non-immunogenic tumors susceptible to ICI.

Results

A combination of oncolytic virus and chemotherapy extends survival of autochthonous tumor-bearing mice.

We previously used TUBO cells (originally isolated from a spontaneous mammary tumor of BALB-NeuT mice[25]) in transplantation experiments to show that oHSV-1 induces markers of ICD and prolongs survival in 50% of tumor-bearing mice[27,28]. To further evaluate the systemic antitumor effects of locally applied ICD-inducing

agents (oHSV-1 and chemotherapeutics) in autochthonous tumors we utilized mouse models of breast carcinogenesis driven by overexpression of the rat oncogene Neu (NeuT)[25,26]. Female mice that express NeuT oncogene acquire highly aggressive mammary tumors[25,26]. In all of the studies, we treated the first palpable tumor and monitored the total tumor burden arising in all of the mammary glands. Although Mitomycin-C (Mito) and Mitoxantrone (MTX) monotherapies slowed tumor growth (Fig. 1b), no monotherapy had significant tumor controlling or survival benefit on their own (Fig. 1a–c). We previously showed that in transplantable TUBO tumors combination therapy of oHSV-1 and MTX results in 69% survival of tumor-bearing mice[29]. Thus, we hypothesized that the combined administration of oHSV-1 with chemotherapeutics would provide multiple danger and pathogen signals for enhanced antitumor effect[27,29–33]. We combined oHSV-1 with ICD-inducing (MTX or Doxorubicin; Dox) or a non-ICD-inducing chemotherapy (Mitomycin-C). Administration of oHSV-1 in combination with MTX or Dox had no significant tumor controlling effect (Fig. 1d) or survival benefit (Fig. 1e). Rather the combined application of oHSV-1 with Mito had a statistically significant tumor controlling effect in BALB-NeuT mice (Fig. 1e, f).

Necroptosis is essential for the therapeutic effect of immunogenic therapy.

To investigate the mechanism by which Mito+oHSV-1 exerts anticancer effect, day 6 tumors were harvested and stained for markers of virus replication, endothelial cell damage, and cell death. Monotherapies of Dox and Mito had insignificant cell death and endothelial cell damage (Fig. 2a–d). Compared with oHSV-1 and Dox+oHSV-1, Mito+oHSV-1 showed a higher level of intratumoral virus replication (Fig. 2a, b) despite in vitro addition of Mito or Dox reducing oHSV-1 replication (Supplementary fig. 1). Mito+oHSV-1 did not alter CD31 levels within tumors (Fig. 2a, c). All the monotherapies (oHSV-1, Dox, Mito) did not affect the level of apoptosis as measured by cleaved caspase 3 (Fig. 2a, d) and necroptosis as assessed by the level of p-MLKL (Fig. 3a). However, Mito+oHSV-1 therapy showed significantly higher apoptosis (Fig. 2a, d) and necroptosis (Fig. 3b, Supplementary fig. 2a, b).

Next, we evaluated which type of cell death is essential for the therapeutic effect of Mito+oHSV-1. Inhibition of caspases using a pan-caspase inhibitor partially offset the therapeutic benefit (Fig. 3c), whereas inhibition of necrosome formation using systemic Nec-1s administration[34] significantly abolished the anticancer effect of Mito+oHSV-1 (Fig. 3c). Nec-1s+Mito+oHSV-1 treatment did not exert a statistically significant change in total lymphocyte numbers (CD4+ and CD8+) in the periphery compared with PBS control and Mito+oHSV-1 treatments (Supplementary fig. 3a, b). An established method for evaluating immune stimulating and antitumor effects of an ICD inducer is to administer in vitro killed cells as a vaccine[35,36]. TUBO cells were previously characterized murine tumor cell line established from spontaneous mammary tumors of BALB-NeuT mice[25]. BALB/NeuT mice vaccinated with dying TUBO cells treated with Mito+HSV-1, but not Dox+oHSV-1, showed significant delay in the growth of spontaneous tumors (Fig. 3d). Moreover, Nec-1s treatment or deletion of MLKL (via CRISPR/Cas9-mediated knockout; supplementary fig. S4) abrogates the antitumor effects of the vaccine (Fig. 3e). Overall, these results suggest that necroptosis is an integral part of Mito+oHSV-1 mediated immunotherapeutic effect.

Combination therapy displays a proinflammatory cytokine signature and T-cell-mediated anticancer effect.

To evaluate the cytokine/chemokine milieu during therapy, we analyzed the level
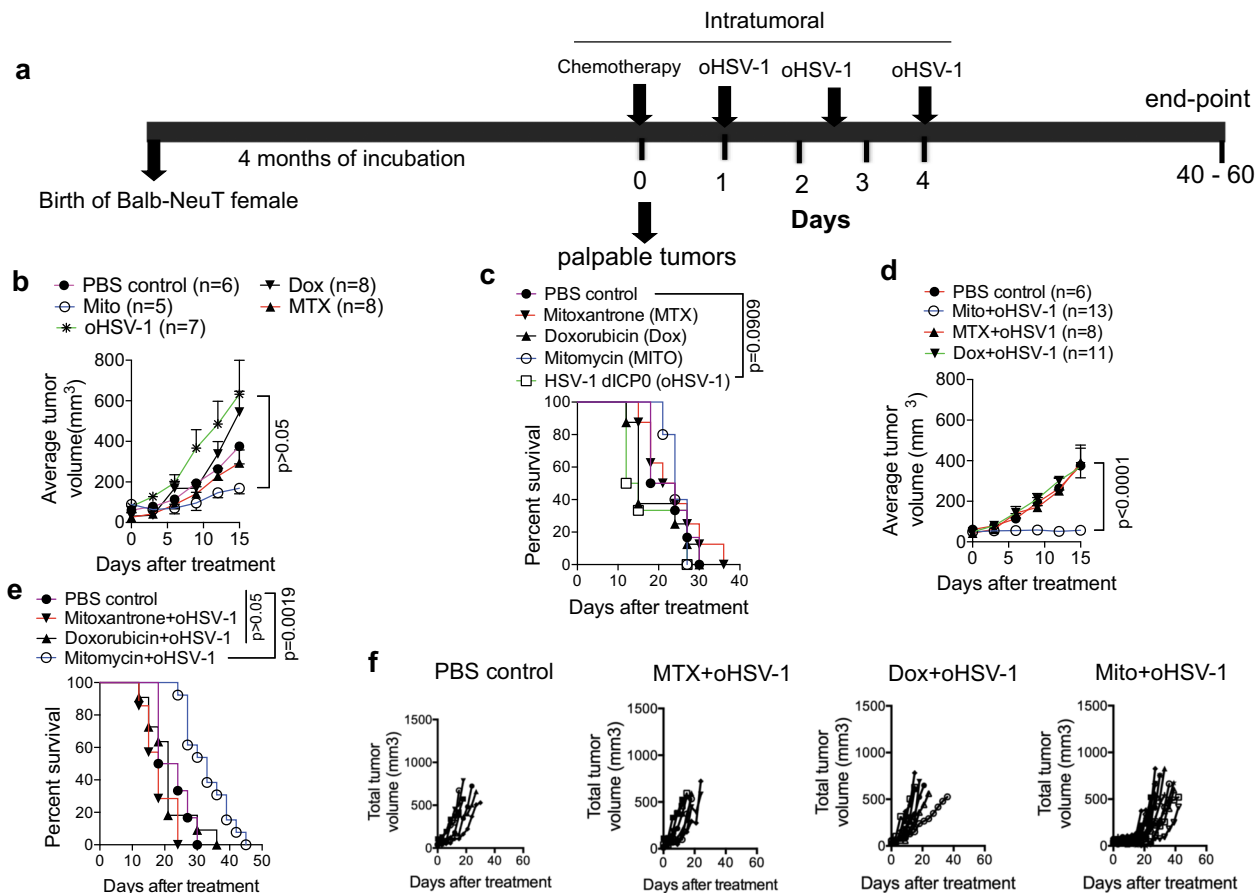
Fig. 1 ICD-inducing monotherapies fail to limit tumor growth while combination therapy of Mito-C+oHSV-1 extends survival. a Outline of treatment regimens for chemotherapy and oHSV-1 in autochthonous tumors of BALB-NeuT mice. **b, c** Treatment with ICD-inducing monotherapies (MTX, Dox, oHSV-1) and non-ICD-inducing (Mito) neither limit tumor growth nor extend survival of tumor-bearing mice. **d, e** Among the various combinations of oHSV-1 and chemotherapy, Mito+oHSV-1 limits tumor growth and extends survival of tumor-bearing BALB-neuT mice. **f** Individual mice total tumor volumes as a proxy for tumor burden of BALB-NeuT mice treated with various combination therapies. Quantitative data are mean±standard deviation of total tumor volumes (Kruskal–Wallis test) **b, d** and Kaplan–Meier survival (Log-rank, Mantel–Cox test) **c, e**. All the data presented in this figure are pooled from at least two independent experiments done at different times.

of 31 cytokines/chemokines in tumor homogenates in treated and control mice. Untreated BALB-NeuT tumors display higher levels of immunosuppressive cytokines (IL-4 and IL-10) that are significantly downregulated after oHSV-1, Dox+oHSV-1, and Mito+oHSV-1 treatment (Supplementary fig. 5a). Other cytokines commonly upregulated by multiple therapies include Eotaxin, IL-6, and IL-15 (Supplementary fig. 5b). Cytokines/chemokines that did not show significant change include GCSF, granulocyte-macrophage colony-stimulating factor (GMCSF), IL-1b, IL-2, IL-3, IL-7, IL-9, IL-12, IP-10, CXCL-1, MIG, and VEGF (Supplementary fig. 5c). Proinflammatory cytokines (RANTES, TNF-alpha, IL-1a, IL-13, IL-17, LIF, MCSF) and chemokines (Macrophage inhibitory protein 1a (MIP1a) (CCL3), MIP1b (CCL4), Monocyte chemoattractant protein 1) (Fig. 4a) were differentially up-regulated in Mito+oHSV-1 treated tumors. Consistent with the chemokine signature, Mito+oHSV-1 treated tumors showed higher Ly6G+ (Fig. 4b, c) and F4/80+ (Fig. 4d, e) immune cell influx 96 h after start of treatment. When Nec-1s is administered along with Mito+oHSV-1, the Ly6G+ population is significantly reduced (Fig. 4b, c). Moreover, both Mito+oHSV-1 treated and untreated control tumors did not show intratumoral Treg infiltration (Supplementary fig. 6). Overall, these studies show that de novo necroptosis activation results in intratumoral myeloid cell infiltrate.

Given the inflammatory cytokine milieu and the influx of myeloid cells during Mito+oHSV-1, we hypothesized that these early events may contribute to cytotoxic T-lymphocyte infiltration within tumor lesions. To evaluate the intratumoral infiltration of CD8+ T cells, we isolated day 10 treated and untreated tumors (within the same mouse) for immunohistochemistry (IHC) staining of CD3/CD8 surface markers. While untreated tumors of BALB-NeuT mice lack T-cell infiltration, Mito+oHSV-1 treated and distant untreated tumors within the same mouse show higher levels of CD3+CD8+ T-cell infiltration (Fig. 5a, b). Quantification of the IHC signal shows that distant tumors are significantly infiltrated with CD3+CD8+ T cells. However, Mito+oHSV-1-treated tumors by day 10 show mostly dying tumor cells and loss of tumor architecture. As a result, the IHC quantification did not show significant T-cell infiltration in treated tumors. Inhibiting necrosome formation during Mito+oHSV-1 results in significant reduction of cytotoxic T-lymphocyte infiltration in distantly located untreated tumors (Fig. 5a, b). The abscopal effects of Mito+oHSV-1 is not an outcome of virus replication in untreated tumors, as there is no detectable virus replication in distant tumors following in vivo imaging of virus replication and IHC staining for HSV-1 structural proteins[27] (Supplementary fig. 7).
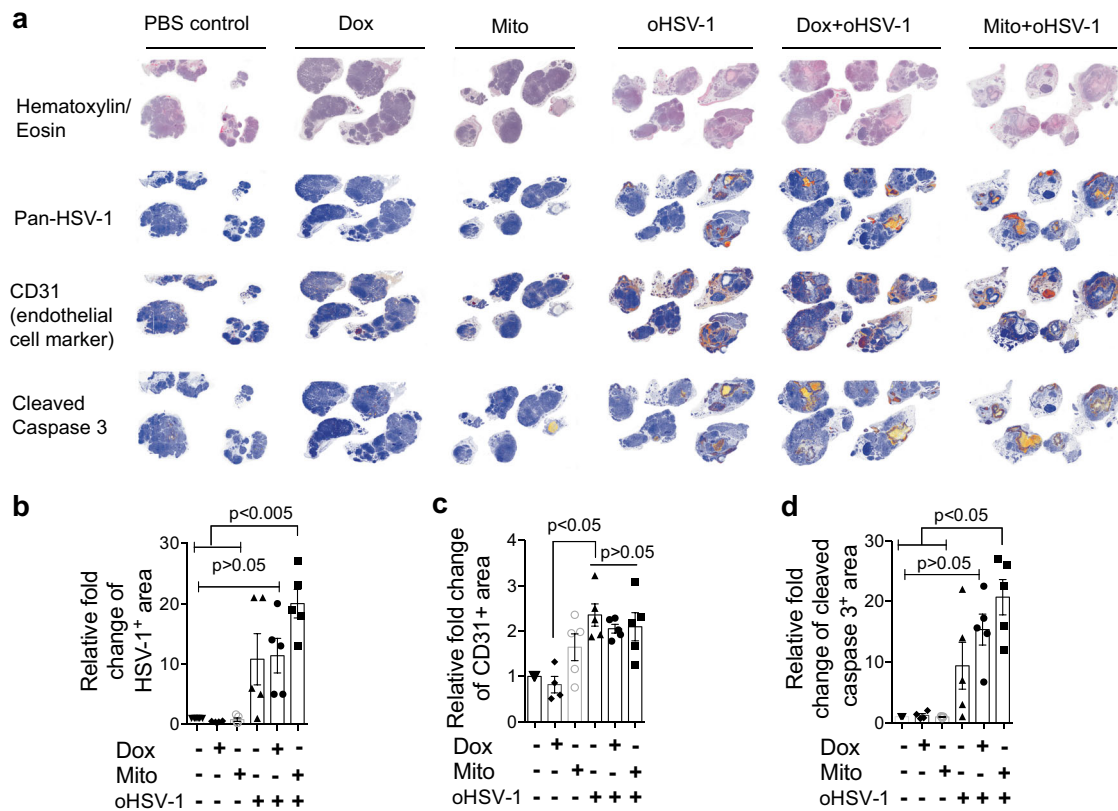
Fig. 2 Mitomycin+oHSV-1 combination increases virus replication and tumor cell death. a Visualization of oHSV-1 replication (using a polyclonal antibody that detects major HSV-1 structural proteins), vascular damage (using endothelial cell marker CD31), apoptotic cell death (using cleaved caspase 3) on day 6 after start of treatment. **b–d** Immunohistochemistry positive signal were quantified and plotted. Quantitative data are mean±standard deviation of fold changes of positive signal relative to PBS control group. Kruskal–Wallis test with Dunn's multiple comparison as a post hoc test was used to test statistical significance.

To evaluate the importance of T lymphocytes in the therapeutic outcome of Mito+oHSV-1, we conducted survival studies in the presence or absence of CD4$^+$ and CD8$^+$ T cells. Depletion of T cells (CD4$^+$ and CD8$^+$) using monoclonal antibodies reduces the efficacy of Mito+oHSV-1 indicating that the therapeutic efficacy of Mito+oHSV-1 is T-cell mediated (Fig. 5c).

Therapy-induced necroptosis renders autochthonous tumors susceptible to ICIs. The extent of tumor-infiltrating CD8$^+$ T-cell density is significantly associated with improved clinical outcome[37], although tumor-infiltrating CD8$^+$ T cells isolated from patient tumors often display exhausted phenotypes characterized by an impaired ability to secrete effector cytokines, high expression of inhibitory receptors and altered signaling pathways[33]. Immunological interventions to block inhibitory receptors at the priming (anti-CTLA-4) and effector (anti-PD-1) stages are able to reinvigorate exhausted T cells, leading to significant therapeutic benefit in multiple human cancer types[3,4,38–40]. Human studies have found that patients harboring non-T-cell-inflamed tumors fare worse during ICI. Moreover, combined anti-CTLA-4+anti-PD-1 therapy has enhanced efficacy that exceeded either of the anti-CTLA-4 and anti-PD-1 monotherapies in melanoma patients[4]. Overall, breast cancer has low mutational load and it has not been intensively investigated for its susceptibility to clinical immunotherapies[41]. ICI using anti-CTLA-4+anti-PDL-1 is undergoing clinical evaluation in breast cancer patients[42]. ICD-inducing agents have a potential to be combined with ICI to treat immunologically cold breast cancer tumors.

As Mito+oHSV-1-mediated necroptosis induce systemic intratumoral cytotoxic T-lymphocyte infiltration, we hypothesized that this treatment may synergize with clinically used anti-CTLA-4+anti-PDL-1 ICI therapy[4]. Untreated tumors of BALB-NeuT mice lack intratumoral cytotoxic T lymphocytes and thus do not benefit from ICI (anti-CTLA-4 and anti-PD-L1). Moreover, Dox+oHSV-1+ICI does not show significant survival or tumor control (Fig. 6a–c, e). However, administration of ICI with Mito+oHSV-1 shows significant anticancer effect evidenced by lower tumor volume (Fig. 6a), tumor multiplicity (Fig. 6b) and prolonged survival of tumor-bearing Balb-NeuT mice (Fig. 6c). Individual mouse tumor plots of Mito+oHSV-1+ICI-treated mice show that a fraction of mice were tumor free for 40 days before relapse (Fig. 6e). Consistent with the essential role of necrosome in Mito+oHSV-1 anticancer immunity, administration of Nec-1s during the triple combination therapy abrogates the survival benefit of Mito+oHSV-1+ICI (Fig. 6d).

Discussion

Tumors hide from immune recognition and subsequent attack by evading immune-stimulatory cell death[43], downregulating MHC expression and secreting suppressive chemokines and cytokines[44]. As a result, cell death insults that activate premortem stress and subsequent expression of immunomodulatory molecules can be one of the ways to reinstate immunosurveillance by promoting tumor antigen uptake and presentation[45]. Using autochthonous tumors, our study demonstrates that (1) combining different classes of "bona fide" ICD-inducing agents (oncolytic virus and chemotherapy)
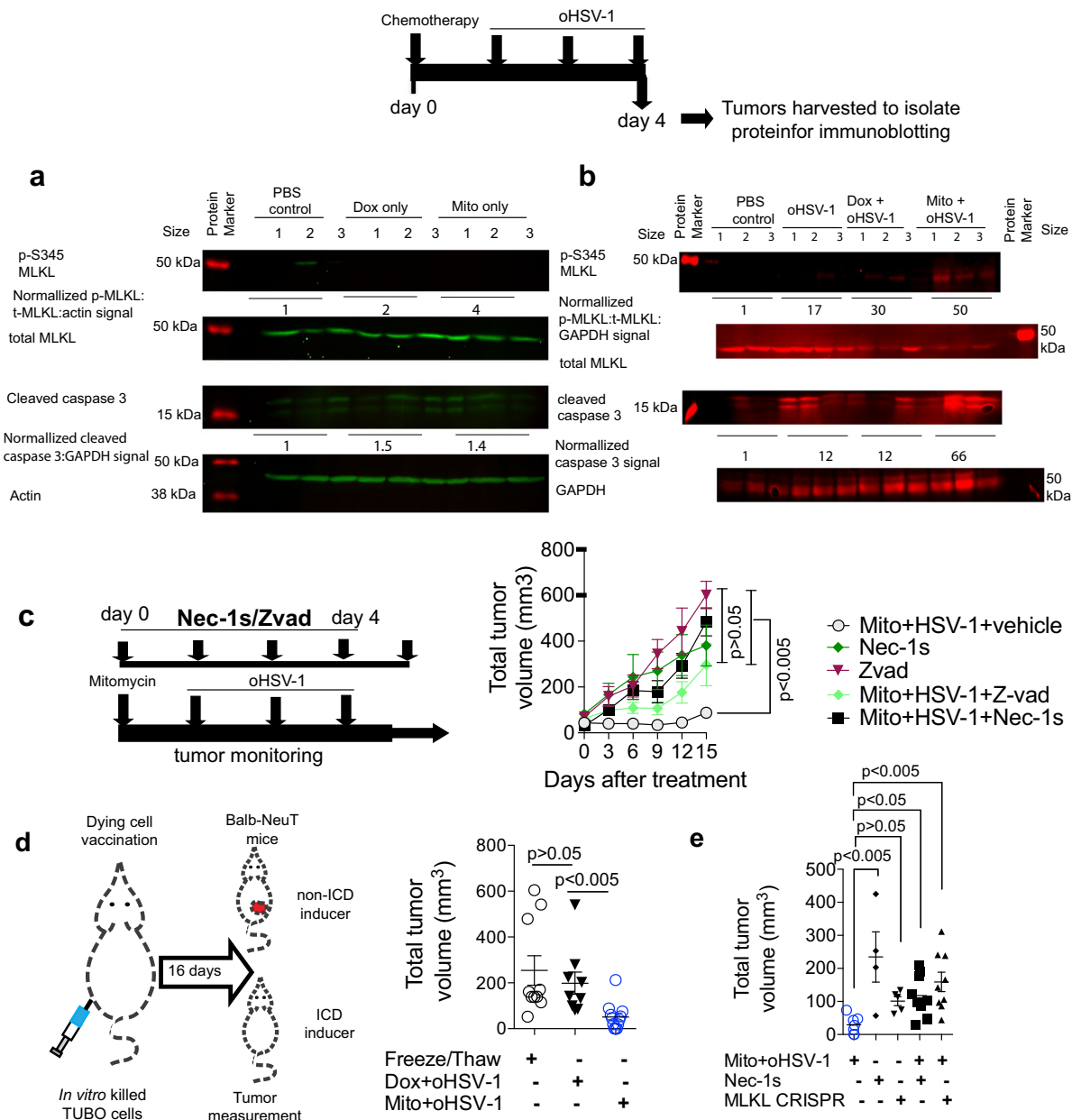
Fig. 3 Therapeutic efficacy of immunogenic combination therapy relies on necroptosis. a, b Immunoblots of protein harvested from tumors at 96 h after start of Mito+oHSV-1 treatment show higher normalized p-MLKL and cleaved caspase 3 fluorescence intensity ($n = 3$ per treatment, labeled 1–3). Uncropped images are displayed in Supplementary Fig. 3. **c** Average total tumor volume of mice treated with Mito+oHSV-1 along with daily intraperitoneal administration of Nec-1s or Pan-Caspase inhibitor (ZVad-FMK) from day 1 to day 5. Quantitative data are mean±standard deviation from $n = 5$ mice per treatment group. **d** Average tumor burden 16 days after vaccination of naïve BALB-NeuT mice with in vitro killed TUBO cells (a cell line isolated from BALB-NeuT spontaneous tumors) (by freeze/thawing ($n = 10$) or 24 h after treatment with Mito+oHSV-1 ($n = 12$) or Dox+oHSV-1 ($n = 9$)). Dying TUBO cells were used to vaccinate 110 days old BALB-neuT mice with small palpable tumors. Quantitative data are mean±standard deviation. **e** Total tumor volume of BALB-NeuT mice 16 days after vaccination with dying wild type TUBO cells 24 h after Mito+oHSV-1 ($n = 7$), Mito+oHSV-1+Nec-1s ($n = 9$), and Nec-1s treatment ($n = 4$). Alternatively TUBO cells lacking MLKL (Supplementary fig. 4) were used for vaccination either 24 h after treatment with Mito+oHSV-1 ($n = 9$) or freeze/thawing ($n = 5$). Quantitative data are mean±standard deviation. Statistical significance of mean tumor volumes were tested using Kruskal–Wallis test with Dunn's multiple comparison as a post hoc test.

provides a rational combinatorial therapy that activates antitumor immune response in necroptosis dependent fashion (2) immunogenic Mito+oHSV-1 therapy activates an inflammatory immune contexture associated with proinflammatory cytokine/chemokine signature and influx of myeloid cells and cytotoxic T-lymphocyte infiltrate in distant tumors, and (3) therapy-induced necroptosis is an essential aspect of Mito+oHSV-1 therapy to render tumors susceptible to ICIs.

Autochthonous tumors are highly immune suppressive and lack an intratumoral T-cell infiltrate and thus often monotherapies fail to show immunotherapeutic effect. We found out that locally administered monotherapies of Dox, Mitoxantrone, Mitomycin-C[13], and oHSV-1[27] fail to extend survival of autochthonous tumor-bearing mice. In support of this, a previous study showed that the anticancer effect of chemotherapies in autochthonous tumors of BALB-NeuT and conditional
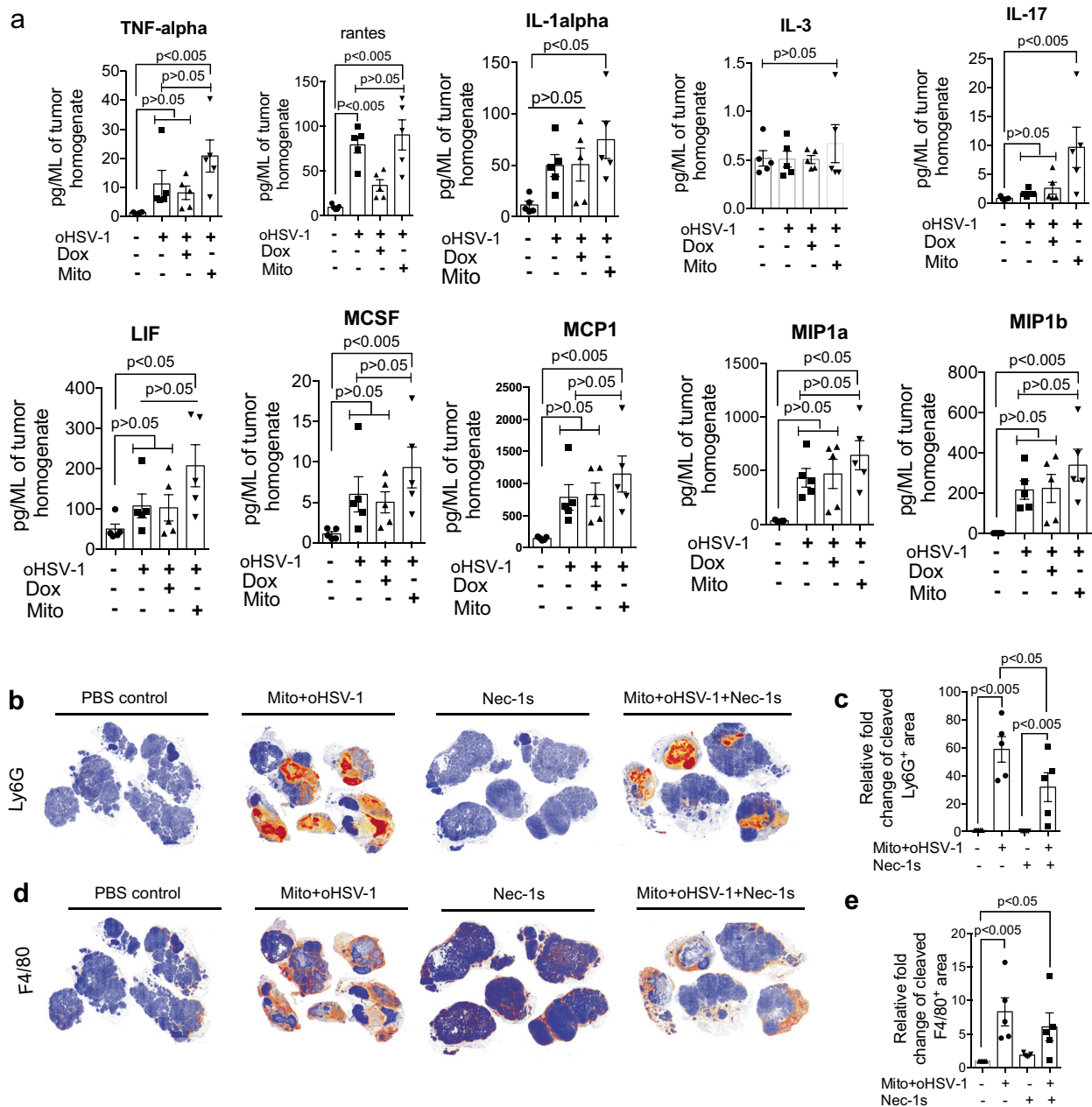
Fig. 4 Mito+oHSV-1 shows necrosome-dependent proinflammatory signature. a Mito+oHSV-1 treated mammary tumors were harvested 96 h post start of treatment and tumor homogenates assayed for expression of 31 cytokines and chemokines. **b–e** Therapy-induced inflammation during Mito+oHSV-1 shows necrosome-dependent Ly6G+ infiltration. Autochthonous tumors of BALB-NeuT mice treated with Mito+oHSV-1 and/or Nec-1s were harvested 96 h post start of treatment and stained for Ly6G **b**, **c** and F4/80 **d**, **e**. Quantitative data are mean±standard deviation. Statistical significance was tested with one-way ANOVA or non-parametric Kruskal–Wallis test with Dunn's multiple comparison as a post hoc test **a**, **c**, **e**. All the experiments in this figure are generated from five mice per treatment group.

$K14cre$; $Cdh1^{flox/flox}$; $Trp53^{flox/flox}$ mice operates independent of the adaptive immune system[23]. However, both of the chemotherapies were able to show intratumoral CD3+ T-cell infiltrate, although the absence of lymphocytes in Rag−/− mice did not change the outcome of mammary tumorigenesis. Among the three combination therapies we tested, only Mito+oHSV-1 treatment showed adaptive immune response in a therapeutic and prophylactic vaccination setting. In support of this findings, a systemic immunogenic combination chemotherapy (cyclophosphamide–oxaliplatin) in $Kras^{LSL-G12D/+}$; $Trp53^{flox/flox}$ lung adenocarcinoma tumors leads to ICD-dependent CD8+ T-cell infiltration and synergy with ICI[45]. Collectively, these

findings suggest that certain combinations of ICD-inducing therapies can exert immune-mediated anticancer effects in autochthonous tumors.

Compared with transplanted TUBO tumors[29], autochthonous BALB-NeuT tumors are highly resistant to therapy. The median survival days and percentage of tumor-free mice after combination therapy are higher in transplanted tumors[29] compared with autochthonous tumors in this study. These differences can be attributed to the tumor cell's ability to sense cell death stimuli and release immunomodulatory molecules[30], and the tumor immune landscape prior to therapy[46]. Moreover, tumor intrinsic oncogenic events may also dictate the differences in therapeutic
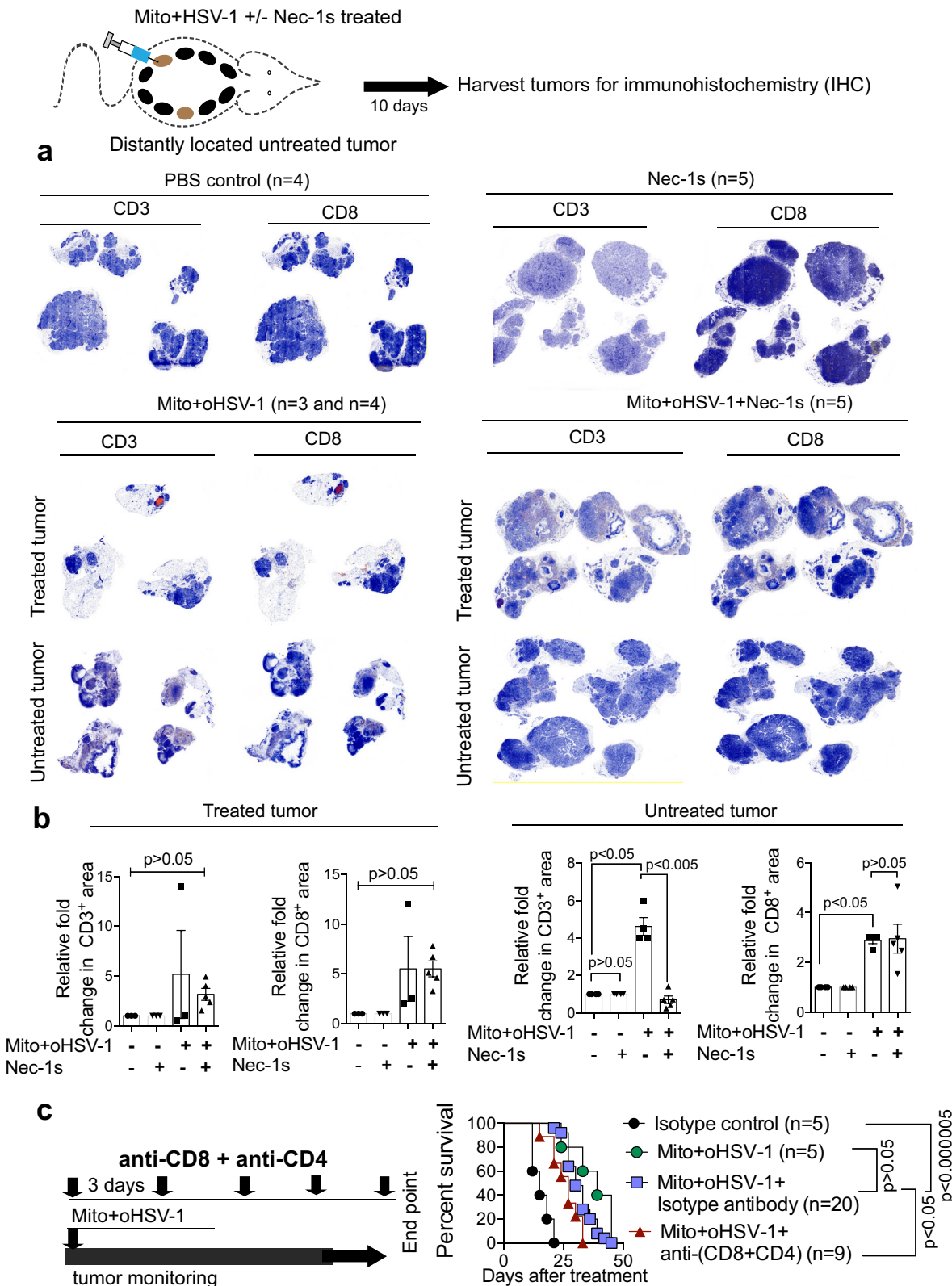
Fig. 5 Immunogenic Mito+oHSV-1 therapy CD8+ T-cell infiltration in distant tumor lesions. a, **b** Ten days after Mito+oHSV-1 treatment treated and distant untreated tumor lesions were processed for IHC staining using CD3 and CD8 antibodies. Fold change in positive signal was calculated relative to the PBS controls. Quantitative data are mean±standard deviation from each treatment group ($n = 5$) (Statistical significance was tested with one-way ANOVA or non-parametric Kruskal–Wallis test with Dunn's multiple comparison as a post hoc test). **c** BALB-neuT mice were treated with Mito+oHSV-1 along with monoclonal antibodies to deplete T cells (CD4 and CD8) or isotype control antibody. Quantitative data are Kaplan–Meier survival (Log-rank, Mantel–Cox test).

Fig. 6 Immunogenic Mito+oHSV-1 therapy renders tumors susceptible to immune checkpoint inhibitors in necrosome-dependent manner. BALB-neuT mice treated with Mito-C+oHSV-1/Dox+oHSV-1 were concurrently treated with antibodies targeting CTLA-4 and PDL-1 (immune checkpoint inhibitor; ICI). Antitumor effect was evaluated by tumor volume reduction **a**, multiplicity of autochthonous mammary tumors **b** as well as prolonged Kaplan–Meier survival **c**. The statistical significance of tumor volume differences and multiplicity of tumors were analyzed using Kruskal–Wallis test. **d** Inhibition of necrosome using Nec-1s abrogates the efficacy of Mito+oHSV-1+ICI. Quantitative data are Kaplan–Meier survival (Log-rank, Mantel–Cox test). The statistical significance of the survival differences in **c** and **d** was analyzed using Log-rank (Mantel–Cox). **e** Individual mice total tumor volumes as a proxy for tumor burden of BALB-NeuT mice after therapy. All the data presented are pooled from at least two independent experiments done at different times.

efficacy by modulating tumor cell death signaling[47] and influencing the tumor immune microenvironment[48].

Depending on the models used necroptosis has immune-stimulatory[21,49,50] as well as immunosuppressive effects[51]. During oHSV-1 monotherapy, we did not observe intratumoral necroptosis at multiple time points, although HSV-1 activates necroptosis in mouse cells while it uses its viral protein ICP6 to block necroptosis in human cells[52,53]. Our findings support that ICD-inducing properties arising from necroptosis correlates with efficacy of Mito+oHSV-1 treatment. Using Nec-1s to inhibit RIPK1-mediated necrosome formation in the context of Mito+oHSV-1 treatment leads to the loss of immunotherapeutic effect. Although Nec-1s is the preferred inhibitor of RIPK1 for in vivo use[54], there are limitations associated with the systemic use of RIPK1 inhibitors owing to pleotropic effects of a related inhibitor Nec-1 in multiple inflammatory pathways and T-cell metabolism[54,55]. To complement experiments involving systemic Nec-1s administration, we vaccinated with dying tumor cells lacking MLKL and show that immunogenic effects of Mito+oHSV-1 treated dying cells are lost. Overall, our finding is in accordance with studies that showed antitumor immune effects of dying necroptotic cells against immunogenic tumors[21,49,50].

Despite heterogeneity of breast cancer, targeted therapies provide durable responses with the exception of metastatic and triple negative breast cancer (TNBC)[56]. In general, breast cancer is not well infiltrated by tumor-infiltrating lymphocytes (TILs) compared with immunogenic lung and melanoma tumors. However, TNBC can display substantial immune infiltrate and patients that harbor tumors with enhanced immunomodulatory genes and/or intratumoral antitumor lymphocytes have a better prognosis[57–59]. A significant proportion of TNBC patient tumors have higher PD-L1 levels. As a result, treatment of TNBC patients

with PD-1 blockade antibody (pembrolizumab) shows an overall response rate of 18.5%[60]. Use of ICI with inflammation-inducing chemotherapy and oncolytic viruses[61–68] has gained conceptual popularity in preclinical and clinical studies[69]. This is evidenced by FDA's recent approval of atezolizumab, a monoclonal antibody targeting PD-L1, plus chemotherapy (abraxane; nab-paclitaxel) for the treatment of adults with PD-L1-positive, unresectable, locally advanced or metastatic TNBC[69]. Moreover, different types of oncolytic viruses display additive/synergistic therapeutic efficacy when combined with ICI[61–68] although mechanisms of synergy are not clearly identified.

Intratumoral administration of talimogene laherparepvec, a genetically engineered HSV-1 expressing GMCSF, showed higher levels of systemic and intratumoral cytotoxic T-lymphocyte infiltrate along with high PD-L1 expression in treated melanoma patient tumors[70]. Consequently, a combination of anti-PD-1 and talimogene laherparepvec therapy shows synergy to achieve 33% complete response in metastatic melanoma patients[70]. Although human trials have showed monotherapies of ICD-inducing agents can synergize with ICI[69,70], the proportion of patients with a complete response remain quite low. It remains unknown if combining different classes of ICD inducers can be harnessed to turn on cold human tumors. Moreover, the biological processes that predict therapeutic outcome in combinatorial therapy are currently lacking. Our study provides preclinical insight that a combination of chemotherapy and oncolytic virus activates necroptosis to convert TIL-autochthonous mammary tumors into TIL+tumors that become susceptible to ICI.

In immune-responsive transplanted tumors, necroptotic fibroblasts provide adjuvant signals to facilitate immune-mediated anticancer effects[49,50,71]. Although the feasibility of vaccinating human cancer patients with necroptotic cells is not

yet clear[21,49], utilizing the immunological benefits of therapy-induced necroptosis along with ICIs may have a translational potential. In line with this, several clinically used small molecules, natural compounds and engineered oncolytic viruses that selectively induce necroptosis have a potential for synergy with ICIs[72]. Future studies should aim to characterize the immunotherapeutic potential of therapy-induced necroptosis in human breast cancer patients.

Methods

Study design. The aim of this study was to understand the immune-stimulatory effects of oncolytic virotherapy and various chemotherapeutics in treatment of spontaneously arising mammary tumors. We adopted an autochthonous mouse model that recapitulates a tumor model that fails to undergo spontaneous T-cell priming. We used this model to administer treatments locally at one of the tumor sites and measure systemic effect by measuring tumor volumes arising from all the 10 mammary glands.

For in vivo anticancer studies, the primary endpoint is tumor size. We have conducted several previous studies using transplantable and autochthonous tumors and determined that $n = 5$–10 is an appropriate sample size per treatment group. There were no samples or animals excluded from the analysis. For most of the experiments reported in this manuscript the efficacy of the treatments were assessed in tumor volume as a proxy for tumor growth. As a result, before animals are allocated to any treatment group their tumor volume was measured and mice were randomized to achieve equal tumor volume per treatment group.

Cells culture. Human osteosarcoma cells (U2OS; American Type Culture Collection; ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS). TUBO is a cell line generated from a spontaneous mammary gland tumor of a BALB-neuT mouse[25]. TUBO cells are a generous gift of Dr. Guido Forni, who developed them originally. All the cell lines were mycoplasma tested and they were free of mycoplasma during the study period. TUBO cells were maintained in DMEM with 10% FBS. All media contained 2 mmol/l l-glutamine, 100U/ml penicillin, and 100 μg/ml streptomycin (Gibco). All cell lines were grown at 37 °C under humidified conditions.

Oncolytic HSV-1. The oHSV-1 has a deletion of the entire *ICP0*-coding region and has been previously described[27]. Viruses were propagated and tittered on U2OS cells in the presence of 3 mmol/l hexamethylene bisacetamide (Sigma; St. Louis, MO). Virus purification and concentration were done using sucrose cushion ultracentrifugation[27].

Western blotting. Protein samples were harvested after lysing cells in radio-immunoprecipitation buffer (10 mM phosphate pH 7.4, 137 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) supplemented with protease inhibitor cocktail (Sigma; St. Louis, MO) and phosphatase inhibitors cocktail II and III (Sigma; St. Louis, MO). Protein extracts were resolved in 10–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore, Billerica, MA). Blots were blocked in Odessey blocking buffer (LI-COR Biosciences, Lincoln, Nebraska). Western blotting detection was done after incubation with primary antibodies (anti-p-MLKL antibody, Cat#ab196436, Abcam; anti-total-MLKL, Cat#AP14272B, Abcepta; anti-cleaved caspase 3 antibody, Cat#9661, Cell Signaling Technology; and anti-beta-actin antibody, Cat#4967, Cell Signaling Technology) infrared dye-conjugated secondary antibody (Donkey-anti-Rabbit IgG-800CW conjugated, Cat#92632213, LI-COR Biosciences) using Odssey Scanner (LI-COR Biosciences, Lincoln, Nebraska).

CRISPR knockout cell line. Design and cloning of sgRNAs targeting mouse MLKL were done as previously described[73]. In brief, the sgRNAs sequences targeting MLKL (Forward 5′-GCACACGGTTTCCTAGACGC-3′, Reverse 5′-GCGTCTAGGAAACCGTGTGC-3′) were cloned into BsmbI digested Lenti-CRISPR v2 plasmid (Addgene, Cambridge, MA). Single guide RNAs-Cas9 cassettes were introduced to TUBO cells by using lentivirus-mediated gene transfer. Lentivirus transduced cells were selected using 1 μg/ml Puromycin for at least 2 weeks and depletion of the protein was verified by immunoblotting.

Treatment with chemotherapeutics and oHSV-1. Mice were maintained at the McMaster University Central Animal Facility and all procedures were performed in full compliance with the Canadian Council on Animal Care and approved by the Animal Research Ethics Board of McMaster University. Weaned mice were genotyped for the presence of the transgenes. Transgenic mice (BALB-NeuT, Gp/PyMT, FVB-NeuT) were allowed to age for 110 days or until the mammary glands start to develop palpable tumors. In the entire study, tumor measurements and treatments were initiated once the first tumor reached palpable size.

Chemotherapeutics were administered once and in the combination setting, chemotherapeutics were administered 1 day prior to intratumoral oncolytic virus treatment (three doses of 2×10^7 pfu[27]). Chemotherapeutic doses were 860 mM (1.25 mg/Kg) Dox hydrochloride (Sigma; St. Louis, MO), 6 mM Mito-C (4 mg/Kg) (Sigma; St. Louis, MO) or 5 μM MTX (Sigma; St. Louis, MO) (6.5 mg/Kg)[13,74], administered intratumorally in 50 μl volume. The RIPK1 inhibitor (Nec-1s) (BioVision, Milpitas, CA) was administered at a dose of 6 mg/kg. The pan-caspase inhibitor ZVAD-FMK (Minneapolis, MN) was used at a dose of 10 mg/kg. Inhibitors or the 5% dimethyl sulphoxide vehicle control were administered intraperitoneally every day for 4 days of Mito+oHSV-1 treatment. All tumors arising from the 10 mammary glands were measured every three days until mice reach endpoint. Mice having a total tumor volume above 525 mm^3 were classified as endpoint and sacrificed[29].

Vaccination with dying TUBO cells. BALB-NeuT mice were vaccinated with dying TUBO cells. The control group was vaccinated with TUBO cells that were freeze-thawed. TUBO cells were infected with HSV-1 dICP0 at MOI of 3 for 1 h and then add back media with 25 μM Dox or 30 μM Mito-C was applied[29]. Nec-1s was used in the context of Mito+oHSV-1 treatment at 100 μM. Cells were incubated for 24 hr before scrapping to pellet the cells for vaccination. Each mouse was vaccinated with three million dying cells. Mammary glands were palpated and measured every 3 days.

Immunohistochemistry. Treated and control mammary tumors were fixed in 10% formalin for 48 h and then transferred to 70% ethanol until histological processing. Tumor sections were processed for Hematoxylin–Eosin staining or immunohistochemistry using antibodies listed in Supplementary Table 1. All the antibodies used in this study were validated by the supplier as well as independent publications from academic laboratories. However, before we use any of the antibodies, we have validated their suitability by titrating the amount of antibody and running positive and negative controls. All antibodies were stained on the Leica Bond RX Automated Stainer with either Epitope Retrieval Buffer 1 (ER1), Epitope Retrieval Buffer 2 (ER2), or with Enzyme 1 (Leica). All antibodies were diluted in Power Vision Super blocker (Leica). The IHC slides were scanned using Aperio ScanScope slide scanner (Aperio Technologies, CA), and the images were analyzed using Positivity Pixel Count 9.0 algorithm within ImageScope software (Aperio Technologies, CA) as described previously[75].

Tumor protein isolation. Mice were anaesthetized and euthanized before resection of the tumors. Tumors were cut into small pieces and homogenized in the presence of tissue extraction solution (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA 2 mM Na3VO4, 1 mM NaF, 20 mM Na4P2O7, 1 mM beta-glycerophosphate, 1% NP-40). Homogenized tumors were incubated on ice for 30 min. Whole-tumor lysates were clarified by two sequential centrifugations at $13,000 \times g$ for 10 min at 4 °C. Twenty to eighty micrograms of total protein was used for western blot analysis.

Cytokine analysis. Cytokine analysis was carried out on tumor homogenates harvested using tissue extraction buffer. Tumor homogenates with equal amounts of protein concentration were shipped to Eve Technologies (Calgary, Alberta, Canada) for 31-Plex murine cytokine/chemokine analysis.

Antibodies. The immune checkpoint blockade antibodies anti-CTLA-4 (clone 9H10, BioXcell), and anti-PDL-1 (clone 10 F.9G2, BioXcell) were used alone or in combination with HSV-1+Mito-C. For ICI treatments, mice with palpable tumors volume below 100 mm^3 were injected with 200 μg of the antibodies every 3 days. In the combination treatments ICI antibodies were applied at the same day as the start of HSV-1 treatment. Treatments were continued until mice reached end point. Monoclonal antibodies targeting CD4 (clone GK1.5, BioXcell) and CD8 (Clone 2.43, BioXcell) T cells were administered intraperitoneally at a dose of 250 μg starting 2 days before treatment and applied every other day for the first two weeks and once a week thereafter. Depletion of cells after antibody administration was verified by flow cytometry.

Statistics and reproducibility. BALB-NeuT female mice tumors take a minimum of 110 days to become palpable and the incidence of macroscopic tumors is a stochastic event making larger experiments difficult to be synchronized. As a result, we conducted experiments with less than five mice per treatment group (while including multiple treatment and controls groups) and pooled experimental results collected from multiple independent experiments.

For each statistical analysis used, normality of the distributions and equality of variance assumptions were tested before running the statistical analyses. One-way analysis of variance, non-parametric Kruskal–Wallis test and t test were used to determine the statistical significance of the differences in means. For analyzing the statistical significance of the difference in Kaplan–Meier survival between treatments, the Log-rank (Mantel–Cox) test was used. All the tests were two-sided. The null hypothesis was rejected for p values <0.05. All data analyses were carried out using GraphPad Prism (La Jolla, CA, USA).

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Author contributions
S.T.W., A.N., Y.W., J.L.B, and K.L.M. conceived the study and designed experiments. S.T.W, A.N., A.V, N.E, A.N., D.N.H., J.W., A.O., Kelly L.M., D.N.H. developed the methodologies, conducted the experiments. S.T.W, K.L.M, A.N, A.O., J.A.N, A.G.M. S.K.S, D.B. analyzed and interpreted the experimental results. S.T.W, A.O., Y.W, J.L.B, and K.L.M. wrote and revised the manuscript.

Competing interests
Authors declare that there are no competing interests.

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