CONCOMITANT DELIVERY OF HISTONE DEACETYLASE INHIBITOR, MS-275,
ENHANCES THE THERAPEUTIC EFFICACY OF ADOPTIVE T CELL THERAPY
IN ADVANCED STAGE SOLID TUMOURS

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TITLE: Concomitant Delivery of Histone Deacetylase Inhibitor, MS-275, Enhances the Therapeutic Efficacy of Adoptive T Cell Therapy in Advanced Stage Solid Tumours

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Abstract

Despite the remarkable success of adoptive T cell therapy in the treatment of melanoma and hematological malignancies, therapeutic capacity in a broad range of solid tumours is impaired due to immunosuppressive events that render tumour-specific T cells unable to persist and kill transformed cells. To address some of the limitations of ACT in solid tumours, our laboratory has developed a therapeutic modality utilizing oncolytic virus, which expresses a tumour-associated antigen, known as an oncolytic viral vaccine (OVV), in combination with tumour specific central memory T cells. With this therapeutic approach (ACT), we can achieve robust *in vivo* expansion of transferred cells resulting in the complete and durable tumour regression in multiple solid murine tumour models. However, we demonstrate that the curative potential is lost when the tumour stage and burden increase as expanded transferred cells differentiate to a dysfunctional state resulting in the progressive decline in the tumour-specific CD8+ T cell response. Thus, we believe that restoring the T cell response in late-stage tumours will lead to enhanced curative potential of ACT in late-stage tumours. We have previously shown that HDACi, MS-275, can enhance the therapeutic capacity of a T cell-based therapy in an aggressive brain tumour model. In addition, concomitant delivery of MS-275 with ACT ensures durable cures through immunomodulatory mechanisms. Strikingly, concomitant delivery of MS-275, a class 1 histone deacetylase inhibitor (HDACi), with ACT in late-stage tumours completely restores the transferred T cell response to similar levels observed in early-stage tumours resulting in the complete regression of advance-stage tumours. Furthermore, MS-275 enhanced the proliferative capacity and tumour-

specific cytotoxic function of transferred cells, independently of tumour stage, type and mouse strain. Interestingly, we did not observe a complete reversal of T cell dysfunction, but rather observed that MS-275 conferred unique properties to T cells as the expression of some markers typically associated with T cell dysfunction was enhanced in addition to persistence and proliferation capacity. Moreover, concomitant delivery of MS-275 also restored the therapeutic capacity of endogenously primed tumour-specific CD8+ T cells expanded by an OVV in late-stage tumours, demonstrating the potential for general use for MS-275 in T cell-based therapies. Our data suggests the use of HDACi may potentiate T cell-based immunotherapies to overcome tumour-mediated T cell dysfunction in advanced stage solid tumours.

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List of Abbreviations

ACT	Adoptive T cell therapy
APC	Antigen Presenting cells
ATP	Adenosine Triphosphate
CAF	Cancer-Associated Fibroblasts
CAR	Chimeric antigen receptor
CFSE	Carboxyfluorescein succinimidyl ester
CRS	Cytokine release syndrome
CTL	Cytotoxic T lymphocytes
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DAMPS	Danger Associated Molecular Patterns
DC	Dendritic Cells
DCT	Dopachrome tautomerase
FOXP3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetyl transferase
HIF	Hypoxia-Inducible Factors
ICD	Immunogenic Cell Death
ICI	Immune checkpoint inhibitor
ICOS	Inducible T-cell Costimulator
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
INF	Interferon
IVIS	In Vivo Imaging Systems
KLRG1	Killer Cell Lectin Like Receptor G1
MDSC	Myeloid-Derived Suppressor cells
MHC	major histocompatibility complex
NK Cell	Natural Killer Cell
NY-ESO	New York - Esophageal Cancer -1
OV	Oncolytic Virus
OVV	Oncolytic Virus Vaccine
PAMP	Pathogen-associated molecular patterns
PD1	Programmed cell death protein 1
PFU	plaque-forming unit
PRR	Pattern Recognition Receptors

PV	Post Vaccination
T1IFN	Type 1 Interferon
TAA	Tumour-Associated Antigen
TAM	Tumour-Associated Macrophages
TAN	Tumour-associated Neutrophils
TCM	Central Memory T cells
TCR	T cell Receptor
TGFB	Transforming growth factor-beta
TIL	Tumour Infiltrating Lymphocytes
TIM3	T cell immunoglobulin and mucin domain-containing-3
TME	Tumour Microenvironment
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
VPA	Valproic Acid
VSV	Vesicular stomatitis virus

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Chapter 1: Introduction

1. Cancer

1.1. The role of the immune system in the progression of cancer

Cancer is described as a collection of diseases characterized by the unregulated division

of somatic cells. Underlying genetic instability of neoplastic cells allows for the

acquisition of hallmark qualities described by Hannah and Weinberg, which contribute to

the progression of this malignancy[1]. One of the hallmarks of cancer that have been the

topic of immense research and discovery is the evasion of immune destruction, which is

based on Paul Erlich's hypothesis revealing that the immune system paradoxically

prevents and is responsible for the development of cancer[2]. This hypothesis led to the

notion that the establishment of clinically relevant tumours proceeds in three phases:

elimination, equilibrium, and escape, known as the cancer immunoediting hypothesis.

During elimination, neoplastic cells that have evaded intrinsic tumour suppressive

mechanisms are recognized and destroyed by the innate (NK, Macrophages and Dendritic

cells) and adaptive (tumour-specific $CD4^+$ and $CD8^+$ T cells) immune systems[3,4].

However, neoplastic cells that escape elimination enter the equilibrium phase, where

tumour cellular growth remains functionally dormant by adaptive immune recognition,

specifically by CD8+ and CD4+ T cells[5]. Occasionally, due to pressures from the anti-

tumour immune system, genetically unstable tumour clones decrease intrinsic pathways

that en sure immune recognition[1]. For example, transformed cells decrease MHC class I

and/or tumour-associated antigen expression while increasing the expression of

checkpoint inhibitors (e.g., PD-L1, PD-L2) to exacerbate immune cell suppression[1,6].

Decreased immune recognition aids in the initiation of unrestrained tumour growth resulting in clinically relevant tumours[3].

1.2. The Immunosuppressive Tumour Microenvironment

The escape phase of immunosurveillance enables the development of an environment comprised of a dynamic relationship between transformed cells and a collection of heterogeneous tumour stromal cells functioning in concert to enhance immune evasion[7,8]. This is based on the "seed and soil" hypothesis, which highlights the synergistic interactions between transformed cells ("seed") and the tumour microenvironment ("soil")[9,10]. The developing tumour mainly consists of transformed cells, fibroblasts, the extracellular matrix, vasculature and immune cells recruited by the tumour microenvironment, contributing to neoplastic disease progression[11]. The developing solid tumour employs strategies such as the development of tumour vasculature, the presence of immunosuppressive cells and cytokines to reduce infiltration and survival of anti-tumour immune cells[12]. To support the high energetic demands of rapidly proliferating tumour cells, pro-angiogenic factors (VEGF-α, IL-8) are secreted by tumour cells to induce the rapid development of new blood vessels from established vascular beds[13]. Pathogenic angiogenesis by the developing tumour results in a highly disorganized and leaky network that hinders infiltration by anti-tumour cells through the downregulation of adhesion molecules (ICAM-1) required for T cell trafficking from lymphoid organs and extravasation [14,15]. The leaky and disorganized nature of the tumour vasculature results in low oxygen delivery and the development of a highly hypoxic and acidic environment.

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Hypoxia induces the increased expression of VGEF-α, IL-10 and hypoxia-induced factor (HIF), and in response, various tumour-promoting cells such as tumour endothelial cells upregulate inhibitory receptors (PD-L1, PD-L2, TIM-3), death receptors (FasL) and secretion of inhibitory molecules (IL-10, TGFB) to further establish a selective immune barrier[16–18]. In addition, cancer-associated fibroblasts (CAFs) also contribute to the development of a selective immune barrier by further promoting angiogenesis along with producing and remodelling of the extracellular matrix[11,19–21]. CAFs also enhance immunosuppression of anti-tumour immunity through the secretion of CXCL12 and TGFB, which inhibits T cell infiltration and activity[1,22–24].

Another strategy employed by the tumour to enhance immune evasion is the recruitment of hematopoietic cells. Factors secreted in response to the developing tumour microenvironment such as VEGF and CCL5 induce the recruitment and development of immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (T$_{regs}$), tumour associated macrophages (TAMs) and neutrophils (TANs)[25–29]. Initially, anti-tumour immune cells are often recruited to the tumour in response to chemokines secreted by tumour cells; however, upon encounter with the cytokine milieu, immune cells differentiate to support tumour growth and immune suppression[25]. For example, neutrophils are recruited by CXCR2 ligands secreted by tumour cells and encounters with factors such as TGFB in the TME cause neutrophils to support tumour growth[30]. The newly differentiated TANs secrete pro-angiogenic molecules to support vasculature growth and induce GM-CSF expression to increase the expression of PD-1 on tumour cells[31]. Similarly, TAMs are initially recruited due to inflammation as an anti-

tumour phenotype; however, like neutrophils, anti-tumour M1 macrophages are converted to pro-tumour M2 macrophages. TAMs secrete immunosuppressive cytokines such as IL-10 and chemokines such as CCL17 and CCL24, which recruit T regulatory cells (T_{regs})[32,33]. Immunosuppression by T_{regs} function to suppress anti-tumour immunity through the depletion of soluble IL-2 by the constitutive expression of the cognate receptor and secretion immunosuppressive cytokines such as IL-10, which suppress T cell activation[34–36]. Furthermore, binding of coinhibitory receptors (LAG3 and CTLA4) on T_{regs} with DC signalling molecules (MHC II and CD80/CD86) inhibits activation and promotes tolerization of DCs characterized by expression of immunosuppressive cytokines (IL-6) and decrease the expression of costimulatory molecules[37–40]. DC tolerization induces T cell anergy as the three signals required for T cell activation, MHC presentation, binding of costimulatory molecules and inflammatory cytokines are not met[41,42].

Immunosuppression of anti-tumour immunity is dynamic and extends beyond the local tumour microenvironment. A comprehensive study performed by Allen *et al.* utilized mass cytometry to exhibit the dynamic composition of systemic anti-tumour immunity of various tumour models and times of tumour development[43]. This provides compelling evidence of the importance of the local and systemic tumour microenvironment to inform treatment strategies.

2. Cancer Immunotherapy

2.1. Rational for utilizing immunotherapy

For the last century, three pillars of cancer treatment: surgery, chemotherapy, and radiation, have been the primary mode of treatment for patients. However, the importance of the immune system in the establishment and progression of cancer has rapidly expanded therapeutics available for cancer treatment[44]. While the three pillars effectively destroy bulk tumours, they are limited in addressing metastasis, are highly toxic as they are indiscriminate of tumour or non-cancerous tissue, and often result in a relapse as some cancerous cells are left undestroyed[45]. Removal of tumours by surgical resection often presents as the first line of defence; however, tumours that have metastasized, are inoperable or incapable of complete resection cannot be treated by surgery[46]. Radiation therapy aids in addressing concerns of tumour inaccessibility by directing high-energy electrically charged particles to induce double-stranded breaks of DNA in tumour cells, abrogating division and proliferation[47]. Radiation therapy permitted the ability to treat tumours inaccessible by surgical intervention; however, radiation therapy remains limited in targeting metastasis[48]. Although the systemic delivery of chemotherapeutic agents addresses challenges associated with metastasis, chemotherapy is highly toxic to the recipient as it is indiscriminate against tumour cells and other rapidly dividing non-transformed cells[49,50]. The three pillars of cancer therapy are integral in cancer treatment; however, research surrounding the importance of the immune system in cancer progression has expanded treatment options, resulting in the development of the fourth pillar of cancer treatment, cancer immunotherapy[51].

Cancer immunotherapy seeks to harness the immune system to recognize and specifically destroy transformed cells through active or passive immunity[52]. Compelling

evidence for the use of the immune system to destroy cancer dates to the 1800s when William Coley observed the regression of inoperable sarcomas following infection with *Streptococcus pyogenes* and subsequent regressions following the administration of Coley's Toxin, a mixture of *S. pyogenes* and *Serratia marcescens*[53]. Insights into the mechanisms of immunological events, unknown at the time, reveal Coley's success was due to the induction of distinct phases in the generation of the specific anti-tumour immune response[52,54]. To initiate the immune response, antigen-presenting cells, specifically DCs, must acquire tumour antigens exogenously through cross-presentation or phagocytosis. Following antigen acquisition, activation signals, such as danger-associated molecular patterns (DAMPS) released from apoptotic cells, must be present in the TME to induce maturation and differentiation required for successful antigen presentation. DC maturation is often characterized by the increased expression of MHC/Peptide complexes, costimulatory molecules (CD80/CD86) and expression of cytokines (IL-12), which represent the three signals required for T cell activation. Subsequently, functional mature antigen-loaded DCs must traffic to secondary lymphoid organs to induce the activation and expansion of tumour-specific CD8+ T cells. Following expansion, tumour-specific T cells must traffic and infiltrate the tumour microenvironment while successfully surmounting immunosuppressive factors, resulting in the destruction of tumour cells and the generation of long-term anti-tumour immunity[55–57].

Strategies employed by tumours to prevent the generation of the immune response have provided various entry points for therapeutic intervention[56]. The initiation of the

specific anti-tumour T cell response is often limited by the presence of immunogenic

tumour antigens and suboptimal activation and maturation by antigen-presenting cells by

DCs[58,59]. Thus, vaccination strategies were developed to introduce a high concentration of

TAAs to the recipient in the form of nucleic acids, peptides, viruses, tumour lysates and

peptide-loaded DCs to enhance successful antigen presentation and T cell activation[60,61].

Strategies such as adoptive T cell transfer passively induces the anti-tumour T cell

response through the infusion of large quantities of tumour-specific T cells[62,63].

Furthermore, therapies such as oncolytic viral therapy[64] and checkpoint therapy[65] aid in

the survival of tumour-specific T cells by converting the immunosuppressive

microenvironment to promote the survival of anti-tumour immune cells. Various

immunotherapies have been developed and expertly reviewed here; however, this

dissertation will focus on the use of adoptive T cell therapy (ACT) and oncolytic viral

therapy (OV).

2.2. Adoptive T cell therapy

Given the importance of functional tumour-specific CD8+ T cells in treating

tumours, adoptive T cell therapy (ACT) serves to amplify tumour recognition through

the infusion of autologous or non-autologous tumour reactive T cells following *ex*

vivo expansion[63]. Primary clinical studies pioneered by Steven Rosenburg's group

expanded tumour-specific T cells from digested tumours in the presence of IL-2[66].

Theoretically, tumour-specific T cells from the tumour are capable of recognizing

various antigens on tumour cells, abrogating the need to identify a single target, thus

overcoming challenges associated with tumour antigen heterogeneity. The use of

tumour-infiltrating T cells in ACT protocols demonstrated success predominantly in the treatment of metastatic melanoma[67]. However, in a broad range of solid tumours, extraction of tumour reactive T cells from the tumour was unsuccessful due to low numbers of tumour reactive T cells at the tumour site, or conversely, heavily infiltrated tumours predominantly contain highly dysfunctional T cells[68].

To increase the infusion of functional tumour-specific cells, T cells present in peripheral blood can be genetically engineered to express a tumour-specific T cell receptor (TCR) or a chimeric antigen receptor (CAR). Clinically, ACT using T cells engineered to express a TCR specific to NY-ESO-1, a cancer/testis antigen, resulted in objective clinical responses to synovial cell sarcoma in 66% of patients and complete and durable regressions of melanoma for one year in 22% of patients treated. Furthermore, targeting the CD19 antigen B cell acute lymphoblastic leukemia by CAR T cells reported 90% complete remission. However, engineered TCR T cells and CAR T cell therapy is limited in the treatment of solid tumours, and strategies were employed to ensure the broad usability of adoptively transferred cells in solid tumours.

2.2.1. Differentiation State of ACT before transfer

The success of ACT is reliant on the ability of a sufficient number of functional T cells to persist long enough to destroy tumour cells. The persistence and functionality can be controlled in two ways, before transfer and post-transfer. Before T cell infusion, the *ex vivo* differentiation state of T cells and enhanced persistence are inversely correlated[69].

Initially, the transfer of terminally differentiated T cells was hypothesized to execute efficient destruction of tumour cells, as *in vitro* cultured terminally differentiated effector cells yielded a large number of extremely cytolytic cells. However, upon *in vivo* administration, terminally differentiated cells exhibited low persistence and cytolytic ability, resulting in limited anti-tumour activity. This finding prompted Restifo *et al.* to determine if adoptively transferring T cells with a lower differentiation status, such as central memory T cells, would yield greater therapeutic capacity[70]. This hypothesis was based on the premise that T cells with a lower differentiation status possess a greater proliferative capacity resulting in a source of effector cells with high cytolytic capacity. Thus, satisfying the requirements of ACT success, the generation of long-lasting, highly functional tumour-specific T cells. To achieve this differentiation status, the conditions utilized in *ex vitro* cultures must be taken into consideration. Historically, IL-2 was utilized to expand T cells; however, this produced terminally differentiated cells characterized by high expression of CD44 and low expression of CD62L, a marker responsible for lymphocyte homing to secondary lymphoid organs[71,72]. It was observed that in comparison to IL-2, IL-21 modulated the differentiation program of T cells resulting in cells with low cytolytic activity, high levels of CD62L, low levels of Granzyme B, CD44, and IL-2R expression. IL-21 treated T cells utilized *in vivo* resulted in complete and durable regression in tumours and exhibited superior secondary expansion of these cells and increased the expression of T-cell factor 1(*Tcf7*) and lymphoid enhancer-binding factor 1(*Lef1*), which are implicated in self-renewal, demonstrating the enhanced proliferative capacity of these cells[73].

In addition, IL-15, a cytokine implicated in the expansion of memory cells, functioned synergistically with IL-21 to further increase the number of memory T cells *in vitro* without altering the differentiation status. *In vivo* administration with central memory differentiated T cells resulted in greater persistence and anti-tumour function[73]. To further ensure the commitment to the central memory phenotype, our laboratory utilizes a combination of IL-21, IL-15 and rapamycin, an mTOR inhibitor yielding a culture of T cells exhibiting a central memory phenotype (CD44$^-$CD62L$^+$)[74–76]. Following T cell activation, the fate commitment to memory or effector T cell phenotype can be attributed to the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K-AKT-mTOR) pathway regulating the ratio of transcription factors: Eomesodermin (Eomes) and T-bet. Thus, inhibition of mTOR by rapamycin promotes the expression of Eomes and decreases the expression of T-bet to ensure a memory phenotype at the end of the culture[77]. T cells treated with rapamycin prior to transfer resulted in superior anti-tumour efficacy and persistence, highlighting the importance of the differentiation status of T cells prior to treatment[78].

2.2.2. Challenges of Adoptive T cell Therapy in the Tumour Microenvironment

Although successful in the treatment of melanoma and hematological tumours, ACT has demonstrated limited success in the treatment of solid tumours due to suboptimal migration, infiltration, persistence and destruction of tumour cells in the solid TME[79]. Contrary to hematological tumours, the solid TME releases chemokines that hinder the migration of tumour-specific T cells[80]. For example, MDSCs in the TME induce the nitration of CCL-2, which is capable of recruiting monocytes; however, N-

CCL2 traps T cells in the extracellular matrix of the tumour stroma, preventing successful T cell infiltration to the tumour core[81]. Furthermore, tumour vasculature expresses increased levels of the Fas ligand-induced apoptosis of T cells. In conjunction with the physical barriers of the solid tumour[17,82].

To support the rapid proliferation of tumour cells, nutrients in the TME are rapidly depleted[83,84]. Thus, tumour-specific T cells must be capable of persisting in an environment void of essential nutrients and oxygen crucial for sustaining the energetic and metabolic demands required for T cell proliferation and function[85]. Tumours also produce high levels of metabolites destined to suppress T cells. Ma *et al.* observed that compared to peripheral organs, tumours had elevated levels of cholesterol within the TME, causing enhanced uptake by T cells, resulting in ER stress and subsequently T cell exhaustion characterized by a loss of function and high expression of inhibitory markers[86]. In addition, immunosuppressive cells like MDSCs, TAMs, TANs and T_{regs} release anti-inflammatory cytokines such as IL-10, TFGB which directly function to suppress T cell functionality[87–89].

Tumour cells work in concert with immunosuppressive cells to evade T cell recognition by employing strategies to abrogate signals required to mount a successful anti-tumour T cell response[42]. Tumour cells also play an active role in immunosuppression by modulating the expression of molecules at the cell surface. Tumour cells co-opt the presence of T cells and induce the enhanced expression of coinhibitory ligands (PD-L1, PD-L2, B7-1) and death receptors (FasL), which upon ligation with the cognate receptor results in T cell exhaustion and induction of

apoptosis[90,91]. Also, tumour cells express the molecule indoleamine 2,3-dioxygenase, which functions to catabolize an essential amino acid for T cell function, tryptophane, resulting in the production of kynurenine that induces the formation of T_{regs}[92–94]. Tumour cells thwart antigen presentation by the reduction of MHC class I expression or the advent of antigen loss variants, abrogating cell recognition by T cells[95,96].

2.2.3. T cell dysfunction Within the Tumour Microenvironment

The chaotic and sinister immunosuppressive environment of solid tumours that infused tumour-specific T cells must surmount results in suboptimal persistence. This has been attributed to tumour-induced dysfunction of T cells, a state akin to virus-induced exhaustion, defined as the progressive decline of T cell function paired with the increased and sustained expression of inhibitory markers[97]. Dysfunctional CD8+ T cells encompass a subset of cells on a spectrum of dysfunction and differentiate to this state by extrinsic and intrinsic means. Extrinsically, the factors mentioned earlier in the TME are implicated in the induction of T cell dysfunction. Emerging studies now reveal that manipulation of intrinsic factors can aid in the reversal of T cell dysfunction[98,99].

In comparison to functional T cells, dysfunctional cells harbour approximately 6,000 distinct open chromatin regions from functional effector and memory T cells, and studies have shown epigenetic signatures define dysfunctional T cells within the tumour. Specifically, the locus for INFɣ expression was inaccessible in dysfunctional cells, and genes encoding inhibitory receptors (*pdcd1, Tigit,* and *ctla4)* displayed high accessibility to NFAT transcription factor binding in dysfunctional cells compared to functional cells. In concert with chromatin states, transcription factors have been implicated in the

differentiation to a dysfunctional state[98]. The acquisition of these epigenetic programs is regulated by the expression of transcription factors, such as HMG-box transcription factor TOX, which is critical for developing exhaustion in T cells[100,101].

The dysfunction of T cells is exacerbated in the presence of an advanced stage tumour, as immunosuppressive programs in the developing tumour are increased with tumour growth[101]. Preclinical and clinical studies have shown that the success of checkpoint and adoptive T cell therapy is dampened as tumour burden and stage advances[102–104]. Huang et al. demonstrated that upon receiving ICI therapy, patients with large late-stage tumours exhibited lower ratios of reinvigorated CD8+ T cells to tumour burden resulting in negative clinical responses when compared to patients with small early-stage tumours[102]. Furthermore, studies have demonstrated that the tumour stage determines the dysfunction of tumour-specific T cells. Schietinger *et al.* posited that local dysfunction of tumour-specific CD8+ T cells occurs in early tumorigenesis and is initially therapeutically reversible; however, this dysfunction ultimately advances to a fixed and therapeutically irreversible state with tumour growth[105].

Furthermore, as the tumour burden increases, systemic anti-tumour immunity is also negatively affected as the function and composition of systemic immune cells are dynamically altered. For example, tumour-specific CD8+ T cells located in distal sites become progressively dysfunctional, specifically exhibiting decreases in proliferation and increases in apoptosis[43]. Thus, the consequence of tumour burden on immunotherapies is critical to consider as it can inform the timing of therapeutics designed to induce local and systemic immune responses.

In line with determining factors that result in suboptimal T cell persistence in ACT, combinatorial approaches have been employed to alter intrinsic T cell mechanisms and/or extrinsically subvert the immunosuppressive TME to an environment favouring T cell survival[106]. A well-known example of this is the combination of ACT and ICI therapy, which results in the greater migration of tumour-specific T cells to the tumour site and reduction in tumour mass due to the reinvigoration of dysfunctional T cells[107–109]. Similarly, IL-21 differentiated transferred T cells in combination with an anti-CTLA4 antibody also enhanced survival and persistence, resulting in effective tumour regression[110]. Combinatorial strategies such as oncolytic virotherapy have also demonstrated promising results to enhance the persistence of T cells in the TME[111,112]. This dissertation will focus on the synergy exhibited by oncolytic virus therapy in combination with adoptive T cell therapy.

2.3. Oncolytic Virus Therapy

In addition to adoptive T cell therapy, the emergence of oncolytic viruses has been an instrumental tool in the progression of novel immunotherapeutics available and advancing knowledge surrounding tumour immunology. OVs are viruses that selectively replicate and kill tumour cells while sparing healthy cells[113,114]. The understanding that viruses could potentially be utilized as a cancer therapeutic stems from observations made in the early 20th century of patients undergoing remission following viral infection[113,115,116]. Currently, a multitude of native and genetically engineered viruses

such as rhabdoviruses[117,118], reoviruses[119–121], herpes simplex[122–124], and vaccinia viruses[125,126], among others, are emerging as potential candidates for cancer therapeutics. Thus, the approval of a modified adenovirus (H101) for the treatment of advanced head and neck tumours in China[127,128] and talimogene laherparepvec (T-VEC), a modified herpes simplex-1 virus for the treatment of advanced melanoma[123], has solidified oncolytic viral therapy as a new class of immunotherapeutics.

Selective and successful replication of OVs in the tumour cells is conferred by both properties of the transformed cells and the virus, specifically, factors that facilitate attachment, entry, and replication of viruses within the cell[129]. Some viruses exhibit a specific tropism for tumour cells due to the overexpression of molecules at the cell surface. For example, enterovirus[130,131] and poliovirus[132] enter transformed cells due to overexpression of integrin α2β1 and CD55, respectively. Upon entry, aberrant mechanisms that tumour cells utilize for immune evasion are exploited by OVs to enhance the proliferation and production of viral progeny. In non-transformed cells, anti-viral mechanisms, including the recognition of pattern-associated molecular patterns (PAMPs) and type I interferon signalling (T1IFN), abrogate successful replication of viral particles. Conversely, studies have demonstrated that various tumour types exhibit defects in T1IFN signalling; thus, providing an environment permissible for viral replication. In addition, this enhances the safety profile of OVs like rhabdoviruses that are potent inducers of the T1IFN pathway. Similarly, the loss of tumour suppressors (p53, RB) and aberrations in Wnt signalling in tumour cells are exploited by OVs, resulting in the lysis of tumour cells[114,133].

2.3.1. Induction and enhancement of the tumour-specific immune response

Upon successful OV replication in neoplastic cells, it was initially recognized that the debulking of tumours by OV was solely attributed to tumour lysis. However, formally seen as a hindrance to oncolytic virotherapy, it is now accepted that oncolytic viruses function in a multimodal fashion by directly lysing malignant cells and transforming an immunologically "cold" tumour into a heavily infiltrated "hot" tumour[134–136].

OV-mediated lysis of tumour cells is recognized as a potent inducer of the anti-tumour response. Successful infection by OVs cause endoplasmic reticulum stress and the production of reactive oxygen species (ROS), resulting in immunogenic cell death (ICD) of tumour cells, characterized by the release of danger-associated molecular patterns (DAMPS) and previously concealed TAAs upon lysis[137]. The release of DAMPs (ATP, HMGB1 and Calreticulin) function to enhance the recruitment of immune cells such as neutrophils and antigen-presenting cells (APCs), specifically DCs. ATP serves as the recruitment signal for DCs to the TME, HMGB1 stimulates the activation and maturation of recently recruited DCs by binding to toll-like receptors, while calreticulin functions as a signal for enhanced phagocytosis and processing of apoptotic tumour cells by dendritic cells and macrophages[138–140]. In addition, viral pattern-associated molecular patterns (PAMPS) such as viral proteins and genetic material (RNA and DNA) are sensed by pattern recognition receptors (PRR) on dendritic cells. PAMP recognition also assists in the activation and maturation of DCs, characterized by augmented expression of MHC class I and II molecules, costimulatory molecules, pro-inflammatory cytokines (IL-6, IL-

12) and chemokines (CCL2), which serve to increase the recruitment of immune cells to the TME[141,142].

The successful acquisition of tumour-associated antigens by DCs is crucial in the induction of the adaptive anti-tumour immune response. Specifically, DCs that express transcription factor basic leucine zipper transcriptional factor ATF-like 3 (BATF3) uptake and present exogenous TAAs on MHC class I through a process called cross-presentation. Antigen-loaded BATF3^{+} DCs subsequently traffic to secondary lymphoid organs to induce the activation, expansion, and differentiation of naïve T cells to effector T cells specific to various TAAs[143]. Subsequently, tumour-specific T cells traffic rapidly to the highly inflamed TME as a result of OV infection[144].

OVs aid the function of T cells by enhancing entry into the tumour, thwarting the immunosuppressive cytokine and cellular environment, and increasing antigen presentation on tumour cells[136,145,146]. OVs have been shown to enhance vascular permeability, disruption of the extracellular matrix, and enhance infiltration of T cells within the tumour. OVs such as vaccinia virus and VSV have been demonstrated to infect and lyse tumour endothelial cells and induce the production of anti-angiogenic effects permitting enhanced extravasation of T cells within the tumour[147–149]. Within the inflamed tumour microenvironment, OVs are an effective option due to the ability to skew the immunosuppressive TME to a pro-inflammatory to enhance immune cell infiltration and activation. Katayama *et al*. demonstrated oncolytic reovirus treatment prevents the inhibition of tumour-specific activity by MDSCs in a TLR3 dependent

manner. Furthermore, OVs enhance the expression of pro-inflammatory cytokines such as IL-8, IL-4, TNFα and IL-6, which enhance immune cell infiltration and activation[150].

Suboptimal antigen presentation due to decreased expression of MHC class I molecules is one of the strategies employed by tumour cells to evade T cell recognition[151,152]. Treatment with OVs has been implicated in enhancing antigen presentation at the level of tumour cells; specifically, the expression of MHC class 1 in response to OV treatment was monitored. It was demonstrated that reovirus administration *in vitro* and *in vivo* increased expression of MHC class I and the transcription of genes associated with antigen presentation (MHC I, β-2 microglobulin, TAP-1 and TAP-2) in a mouse epithelial ovarian cancer ID8 cell line void of MHC class I[153].

2.3.2. Oncolytic virus vaccine

The generation and recruitment of tumour-specific CD8+ T cells by OVs has been accepted as a critical mode of action[154]. One strategy that has been employed to augment this process is the addition of a tumour-associated antigen (TAA) to the backbone of the virus, known as an oncolytic virus vaccine (OVV). This strategy induces a specific and robust expansion of T cells against the encoded TAA[155]. However, the immune response against the virus dominates that of the TAA, given the virus's foreign nature, eliciting a subdued anti-tumour response[118]. Conversely, the generation of an optimal anti-TAA immune response can be achieved using two different viruses encoding a common TAA in a heterologous prime-boost strategy. The priming virus elicits a primary immune

response against both the virus and TAA, resulting in the generation of memory T cells against the priming virus and TAA. The second boosting virus induces a robust recall response against the TAA, which eclipses the primary response against the second virus. This technique was exhibited in an aggressive brain tumour model using vesicular stomatitis virus and adenovirus, both encoding Dopachrome tautomerase (DCT), an antigen found on B16F10 tumours. Boosting with VSV encoding hDCT yielded an hDCT specific CD8+ T cell response 85-fold higher than the T cell response generated by Ad-hDCT alone, resulting in a significant increase in survival[118,156].

Our laboratory has demonstrated that vaccination with an oncolytic virus expressing a TAA can achieve a robust *in vivo* expansion of tumour-specific T cells resulting in complete regression and enhanced survival of challenging murine models[118,156].

Oncolytic viruses present as a remarkable tool for the treatment of cancer, particularly through the stimulation of the tumour-specific CD8+ T cell response. However, its effects are limited due to low counts of functional T cells capable of expansion and differentiation[135]. However, OV therapy can be further enhanced through its combination with other immunotherapies; and we have shown potent synergy with the use of adoptive T cells in combination with OV therapy[74–76].

2.4. Combination Therapy: Adoptive T cell therapy and Oncolytic Viral Vaccination

In our laboratory, we currently utilize the rhabdoviral oncolytic vaccine to induce the expansion and differentiation of adoptively transferred central memory T cells (ACT).

Upon intravenous administration, central memory T cells home to the secondary lymphoid organs due to high expression of L-selectin (CD62L). Twenty-four hours later, we intravenously administer vesicular stomatitis virus encoding the same tumour-associated antigen. The systemic administration of the OVV employs a strategy known as the "push-pull mechanism." Trafficking of the OVV to the secondary lymphoid organs induces a robust *in vivo* expansion and activation of central memory cells (push). Subsequently, the newly differentiated tumour-reactive effector T cells are recruited rapidly to the highly inflamed tumour site as a function of oncolysis (pull)[74]. This therapeutic platform results in a tumour reactive CD8+ T cell response of upwards of 40%, resulting in complete regression of tumours of various strains and models[74,75]. This combinatorial strategy addresses limitations associated with monotherapies like oncolytic virotherapy and ACT. Compared to heterologous prime-boost, this combination therapy nullifies the need for two viruses and decreases the time required to expand tumour-specific T cells. Furthermore, the robust *in vivo* expansion of central memory T cells reduces the possibility for terminal differentiation of T cells in the *in vitro* culture, reduces the number of cells required for infusion and enhances the *in vivo* persistence of transferred cells[76].

3. HDACi: Therapeutic option for potentiation of immunotherapies

3.1. Chromatin structure and Histone proteins

Eukaryotic DNA is organized into chromatin structures that compact the DNA structure. The post-translational modifications on the molecules involved in the

contraction process of DNA dictate the ability for transcriptional machinery to access the DNA, also known as epigenetic modification[157]. Epigenetics is defined as the heritable changes in gene function that do not alter the DNA sequence. These heritable changes include alterations to histone proteins in the form of acetylation, sumoylation, DNA methylation. Epigenetic manipulations are similar to genetic modifications in that they can impose changes on the behaviour of cells; however, contrary to genetic alterations, they are reversible[158]. While epigenetic modifications are essential in many biological processes, aberrations to these modifications are a hallmark of cancer, highlighting the importance of delineating epigenetic disruption in the malignant transformation of a cell[1]. Determining epigenetic changes that occur during tumorigenesis is of interest due to the reversible nature of epigenetic modifications. Many efforts are currently focused on modifying multiple epigenetic mechanisms[159], and this review will focus on histone modifications.

Histone proteins are highly conserved alkaline proteins that condense and order DNA into nucleosomes. Histone molecules have long N-Terminal extensions subject to post-translational modifications such as acetylation, methylation, sumoylation, ubiquitination, phosphorylation, and ADP-ribosylation[160]. Post-translational modifications such as acetylation dictate whether transcriptional machinery can access the DNA and ultimately regulate gene transcription or silencing[161].

3.2. Inhibition of Histone Deacetylase in the Treatment of Cancer

Acetylation of histone proteins results from the activity of two antagonistic enzymes: histone acetylases (HAT) and histone deacetylases (HDAC), responsible for the addition and removal of acetyl groups to lysine residues tails on amino-terminal tails of histones, respectively. The transfer of acetyl groups causes the removal of a positive charge, which decreases the interaction between the negatively charged DNA resulting in transcriptional accessibility[162]. Conversely, deacetylation of lysine residues increases the charge density strengthening the interactions between DNA and core nucleosome proteins resulting in transcriptional repression and decreasing the accessibility of enhancer and promoter regions.

The erasure of acetyl groups catalyzed by HDACs is not solely on histone proteins but also non-histone proteins, including those involved in DNA transcription, such as transcription factors and DNA repair proteins[163]. Due to the broad effects of HDACs, aberrantly deacetylated proteins are involved in various stages of tumorigenesis. In cancer cells, Class 1 HDACs are frequently overexpressed; specifically, high HDAC 1-3 expression is associated with poor prognosis in ovarian cancer, gastric cancer, and poor responsiveness to radiotherapy in breast cancer[164]. Aberrant HDAC activity is involved in the dysregulation of cell proliferation. In pancreatic cancer, HDAC3 promotes proliferation, invasion and migration of pancreatic cells via H3K9 deacetylation resulting in the repression of the transcription of P53, P27 genes involved in cell cycle arrest and Bax genes involved in apoptosis[165].

Due to research surrounding aberrant HDAC expression in tumourigenesis, therapeutics have been generated to inhibit HDAC activity. HDAC inhibitors (HDACis) are classified into four classes based on chemical structure: Hydroxyamates, cyclic peptides, aliphatic acids and benzamides[163]. Depending on the chemical structure, HDACis can be therapeutic against cancer by inducing cell cycle arrest, differentiation and apoptosis. There are currently four HDACis approved by the United States Food and Drug Administration (US FDA) for cancer treatment. The first HDACi approved was suberoylanilide hydroxamic acid (SAHA) to treat cutaneous T-cell lymphoma (CTCL)[166]. Presently, HDACis are being evaluated mechanistically in preclinical and clinical cancer studies. HDACis can induce cell cycle arrest in the G1 phase by either upregulating cyclin-dependent kinase inhibitors or downregulating cyclin-dependent kinases and cyclins. Furthermore, HDACi can also induce apoptosis of tumour cells, specifically, the use of Valproic acid (VPA), an HDAC1 inhibitor demonstrated to induce apoptosis of neuroblastoma derived cells by the degradation of Hypoxia-inducible factor 1 alpha (HIF-1alpha), a non-histone protein implicated in the induction of cancer[167].

3.3. Immunomodulation by Histone Deacetylase Inhibitors

Aside from the direct impact on neoplastic cells, HDACi can induce potent immunomodulatory effects. HDACis have been reported to increase tumour infiltration by immune cells via augmented MHC costimulatory marker expression on tumour cells and APCs[168,169]. In addition, it has been reported that the use of HDACi increases the

expression of tumour-associated antigens on tumour cells, also enhancing immune infiltration[168,170]. HDACi administration has been demonstrated to alter immunosuppressive cells within the tumour microenvironment. We have reported that concomitant administration of MS-275 induced the polarization of tumour-infiltrating MDSCs from the M2 immunosuppressive pro-tumour phenotype to the M1 anti-tumour immunostimulatory phenotype resulting in the direct killing of antigen loss variants within tumour cells[75].

Furthermore, HDACi administration directly increases the functionality of cytotoxic CD8+ T cells. Zhang *et al*. reported that the treatment of exhausted CD8+ T cells with Valproic Acid resulted in the restoration of CD8+ T cell function[171]. Furthermore, we previously reported that concomitant delivery of MS-275 during the boosting phase of the heterologous prime-boost vaccination protocol generates tumour-reactive T cells with enhanced cytotoxic function. This strategy resulted in durable cures in 64% of mice bearing aggressive intracranial tumours[172]. With evidence, it is of interest to utilize HDACis to modulate the subversion of tumour-induced immunosuppression to prevent or reverse T cell dysfunction.

4. Hypothesis and goals

We have demonstrated the ability for a viral vaccine to induce a robust *in vivo* expansion of adoptively transferred tumour-specific T cells (ACT) (Figure 1), resulting in complete regression and survival of small early-stage tumours[74,75]. However, when this therapy is applied to late-stage tumours with an increased tumour burden, the transferred

tumour-reactive CD8+ T cell response is severely dampened, resulting in the loss of

tumour control and survival (Figure 1). Thus, we believe that the loss of therapeutic

efficacy in late-stage tumours is due to the loss of tumour-reactive T cells before tumour

regression. **Therefore, we hypothesize that the restoration of the tumour-specific**

CD8$^+$ T cell response will ensure the therapeutic capacity of ACT in late-stage

tumours. We have previously demonstrated the effect of MS-275 as we have previously

observed its ability to enhance the cytotoxic function of tumour-specific CD8+ T cells

following oncolytic vaccination. The report will assess the following goals: firstly,

determine if the concomitant administration of MS-275 with ACT can prevent the

dampening of the tumour-specific T cell response to ensure the restoration of therapeutic

efficacy in late-stage tumours. Secondly, characterize the phenotypic and functional

alterations of tumour reactive CD8+ T cells in response to MS-275. Thirdly, evaluate the

general use of MS-275 in the potentiation of T-cell-based therapies affected by increased

tumour stage. Specifically, to evaluate if MS-275 can potentiate the therapeutic efficacy

of oncolytic vaccination in late-stage tumour models.

Chapter 2: Materials and Methods

1. **Mice**

Female Balb/c and C57Bl/6 mice aged 6-8 weeks old were purchased from

Charles River Laboratories and housed in a pathogen-free room in Central Animal

Facility at McMaster University. DU18 Thy1.1 mice were kindly provided by Lyse

Norian from the University of Iowa. P14 mice were purchased from Taconic Breeding

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

2. **Vesicular Stomatitis virus**

VSV-ErkM is a wild-type vesicular stomatitis virus that was engineered to express ErkM$_{136-144}$, an H-2K^d- restricted CD8+ T cell epitope of the mutated mouse mitogen-activated protein kinase (ERK2). VSV-Gp33 expresses an H-2D^b-restricted epitope from the lymphocytic choriomeningitis virus glycoprotein (LCMV GP$_{33-41}$)

3. **Peptides**

ErkM (QYIHSANVL) and Gp33(KAVYNFATM) peptides were purchased from Biomer Technologies. They were dissolved in PBS with 0.5% BSA

4. **Tumour cell lines**

All cells were maintained at 37 degrees Celcius and 5% CO2. CMS-5 cells (gifted by Lyse Norian) and MC38-gp33 cells were maintained in a T150 culture dish with Rosewell Park Memorial Institute media (RPMI) supplemented with 10% FBS, 1% L- glutamine, 1% penicillin/streptomycin, 0.1% Beta-mercaptoethanol (Sigma-Aldrich)

5. **In vitro T cell differentiation**

Bulk splenocytes derived from transgenic DUC18 and P14 mice were isolated and processed into a single cell suspension by mechanical disruption and centrifuged at 1500 rotations per minute(rpm) for 5 minutes. The resultant pellet was resuspended in ACK lysis buffer for 5 minutes and inactivated by Hanks buffer. The cells were cultured for seven days in cRPMI in a 24 well dish at a concentration of 3x106cells/mL. The

splenocytes DUC18 and P14 were stimulated with 1μg/ml ErkM or Gp33peptide, respectively. The splenocytes were cultured in the presence of IL-21 (10ng/mL), IL-15 (10ng/mL) rapamycin (20ng/mL) to ensure memory differentiation. At days 2 and 5 of the culture, the splenocytes were expanded at a dilution of 1:2.5 in a master mix of cRPMI, IL-21 (10ng/mL), IL-15 (10ng/mL) rapamycin (20ng/mL).

6. **Tumour challenge, Adoptive Transfer and Oncolytic Viral Vaccination**

Once confluent, CMS-5 or MC38-gp33 cells were washed in PBS twice, trypsinized and resuspended in PBS at a concentration of 1×10^6 cells/30μL or 2×10^5 cells/30μL of PBS, respectively. Balb/c (CMS5) and C57BL/6 (MC38-gp33) mice were then intradermally challenged. CMS-5 tumours were grown to a volume of 50-100mm^3 for 6 days or 400-600 mm^3 in 12-14 days, and MC38-gp33 tumours were grown to a volume of 50-100 mm^3 for 6 days or 300-400 mm^3 in 14-17 days. Central memory DUC18 ErkM and P14 Gp33 specific CD8+ T cells were harvested and resuspended in PBS at a final concentration of 10×10^6 cells/200μl. The cells were intravenously injected into the tail vein of the recipients. Twenty-four hours later, VSV-ErkM or VSV-Gp33 was intravenously injected into the tail vein at a concentration of 5×10^8 PFU/ 200 μl.

7. **Histone deacetylase inhibitor treatment**

Four hours following vaccination, all three HDACis were administered via intraperitoneal injection on the day of vaccination and subsequently daily for five days at the following doses: MS-275 at a dose of 100μg/mouse in 50 μl of PBS, Valproic acid (VPA) at a dose of 8.5mg/mouse in 500μl of PBS and RGFP966 at a dose of 0.25mg/mouse in 50μl of DMSO.

8. Blood collection

Blood was collected from the retro-orbital vein of the treated mice with heparinized capillary tubes. The blood was collected into Eppendorf tubes with 50µl of heparin. The blood volumes were then calculated and transferred into FACS tubes. ACK lysis buffer was then added and incubated for 5 minutes at room temperature and then inactivated with Hanks buffer and centrifuged at 1500rpm. This lysis step is then repeated. The remaining pellet is then resuspended into cRPMI, and 50µl is transferred into a 96 well plate. ErkM and Gp33 peptide at a concentration of 0.5µg/ml in 100 µl of cRPMI is added to each well and left to incubate for 1 hour at 37 degrees Celsius. After one hour, Golgi plug dissolved in 50ul of cRPMI is added to each well and left to incubate in the same conditions for four additional hours.

9. Flow Cytometry, Intracellular, and Extracellular Staining

Filipin III was purchased from Cayman Chemical, and Granzyme B, Ki67 and TNFα were purchased from eBioscience ThermoFisher. Unless indicated otherwise, all antibodies used for flow cytometry were purchased from BD Biosciences (Table 1). The peripheral blood mononuclear cells in the 96 well plate were pelleted and incubated with FC block for 10 minutes at 4 degrees Celsius, then the 2X surface antibodies were added at a volume of 25 µl in FACS buffer. Then incubated at 0 degrees Celsius for 20 minutes, washed twice with FACS buffer and incubated with 100µl of CytoFix or eBioscience Foxp3/Transcription factor staining buffer kit for 20 or 45 minutes, respectively. The cells were washed twice, and antibodies were incubated in a final volume of 25 µl for 20 minutes or 45 minutes for Filipin III and Ki67 at 4 degrees Celsius. After washing twice,

the cells were then resuspended in 200μl of FACS buffer, filtered and analyzed

by BDfortessa flow cytometry machine. The plots were then further analyzed

using FlowJo 10 software.

Marker	Fluorophore	Clone	Company
CD8a	BV711	53-6.7	BD Biosciences
Thy1.1	APC-CY7, PerCP-Cy5.5	OX-7	BD Biosciences
CD62L	APC-CY7	MEL-14	BD Biosciences
CD44	BV786	IM7	BD Biosciences
Tim3	BV421	5D12	BD Biosciences
KLRG1	PE-CF594	2F1	BD Biosciences
PD-1	BV711	J43	BD Biosciences
Fixable viability Stain	BV510		BD Biosciences
KI67	PE-CY7	SolA15	eBioscience ThermoFisher
Interferon- Gamma	APC	XMG1.2	BD Biosciences
Granzyme B	PE	NGZB	eBioscience ThermoFisher
Tumour necrosis factor Alpha	PE-Cy7	MP6-XT22	eBioscience ThermoFisher
Filipin III			Cayman Chemical

Table 1. Antibody chart for flow cytometry

10. In vitro treatment with MS-275

Bulk splenocytes derived from P14 mice were cultured *in vitro* to obtain a central

memory phenotype (see In vitro T cell differentiation). DC2.4 cells are diluted in cRPMI

to at least $1x10^6$ cells/mL with 2 ug/mL of gp33 peptide in a 50 mL falcon tube for 2

hours at 37 degrees Celsius. Following DC pulsing, $2x10^6$ P14 TCM were co-cultured

with gp33-pulsed DC2.4s at a ratio of 0.1:1 DCs: P14 T cells or 1uM of MS-275 in a 24

well plate with a final cRPMI volume of 2mL.

11. In vitro killing assay

Purify bulk splenocytes using Thy1.1 positive selection kit and co-culture purified splenocytes with MC38-gp33 cells labelled with 2μL of V450 proliferation dye in a 96 well plate at the following ratios of T cells: Tumour cells (0.5:1, 1:1, 2:1, 5:1) for 8-10 hours. The cells will be stained for viability dye, CD8, Thy1.1 and analyzed by flow cytometry. The following equation calculated specific lysis: (100x (% specific lysis - % basal lysis) /(100 - %basal lysis)) with the basal lysis indicating the viability dye on labelled tumour cells without effector cells.

12. Statin Treatment

Four hours following vaccination, 500ug/mL per mouse at a final volume of 50 uL of Pitavastatin (Cayman Chemical) was administered via intraperitoneal injection on the day of vaccination and subsequently daily for five days.

13. Statistics

GraphPad Prism for Macintosh was utilized for statistical analysis and graphing.

Chapter 3: Results

1. Concomitant delivery of MS-275 rescues the therapeutic efficacy of Adoptive T cell (ACT) therapy as tumour burden increases.

The development of immunotherapies, specifically adoptive T cell therapy and oncolytic virus therapy, has radicalized cancer treatment. Despite its success over conventional forms of treatment, clinical studies indicate the solid tumour stage and

increased tumour burden has a detrimental effect on the therapeutic efficacy of immunotherapies reliant on the function of a tumour-specific CD8+ T cell response [102]. In our laboratory, we have demonstrated that utilizing an oncolytic vaccine expressing a TAA can induce a robust *in vivo* expansion of adoptively transferred tumour-specific central memory CD8+ T cells (Tcm) in the periphery and the TME resulting in enhanced survival and the complete regression of solid tumours. However, the therapeutic success of ACT has only been observed in mice bearing early-stage tumours[74,75]. Studies have reported that tumour burden can be a factor that determines the success of immunotherapies such as ICI and ACT. Specifically, a study demonstrated that the ratio of T cell reinvigoration by PD1 blockade to tumour burden determines success, and patients with larger tumours demonstrate lower rates of success[102]. Thus, we sought out to evaluate the effect of the tumour stage on the therapeutic efficacy of ACT.

WT Balb/c mice were intradermally challenged with CMS5 fibrosarcoma cells expressing the mutant form of the mouse mitogen-activated protein kinase, ErkM[173,174]. When tumours reached a volume of 100-300 mm^3 (Early-stage CMS5 tumours) grown in 6 days, 1×10^6 ErkM-specific CD8+ T cells from DUC18 transgenic mice cultured to achieve a central memory phenotype were administered intravenously[74]. Twenty-four hours later, 5×10^8 PFU of vesicular stomatitis virus expressing ERK antigen (VSV-Erk) was administered intravenously (i.v) (Fig. 1A). We have previously demonstrated that i.v. administration of a rhabdoviral vaccine is the optimal route for expanding T$_{CM}$ in secondary lymphoid organs[74,118,156]. We observed that ACT treatment in mice with small early-stage CMS5 tumours resulted in a robust ERK specific CD8$^+$ T cell response on

day 5 post-vaccination (PV) (Fig. 1B), complete tumour regression (Fig. 1D) and 100% survival (Fig.1E), which is consistent with our previous findings [74]. Furthermore, the frequency of the transferred T cell response in small CMS5 tumours is similar to that observed in tumour-free animals receiving ACT treatment (Fig. 1C).

Following the success of ACT in small early-stage CMS5 tumours[74–76], we sought to evaluate if therapeutic success is maintained as tumour stage and burden advances. We observed that when ACT is repeated in CMS5 tumours with a volume of 400-600mm^3 (Late-stage CMS5 tumours) grown for 12 days, the therapy completely fails. The ERK-specific transferred CD8+ T cell response was not detected at day 5PV (Fig. 1B); there is a loss of tumour control (Fig. 1D), and the survival rate is 0% (Fig. 1E). This data insinuates that increased tumour burden and stage at the onset of ACT treatment imposes more significant immunosuppression of transferred cells compared to the early-stage tumour as transferred cells are undetected at day 5 PV, which represents the peak T cell response in this model[76]. This data corroborates previous findings we have made demonstrating that the success of ACT relies on the presence of tumour-specific CD8+ T cells. We demonstrated the loss of tumour control and survival following the depletion of tumour-reactive CD8+ T cells [76].

Suboptimal persistence of tumour-specific CD8+ T cells has been associated with T cell dysfunction due to enhanced immunosuppression within the tumour microenvironment[105]. Thus, we believe that utilizing an agent capable of subverting immunosuppression associated with tumour growth to enhance T cell persistence may potentiate our therapeutic approach. We have previously shown that MS-275, a Class 1

and 3 histone deacetylase inhibitor (HDACi), displays immunomodulatory properties to subvert immunosuppression within the tumour microenvironment in addition to enhancing the function of tumour-specific T cells expanded by an OVV[75,172]. Thus, we evaluated the possibility of MS-275 to rescue the therapeutic efficacy of ACT in large late-stage CMS5 tumours. To do this, we intraperitoneally (i.p) administered MS-275 at a dose of 100ug/ml starting on the day of vaccination and daily for a total of 5 days, as previously optimized (Fig. 1A)[75,172]. Interestingly, concomitant delivery of MS-275 with ACT+ OVV restored the transferred T cell response to similar levels seen in tumour-free and small tumour-bearing animals (Fig. 1B and C), completely regressed large tumours (Fig. 1D) and restored the curative effect (Fig. 1E). Thus, this suggests that MS-275 may alter immunosuppressive factors of the late-stage tumour to ensure enhanced persistence of tumour-specific T cells.
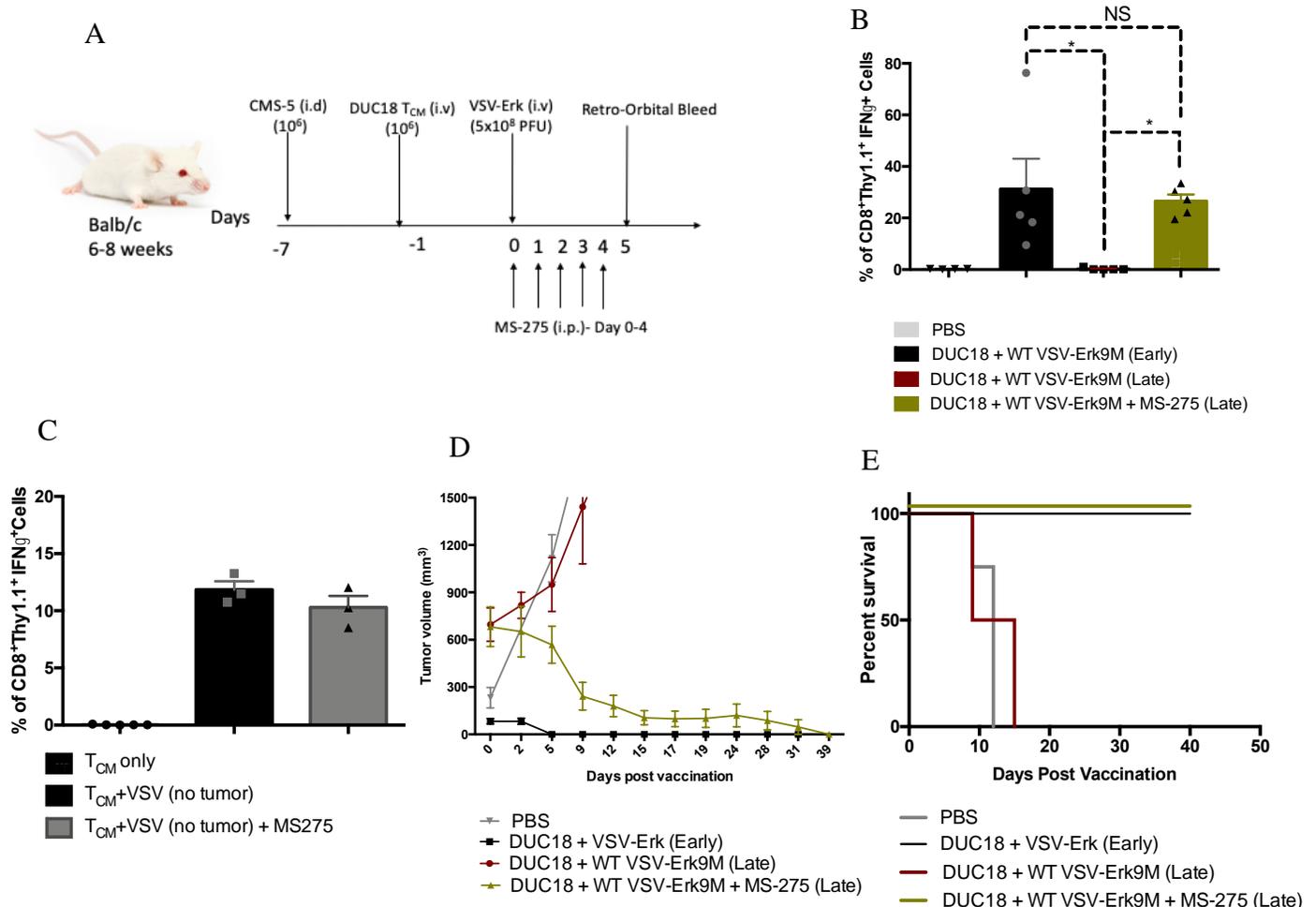
Figure 1. Concomitant delivery of MS-275 rescues the therapeutic efficacy of Tcm + VSV-ErkM in late-stage tumours.

A) Experimental schematic. Balb/c mice were inoculated with 10^6 CMS-5 cells/30 μL 6 days with a tumour volume of 50-100mm^3 (early-stage CMS5 tumours) or 12 days with a tumour volume of 600mm^3 (late-stage CMS5 tumours) before injecting 10^6 DUC18 Tcm cells i.v, followed by 5x10^8 PFU VSV-Erk9M 24 hours later. Mice treated with MS-275 were administered 0.1mg intraperitoneally (i.p) 4 hours following vaccination and repeated daily for a total of 5 days. Peripheral blood lymphocytes were extracted retro-orbitally from mice 5 days post vaccination. The quantity of transferred DUC18 T cells from tumour-bearing (B) and tumour-free (C) mice expressing INFγ following ex vivo stimulation with ERK peptide. Tumour volumes (D) and survival (E) were calculated on the specified days following vaccination. Figure B and C data was generated by Andrew Nguyen.

2. MS-275 enhances the persistence of transferred cells in late-stage CMS5 tumours

Next, we sought to determine if the absence of transferred cells at day 5 in the late-stage tumour was due to the inability for transferred T cells to be expanded upon vaccination or if T cell absence is due to a progressive loss of persistence following expansion. To track the *in vivo* kinetics of transferred T cells under the influence of large late-stage CMS5 tumours, we transduced transferred ErkM-specific CD8$^+$ T cells with luciferase and monitored the *in vivo* trafficking and proliferation by In Vivo Imaging System visualization (IVIS) from day 1PV to day 5PV (Fig. 2A, B). Transferred CD8$^+$ T cells are detected in the tumour, lymph nodes and spleen as early as day 2PV in the presence or absence of MS-275. For animals treated without MS-275, the peak T cell response in circulation, tumour, lymph nodes, and spleen was at day 3PV, followed by the progressive decline at day 4PV and complete loss of T cells by day 5PV. Conversely, animals treated with MS-275 demonstrate a progressive increase in ERK-specific T cells at days 3 and 4, with a peak at day 5 PV (Fig. 2). This demonstrates that the absence of transferred cells at day 5 PV in the late-stage tumour is not due to ineffective expansion in response to oncolytic vaccination, but the progressive loss of transferred cells may be due to heightened tumour-induced immunosuppression accompanied by increased tumour stage and size. However, this suggests that concomitant delivery of MS-275 allows for sustained proliferation of transferred cells in the presence of the late-stage tumour.
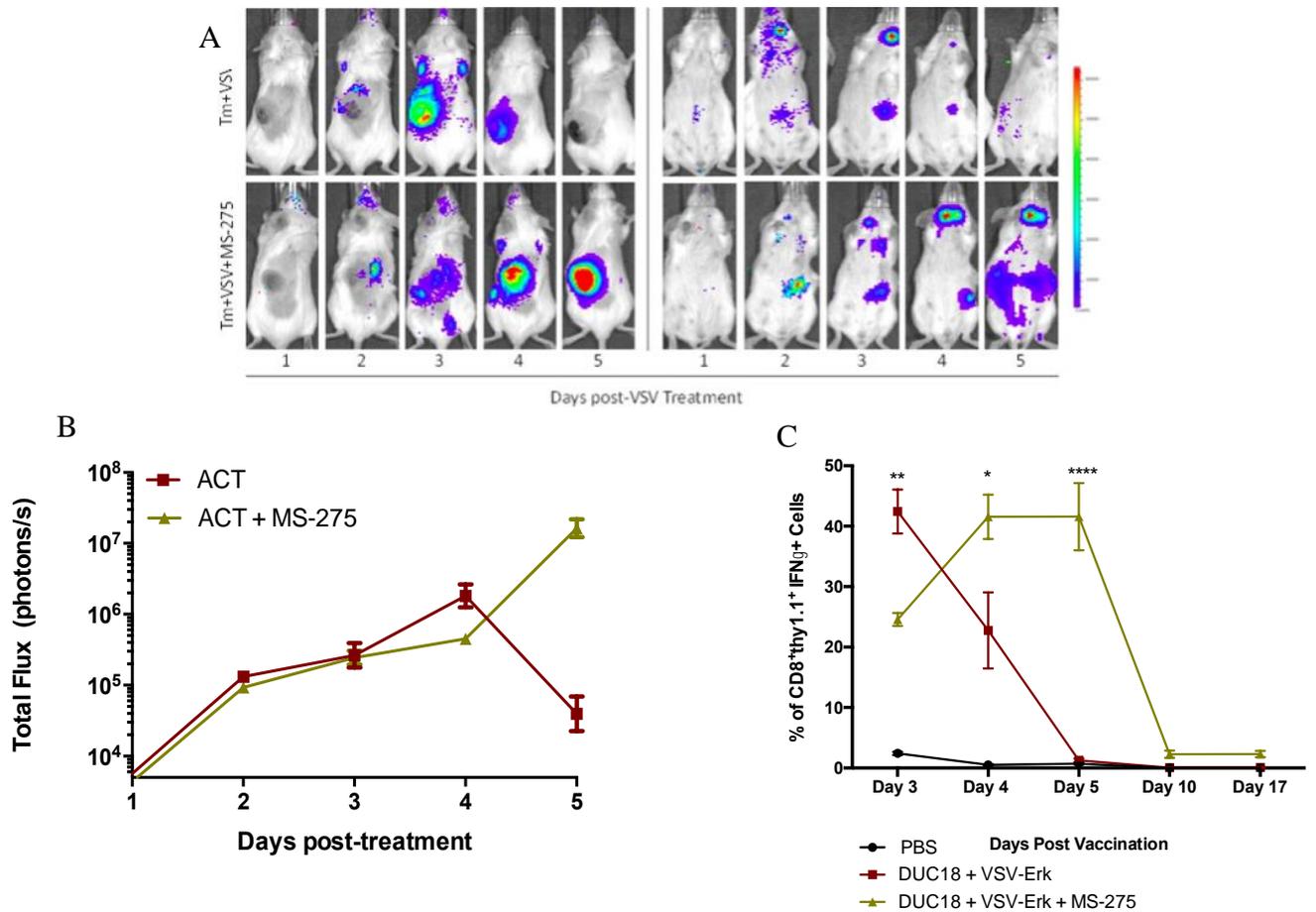
Figure 2. MS-275 enhances the persistence of transferred cells in late-stage CMS5 tumours.

Kinetics of the DUC18 transferred cell response in late-stage CMS5 Tumours following ACT +/- MS-275 treatment. A) and B) IVIS was utilized to track the fate of DUC18 CD8+ T cells transduced with luciferase following treatment on the indicated days C) The frequency of transferred DUC18 CD8+ T cells was analyzed in peripheral blood on the days indicated. Figure A and B data was generated by Andrew Nguyen.

3.Tumour burden results in decreased proliferative capacity and increased expression of exhaustion markers on adoptively transferred T cells.

Suboptimal *in vivo* persistence of adoptively transferred cells in solid

malignancies have been attributed to tumour-induced dysfunction of T cells[70,98,175]. This

state, akin to T cell exhaustion observed following chronic viral infection, is often described as a high expression of markers associated with exhaustion such as TIM3, PD1, an increase in KLRG1, a marker for terminal differentiation and a decrease in proliferation [98,176–178]. To explore if the disappearance of transferred cells is attributed to T cell dysfunction, we monitored the expression of PD1, KLRG1 and TIM3, markers associated with dysfunction, in addition to KI67, a marker of proliferative capacity (Fig. 3, Appendix Fig. 7)). To ensure the recovery of a sufficient number of transferred cells for analysis, we extracted and compared the expression of PD1, TIM3, KLRG1 and KI67 on transferred cells from the spleen, blood and tumours of animals bearing early or late-stage tumours on day 4.5PV, prior to transferred cell disappearance on day 5PV. We observed that the expression of PD1, TIM3, KLRG1 and KI67 on transferred cells within the spleen of early and late-stage tumours does not exhibit any significant changes. However, in the blood, transferred cells in animals with late-stage CMS5 tumours demonstrate a decreased expression of KI67 and an increased expression of TIM3 and PD1. Similarly, transferred cells extracted from the late-stage tumour demonstrate a decrease in KI67 expression and an increase in KLRG1, PD1, and TIM3 expression compared to tumour infiltrating transferred cells in animals bearing early-stage CMS5 tumours. This observation demonstrates that transferred cells in the late-stage tumour exhibit hallmarks of classically dysfunctional T cells. This suggests that increased tumour burden and stage induce a progressive differentiation of transferred T cells to a dysfunctional phenotype, which is heightened by trafficking to the tumour microenvironment (Fig. 3).
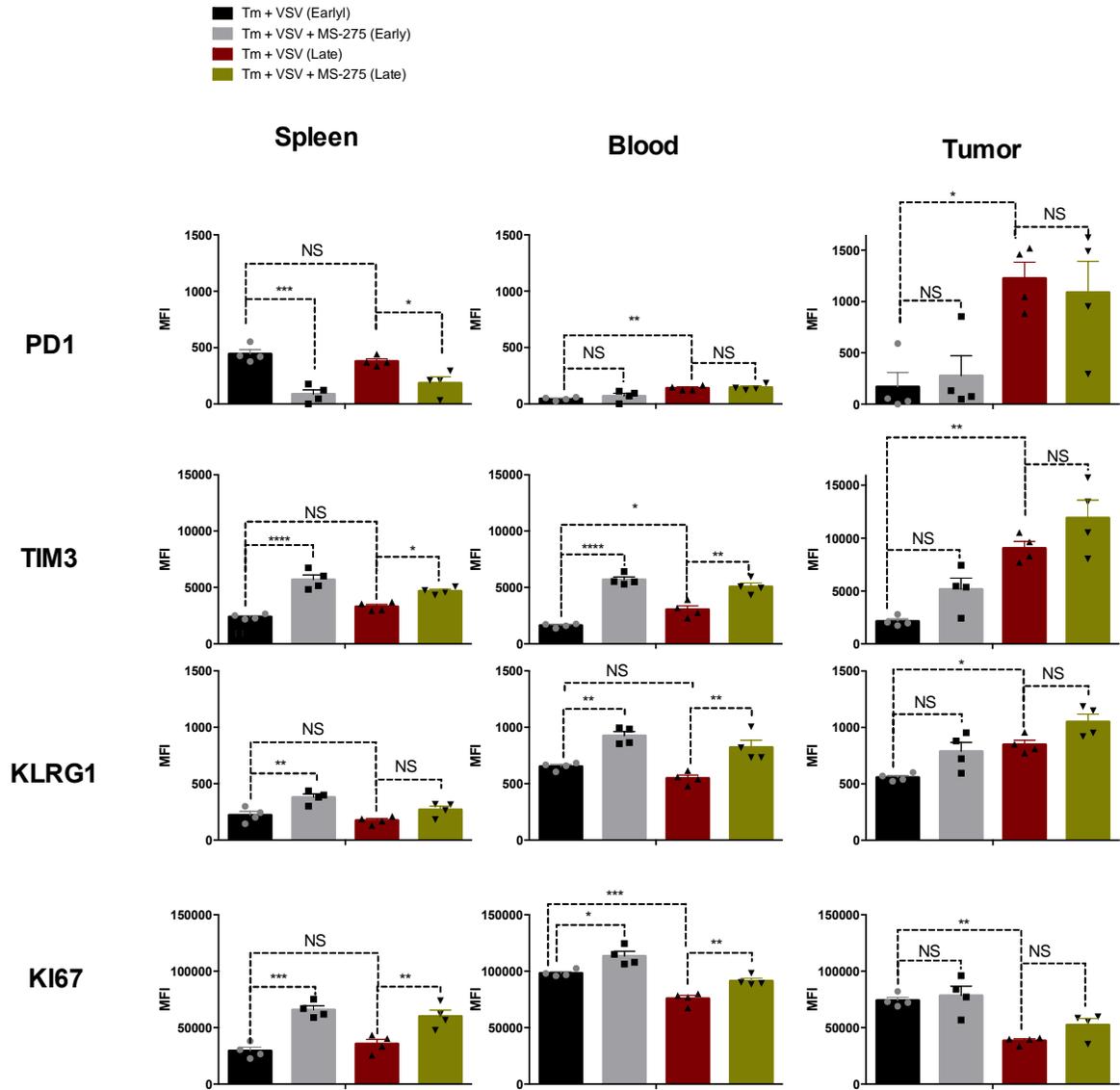
Figure 3. Adoptively transferred T cells exhibit a dysfunctional phenotype in late-stage tumours.

DUC18 CD8+ T cells were extracted on day 4.5 post vaccination from the spleen, peripheral blood and tumours of mice bearing early or late-stage CMS5 Tumours following ACT treatment. DUC18 T cells were analyzed for the expression of PD1, TIM3, KLRG1, and KI67 expression by flow cytometry. Figure flow cytometry data was generated by Andrew Nguyen.

4.MS-275 alters the proliferative capacity and the expression of exhaustion markers of transferred cells in late-stage CMS5 tumours

Next, we sought to determine if the concomitant delivery of MS-275 rescues the presence and persistence of adoptively transferred cells in the late-stage tumour by reversing the hallmarks of T cell dysfunction observed in the late-stage tumour. Therefore, we monitored the expression of TIM3, PD1, KLRG1 and KI67 expression on transferred cells in response to the presence or absence of concomitant MS-275 treatment within late-stage CMS5 tumours on day 4.5 PV (Fig. 3). In animals treated with MS-275, PD1 expression was decreased on transferred cells located in the spleen. Conversely, KI67 expression was significantly increased on transferred cells located in the spleen and blood of animals treated with MS-275. Based on this data, we were under the impression that MS-275 treatment may maintain transferred T cell presence by reversing tumour-induced T cell dysfunction. However, surprisingly, we observed the increased expression of TIM3 with MS-275 in the spleen and blood, and KLRG1 increased in the blood. This implies that MS-275 treatment may not reverse T cell dysfunction to ensure the persistence of T cells in the late-stage tumour as markers associated with dysfunction were increased. Furthermore, MS-275 treatment may confer properties to the T cell that allows for efficient proliferation in the presence of heightened immunosuppression due to enhanced tumour burden and stage. Although upon MS-275 treatment, there is a higher percentage of transferred cells in the periphery and within the TME (Fig 2), the phenotypic changes mediated by MS-275 are only observed in the periphery, with the most significant effect occurring in the spleen in comparison to the blood, and alterations to T cell phenotype are absent in the tumour (Fig. 3). This data suggests that there may be compartmental restrictions on where MS-275 exerts phenotypic changes, implying that

only transferred cells in the periphery are subject to phenotypic alterations induced by MS-275, while tumour infiltrating transferred cells are not.

Interestingly, administration of MS-275 exerts the same phenotypic changes on transferred cells in the blood and spleen of early-stage CMS5 tumours as transferred cells in the periphery of late-stage CMS5 tumours (Fig 3). This demonstrates that MS-275 can alter the phenotype of systemic transferred cells independently of tumour size.

5. MS-275 enhances the therapeutic efficacy of OVV therapy as tumour burden increases

The success of various immunotherapies currently available, like checkpoint inhibition, is dependent on the presence and function of endogenous tumour-specific CD8+ T cells[74,181]. Since the therapeutic capacity of ACT is rescued with MS-275 in late-stage CMS5 tumours (Fig. 1) and seemingly has a direct effect on dysfunctional T cells (Appendix 2.1, 2.2), we sought to explore if MS-275 can enhance therapies reliant on the function of endogenous tumour primed CD8+ T cells in an aggressive tumour model. To broadly evaluate the potential of MS-275 on T cell-based therapies, we used a well-characterized model in our laboratory that is reliant on the expansion of pre-existing tumour-specific memory T cells by an OVV and, like the CMS5 model, is unsuccessful in late-stage tumours.

C57BL/6 mice were intradermally implanted with Murine Colon Adenocarcinoma cells (MC38) expressing Gp33, an epitope from the lymphocytic choriomeningitis virus glycoprotein (LCMVGP$_{33-41}$)[182] (MC38-Gp33) and grown for six days with a tumour

volume of 50-100 mm^3 (early-stage MC38 tumours). VSV expressing gp33 (VSV-Gp33) is administered intravenously, resulting in a Gp33 specific CD8$^+$ T cell responses of around 38% of the total circulating CD8$^+$ T cells (Fig. 4.1A), complete tumour regression (Fig. 4.1B) and a survival rate of 100% (Fig. 4.1C). However, when tumours are grown for 14-17 days and have a tumour volume of around 300 mm^3 (late-stage MC38 tumours), and VSV-GP33 is administered, the gp33 specific T cell response is dampened to around 15 % of total circulating CD8+ T cells (Fig. 4.1A), regression of tumours is incomplete (Fig. 4.1B), and the curative potential is lost (Fig. 4.1C). However, like in the CMS5 model, concomitant delivery of MS-275 for five consecutive days, starting at the day of vaccination, rescues the transferred T cell response to around 30% (Fig. 4.1A), resulting in tumour regression (Fig. 4.1B) and enhanced survival (Fig. 4.1C). This data suggests that increased tumour burden and stage induces the decline of T cell presence following expansion with an OVV and is indiscriminate of the source of T cells, transferred or endogenous. Similarly, MS-275 can restore the transferred or endogenous tumour-specific T cell response in late-stage tumours following oncolytic vaccination.
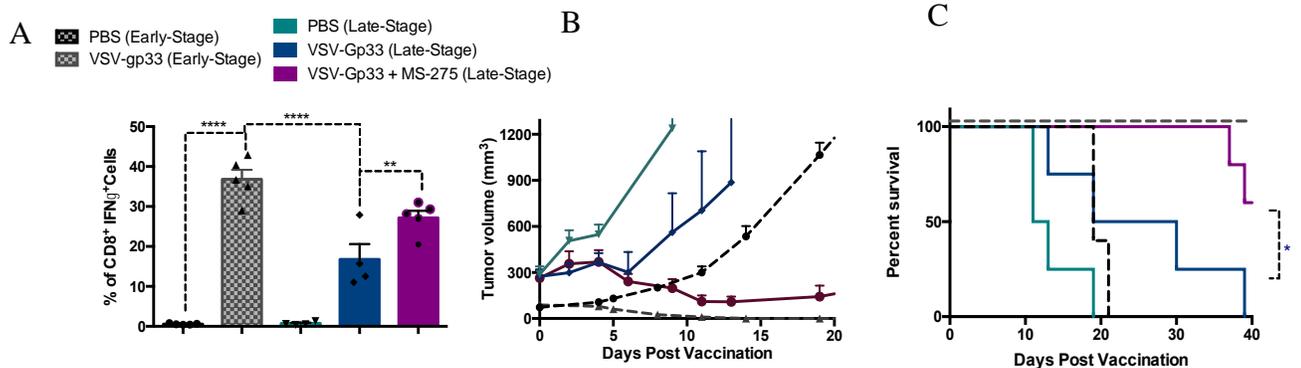
Figure 4. 1. Concomitant delivery of MS-275 rescues the therapeutic efficacy of OVV therapy in late-stage MC38 tumours.

C57BL/6 mice were inoculated with 2×10^5 MC38-Gp33 cells for 6 days with a tumour volume of 50 mm^3 (early-stage MC38 tumours) or 14-17 days with a tumour volume of 300mm^3 (late-stage MC38 tumours) before intravenously administering 2×10^8 PFU VSV-Gp33. Mice treated with MS-275 were administered 0.1mg intraperitoneally (i.p) 4 hours following vaccination and repeated daily for a total of 5 days. A) Peripheral blood lymphocytes were extracted retro-orbitally 5 days post vaccination. B) Tumour volumes and C) survival were monitored on the indicated days following vaccination. Early-stage tumour data was generated by Dr. Li Deng

Following the observation that MS-275 can potentiate OVV therapy in late-stage

MC38 tumours, we sought to monitor any phenotypic similarities between transferred

cells and endogenous cells expanded by an OVV in the presence of MS-275. Thus, we

monitored the expression of TIM3 and KLRG1 and KI67 on endogenous gp33 specific T

cells on day 5 PV within the blood (Fig. 4.2). Interestingly, similarly to T cells treated

with MS-275 in early or late-stage CMS5 tumours, MS-275 enhanced the number of

tumour-specific T cells (Fig. 4.1a) and the expression of KI67, TIM3, and KLRG1 on

these cells (Fig. 4.2). Specifically, it is interesting to observe the same changes induced

by MS-275 on T cells regardless of how T cells are primed, either through ideal *in vitro* conditions or in the presence of immunosuppressive factors of the developing tumour. This may suggest that even though there exist subtle therapeutic differences between the CMS5 and MC38gp33 models exhibiting complete T cell absence or a dampened response, respectively, there may be a standard mode of immunosuppression of OVV expanded T cells in the late-stage tumour that MS-275 alters.
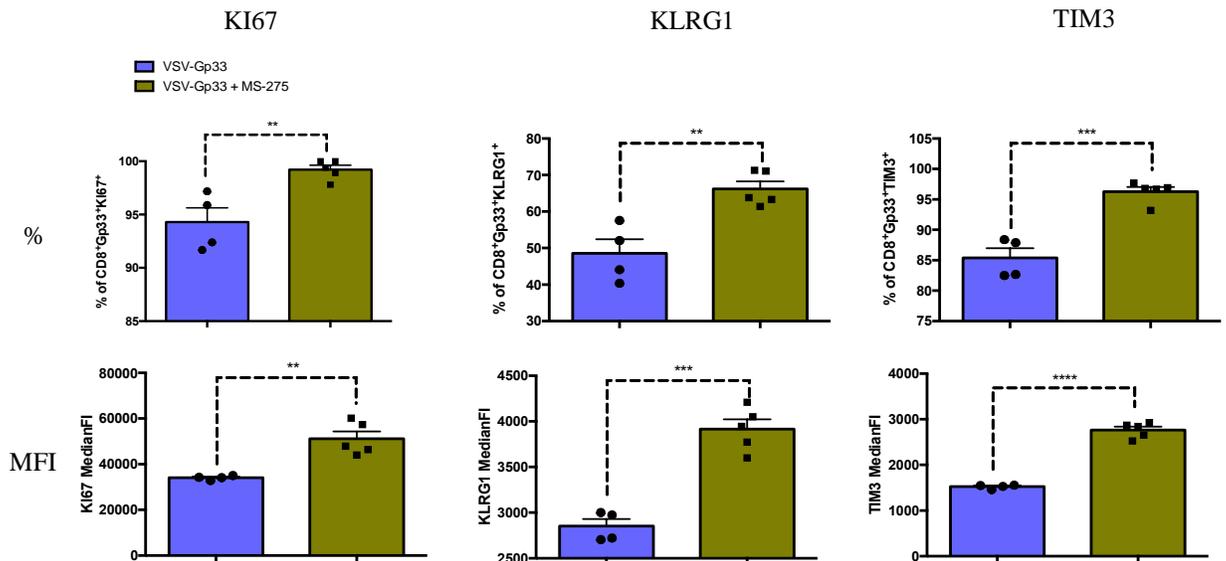
Figure 4. 2. MS-275 increases the expression of TIM3 and KLRG on endogenous tumour-primed cells following vaccination in late-stage MC38 tumours.

Gp33 specific CD8+ T cells were extracted on day 5 post vaccination from the peripheral blood of mice bearing late-stage MC38-gp33 tumours following OVV treatment +/- MS-275. Gp33 specific T cells were analyzed for the expression of KI67, KLRG1, and TIM3 expression by flow cytometry.

Next, we sought to evaluate the magnitude and phenotype of T cells in transit to

the TME of late-stage MC38-Gp33 tumours. Specifically, we monitored T cells in the
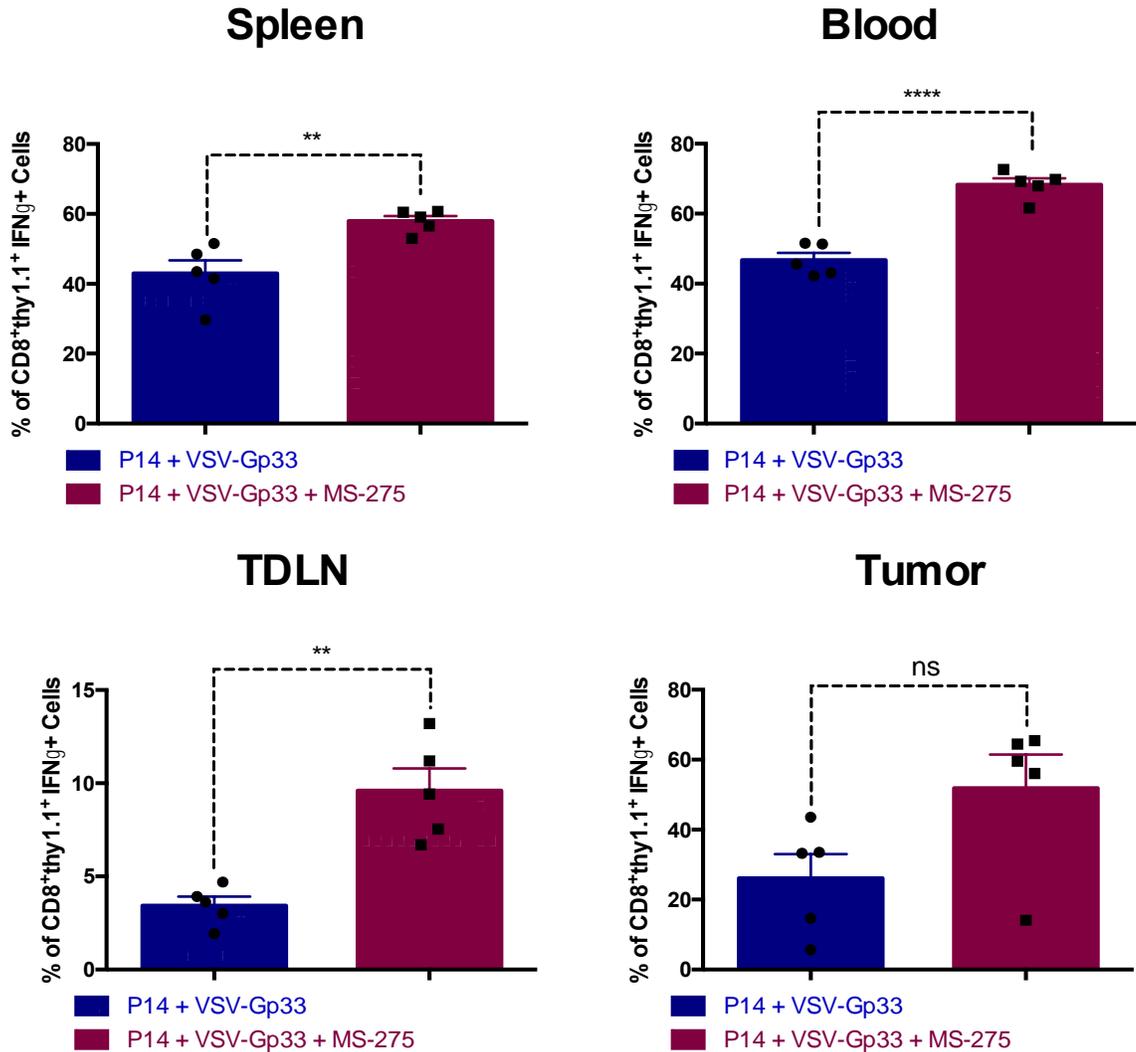
Figure 5.Concomitant delivery of MS-275 increases the percentage of tumour reactive cells locally, and in the periphery in late-stage tumours.

C57BL/6 mice were inoculated with $2x10^5$ MC38-Gp33 cells for 14-17 days with a tumour volume of $300mm^3$ (late-stage MC38 tumours) and intravenously administered $1X10^6$ central memory P14 T cells 24 hours before intravenously injecting $2x10^8$ PFU VSV-Gp33. Mice treated with MS-275 were administered 0.1mg intraperitoneally (i.p) 4 hours following vaccination and repeated daily for a total of 5 days. Transferred CD8+ T cells were extracted from the spleen, blood, tumour draining lymph nodes and tumours of mice with and without MS-275 treatment and analyzed by flow cytometry.

spleen, blood, tumour draining lymph nodes and the tumour of late-stage MC38-Gp33 tumours. To ensure adequate recovery of tumour-specific T cells to evaluate the effect of MS-275 in multiple organs effectively, we transferred gp33 specific central memory T cells 24 hours prior to vaccination and extracted transferred cells from the organs mentioned above on day 5PV. We observed that MS-275 significantly increased the percentage of transferred cells present in various organs (Fig. 5). Next, we monitored the expression of markers associated with exhaustion (TIM3, KLRG1) and proliferation (KI67). In the spleen, blood and tumour draining lymph nodes, we observed significant increases in KI67, TIM3 and KLRG1on transferred cells in animals treated with MS-275. Conversely, although we observed a significant increase in the quantity of tumour-specific T cells within the TME with MS-275 treatment, we did not observe alterations in the expression of TIM3 and KI67 and observed minimal increases with the expression of KLRG1 on transferred cells in the tumour. (Fig. 6). This suggests that MS-275 can also enhance the therapeutic efficacy of OVV within late-stage MC38 tumours by increasing the proliferative capacity and altering the phenotype of tumour-specific CD8+ T cells expanded by the OVV seen similarly in the CMS5 model.

Figure 6. MS-275 enhances the expression of KLRG1 and TIM3 on endogenous tumour reactive T cells within late-stage MC38 tumours.

P14 cells were extracted on day 5 post vaccination from the spleen, peripheral blood, tumour draining lymph nodes, and tumours of mice bearing 14–17-day MC38 tumours following OVV treatment +/- MS-275. Transferred cells specific T cells were analyzed for the expression of KI67, KLRG1, and TIM3 expression by flow cytometry.
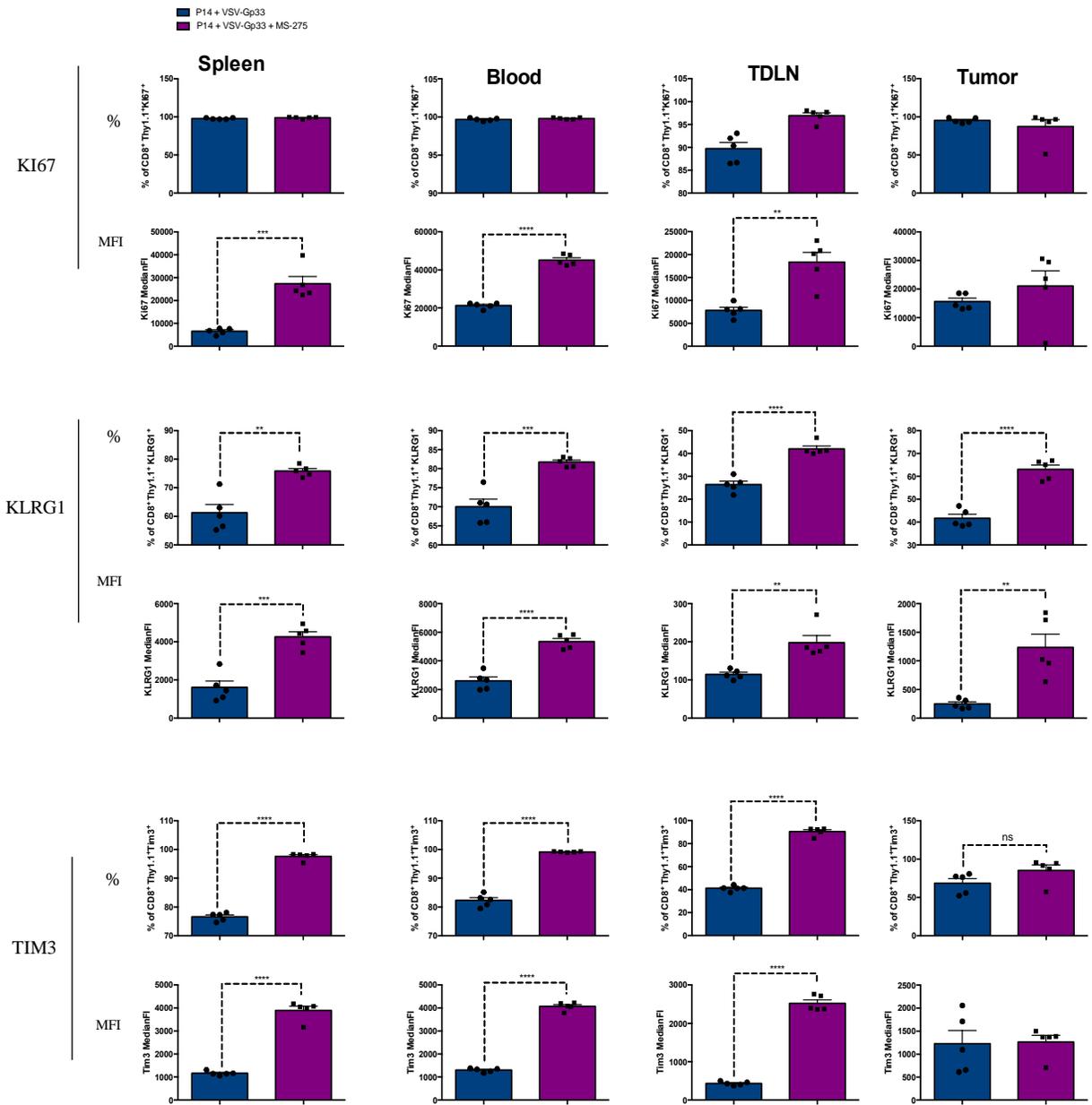
6.MS-275 can alter the cytotoxic function of CD8+ T cells in advance stage tumours

To elicit tumour debulking, tumour-specific T cells must be capable of recognizing tumour cells, followed by the release of cytotoxic molecules such as granzyme B and TNFα to promote tumour cell destruction[183,184]. It has been demonstrated that dysfunctional tumour-specific T cells have a reduced ability to secrete pro-inflammatory cytokines and cytotoxic molecules[185,186]. Thus far, the data has demonstrated that MS-275 can restore the T cell presence and persistence in late-stage tumours and can alter the phenotype of transferred cells not to reverse markers associated with exhaustion but to promote the expression of TIM3 and proliferation. However, although the number is increased overall, we sought to examine the functional status of T cells in response to MS-275 in the late-stage tumour. Thus, we examined if MS-275 can also alter the function of tumour-reactive CD8+ T cells in late-stage MC38 tumours in addition to the T cell number and phenotype. To do this, we monitored the secretion of inflammatory cytokines, interferon-gamma (INFɣ) and tumour necrosis factor-alpha (TNFα) in transferred tumour-reactive CD8+ T cells located in the spleen blood, tumour draining lymph nodes and tumour of animals treated with ACT in the presence and absence of MS-275 (Fig. 7). Surprisingly, we observed that the expression of inflammatory cytokines INFɣ and TNFα were unchanged in transferred cells located in the tumour but were decreased in the blood and further decreased in the spleen (Fig. 7).
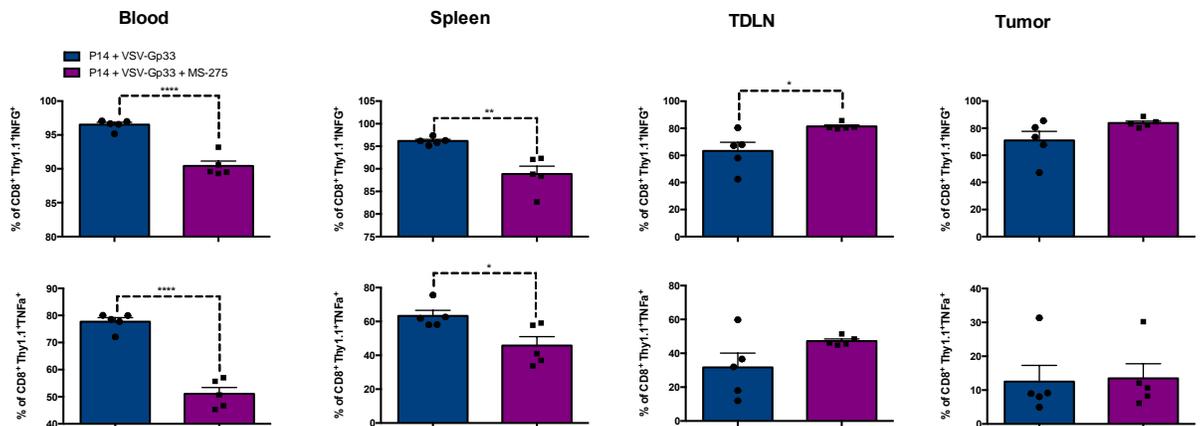
Figure 7. MS-275 alters the expression of inflammatory molecules of tumour reactive CD8+ T cells in late-stage tumours.

P14 CD8+ T cells were extracted on day 5 post vaccination from the spleen, peripheral blood and tumours of mice bearing late-stage MC38-gp33 tumours following OVV +/- MS-275 treatment. P14 CD8+ T cells were analyzed for the expression of Interferon gamma (INFɣ), and Tumour-necrosis factor alpha (TNFα) expression by flow cytometry.

This suggests that transferred cells located in the periphery in the presence of MS-275 have a reduced ability to secrete inflammatory cytokines. The decreased expression of pro-inflammatory cytokines from T cells is typically associated with dysfunction[186,187], but with the use of MS-275, we observed increased therapeutic efficacy through the restoration of the T cell response. Thus, we sought to assess the direct cytotoxic capabilities of transferred cells in the presence of MS-275. To monitor if there were alterations to the function of T cells in the presence of MS-275, we took two approaches, firstly, we monitored the expression of cytotoxic molecules of transferred cells located in the spleen, blood tumour draining lymph nodes and the tumour upon *ex vivo* peptide stimulation. Secondly, we harvested transferred cells from the spleens of mice treated with ACT +/- MS-275 and monitored specific tumour cell killing *in vitro.*

One of the ways that cytotoxic T cells eliminate target cells is by granule-dependent cytotoxicity. Lytic granules are pre-formed granules consisting of granzymes and perforins encapsulated by a lipid bilayer consisting of lysosomal associated membrane glycoproteins (LAMPs) such as CD107a. Upon degranulation, LAMPS are detected at the cell surface following fusion of the granule membrane with the plasma membrane[188–190]. Thus, to evaluate the cytotoxic ability of T cells in the presence of MS-275 in late-stage MC38-GP33 tumours, we monitored the expression of CD107a and granzyme B in transferred cells extracted from the spleen, blood, tumour draining lymph nodes and tumour. We observed that transferred CD8+ T cells from the spleen and the blood of MS-275 treated animals demonstrated a three-fold increase in granzyme B expression and a significant increase in the expression of CD107a in the tumour draining lymph nodes and the tumour (Fig. 8A). This suggests that with MS-275, there is a greater production of granzyme B expression in the periphery; however, as T cells enter the TME, there are similar granzyme B expression levels but enhanced degranulation of lytic granules in the TME.

Although with MS-275, we observed enhanced degranulation, we sought to evaluate further the specific killing ability of tumour cells by tumour-reactive T cells treated with and without MS-275 by conducting an *in vitro* killing assay. To do this, we extracted and purified transferred T cells from the spleens of animals treated with and without MS-275 and co-cultured the extracted cells with MC38-gp33 tumour cells to monitor the death of tumour cells (Fig. 8B). We observed that transferred T cells in the spleens of animals treated with MS-275 have an enhanced ability to specifically destroy

MC38gp33 tumour cells (Fig. 8B). This suggests that in addition to enhanced

degranulation, transferred T cells in the presence of MS-275 have enhanced ability

specifically to recognize and destroy MC38-gp33 tumour cells. Overall, this data suggests

that MS-275 confers properties to transferred T cells that result in the increased and

sustained expression of inhibitory receptors and enhancing the tumour-specific cytotoxic

function.

Figure 8. MS-275 enhances the cytotoxicity of tumour reactive CD8+ T cells in late-stage tumours.

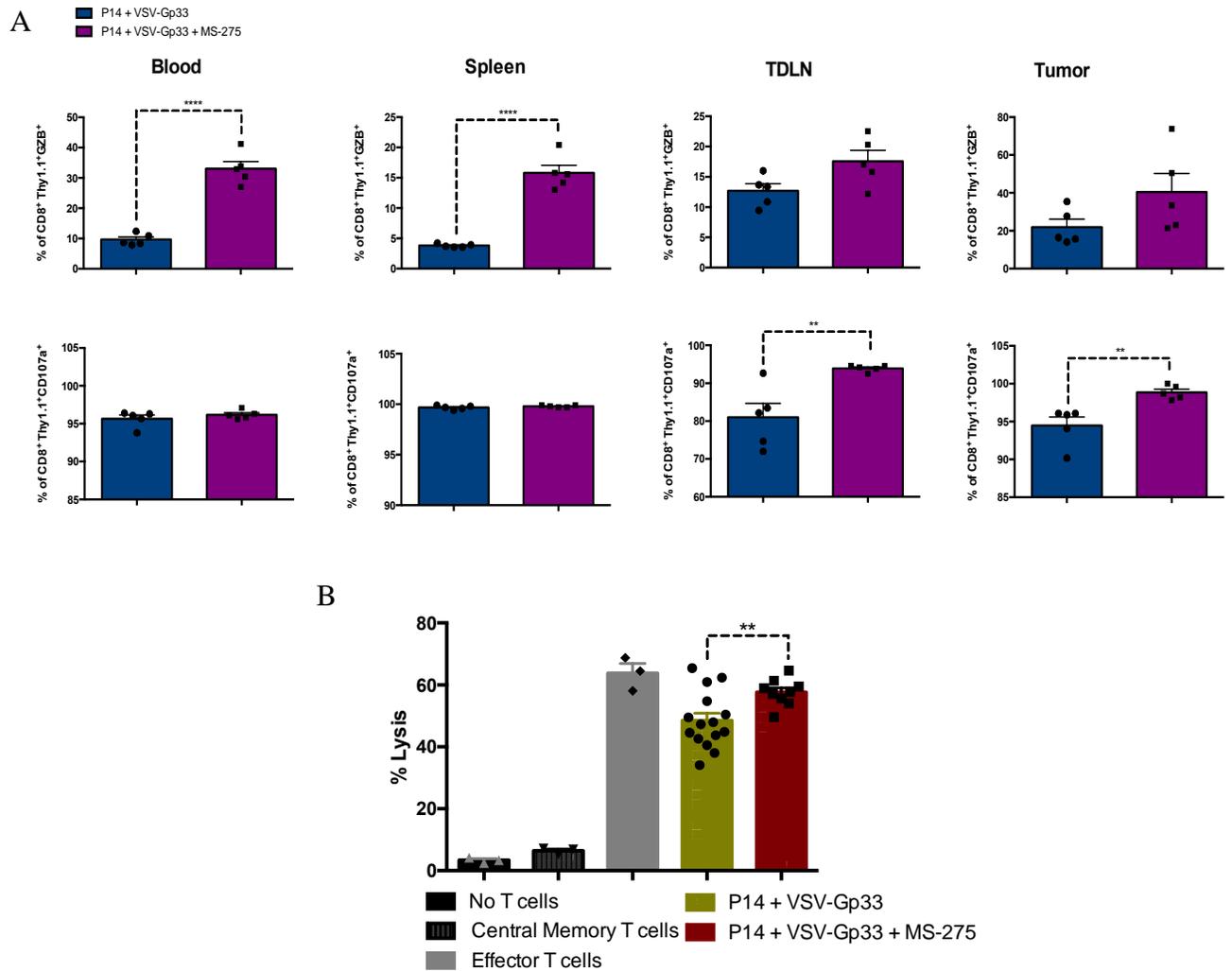**A)** P14 CD8+ T cells were extracted on day 5 post vaccination from the spleen, peripheral blood and tumours of mice bearing late-stage MC38-gp33 tumours following OVV +/- MS-275 treatment. P14 CD8+ T cells were analyzed for the expression of degranulation marker CD107a, and granzyme B expression by flow cytometry. **B)** Purified Thy1.1 T cells from the splenocytes from mice bearing late-stage tumours +/- MS-275 treatment were co-cultured with MC38-gp33 cells and the specific lysis of tumour cells were calculated at a ratio of 0.5 T cells to Tumour cells. Controls included p14 splenocytes cultured with IL-15, IL21, Rapamycin or IL-2 to obtain a central memory or effector phenotype, respectively. Killing assay preformed by Dr. Li Deng

Chapter 4: Discussion

Adoptive T cell therapy has demonstrated immense strides in the treatment of cancer. CAR-T cell therapy and TIL therapy has demonstrated unprecedented success in the treatment of hematological tumours[191–193] and melanoma[62,80], respectively, demonstrating the curative potential of the infusion of functional tumour-reactive CD8+ T cells. However, despite this success, poor tumour recognition, infiltration and persistence within the immunosuppressive tumour microenvironment are some of the factors that render the limited efficacy of ACT when extended to other tumour types beyond hematological tumours and melanoma but specifically solid tumours[68,80,194,195]. Research surrounding tumour immunology has revealed that the ability to remove inhibitory mechanisms utilized by the TME to restrain tumour-reactive T cell activity has permitted the unprecedented success of checkpoint inhibition therapy in a broad range of malignancies[103]. However, checkpoint inhibition therapy is successful in less than half of patients due to adequate numbers of tumor-specific T cells[196–198]. Thus, ACT can benefit from combinatorial approaches that simultaneously expand transferred cells and thwart the immunosuppressive microenvironment to favour the recruitment, proliferation and survival of cells. Our laboratory has developed a therapeutic platform that demonstrated the potent synergy between the adoptive transfer of antigen-specific central memory T cells followed by an oncolytic vaccine to achieve a robust *in vivo* expansion of transferred cells and tumour infiltration resulting in the complete and durable regression of multiple preclinical solid tumour models[74–76]. However, like the majority of preclinical

studies, our model has demonstrated success in early-stage preclinical tumours and emerging preclinical and clinical data with checkpoint inhibition has demonstrated that advanced tumour stage and burden results in negative clinical outcomes[102,199]. Similarly, in this study, we observed the complete loss of tumour-reactive transferred T cells, tumour control and survival when this therapy is repeated in late-stage tumours (Fig. 1). In the case of T cell-based therapies, the tumour-specific T cells are often rendered dysfunctional due to the heightened immunosuppressive tumour microenvironment associated with advanced tumour growth and stage[102,105]. Thus, in this study, we evaluated the ability of a histone modifier, specifically an HDACi that has immunomodulatory properties, to rescue the loss of therapeutic efficacy of our combination therapy, ACT treatment, in late-stage tumours.

In earlier studies, we have demonstrated that the therapeutic efficacy of ACT is reliant on the persistence of transferred tumour-specific CD8+ T cells as tumour control and survival is lost upon the depletion of CD8+ T cells[76]. In this study, we observed that transferred cells in late-stage tumours are indeed expanded due to vaccination and can infiltrate the tumour as early as day 2 post-vaccination. However, by day 3-4 PV, we observed a progressive decline in the number of transferred cells from the system and followed by a complete absence by day 5 post-vaccination (Fig. 2). This suggests that altered systemic and tumour environments associated with the advanced tumour stage is responsible for the loss of therapeutic efficacy, as the transferred T cells are unable to surmount the heightened immunosuppression posed by the late-stage tumour. This observation is corroborated by the findings made by Huang *et al.* where they described

that the therapeutic efficacy of PD1 blockade is dependent on the ratio of reinvigorated T cells to tumour burden. Additionally, in the context of adoptive T cell therapy, preclinical and clinical data demonstrates that patients with a high solid tumour burden at the onset of treatment demonstrate adverse clinical outcomes following the adoptive transfer of CAR T cells[200]. A possible explanation for the loss of therapeutic efficacy in late-stage tumours is that during tumour growth, systemic and local immune landscapes are dynamically altered, resulting in the accumulation of immunosuppressive cells systemically and locally[43,201]. Thus, transferred cells in our model may not surmount the immunosuppressive burdens present in the late-stage tumour when compared to the early-stage tumour. However, further experiments need to be conducted to determine the specific alterations to systemic and peripheral immune landscapes associated with the late-stage tumour.

Studies have also demonstrated that suboptimal persistence of transferred tumour-specific T cells in the tumour microenvironment can be due to intrinsic T cell deficiencies[187,200]. In our model, the rapid decline of T cells from the system following vaccination in the late-stage tumour may be due to tumour-induced T cell dysfunction. The hallmarks of this state include increased expression of inhibitory receptors, decreased proliferation and function of T cells, resulting in decreased persistence and anti-tumour efficacy[97,187]. The phenotype of transferred T cells in late-stage tumours seems to be consistent with a dysfunctional phenotype as we observed a higher density of inhibitory receptors (PD1 and TIM3) and decreased proliferation (KI67) compared to transferred cells in early-stage tumours (Fig. 3, Appendix Figure 7). This implies that in our model,

transferred cells are expanded (push) and recruited to the tumour (pull) by vaccination, but differentiate into a dysfunctional state in the presence of the late-stage tumours, resulting in decreased persistence[74]. Although the direct difference between early and late-stage tumours is not directly addressed in the study, tumours with a larger tumour burden are correlated with increased immunosuppression. The increased size and stage of the tumour may result in faster replication of tumour cells than the replication of transferred cells allowing for the conservation of an environment unsuited for efficient infiltration and persistence of tumour-specific T cells[202]. This suggests that although tumour-specific T cells can recognize and kill tumour cells, the replication of tumour cells outweighs the destruction done by the T cells in the presence of an immunosuppressive environment, resulting in dysfunction and ultimately death. Specifically, work performed by Andrew Nguyen (data not shown) demonstrates that T cell proliferation and presence can be restored upon surgical resection of the late-stage tumour. This further demonstrates that the enhanced tumour burden curates an immunosuppressive environment that prevents the persistence of transferred cells and loss of therapy. This may also insinuate that the systemic immunosuppression induced by the late-stage tumour is reversible. Similar observations were made where T cell proliferation and granzyme B production in response to *Listeria monocytogenes* is restored to similar levels observed in tumour-free animals upon surgical resection of late-stage tumours[43]. Upon further analysis, the composition of the immunosuppressive peripheral immune landscape also returns to similar states observed in the control group following surgical resection[43]. This demonstrates that the presence of a rapidly

developing tumour maintains immunosuppression locally, which extends to the periphery to dampen anti-tumour immune responses, and this immunosuppression is amplified with tumour stage and burden.

The primary goal of this study was to determine if we can potentiate our therapy in large late-stage tumours and how that potentiation is possibly achieved. For this study, we monitored the therapeutic outcomes of our therapy in late-stage tumours when MS-275 was concurrently administered in two therapeutic modalities; an viral vaccine expanding adoptively transferred T cells (Fig. 1) or endogenously primed tumour specific T cells (Figure 4.1). The previous observations made in the large late-stage tumour indicate that suboptimal T cell persistence might be due to T cell dysfunction. Thus, we hypothesized that the immunomodulating properties of MS-275 might therapeutically reverse T cell dysfunction in late-stage tumours. We have also previously utilized MS-275 and observed durable cures of tumours following ACT treatment [172], and MS-275 has been utilized in our laboratory to enhance T cell persistence in the context of ACT (data not shown) further rationalizing its use. In this study, concomitant delivery of MS-275 with ACT in late-stage tumours completely restores the T cell response to similar levels observed in small tumours on day 5PV, resulting in the complete restoration of therapeutic efficacy in different tumour models and different strains of mice (**Figure 1, 4**).

To further evaluate if the restoration of therapy can be extended to other HDACi, we administered Valproic acid and RGFP966, class 1 and class 3 HDACi, respectively[203,204] (**Appendix Figure 1**). We observed the ability of VPA to regress late-

stage tumours and completely restore the T cell response on day 5PV. Conversely, RGFP966 was capable of moderate tumour control and the T cell response observed at day 5 PV was comparable to that observed in animals treated without an HDACi (**Appendix Figure 1**). This could suggest that the differentiation of tumour-specific T cells within the tumour microenvironment to a dysfunctional state can be altered by epigenetic modulation. The observations made with MS-275 and VPA may insinuate that class 1 HDAC dysregulation may play a specific part in T cell dysfunction; however, further studies will need to be performed to delineate the mechanism.

Based on the potentiation of our therapy in late-stage tumours, we hypothesized that MS-275 restores the T cell response by reversing all the hallmarks of dysfunction induced by the late-stage tumour. To evaluate this, we monitored the magnitude of the T cell response, the expression of classical phenotypic markers associated with T cell dysfunction, proliferative capacity, the secretion of inflammatory molecules and the killing capacity of tumour-specific CD8+ T cells located in the spleen, blood, tumour draining lymph nodes and the tumour. We observed a significant increase in the magnitude of transferred cells in the organs monitored in the different models treated with MS-275. In conjunction with this, we observed a significant increase in the proliferative capacity of transferred cells in the presence of MS-275 within the late-stage tumour. We utilized KI67 as a measure of proliferation. KI67 is an intracellular nuclear protein that is only expressed in actively dividing cells[205]. Historically, KI67 has been used as a binary marker for proliferation; however, recent studies have demonstrated that its expression can be used to determine the rate of replication. Emerging evidence has

shown that the density of KI67 in a cell can determine the rate of replication of that cell at that time; specifically, a high density of KI67 in tumour cells demonstrates a cell that is rapidly dividing. In contrast, a cell that is not proliferating as rapidly demonstrates background levels of KI67[206,207]. Thus, this could suggest that T cells in the presence of MS-275 are proliferating at a much higher rate than cells treated without MS-275 and this was also demonstrated *in vitro.* However, further experiments, such as an experiment with carboxyfluorescein diacetate succinimidyl ester (CFSE), can be utilized to accurately determine the proliferative capacity of T cells in the presence of MS-275 in the late-stage tumour[208,209]. This suggests that the increased magnitude that we observed with MS-275 may be due to the increased ability for T cells to proliferate in the immunosuppressive environment imposed by the late-stage tumour.

Upon analysis, our initial observations lead us to believe that the increase in T cell presence by MS-275 was due to the reversal of the phenotype associated with T cell dysfunction, as we observed the decreased expression of PD1 and increased KI67 expression **(Figure 3).** However, contrary to this hypothesis, we observed that T cells treated with MS-275 expressed higher levels of TIM3 (**Figure 3, 4.2, 6**) , which like PD1, are typically used to identify severely exhausted tumour-specific T cells[210–214]. However, unlike PD1, TIM3 has been demonstrated to paradoxically be involved in enhanced T cell activation[215–218]. Specifically, TIM3 is involved in enhancing TCR signalling through the interactions with Src-family kinase Lck on the CD8 co-receptor[215,219]. Interestingly, we observed that T cells in late-stage tumours exhibited a significant decline in the level of the CD8 co-receptor from day 3-5PV. Conversely, CD8 co-receptor expression was

maintained without decline with the use of MS-275 (**Appendix Figure 3).** This was an interesting observation because the levels of CD8$^+$ decline following activation, so there could be a possibility that MS-275-induced TIM3 and CD8+ co-receptor expression could transduce signals to enhance T cell activation and proliferation. Furthermore, reports generated by Avery *et al.* demonstrate that the expression of TIM3 was indispensable for exhaustion development and was associated with enhanced T cell activation through enhanced TCR signalling by cell-intrinsic mechanisms[215]. Thus, this could suggest that the downregulation of PD1, combined with the upregulation of TIM3, could indicate an altered phenotype induced by MS-275 to enhance T cell activation. In addition, this altered phenotype by MS-275 may occur at the moment of activation, as phenotypic changes by MS-275 were observed exclusively after stimulation (**Appendix Figure 2**). This may imply that MS-275 may not reverse T cell dysfunction in the classical sense through the reduction of coinhibitory markers. MS-275 may confer an altered phenotype during reactivation to enhanced proliferation and persistence in the presence of heightened immunosuppression associated with the late-stage tumour.

We observed the increase in markers associated with exhaustion in two settings, first, when T cells are compared to each other in the context of early versus late-stage tumour and secondly, when T cells in late-stage tumours are in the presence or absence of MS-275. However, what differentiates these two observations, is that although the expression of exhaustion markers is increasing in both settings, we observe two very different therapeutic outcomes. On the one hand, with transferred cells in the late-stage tumour, we observed the dysfunction of T cells (**Figure 1**)**;** but the opposite is

demonstrated with MS-275 as we observed the enhanced function of T cells **(Figure 1)**. Specifically, we monitored if MS-275 can enhance the cytotoxic ability of T cells in the late-stage tumour. With MS-275, T cells exhibited an increased ability to release the contents of lytic granules successfully, increased production of cytotoxic molecules (granzyme B) and enhanced specific killing of tumour cells when compared to T cells in late-stage tumours without MS-275 (**Figure 8**). The use of an HDACi has been demonstrated to restore the function of dysfunctional T cells by altering the expression of diacetylated histone H3 (diAcH3)[171]. Thus, it could be that although without MS-275, transferred T cells in the late-stage tumour are capable of activation and expansion by vaccination, the epigenetic modifications that occur during differentiation may program the cell to become dysfunctional. Conversely, the use of MS-275 could alter the epigenetic programs on transferred cells to allow for greater activation and differentiation into functional effector cells capable of enhanced persistence and cytotoxicity in the late-stage tumour. However, further studies need to be conducted to monitor the exact epigenetic alterations on T cells by MS-275, which confer a prolonged-expression of exhaustion markers without the decline in function and persistence.

The spatial location of T cells has been reported to determine the degree of T cell dysfunction, with the tumour serving as the focal point of immunosuppression that extends to the periphery[43,86]. Following the priming of T cells, the state of T cell exhaustion becomes progressively irreversible as cells are chronically stimulated[176]. Ma *et al.* demonstrated that tumour-infiltrating T cells exhibit the highest degree of exhaustion compared to T cells in the periphery[86,220]. Similar observations were made in

the late-stage CMS5 tumour, as transferred cells progressively declined in number and exhibited the highest expression of coinhibitory molecules in the TME. Conversely, with MS-275, we observed progressive phenotypic (**Figure 3, 6**) and functional (**Figure 8**) changes on transferred cells, with the most significant change in the spleen, followed by the blood. Although we observed an increased percentage of tumour-specific T cells within the tumour (**Figure 2**), we did not observe any phenotypic or minimal functional alterations to tumour-infiltrating transferred cells with MS-275 treatment. This is corroborated by an observation made by Allen *et al.* that in late-stage tumours, terminally dysfunctional CD8+ T cells were only found within the tumour and not in the spleen[43]. This observation may indicate that transferred cells may possess different alterations within the tumour and the periphery. Specifically, the dysfunction of transferred cells within the periphery can be reversed therapeutically while transferred cells in the tumour cannot. Therefore, as transferred cells traffic to the tumour microenvironment, the effect of MS-275 on T cells decreases. This may be due to particular epigenetic modifications; however, this requires further experiments to delineate the exact mechanism of action.

It has been demonstrated that the success of therapies reliant on the tumour-specific CD8+ T cell response, namely PD1 blockade, is attributed to the reversal of T cell dysfunction within the TME [187,221]. However, recent reports have stated that clinical success may be due to the enhanced supply and function of T cells within the periphery, as this serves to continually replenish the tumour microenvironment with functional cells[222]. In addition to phenotypic changes observed and enhanced proliferative capacity, MS-275 also confers properties that enhances T cell function by increasing granzyme B

production in transferred T cells exclusively located in the periphery (blood and spleen). Although no significant increases in granzyme B expression were observed in the TME, transferred cells treated with MS-275 in the tumour demonstrated elevated levels of successful degranulation and increased specific killing ability of tumour cells (**Figure 8**), suggesting that enhancing T cell function in the periphery elicits superior anti-tumour effects upon infiltration into the TME. This suggests that the improved therapeutic outcomes by MS-275 is reliant on the enhanced number of T cells present on the system, and MS-275 may induce alterations made on transferred T cells in the periphery.

Clinically, a side effect commonly associated with adoptive T cell transfer is a massive release of cytokines following peripheral T cell activation, triggering an uncontrolled systemic inflammatory response, known as cytokine release syndrome (CRS) and is commonly seen higher in patients with late-stage tumours[223–225]. Clinical symptoms include fever, and some cases can quickly progress to symptoms, including life-threatening capillary leakage resulting in organ failure and death[192]. Thus, it is of interest to develop mildly inflammatory and highly cytotoxic T cells for ACT. In our system, the release of inflammatory molecules from activated tumour reactive T cells combined with the increased release of pro-inflammatory molecules as a result of oncolytic vaccination may pose a negative side effect. However, with MS-275, we observed that transferred cells decrease the percentage of inflammatory molecules secreted while maintaining superior function (**Figure 7, 8**). Although the current understanding is that the decreased expression of these cytokines is associated with T cell dysfunction[226–229], this may be advantageous in the context of ACT. This may suggest

that tumour-specific T cells in the presence of MS-275 have a better killing capacity but are not as inflammatory.

The phenotypic and functional alterations on tumour-specific T cells observed in response to MS-275 led us to question if MS-275-induced changes on T cell resulted from a T cell-intrinsic or extrinsic manner. HDACi, specifically MS-275, can alter various cells and metabolic processes within the recipient, resulting in a tumour microenvironment that favours the survival of T cells[172,179,230]. For example, we have reported that MS-275 can alter the fate of myeloid cells within the tumour microenvironment from a pro-tumour to an anti-tumour inflammatory status[75]. We have also reported that MS-275 can reduce the amount of T regulatory cells present within the system[75,172]. The changes to these immunosuppressive cells could contribute to the enhanced survival and persistence of expanded transfer cells. In addition, HDACi has been demonstrated to induce apoptosis of tumour cells and increase MHC levels to enhance immune infiltration[231]. The metabolic, phenotypic and functional changes observed on T cells in the presence of MS-275 *in vitro* allow it to be plausible that in addition to external alterations, MS-275 may directly alter tumour reactive T cells to ensure T cell persistence (**Appendix Figure 2, 5**)**.** Further experiments need to be performed to comprehensively analyze the extrinsic immunological and metabolic processes associated with late-stage tumours and MS-275 treatment. However, with MS-275, this presents an exciting opportunity to elucidate specific mechanisms preventing tumour-reactive CD8+ T cell persistence in late-stage tumours.

Initially, we made efforts to determine how the large tumour induces transferred cell dysfunction. In our laboratory, we have previously shown that there are various genetic differences between early and late-stage tumours; however, one that was of interest was the increase in cholesterol accumulation (work done by Andrew Nguyen, data not shown). A study done by Ma *et al*. reported that cholesterol accumulation by T cells induces the exhaustion of T cells within the tumour microenvironment, and the extent of this exhaustion is increased upon tumour infiltration due to elevated levels of cholesterol within the TME[86]. Based on the hypothesis that an increase in cholesterol may cause an increase in exhaustion and dysfunction of tumour-specific T cells in late-stage tumours, we treated tumours with ACT in combination with Pitavastatin, a drug that inhibits HMG-CoA reductase, the rate-limiting step in cholesterol synthesis[232]. We observed enhanced survival of mice treated with ACT+ Pitavastatin; however, unlike with MS-275, the frequency and phenotype of transferred cells remained unchanged compared to the control (**Appendix Figure 4**). This may suggest that altering environmental cholesterol may enhance the therapeutic efficacy of adoptive T cell therapy by extrinsic means. To further evaluate the cholesterol metabolism of T cells within the tumour microenvironment with the use of MS-275, we monitored the level of intracellular unesterified cholesterol in tumour-specific T cells. To do this, we utilized Filipin III, which is a fluorescent antibiotic that binds explicitly to unesterified cholesterol[233]. Upon monitoring the levels of expression on T cells both *in vivo* and *in vitro* (**Appendix Figure 5, 6**), we observed that MS-275 significantly increased the levels of intracellular unesterified cholesterol upon reactivation of transferred cells. This implies

MS-275 may alter the cholesterol metabolism of T cells to aid in the restoration of the T cell response in late-stage tumours.

There is currently controversy surrounding the cholesterol metabolism of T cells in the context of the tumour microenvironment. One study performed by Ma *et al.* states that the accumulation of extracellular cholesterol in T cells from the tumour microenvironment is associated with the dysfunction of T cells[86]. Conversely, a study performed by Yang *et al.* reported that higher levels of unesterified cholesterol correlated with more significant TCR signalling as cholesterol concentration was enhanced at the plasma membrane, allowing for a stronger TCR signal and resulting in greater anti-tumour CD8+ T cell responses[234]. In addition, another study stated that inhibiting PCSK9, a protein that regulates cholesterol in tumour cells, enhances PD1 checkpoint blockade in a T cell-dependent mannerr[235]. However, observations made with MS-275 may align closer to reports on enhanced T cell responses. This may suggest that MS-275 can alter cholesterol metabolism within reactivated T cells; however, further experiments and research are required to determine the role this plays in T cell therapy. For this study, we chose not to include cholesterol metabolism as it lies outside the scope of my project; however, it presents as an exciting interdisciplinary approach to evaluate the role of cholesterol metabolism on T cells in the context of cancer immunotherapy.

Conclusion

With this information, we demonstrate that tumour burden negatively affects ACT therapy; however, the curative potential of this therapy can be rescued by concomitant

delivery of MS-275 due to the restoration for the T cell response. We demonstrate that potentiation by MS-275 is a result of the enhanced persistence, proliferation of transferred tumour reactive CD8+T cells. The new properties conferred to T cells by MS-275 renders tumour-specific CD8+ T cells capable of proliferating and persisting for extended periods while exhibiting extremely cytolytic properties and high expression of inhibitory markers. However, the role that MS-275-induced expression of exhaustion markers may play in the enhanced proliferation and function of T cells requires further exploration. This data provides the rationale that MS-275 can be used in combination with T cell-based therapies to potentiate T cell persistence in aggressive tumour models.

A

B
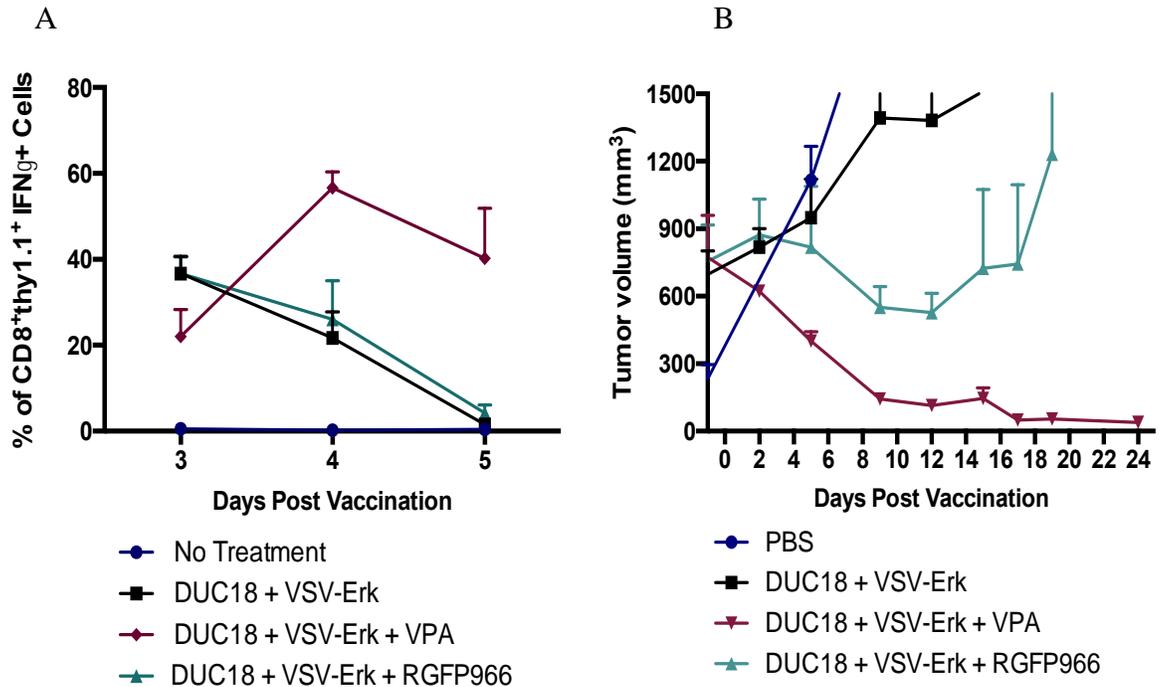
Appendix Figure 1. Other HDACi can potentiate ACT in late-stage CMS5 tumours.

Balb/c mice were inoculated with 10^6 CMS-5 cells/30 µL for 12 days with a tumour volume of 600mm^3 (late-stage CMS5 tumours) before injecting 10^6 DUC18 T$_{cm}$ cells i.v, followed by 5x10^8 PFU VSV-Erk9M 24 hours later. Mice treated with RGFP966 (250ug/mouse) and Valproic Acid (8.5mg/mouse) were administered intraperitoneally (i.p) 4 hours following vaccination and repeated daily for a total of 5 days. Peripheral blood lymphocytes were extracted retro-orbitally from mice on days 3-5 days post vaccination. **A)** The quantity of transferred DUC18 T cells expressing INFγ following *ex vivo* stimulation with ERK peptide. **B)** Tumour volumes were calculated on the specified days following vaccination
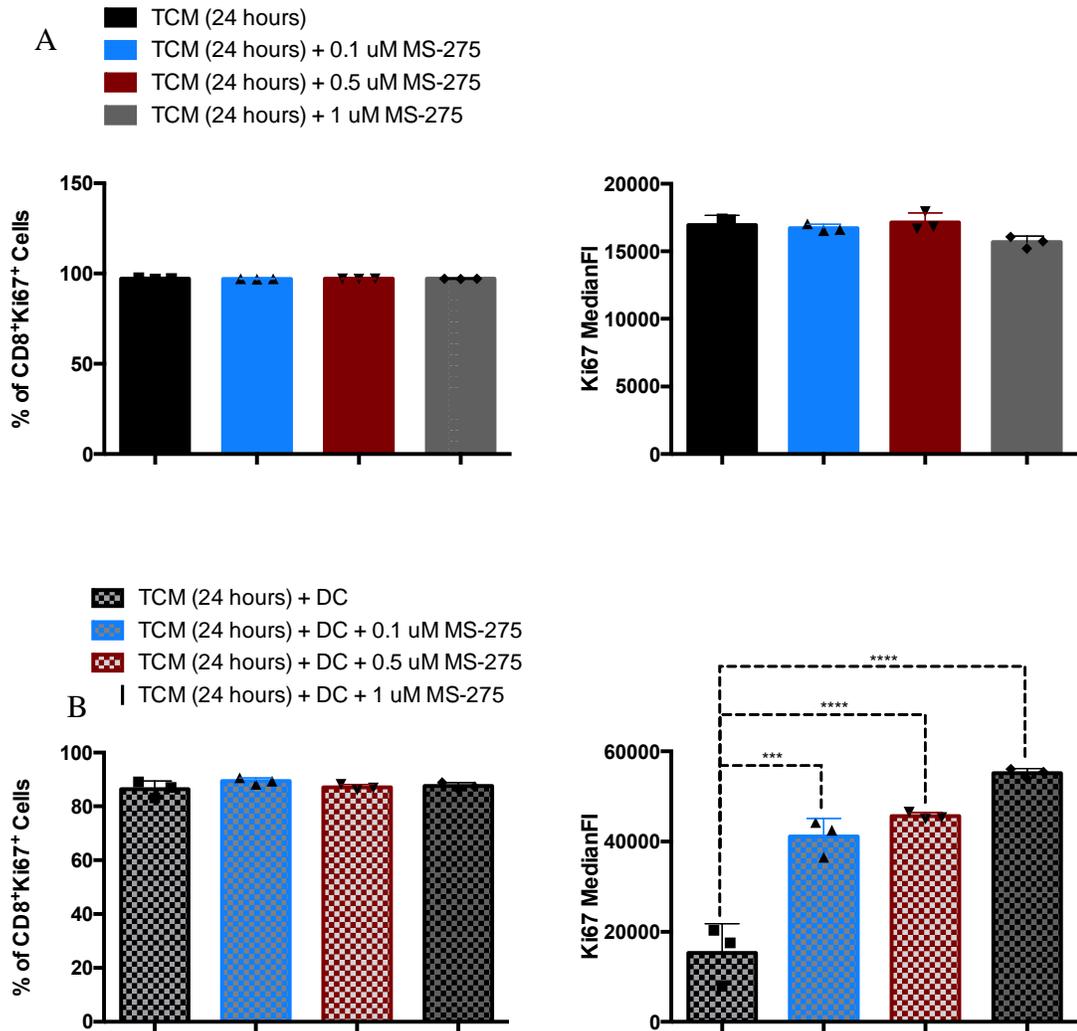
Appendix Figure 2.1: MS-275 increases the expression of KI67 *in vitro*.

 P14 T cells were cultured *in vitro* for 7 days in the presence of Gp33 peptide, IL15, IL21 and Rapamycin, to achieve a TCM phenotype. TCM P14 T cells were cocultured without **A)** and with **B)** Gp33-peptide pulsed DC2.4 in the presence of MS-275 at varying doses (0, 0.1, 0.5 and 1uM) for 24 hours. KI67 expression was analyzed on Thy1.1+CD8+ T cells by flow cytometry.
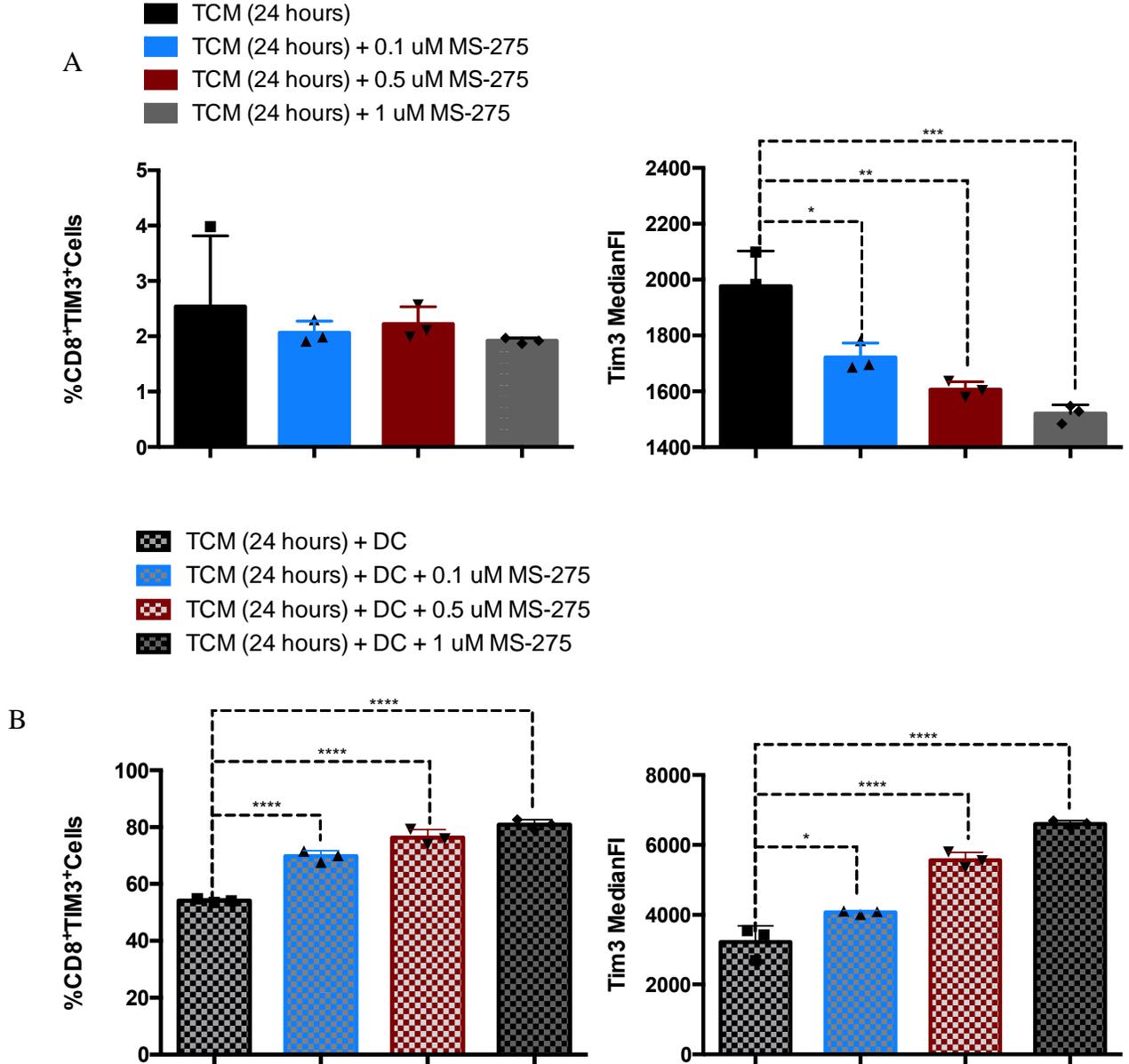
Appendix Figure 2.2 : MS-275 increases the expression of TIM3 *in vitro*.

P14 T cells were cultured *in vitro* for 7 days in the presence of Gp33 peptide, IL15, IL21 and Rapamycin, to achieve a TCM phenotype. TCM P14 T cells were cocultured without **A)** and with **B)** Gp33-peptide pulsed DC2.4 in the presence of MS-275 at varying doses (0, 0.1, 0.5 and 1uM) for 24 hours.TIM3 expression was analyzed on Thy1.1^{+}CD8^{+} T cells by flow cytometry.
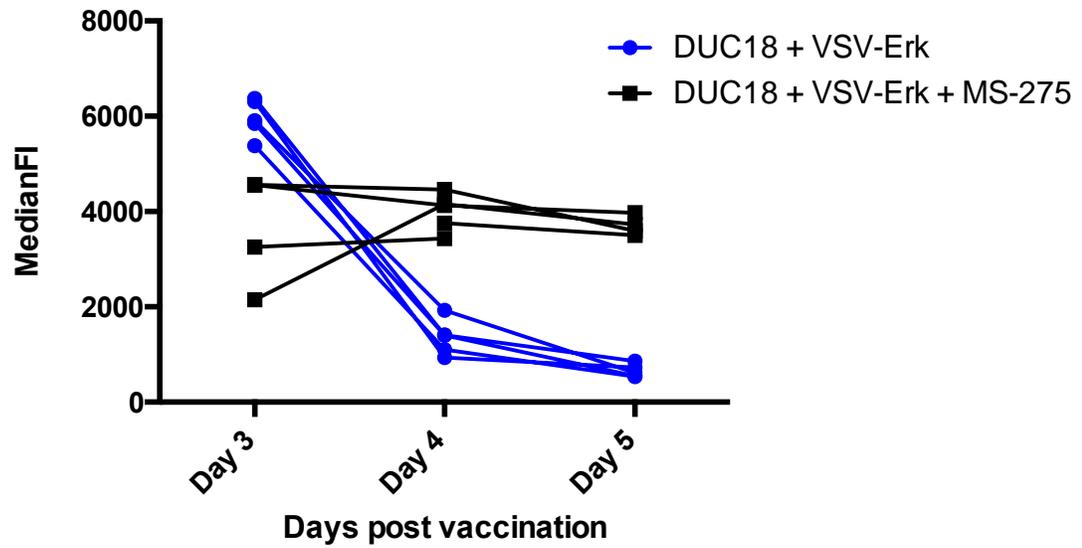
Appendix Figure 3. MS-275 maintains the expression of CD8+ co-receptor on transferred cells.

The expression of CD8 was monitored by flow cytometry on DUC18 transferred cell response in circulation of large late-stage CMS5 tumours following ACT +/- MS-275 treatment on the days indicated.
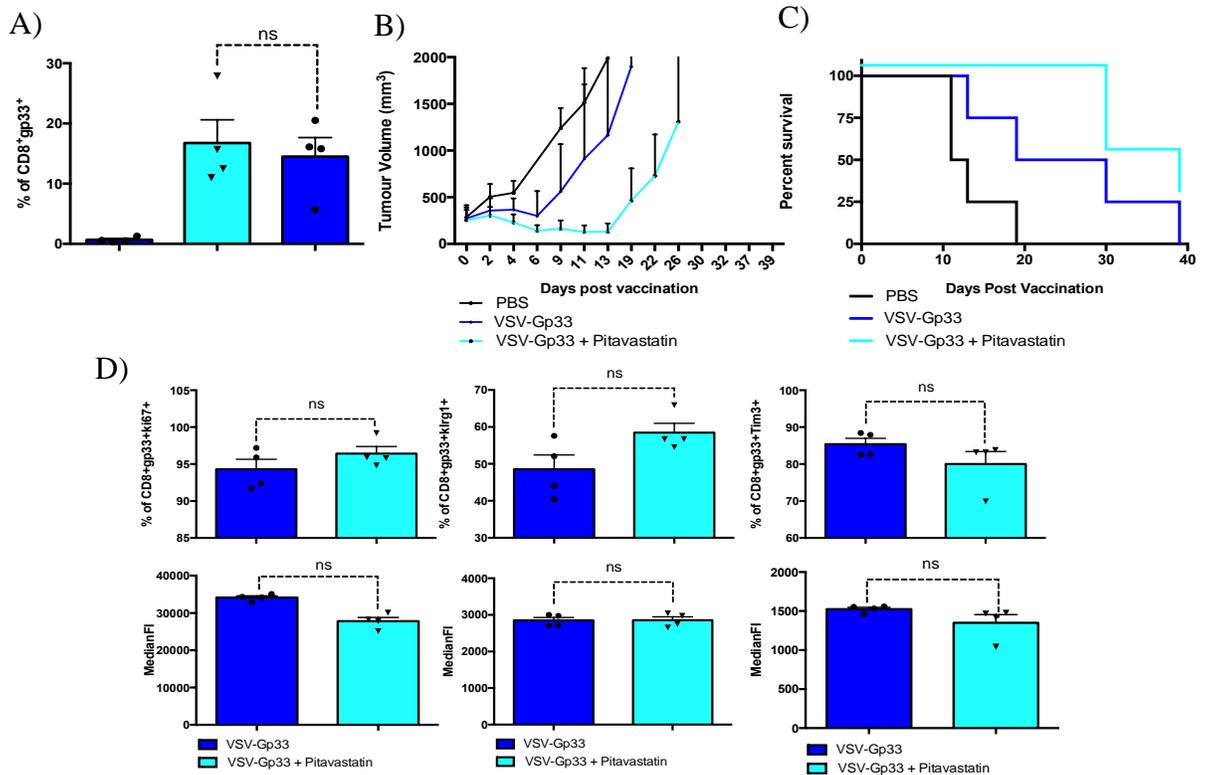
Appendix Figure 4. Statin treatment enhances OVV therapy in late-stage tumours without altering the tumour reactive CD8+ T cell phenotype.

Mice bearing large MC38 tumours were treated with VSV-gp33 followed by Pitavastatin (500ug/mL) and daily for 5 days. **A)** Peripheral blood lymphocytes were extracted retro-orbitally on day 5post vaccination. **B)** Tumour volumes and **C)** survival was monitored on the days indicated. **C)** P14 TCM CD8+ T cells were intravenously administered 24 hours prior to OVV +/- Pitavastatin treatment. **D)** P14 CD8+T cells were extracted from peripheral blood and analyzed for KI67, TIM3, and KLRG1 expression by flow cytometry.
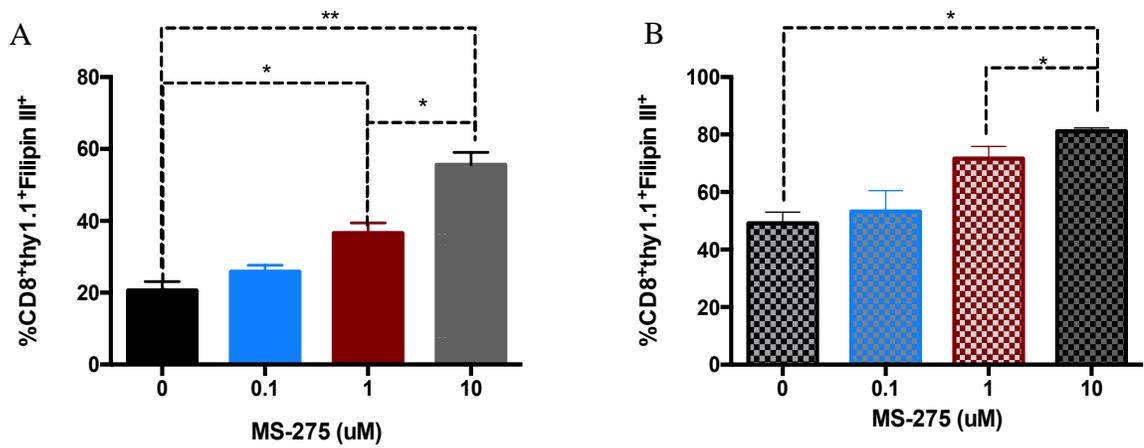
Appendix Figure 5: MS-275 increases unesterified intracellular cholesterol in T cells in a dose dependent manner *in vitro*.

DUC18 T cells were cultured *in vitro* for 7 days in the presence of ERK peptide, IL15, IL21 and Rapamycin, to achieve a TCM phenotype. TCM T cells were cocultured **A)** without or **B)** with 0.1 ug/mL of ERK peptide in the presence of varying doses of MS-275 (0, 0.1, 1, and 10 uM) for 24 hours. Filipin III expression was analyzed on Thy1.1+CD8+ T cells by flow cytometry.
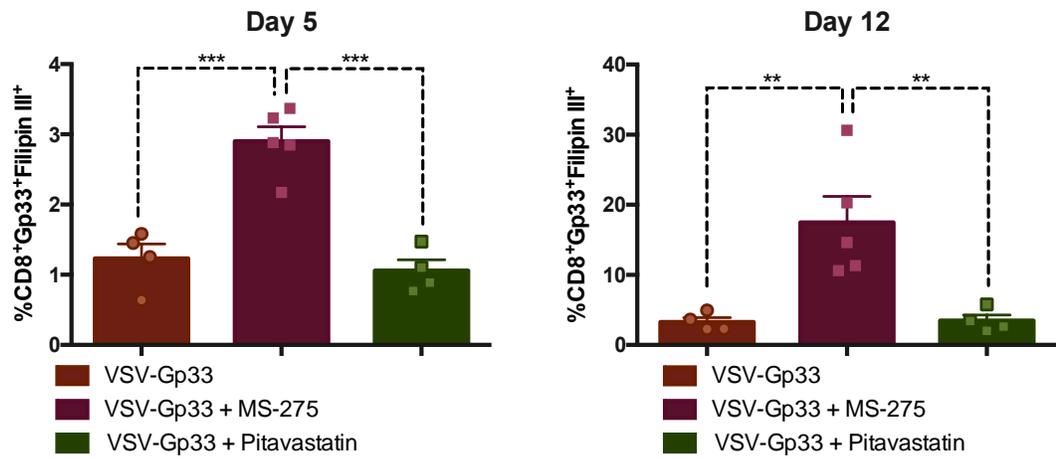
Appendix Figure 6: MS-275 increases unesterified intracellular cholesterol in T cells in a dose dependent manner *in vitro*

 C57BL/6 mice were inoculated with 2×10^5 MC38-Gp33 cells for 14-17 days with a tumour volume of 300mm^3 (late-stage MC38 tumours) before intravenously administering 2×10^8 PFU VSV-Gp33. Mice treated with MS-275 were administered 0.1mg intraperitoneally (i.p) 4 hours following vaccination and repeated daily for a total of 5 days. Peripheral blood lymphocytes were extracted retro-orbitally 5 and 12 days post vaccination and analyzed for Filipin III expression by flow cytometry.
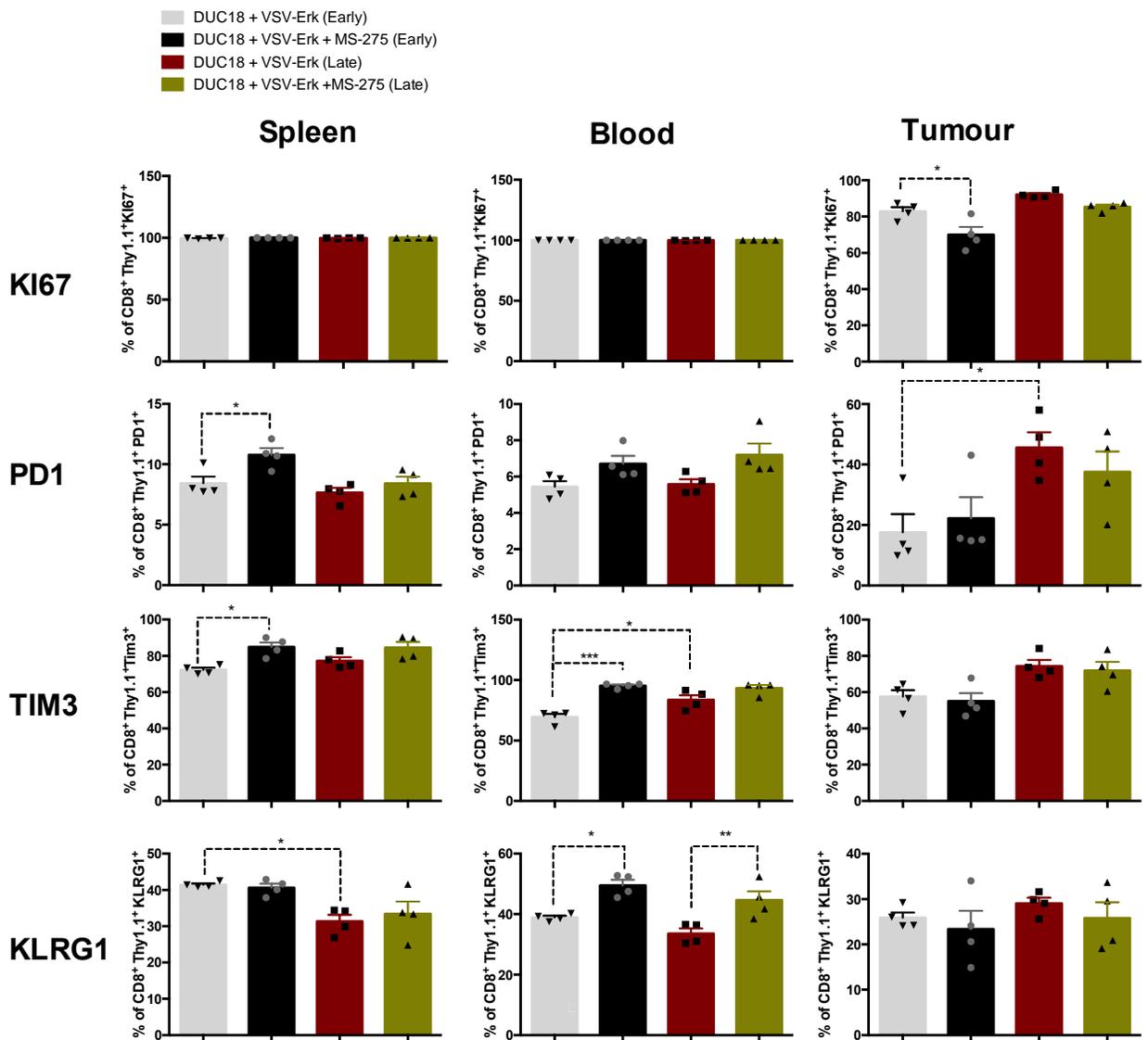
Appendix Figure 7: Expression of Inhibitory receptors and Proliferation Markers on transferred cells in early and late-stage tumours in the presence and absence of MS-275

DUC18 CD8+ T cells were extracted on day 4.5 post vaccination from the spleen, peripheral blood and tumours of mice bearing early and late-stage CMS5 Tumours following ACT +/- MS-275 treatment. DUC18 T cells were analyzed for the percentage of transferred cells that expressed PD1, TIM3, KLRG1, and KI67 expression by flow cytometry. Figure flow cytometry data was generated by Andrew Nguyen.

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