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Immunotherapy of Cancer: Reprogramming Tumor/Immune Cellular Crosstalk to Improve Anti-Tumor Efficacy

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy at Virginia Commonwealth University

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Dedication

This work is dedicated to my family and loved ones; their eternal love and strength illuminates the darkness.

Table of Contents

	Page
List of Figures.....	viii
List of Abbreviations.....	xi
Abstract.....	xvi
Preface.....	xviii
Chapter One.....	1
Introduction	1
Status of current cancer therapeutics.....	1
Immune suppression in the tumor microenvironment.....	2
Modulation of the TME to overcome tumor-induced immune suppression.....	4
ACT of breast cancer utilizing reprogrammed memory T cells and CD25+ NKT cells....	7
Activation of antigen-sensitized T cells: An antigen-independent protocol using Bryostatins 1 and Ionomycin.....	7
Immune cell phenotypic manipulation using the γ -c cytokines.....	9
Epigenetic enhancement of tumor immunogenicity: Dec as an <i>in situ</i> vaccine.....	10
Materials and Methods	14
Mouse model.....	14
Tumor cell lines.....	14
<i>Ex vivo</i> reprogramming and expansion of splenocytes.....	14
Adoptive cellular therapy.....	15
Characterization of splenocytes.....	15
<i>In vitro</i> and <i>in vivo</i> induction of CTA expression in MMC cells and cDNA synthesis...	16
Quantitative Real-Time Polymerase Chain Reaction for the detection of CTA expression.....	16
IFN- γ ELISA.....	17

Sample size and power calculation justification for patient CTA expression.....	17
Clinical specimens.....	18
Statistical analysis.....	18
Results	21
ACT utilizing reprogrammed murine splenocytes which are resistant to MDSCs fails to reject established primary tumors in the FVBN202 mouse.....	21
Dec induces CTA expression in human breast cancer cells.....	21
Dec upregulates CTA expression in primary murine tumor cells <i>in vitro</i> and <i>in vivo</i> , and reduces the frequency of splenic MDSCs.....	27
Splenocytes harvested from mice bearing CTA-expressing primary tumor demonstrate expansion and phenotypic characteristics typical of successful reprogramming, and demonstrate enhanced anti-tumor immune responses <i>in vitro</i>	30
ACT fails to reject CTA positive established primary tumors in FVBN202 mice.....	31
Discussion	35
Chapter Two.....	41
Introduction	41
Stem cell transplantation following the modulation of tumor-immune crosstalk.....	41
Material and Methods	45
Patients.....	45
Investigational regimen.....	45
qRT-PCR for the detection of CTA expression.....	46
IFN- γ ELISA for the detection of CTA-specific T cell responses.....	50
Study design.....	50

Results	52
Patient demographics.....	52
Response to Aza-Rev and autologous lymphocyte collection.....	52
<i>In vivo</i> induction of CTA expression with Aza-Rev in MM.....	53
CTA-specific T cell response following Aza-Rev therapy.....	58
Loss of CTA expression following immunotherapy was associated with disease relapse.....	59
Discussion	63
Chapter Three.....	67
Introduction	67
Material and Methods	71
Mouse Model.....	71
Tumor cell lines.....	71
<i>Ex vivo</i> reprogramming and expansion of splenocytes.....	71
Adoptive cellular therapy.....	72
Characterization of splenocytes and tumor-infiltrating leukocytes.....	72
Sorting of myeloid cells by FACS.....	73
IFN- γ ELISA.....	73
Isolation and characterization of lung metastases.....	74
Characterization of metastatic tumor-infiltrating leukocytes.....	74
Establishment of <i>in vitro</i> tumor cell dormancy.....	74
Cytotoxicity assay.....	75
Statistical analysis.....	75

Results	77
Immunotherapy of metastatic tumor can prolong animal survival but fails to cure cancer.....	77
ACT utilizing reprogrammed T cells and CD25+ NKT cells sustains an anti-tumor memory response, <i>in vivo</i>	87
ACT shifts splenic myeloid cells from Gr1+CD11b+ MDSCs to Gr1-CD11b- myeloid cells.....	87
ACT modulates the phenotype of Gr1+CD11b+ MDSCs in the spleen.....	88
ACT generated splenic Gr1-CD11b- myeloid cells boost anti-tumor immune responses.....	89
ACT generated splenic GR1-CD11b- myeloid cells are unique immune stimulatory cells, which differ from conventional APC.....	95
Immunotherapy induces tumor escape in proliferating tumor cells and indolent dormant cells, but not in quiescent dormant cells.....	96
Dormant MMC cells established by ADR become resistant to chemotherapy, but remain sensitive to immunotherapy.....	109
Discussion	114
Conclusions	121
Future Perspective	123
Literature cited.....	124
Vita.....	154

List of Figures

	Page
Figure 1: ACT utilizing reprogrammed murine splenocytes, which are resistant to MDSCs, fails to reject established primary tumors in the FVBN202 mouse.....	23
Figure 2: Human breast cancer cells upregulate CTA expression upon treatment with Decitabine, <i>in vitro</i>	24
Figure 3: Breast cancer patients remaining free of tumor relapse display a trend of elevated CTA expression.....	26
Figure 4: Decitabine upregulates CTA expression in MMC tumor cells <i>in vitro</i> and <i>in vivo</i>	28
Figure 5: Decitabine reduces the frequency of splenic MDSCs in FVBN202 mice bearing established primary tumors.....	29
Figure 6: Expansion and phenotypic reprogramming of tumor-reactive splenocytes is similar between animals bearing primary cancer with and without Decitabine preconditioning.....	32
Figure 7: CTA-expressing MMC cells promote enhanced IFN-g release from CTA-sensitized reprogrammed splenocytes.....	33
Figure 8: ACT fails to reject CT-positive established primary tumors in FVBN202 mice.....	34
Figure 9: Multiple Myeloma Study Schema.....	48
Figure 10: Aza-Rev does not modulate numbers of circulating lymphocytes, but ALI results in a transient increase CD3+ and CD8+ cells.....	56
Figure 11: Induction of a panel of CTA expression in bone marrow of patients with MM following a 3-cycle administration of Aza-Rev.....	57
Figure 12: Induction of NY-ESO-1-reactive T cell responses in patients with MM.....	60
Figure 13: Induced expression of CTAs in bone marrow of patients is transient in one patient experiencing disease progression.....	61
Figure 14: Combined ACT and Decitabine therapy prolongs survival in mice with circulating tumor cells.....	80
Figure 15: Decitabine induces downregulation and loss of neu expression on MMC cells, <i>in vivo</i>	81
Figure 16: Decitabine induces downregulation and loss of neu expression on MMC cells, <i>in vivo</i>	82

Figure 17: Induction of CTA expression is transient in human breast cancer cells.....	83
Figure 18: Expansion and phenotypic reprogramming of tumor-reactive splenocytes is similar between animals bearing primary and metastatic cancer.....	84
Figure 19: Reprogrammed immune cells derived from animals bearing primary cancer or experimental metastasis and used for ACT demonstrate similar functional efficacy, in vivo....	85
Figure 20: The efficacy of ACT is limited against circulating MMC despite reductions in tumor challenge.....	86
Figure 21: ACT modulates the splenic myeloid cell compartment.....	91
Figure 22: ACT modulates the phenotype of splenic MDSCs.....	92
Figure 23: ACT modulates the phenotype of splenic MDSCs.....	93
Figure 24: ACT generated Gr1- CD11b- promote tumor cell-mediated IFN-g release by endogenous splenocytes.....	94
Figure 25: ACT modulates the phenotype of splenic CD11b-Gr1- myeloid cells.....	99
Figure 26: Splenic CD11b-Gr1- myeloid cells demonstrate characteristics of APC maturation upon stimulation with LPS.....	100
Figure 27: CD11b negative myeloid cells are not enriched with CD11c+ DCs upon ACT.....	101
Figure 28: CD11b negative myeloid cells are not enriched with F4/80+ macrophages upon ACT.....	102
Figure 29: ACT promotes immunoediting of lung metastatic lesions.....	103
Figure 30: Reprogrammed splenocytes express PD-1.....	104
Figure 31: The frequency of tumor-infiltrating CD8+ T cells increases upon ACT, yet retain PD-1 expression.....	105
Figure 32: Splenic lymphocytes retain PD-1 expression after ACT.....	106
Figure 33: ADR-treatment results in the emergence of a viable, dormant MMC cells.....	107
Figure 34: IFN- γ induces upregulation of PD-L1 on Ki67+ but not on Ki-67- tumor cells.....	107
Figure 35: Immunotherapy displays cytotoxic function against treatment- refractory dormant tumor cells, <i>in vitro</i>	111

Figure 36: Immunotherapy displays cytotoxic function against treatment- refractory dormant tumor cells, *in vitro*.....112

Figure 37: ACT induces regression of ADR-induced established indolent primary breast cancer.....113

List of Abbreviations

ACT - Adoptive cellular therapy

ADH - Atypical ductal hyperplasia

ADR - Adriamycin

AKAP - A-kinase anchor proteins

ALI - Autologous lymphocytes infusion

allo-SCT - Allogeneic stem cell transplantation

APC - Antigen presenting cell

ASCT - Autologous stem cell transplantation

Aza - Azacitidine

B/I - Bryostatin 1/ionomycin

BCSC - Breast cancer stem cells

CAR - Chimeric antigen receptor

CR - Complete remission

CTA - Cancer testis antigen

CTLA-4 - Cytotoxic T-lymphocyte-associated protein 4

CYP - Cyclophosphamide

DCIS - Ductal carcinoma in situ

DCK - Deoxycytidine kinase

DCs - Dendritic cells

Dec - Decitabine

DNMT - DNA-methyltransferase

ELISA - Enzyme-linked immunosorbent assay

EMT - Epithelial to mesenchymal transition

ER - Estrogen receptor

ESX1 - Extraembryonic, spermatogenesis, homeobox 1

FACS - Fluorescence-activated cell sorting

FBS - Fetal bovine serum

FDA - Food and Drug Administration

FITC - Fluorescein isothiocyanate

FVS - Fixable viability stain

G-CSF - Granulocyte colony-stimulating factor

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GM-CSF - Granulocyte-macrophage colony-stimulating factor

HER-2/neu - Human epidermal growth factor receptor-2

HLA - Human leukocyte antigen

HPV - Human papilloma virus

ICD - Immunogenic cell death

IFN- γ - Interferon gamma

JAK - Janus kinase

Lipopolysaccharide - LPS

MAGE - Melanoma-associated antigen

MDS - Myelodysplastic syndrome

MDSC - Myeloid-derived suppressor cells

MFI – Median Fluorescence Intensity

MHC - Major histocompatibility complex

MICA - Major histocompatibility complex class I-related chain A

MM - Multiple myeloma

MMC - Mouse mammary carcinoma

MMSET - Multiple myeloma SET domain

MRD - minimal residual disease

Mregs - Myeloid regulatory cells

NY-ESO-1- New York esophageal squamous cell carcinoma-1

PR - Partial Remission

PBMCs - Peripheral blood mononuclear cells

PD-1 - Programmed cell death protein 1

PDL-1 - Programmed death ligand 1

PE - Phycoerythrin

PI - Propidium iodide

PKC - Protein kinase C

PKC- θ - Protein kinase C theta

PR - Progesterone receptor

qRT-PCR - Quantitative real-time polymerase chain reaction

Rev - Revlimid

ROS - Reactive oxygen species

RT - Radiation therapy

SEM - Standard error of the mean

SLLP1 - Sperm intra-acrosomal, lysozyme like, non-bacteriolytic protein

SPA17 - Surface protective antigen 17

SPANXB1 - The sperm protein associated with the nucleus on the X chromosome B1

STAT-1 - Signal transducers and activators of transcription-1

TAAAs - Tumor-associated antigens

TBI - Total body irradiation

Tcm - T central memory

TcR - T cell receptor

TIL - Tumor-infiltrating lymphocytes

TME - Tumor microenvironment

TLR - Toll-like receptor

VGPR - Very good partial remission

γ -c - Gamma-chain

γ/δ T cells - gamma/delta T cells

Abstract

IMMUNOTHERAPY OF CANCER: REPROGRAMMING TUMOR/IMMUNE CELLULAR CROSSTALK TO IMPROVE ANTI-TUMOR EFFICACY

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
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Virginia Commonwealth University, 2015

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Immunotherapy of cancer has been shown to be promising in prolonging patient survival. However, complete elimination of cancer and life-long relapse-free survival remain to be major challenge for anti-cancer therapeutics. We have previously reported that *ex vivo* reprogramming of tumor-sensitized immune cells by bryostatin 1/ionomycin (B/I) and the gamma-chain (γ -c) cytokines IL-2, IL-7, and IL-15 resulted in the generation of memory T cells as well as CD25+ NKT cells and CD25+ NK cells. Adoptive cellular therapy (ACT) utilizing these reprogrammed

immune cells protected FVBN202 mice from tumor challenge, and overcame the suppressive functions of myeloid-derived suppressor cells (MDSCs). We then demonstrated that the presence of CD25+ NKT cells was required for anti-tumor efficacy of T cells as well as their resistance to MDSCs. Similar results were obtained by reprogramming of peripheral blood mononuclear cells (PBMC) from patients with early stage breast cancer, demonstrating that an increased frequency of CD25+ NKT cells in reprogrammed immune cells was associated with modulation of MDSCs to CD11b-HLA-DR+ immune stimulatory cells. Here, we tested the efficacy of immunotherapy in a therapeutic setting against established primary breast cancer (Chapter One), experimental metastatic breast cancer (Chapter Three) as well as against minimal residual disease (MRD) in patients with multiple myeloma (Chapter Two). We evaluated the ability of reprogrammed immune cells, including CD25+ NKT cells, to convert MDSCs to myeloid immune stimulatory cells, *in vivo*; this resulted in the identification and characterization of a novel antigen presenting cell (APC). These novel immune stimulatory cells differed from conventional APCs, including dendritic cells (DCs) and macrophages. We have also demonstrated that enhancing immunogenicity of mammary tumors by treatment with Decitabine (Dec) along with overcoming MDSCs by utilizing reprogrammed T cells and NKT cells in ACT prolongs survival of animals, but fails to eliminate the tumor. However, targeting cancer during a setting of MRD, when tumor cells are dormant, results in objective responses as evidenced in our multiple myeloma studies. This suggests that targeting breast cancer with immunotherapy following conventional therapies, in a setting of residual disease when tumor cells are dormant, may be effective in eliminating such residual cells or maintaining dormancy and extending time-to-relapse for breast cancer patients.

PREFACE

Despite tumor immune surveillance, individuals with an intact immune system still develop cancer. This is due to escape mechanisms developed by tumors under pressure from the host's immune system. Therefore, modifying tumor cells and reprogramming tumor-sensitized immune cells may establish a novel crosstalk between tumor cells and cells of the immune system which could lead to tumor rejection. The use of demethylating drugs such as Azacitidine (Aza) or Decan can induce the expression of highly immunogenic cancer testis antigen (CTAs) in the tumor, thereby increasing the immunogenicity of tumor cells and rendering them more vulnerable to immune recognition and attack. We have also established an antigen-free *ex vivo* protocol for reprogramming and expansion of tumor-sensitized immune cells by using bryostatin 1/ionomycin (B/I) and common gamma chain cytokines, IL-2, IL-7, and IL-15. Such reprogrammed immune cells consisted mainly of memory T cells and CD25+ NKT cells, which overcame MDSC suppression. Previous work demonstrated that ACT utilizing reprogrammed immune cells protected animals from primary tumor challenge. Here, we sought to determine the therapeutic efficacy of ACT against established primary breast cancer. In **Chapter One**, we evaluated ACT with or without Dec against established primary mammary carcinoma. This strategy failed to eliminate established tumors. In **Chapter Two**, we participated in a Phase II randomized clinical trial using Aza combined with an immunomodulator Revlimid (Rev) and ACT in patients with multiple myeloma who harbored MRD. This strategy was effective in generating responses against multiple myeloma. In **Chapter Three**, we tested the therapeutic efficacy of ACT with or without Dec against experimental metastatic mammary carcinoma. These studies identified novel APCs, as well as chemotherapy-induced dormant tumor cells that were sensitive to immunotherapy.

Chapter One

Introduction

The role of the immune system in maintaining equilibrium or eliminating neoplastic cells has become appreciated within the last four decades. Anti-tumor immune surveillance is supported by observations of spontaneous tumor regression associated with anti-tumor immune function (1), as well as the observation of a higher incidence of virally induced cancers and a worse prognosis for other cancers in immunocompromised or immunodeficient patients (2, 3). Such observations have incited the development of immune-based therapies for use against cancer in order to harness naturally occurring anti-tumor immune response.

Status of current cancer therapeutics

Despite advances in conventional cytotoxic therapies of early-stage breast cancer (4, 5) there remains no therapeutic strategy that can ensure relapse-free survival. Furthermore, studies have shown that 20% of clinically disease-free early-stage breast cancer patients relapse within 10 years after conventional therapies (6); indeed, most cancer-related deaths within the United States are attributed to relapse (7). Thus, there is an urgent need to develop more effective therapies to overcome breast cancer relapse and to treat the advanced stages of the disease. To this end, immunotherapy emerges as a promising strategy for the prevention of tumor relapse, when combined with conventional therapies.

Thus far, advances in the immunotherapy of cancer have also been met with a number of setbacks. Several vaccination strategies used against breast cancer have been successfully employed to induce tumor-specific CD8+ and CD4+ T-cell responses; however, such

immunological responses have rarely been potent enough to achieve objective results (8-10). Additionally, it has been demonstrated by several groups that adoptive cellular therapy (ACT) directed against highly immunogenic melanoma-associated antigens results in objective responses in animal models as well as in some melanoma patients (11, 12). ACT has also been tested against breast cancer both in preclinical and clinical studies (13, 14); however, unlike melanoma, ACT has not produced promising results in breast cancer patients and has only displayed effectiveness in animal models in prophylactic settings (15, 16), rather than against well-established, vascularized tumors. Such failure has been attributed, in part, to (i) the lack of a robust antitumor immune response as a result of the expression of weakly immunogenic tumor antigens coupled with the presence of low frequency and low affinity T cells and (ii) the suppression of antitumor immune responses through the activity of immunosuppressive mechanisms. Indeed, distant recurrence of breast cancer may occur even in the presence of tumor-specific immune responses. The ability to overcome these barriers will likely improve the efficacy of immunotherapy directed against cancer.

Immune suppression in the tumor microenvironment

The immunosuppressive tumor microenvironment (TME) has come to be appreciated as a major facilitator in the progression of solid tumors. It is now clear that the escape of malignant cells from immune destruction is due in part to immunosuppressive mechanisms within the TME, as reviewed by Hanahan and Coussens (17). The expression of immunoregulatory molecules, such as cytotoxic T lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) as well as the ectoenzyme, CD73, inhibits the proliferation and function of conventional T cells with anti-tumor activity (18, 19). Furthermore, immunosuppressive cells such as

alternatively activated M2 macrophages, type II NK cells, and regulatory T cells have been also been demonstrated to antagonize tumor immunosurveillance (20-24). Results from clinical studies of breast cancer patients indicate that another critical regulator of tumor immunosurveillance, the myeloid-derived suppressor cell (MDSC), was found to be the most abundant type of suppressor cell (25, 26) and thus represent a major hurdle in overcoming antitumor immune suppression. MDSCs play a major role in the suppression of anti-tumor immune responses as the failure of endogenous anti-tumor immune responses results, in part, from the increased activity of these cells (27-29). MDSCs consist of immature cells of myeloid origin that exert suppression of T cells via modulation of the local T-cell environment, direct cell-to-cell contact, as well as by their ability to generate inducible Tregs (15, 30-33). These cells have been found in tumor-bearing mice as well as cancer patients and have been shown to possess multiple mechanisms to suppress the antitumor immune response (34, 35). Such responses include disrupting T cell receptor (TcR) antigen recognition and T-cell mediated interferon gamma (IFN- γ) production (36, 37), depletion of essential amino acids within the tumor microenvironment (38), and overproduction of reactive oxygen species (ROS) (39). Murine MDSCs are defined as coexpressing Gr-1 and CD11b, with two subsets commonly being described: granulocytic (CD11b+Ly-6G+Ly-6C-) and monocytic (CD11b+Ly-6G-Ly-6C+) (40). Human MDSCs, on the other hand, have been difficult to identify as initial studies revealed that these cells express varied phenotypes and suppressive patterns (34). It is now regarded, however, that human MDSCs fall into two main subsets: a monocytic population characterized by expression of CD14 and a granulocytic population characterized by CD15 expression; both subtypes have been reported to express the common myeloid markers CD11b and CD33, with minimal expression of myeloid maturation markers such as HLA-DR (41). The accumulation of

these cells in association with cancer development is corroborated by experimental mouse models (15, 42), and human (25, 34, 43) studies, indicating that MDSCs develop as a function of tumor progression. For instance, our group has previously reported that FVBN202 mice, which overexpress the rat neu oncogene in their mammary glands, develop atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS) in mammary epithelial cells prior to the formation of spontaneous mammary tumors (44). DCIS of the breast is conventionally regarded as a precursor of invasive breast cancer, and ADH is a risk factor for the development of the disease (45, 46). Compromised anti-neu immune responses occur as a result of the emergence of such premalignant events which are characterized by an accumulation of MDSCs in the blood, bone marrow, secondary lymphoid tissues, and within tumor lesions due to an increased production of tumor-derived soluble factors (44, 47-51). Such findings provide evidence that MDSCs function as potent inhibitors of antitumor immunity in breast cancer models. Likewise, human MDSCs have been observed to negatively regulate both adaptive and innate immunity during cancer development and progression, with accumulation having been observed in peripheral blood and lymphoid tissues as well as draining tumor sites of cancer-bearing patients (40). In addition to breast cancer, the accumulation of MDSCs has been observed in hepatocellular, pancreatic, esophageal, and colorectal cancers (35).

Thus, MDSC accumulation is paramount in the ability of cancer to evade effective immune responses; devising strategies to overcome MDSCs is critical to improving effective anti-tumor immunity.

Modulation of the TME to overcome tumor-induced immune suppression

As MDSCs are immature cells of myeloid lineage, one may hypothesize that providing the correct signal to these cells may support their maturation into cells which may support anti-tumor function, such as dendritic cells (DCs) or M1 macrophage subsets. Indeed, studies in animal models suggest that activated NKT cells may have a role in modulating the immunosuppressive TME by promoting the conversion of MDSCs into DCs (16, 52, 53), yielding enhanced, rather than suppressed anti-tumor immune responses, while also potentially decreasing the pool of environmentally induced Tregs (31-33). Our group has recently observed that activated CD25⁺ NKT cells can promote the maturation of human MDSCs *in vitro*, which renders them immunostimulatory in early stage breast cancer patients (54). The physiological role of NKT cells in tumor immunity is yet to be fully defined, but it appears a primary function of these cells is to activate DCs to promote antigen-specific T-cell responses (55-57). Furthermore, the ability of NKT cells to direct myeloid lineage cellular differentiation is becoming apparent. Hedge and colleagues (58) have demonstrated the ability of NKT cells to induce the differentiation of monocytes into DCs as a result of secretion of granulocyte–macrophage colony-stimulating factor and IL-13 and, similarly, others have reported on the ability of innate immune cells to induce DC maturation (59-61).

NK cells, traditionally considered to function as effector cells in the innate arm of the immune system, have also recently been shown to play immunoregulatory ‘helper’ functions through an ability to activate and enhance the ability of DCs to produce proinflammatory cytokines and to stimulate Th1 and cytotoxic T lymphocyte responses of tumor-specific CD4⁺ and CD8⁺ T cells (62). Furthermore, Wong *et al.* (63) have recently described an ability of IL-18 primed human NK cells to promote DC activation and DC-mediated induction of type-1 immune responses against cancer via a chemokine-dependent mechanism. Similarly, a study by Srivastava *et al* (64)

has demonstrated that cetuximab, a chimeric monoclonal antibody that targets the EGF receptor and is approved for use in colorectal as well as head and neck cancers, also functions to activate NK cells and support dendritic cell maturation through NKG2D/MICA binding, with subsequent priming of tumor-antigen specific T cells. Such findings emphasize a role for NK cells functioning in a ‘helper’ role in which DCs are activated and result in the priming of anti-tumor T-cell responses.

Much attention in the field of cellular immunotherapy of cancer has recently focused on the use of genetic engineering of the T cell receptor to improve T cell specificity for MHC class I restricted tumor antigen peptides (65), or the use of chimeric antigen receptor (CAR) modified T cells to target MHC-unrestricted native cell surface antigens (66). These approaches, which consist of a purified T cell product, have had some success in the treatment of hematological cancer, but have been of limited therapeutic efficacy in the treatment of solid cancer (67-72). The gold standard for ACT of solid cancer remains the use of tumor-infiltrating lymphocytes (TIL). TIL are harvested from surgically excised tumor and expanded *ex vivo* in the presence of IL-2. This technique was developed by Steven Rosenberg and colleagues in the late 1980s (73, 74), and has resulted in overall response rates of 48–72% for the treatment of advanced melanoma (75, 76). It is important to appreciate that the cellular composition of TIL is most certainly enriched with effector lymphocytes beyond traditional α/β T cells. While $CD4^+$ and $CD8^+$ T cells do indeed comprise a large percentage of the TIL product, NK cells, NKT cells as well as γ/δ T cells have also been detected (77-79). Given the superior responses observed using TIL, rather than purified T cells in solid cancers (80), such data must lead us to pursue an understanding of the mechanistic crosstalk among the various lineages of leukocytes in order to manipulate that communication network to lead to a more effective eradication of tumor cells.

Therefore, developing a cellular based immunotherapy for cancer which includes T cells as well as NK and NKT cells will likely lead to the orchestration of more effective activation of DCs and other APCs which may further promote endogenous T cell responses in addition to the transferred T cells and may have the benefit of reducing the frequency of MDSCs.

ACT of breast cancer utilizing reprogrammed memory T cells and CD25+ NKT cells

Our group has recently observed that the expression of molecules involved in the activation signaling of NK cells as well as NK cell:target interactions is increased in breast cancer patients with a favorable prognosis (81). Additionally, we have demonstrated that immune function genes involved in the crosstalk between adaptive and innate immune responses were exclusively upregulated in breast cancer patients with relapse-free survival (82). These findings suggest that innate immune signals within the TME may function to support adaptive anti-tumor immune responses.

Therefore, a strategy to enhance the cross-talk between innate and adaptive immune cells is one of immune cell reprogramming, *ex vivo*, and infusing these cells as ACT in order to increase the number of functionally superior tumor-sensitized T cells as well as to induce the activation of NKT cells to provide signals to modulate myeloid cells in secondary lymphoid tissues and the TME. Immune cell reprogramming requires activation of antigen experienced immune cells, followed by phenotypic manipulation using γ -c cytokines.

Activation of antigen-sensitized T cells: An antigen-independent protocol using Bryostatin 1 and Ionomycin

Circulating tumor-reactive T cells have been identified in the peripheral blood as well as draining lymph nodes of cancer patients (83-88) and, therefore, represent a potential source of cells with endogenous reactivity against tumor-associated antigens (TAAs) for use in ACT. Stimulation of CD3 and CD28 using beads or monoclonal antibodies without the presence of antigen has been widely used as a method to induce T-cell activation and expansion, which may then be used for ACT (89-92). This method, however, leads to nonspecific polyclonal T-cell proliferation, and depending upon the anatomical location from which the cells were harvested, may lead to insufficient frequencies of expanded tumor-sensitized T cells for use in ACT. As an alternative approach, preclinical studies have reported that the combined use of the pharmacological agents bryostatin 1 and ionomycin (B/I) selectively stimulates tumor-sensitized T cells *in vitro*, without requiring the presence of antigen; lymphocytes from sarcoma-bearing mice activated with B/I and expanded with IL-2 underwent tumor-specific T-cell expansion by orders of magnitude (93). Bryostatin 1 functions by activating T cells through protein kinase C (PKC) activity (94), which leads to the secretion of IL-2 and the generation of a proliferative response when combined with ionomycin (95). Naive T cells, in contrast to primed T cells, are impaired in TcR downregulation upon exposure to the classical PKC activator phorbol 12, 13-dibutyrate (96), suggesting PKC activators may differentially regulate naive and primed T cells. In fact, TcR downregulation has been attributed to PKC- θ activity in primed T cells (97). Von Essen *et al.* (96) have demonstrated that the majority of PKC- θ exists as a high-molecular disulfide-linked inactive form in the plasma membrane of naive T cells, whereas primed T cells express an active form of PKC- θ ; activation of PKC- θ is redox dependent and requires *de novo* synthesis of the major redox regulator glutathione during T-cell activation. Furthermore, differential localization of PKC- θ occurs following activation of primed T cells and naive T cells (98, 99). PKC- θ has also been

implicated in having a central role in the anti-tumor activity of NK cells (100). Such observations underscore the ability of PKC activators to elicit distinct TcR signaling in naive versus primed T cells. This suggests that the activity of bryostatin 1 preferentially mimics TcR signaling in primed T cells, theoretically activating T cells with specificity for several different TAAs expressed within the same tumor, and may stimulate innate immune cells; this results in their collective activation and subsequent responses to treatment with cytokine cocktails as discussed below. Importantly, it has been reported that *in vivo* administration of bryostatin 1 does not result in high-grade toxicities (101).

Immune cell phenotypic manipulation using the γ -chain cytokines

The selective stimulation of primed T cells and innate immune cells isolated from peripheral blood or draining lymph nodes with B/I followed by *ex vivo* culture with homeostatic cytokines is a potential approach to 'reprogram' lymphocytes to elicit more efficacious anti-tumor immune responses upon reinfusion during ACT. Lymphocyte homeostasis is mediated in large part by the activity of γ -c dependent signaling. The γ -c cytokines are so named due to their sharing of the cytokine receptor γ chain. The γ chain is coupled with cytokine-specific receptor subunits which then compose the receptor motif. In particular, the γ -c cytokines, IL-2, IL-7 and IL-15 have been widely reported to support memory T-cell homeostasis as well as enhancement of NK cell function and terminal NKT-cell maturation (102-106). Others have previously reported that T central memory (Tcm) cells are more effective than the T effector phenotype in generating long-lasting protection against tumor cells *in vivo* (107, 108). Importantly, our group has made similar observations in the FVBN202 mouse model using ACT which contained an enriched component of Tcm cells that had been reprogrammed using B/I and the γ -c cytokines IL-2, IL-7 and IL-15

protected animals from tumor challenge as well as tumor rechallenge two months later, demonstrating the functionality of the *ex vivo* reprogrammed memory T cell response (12, 16, 109).

Importantly, we have previously demonstrated that immune cell activation and phenotypic reprogramming using B/I and γ -c cytokines also robustly increased the frequency of CD25⁺ NKT cells which rendered T cells resistant to MDSC suppression in *in vitro* studies in both mouse models as well as breast cancer patients (16, 54), and induced *in vitro* maturation of human MDSCs (54).

These data suggest that signals provided by CD25⁺ NKT cells may function in the secondary lymphoid tissue and the TME of tumor-bearing hosts to support adaptive immune responses by modulating cells of myeloid lineage. We therefore hypothesized that ACT using both Tcm and CD25⁺ NKT cells may therefore have therapeutic efficacy against primary breast cancer.

Epigenetic enhancement of tumor immunogenicity: Dec as an *in situ* vaccine

An additional barrier to successful immunotherapy of breast cancer is the weak immunogenicity of tumor cells, for example, expression of tumor associated antigens to which the immune system is tolerant. Therefore, improving the immunogenicity of breast tumor cells is essential to improving tumor immunotherapy. To this end, *in situ* induction of foreign-like antigens, such as cancer testis antigens (CTAs), to which adaptive immunity is not tolerized, is a promising option. CTAs are highly immunogenic with no natural self-tolerance due to the observation that they are normally only expressed during embryonic development; after birth, expression is generally limited to immunologically privileged germ cells and the placenta (110). Aberrant CTA

expression was first described in melanoma; as such, this expression was found to generate CTA-specific cytotoxic T-cell responses (111). Recently, it was reported that treatment of metastatic melanoma with autologous CD4+ T cells specific for the CTA, NY-ESO-1, mediated a durable clinical remission (112). In addition to melanoma, CTA expression has also been observed in hematological malignancies (113) as well as solid tumors, including breast cancer (114, 115). Furthermore, CTA expression in breast cancer has been shown to elicit a broad range of cellular and humoral immune responses (114, 116, 117); both CD8+ T cell and CD79+ B cell infiltration has been observed in primary and metastatic NY-ESO-1 expressing breast cancer (118). Of note, a significantly elevated expression of NY-ESO-1 and MAGE-A was detected in triple negative breast cancers (119), which therefore represent antigenic targets in an otherwise perceived immunologically refractory breast cancer subtype.

The expression of CTAs is normally silenced by methylation within the promoter region of these genes. Methylation at the C-5 position of cytosine bases within DNA is a covalent chemical modification which characterizes a key, biologically functional, epigenetic modification of the animal genome (120). This action primarily occurs at CpG dinucleotides in mammals, where DNA-methyltransferases (DNMTs) mediate the transfer of methyl groups to cytosine, thereby generating 5-methylcytosine (5mC) that has been shown to play a critical role in cellular protein expression by transcriptional silencing of genes (121). Aberrant CTA expression likely occurs due to epigenetic molecular alterations which arise during tumor progression; cancer cells display drastic changes in DNA methylation status, typically exhibiting global DNA hypomethylation as well as region-specific hypermethylation (122), resulting in irregular expression of CTAs.

While CTAs are an attractive target, the aberrant expression of these proteins within a heterogeneous tumor may limit the efficacy of any CTA-specific immunotherapy to only those tumor clones which are CTA-positive. Therefore, in order to induce and/or increase expression of CTAs to function as target antigens, it is possible to modulate the tumor epigenome to initiate the cellular CTA transcriptional program; such an approach will serve to impart a more immunogenic tumor cell phenotype. Aza and Dec are both hypomethylating agents employed in epigenetic therapy to modify cellular methylation patterns; both of these agents have been approved for clinical use in the treatment of myelodysplastic syndrome. Aza and Dec function as cytosine analogs, which lead to their incorporation into newly synthesized DNA strands during S phase of the cell cycle; these agents have been shown to induce and/or increase the expression of various CTAs in a variety of *in vitro* and *in vivo* tumor models (113, 116-118). Both Aza and Dec have demonstrated the ability to induce the expression of CTAs, as well as the tumor suppressor gene p53 (123) and the death receptor Fas (124) on tumor cells. These are attributed to their capacity to function as potent DNMT inhibitors through the formation of a covalent complex with a cysteine residue at the active site of DNMT1, which therefore results in CpG island demethylation during cellular proliferation. This, in turn, results in hypomethylation within the promoter of tumor suppressor genes as well as highly immunogenic CTAs (120, 125-127), thereby rendering tumor cells susceptible to CTA-reactive immune responses and potentially reducing the proliferative capacity of tumor cells by restoring p53 expression. Others have demonstrated the feasibility to induce CTA expression *in vivo* using Dec in the 4T1 model of murine breast carcinoma, resulting in greater tumor cell cytotoxicity upon treatment with CTA-specific T cells (128).

Dec is a particularly attractive option to induce CTA expression as it functions as a prodrug which requires activation by deoxycytidine kinase (DCK), an enzyme preferentially expressed in tumor cells and myeloid cells. Thus, the effects of Dec are likely tumor specific, thus protecting T and B cells from the potentially deleterious demethylating effects of this agent. In addition, DCK has been found to be overexpressed in poor outcome breast cancer (129), suggesting that epigenetic therapy to induce CTA expression may prove to be an efficacious approach in breast cancer patients with poor prognosis.

The aim of Chapter One was to determine the ability of ACT to induce regression of established breast cancer in the FVBN202 mouse model by utilizing reprogrammed immune cells which were resistant to MDSCs. Induction of CTAs in these tumors and reprogramming CTA-sensitized immune cells was tested to determine the ability of CTA-targeted cellular therapy to enhance the response to ACT.

MATERIALS AND METHODS

Mouse model

FVBN202 transgenic female mice (The Jackson Laboratory; Bar Harbor, ME) were used between 8 and 12 weeks of age throughout these experiments. These mice overexpress non-mutated, non-activated rat neu transgene under the regulation of the mouse mammary tumor virus promoter (130). These mice develop premalignant mammary hyperplasia similar to ductal carcinoma *in situ* prior to the development of spontaneous carcinoma (44). Premalignant events in FVBN202 mice include the accumulation of endogenous MDSCs (44). These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Tumor cell lines

The neu overexpressing mouse mammary carcinoma (MMC) cell line was established from a spontaneous mammary tumor harvested from FVBN202 mice. Tumor cells were maintained in RPMI 1640 supplemented with 10% FBS.

Ex vivo reprogramming and expansion of splenocytes

FVBN202 transgenic mice were inoculated in the mammary fat pad with 3×10^6 MMC cells. Tumor growth was monitored by digital caliper, and tumor volumes were calculated by volume (v) = $(L$ [length] \times W [width] 2)/2. As previously described (16, 54), splenocytes were harvested 21–25 days after tumor challenge, when the tumor had reached $\geq 1000\text{mm}^3$. Splenocytes were

then cultured in complete medium (RPMI 1640 supplemented with 10 % FBS, L-glutamine (2 mM), 100 U/ml penicillin, and 100 µg/ml Streptomycin) and were stimulated with Bryostatin 1 (2 nM) (Sigma, Saint Louis, MO), Ionomycin (1 µM) (Calbiochem, San Diego, CA), and 80 U/ml of IL-2 (Peprotech) for 16–18 h. Lymphocytes were then washed thrice and cultured at 10^6 cells/ml in complete medium with IL-7 and IL-15 (20 ng/ml, Peprotech, Rocky Hill, NJ). After 24 h, 20 U/ml of IL-2 was added to the complete medium. The following day, the cells were washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml of IL-2. After 48 h, cells were washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml of IL-2. Twenty-four hours later, lymphocytes were washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml of IL-2. Lymphocytes were harvested 24 h later on the sixth day and were then either used for *in vitro* studies or *in vivo* as ACT.

Adoptive cellular therapy

Twenty-four hours prior to ACT, FVBN202 mice were injected i.p. with CYP (100 mg/kg) to induce lymphopenia. Individual groups of mice were challenged intradermally with 3×10^6 MMC cells. Individual groups of mice then received reprogrammed splenocytes i.v. at a dose of 70×10^6 /mouse once the tumor became palpable ($50\text{-}70\text{mm}^3$) (+ACT), or remained untreated (Control).

Characterization of splenocytes

Spleens of FVBN202 mice bearing primary tumors that served as donors of ACT were harvested when the tumor was $\geq 1000\text{mm}^3$, and were then homogenized into a single cell suspension as previously described (16); splenocytes were then characterized using flow cytometry on day 0,

day 1 and day 6 of the expansion and reprogramming procedure. Reagents used for flow cytometry: anti-CD16/32 Ab (93), FITC-CD3 (17A2); FITC-CD11b (M1/70); PE-GR-1 (RB6-8C5); PE-CD25 (3C7); Allophycocyanin-CD49b (DX5); Allophycocyanin-CD62L (MEL-14); PercP/CY5.5-CD4 (GK1.5); PE/CY7-CD8 α (53-6.7); all of which were purchased from Biolegend (San Diego, CA). All reagents were used at the manufacture's recommended concentration. Cellular staining was performed as previously described by our group (16, 54). Multicolor data acquisition was performed using a FACSCanto II (BD Biosciences). Data was analyzed using FCS Express v4.07 (De Novo Software; Glendale, CA).

***In vitro* and *in vivo* induction of CTA expression in MMC cells and cDNA synthesis**

MMC cells were cultured in the presence of 3 μ M Dec (Sigma-Aldrich; St. Louis, MO) for 72 hours. Medium was then removed and cells were washed with PBS, and then treated with TRIzol® (Life Technologies) per the manufacturer's instructions. *In vivo*, FVBN202 mice bearing primary tumor $\geq 1000\text{mm}^3$ were injected with Dec (2.5mg/kg) once daily for five days. Mice were euthanized and tumors were harvested 3 days later, minced, then treated with TRIzol® per the manufacturer's instructions. Contaminate DNA was then removed by Dnase I digestion from both the *in vitro* and *in vivo* specimens; RNA was then purified, followed by cDNA synthesis as previously described (131).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) for the detection of CTA expression

Quantitative reverse-transcriptase PCR was performed in triplicate wells using SensiMix SYBR and Fluorescein Kit according to the manufacturer's procedure (BIOLINE) with the BioRad CFX96 Real-Time PCR Detection System. qRT-PCR was performed using primers specific for six murine CTAs and murine GAPDH (Table 1). The reaction was initiated by a denaturing period of 10 minutes at 95°C, followed by 40 cycles of 95°C for 0:15 minutes, 60°C for 0:30 minutes, and 72C for 0:15 minutes. Relative CTA expression was computed after normalization to GAPDH using the $\Delta\Delta Cq$ method.

IFN- γ ELISA

Splenocytes from FVBN202 mice bearing primary tumors were reprogrammed as described above. Reprogrammed immune cells were then cultured in complete medium with irradiated (140Gy) MMC cells or irradiated CTA-expressing MMC (as described above) at a 10:1 ratio for 20 hours. Supernatants were then collected and stored at -80°C until assayed. IFN- γ was detected using a Mouse IFN- γ ELISA kit (BD Biosciences), according to the manufacturer's protocol.

Sample size and power calculation justification for patient CTA expression

The observed gene expression data were transformed into a natural logarithm for normality.

Assume that the actual true difference between the two groups (recurrence vs non-recurrence) in a gene expression and the actual standard deviation were observed in our preliminary results.

Also assume that the ratio of the size of the two groups is 1:1. Therefore, by a two-sample z test,

the sample size per group and the total sample size (Table 2) are required to gain at least 80% power to detect the difference between the two groups at a two-sided significance of 5%.

Clinical specimens

Tissue specimens had been collected from female breast cancer patients and maintained in the VCU Massey Cancer Center Tissue & Data Acquisition and Analysis Core (TDAAC). Frozen tissue was used for RNA extraction. These studies have been reviewed and approved by the Institutional Review Board (HM10920 and 2471-Tissue Acquisition System for Cancer Research) at Virginia Commonwealth University.

Statistical analysis

Outcomes are summarized by basic descriptive statistics such as mean and standard error of the mean (SEM); differences between groups are illustrated using graphical data presented as mean \pm SEM. Statistical comparisons between groups were made using one-tailed and two-tailed Student *t* test per the specific hypothesis. P-value ≤ 0.05 was considered statistically significant.

Table 1

Primer Sequence	Sense or Antisense Strand	Gene	Fragment Size
ACTGGAACCATCCGCACCAGC	Sense	SPA17	448bp
CACGTGTCCCCGGAAGAGGGA	Antisense		
CCGCGCTCCGGTCATGGAAT	Sense	ESX1	392bp
CCCCAGCTGAGCGTTGGAC	Antisense		
GCCCTGGTAGGCAGTGGCTC	Sense	AKAP4	329bp
GCCATGTTGCCACGGCTTC	Antisense		
GAGCGGTGGGAACAGAAGG	Sense	PEM5	203 bp
GAGGGGCATCTGCCTACCCCC	Antisense		
GTAGTCACCATGCCAGGGGT	Sense	MAGEB5	269bp
CCACAAACAGTGGCAGGCGA	Antisense		
AAGGCAGTGCTCGGAGCCAA	Sense	MAGEA4	194bp
AGCTTCCTCAGATGGGCCTTCA	Antisense		
ACCACAGTCCATGCCATCAC	Sense	GAPDH	452bp
TCCACCACCCTGTTGCTGTA	Antisense		

Table 2

Variable	Mean difference	Std Dev of diff	N per Group	Total sample size
MageA4	0.5402	2.8162	426.576	854
MageA5	0.6705	2.4265	205.577	412
AKAP	1.5319	2.5428	43.251	86
NY-ESO-1	0.4452	1.5713	195.541	392
SLLP1	1.6315	1.3921	11.428	22

RESULTS

ACT utilizing reprogrammed murine splenocytes which are resistant to MDSCs fails to reject established primary tumors in the FVBN202 mouse

It was demonstrated that tumor-derived factors increase the accumulation of MDSCs (27), thereby inhibiting the therapeutic efficacy of anti-tumor immune responses. We have previously established an *ex vivo* protocol for reprogramming tumor-sensitized immune cells in which tumor-reactive T cells became resistant to the suppressive functions of MDSCs due to the activity of CD25+ NKT cells (16, 54). In order to determine if reprogrammed, tumor-sensitized, immune cells could enhance the anti-tumor efficacy of immunotherapy by overcoming MDSCs, FVBN202 mice bearing primary tumors received ACT when the tumor had reached 50-70mm³, or remained untreated. As shown in Figure 1, ACT did not slow the rate of tumor growth (left panel) or improve overall survival (right panel) in recipient mice compared to untreated control mice.

Dec induces CTA expression in human breast cancer cells.

Since overcoming MDSCs was not sufficient to enhance therapeutic efficacy of ACT, we sought to determine whether the immunogenicity of mammary tumor cells can be enhanced by inducing the expression of CTAs. First, we sought to determine the translational capability of epigenetically modifying tumor cells to express CTA. To do this, we utilized two human breast cancer cell lines, MCF-7 and SKBR3, for *in vitro* treatment with Dec; control cells remained untreated. We observed a greater than two-fold increase in 4/9 CTA-specific transcripts for both

cell lines (Figure 2). This observation suggests the use of Dec may have clinical applicability to induce CTA expression within human breast tumors. We then questioned whether CTA expression may have a role in protecting breast cancer patients from disease recurrence; the hypothesis being that CTA expression within the tumor generates robust adaptive immune responses that may eliminate residual tumor cells after the completion of conventional cancer therapies. Therefore, CTA expression in primary tumor lesions might have prognostic value. We performed retrospective studies by using tumor biopsies that were collected from 10 breast cancer patients at the time of initial therapy, 5 of which had remained relapse free at least 62 months and 5 which had relapsed within 76 months (Table 3). Upon RNA extraction from the tissue samples, qRT-PCR was performed to determine the trend of CTA expression in patients who experienced disease relapse, or who had remained relapse free. As shown in Figure 3, five of the CTAs investigated demonstrated a strong trend toward increased expression in patients that remained relapse-free, suggesting there may be a positive correlation between increased CTA expression and survival after initial therapy. Statistically significant p-values were not achieved, however; this is likely due to the limited sample size of patients available for this study. Within this limited sample size, patients possessed diverse characteristics in terms of age, neoadjuvant therapy, tumor stage, hormone receptor and HER-2 status (Table 3). The sample size required to gain at least 80% power at a two-sided type I error of 5% to detect the difference in CTA expression between such diverse patients who either relapsed or remained relapse-free is shown in Table 2; this ideal sample size ranges from 11 patients per group (SLLP1) to 427 patients per group (MAGE A4). Therefore, understanding the role of CTAs in relapse-free survival will require studying a larger pool of patients and stratifying CTA expression with other known prognostic factors.

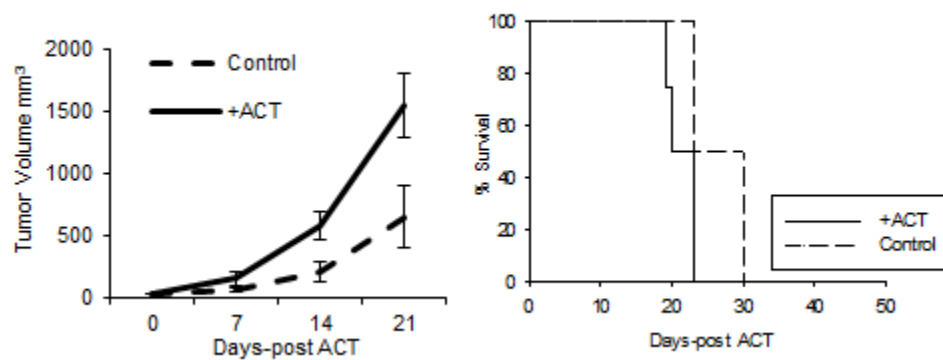


Figure 1. ACT utilizing reprogrammed murine splenocytes, which are resistant to MDSCs, fails to reject established primary tumors in the FVBN202 mouse. Animals were challenged with MMC (3×10^6) intradermally in the mammary gland region; upon the tumor reaching 50-70mm³, animals were conditioned with CYP (100 mg/kg) the next day. The following day mice remained untreated (Control; n=4) or received ACT (+ACT; n=4). Error bars represent mean \pm SEM.

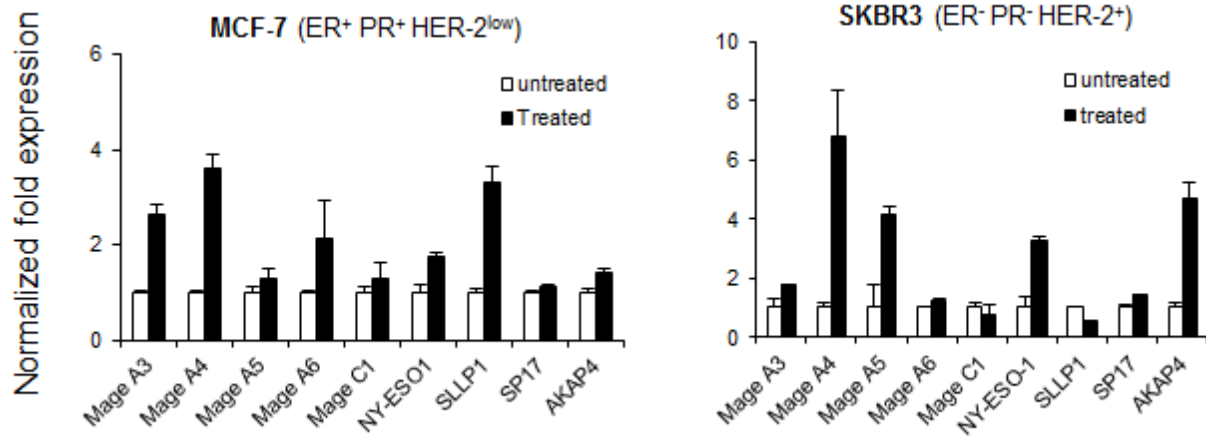


Figure 2. Human breast cancer cells upregulate CTA expression upon treatment with Decitabine, *in vitro*. MCF-7 and SKBR3 cells were cultured with (Treated) or without (Untreated) Decitabine (3 μ M) for 72 hours; RNA was then extracted and converted to cDNA followed by qRT-PCR using primers specific for nine human CTAs. Expression was normalized to GAPDH. Data represent mean \pm SEM.

Table 3

Patient	Age/Sex	Neoadjuvant Therapy	Stage	Hormone Status	HER2 Status	Relapse
1	39/F	No	IA	Negative	Positive	No - 110 mo
2	63/F	No	IIB	Positive	Positive	No - 91 mo
3	49/F	Yes	IIIC	Positive	Negative	No - 87 mo
4	77/F	No	IIB	Positive	Negative	No - 76 mo*
5	44/F	No	IA	Positive	Positive	No - 62 mo*
6	49/F	No	IA	Negative	Negative	Yes - 76 mo
7	45/F	No	IIA	Negative	Borderline	Yes - 60 mo
8	57/F	Unknown	IIA	Negative	Negative	Yes - 21 mo
9	75/F	No	IIA	Negative	Negative	Yes - 11 mo
10	29/F	Unknown	IA	Negative	Negative	Yes - 9 mo

*Lost to follow-up.
All patients received surgical resection of tumor. All information as of July 2014.

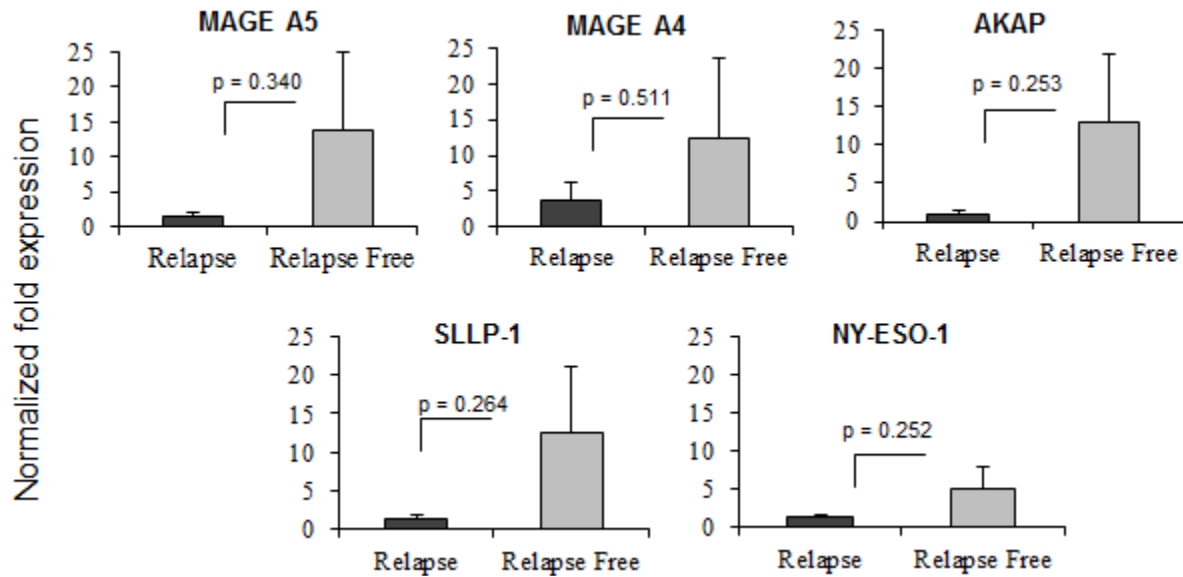


Figure 3. Breast cancer patients remaining free of tumor relapse display a trend of elevated CTA expression. RNA was extracted from breast tumor biopsy specimens from patients that eventually experienced relapsed relapse within six years (n=5), or patients who remained relapse free up to nine years (n=5). cDNA from each specimen was synthesized, followed by qRT-PCR using primers specific for five human CTAs. Expression was normalized to GAPDH. Data represent mean \pm SEM.

Dec upregulates CTA expression in primary murine tumor cells *in vitro* and *in vivo*, and reduces the frequency of splenic MDSCs.

Due to the promising results as to clinical translatability of the induction of CTA expression in human tumor cells (Figures 2 and 3), we began to perform preclinical studies. It was hypothesized that poor immunogenicity of the tumor allowed tumor progression in the ACT recipient mice (Figure 1); thus, improving tumor cell immunogenicity may improve the efficacy of ACT and lead to tumor regression. Therefore, the DNA demethylating agent, Dec, was utilized to determine if CTA expression could be induced and/or upregulated in MMC cells *in vitro* and in a setting of primary disease *in vivo*. As seen in Figure 4, treatment of MMC cells *in vitro* with Dec lead to an observed increase in levels of 3/5 CTA coding transcripts. Additionally, following five sequential injections of Dec to mice bearing primary MMC tumor all five CTA genes examined displayed upregulation compared to untreated tumor-bearing mice, with AKAP4, ESX1 and MAGE A4 increasing expression 10-fold or greater.

Furthermore, we observed that the administration of Dec to primary breast cancer-bearing mice significantly reduced the frequency of Gr1+CD11b⁺ MDSCs (Figure 5), suggesting Dec may have a dual role in enhancing tumor cell immunogenicity while also reducing the frequency of suppressive myeloid cells.

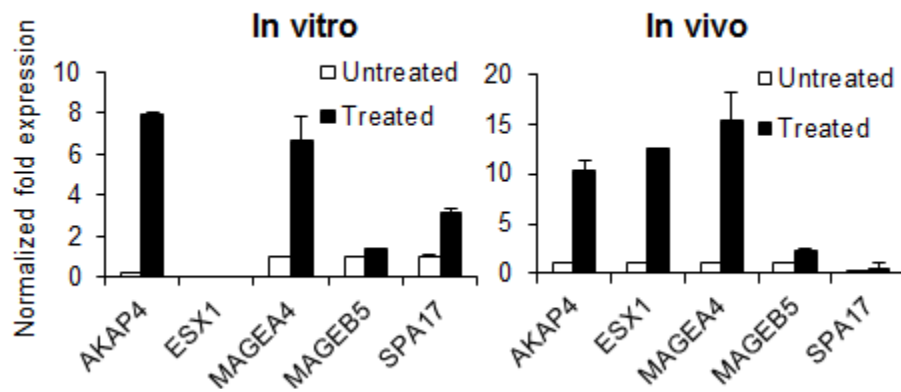


Figure 4. Decitabine upregulates CTA expression in MMC tumor cells *in vitro* and *in vivo*. A) MMC cells were cultured with (Treated) or without (Untreated) Dec ($3\mu\text{M}$) for 72 hours; RNA was then extracted and converted to cDNA followed by qRT-PCR using primers specific for six murine CTAs. B) Intradermal tumor-bearing ($\sim 1000\text{mm}^3$) FVBN202 mice received 5 injections of Dec, one per day, (Treated; 2.5mg/kg ; $n=1$) or remained untreated ($n=1$); the tumors were harvested 3 days later and cDNA was generated to quantify CTA expression. Expression was normalized to GAPDH.

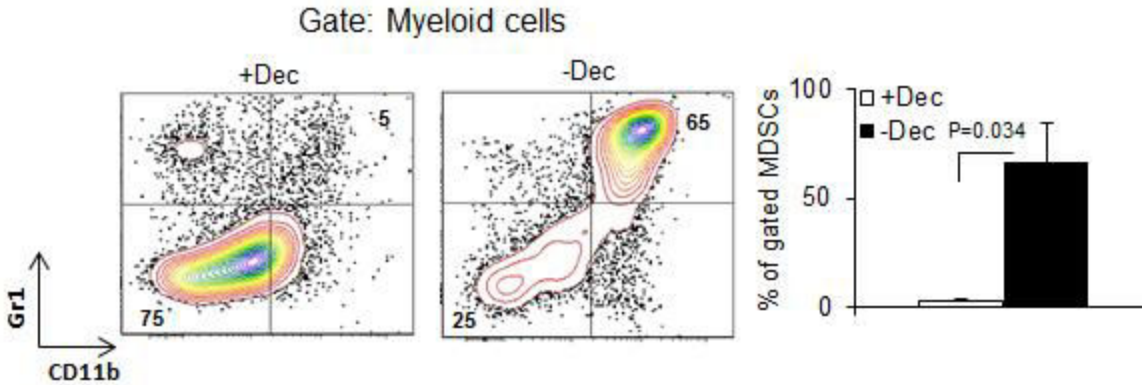


Figure 5. Decitabine reduces the frequency of splenic MDSCs in FVBN202 mice bearing established primary tumors. Animals were challenged intradermally with MMC (3×10^6) in the mammary gland region; after tumors reached $50-70 \text{ mm}^3$ all animals were treated with Decitabine (Every other day for 3 total injections; 2.5 mg/kg ; i.p.) ($n=3$), or remained untreated ($n=3$). Seven days later mice were euthanized and MDSCs were analyzed in the spleen. Data represent mean \pm SEM.

Splenocytes harvested from mice bearing CTA-expressing primary tumor demonstrate expansion and phenotypic characteristics typical of successful reprogramming, and demonstrate enhanced anti-tumor immune responses *in vitro*.

Next, we hypothesized that the induction of CTA expression in tumor-bearing mice would sensitize their splenocytes against these highly immunogenic antigens and therefore may prove to be superior donors for ACT. Thus, characterization of the ability of CTA-sensitized splenocytes to expand after B/I stimulation and culture with IL-2, IL7 and IL-15 was performed. As seen in Figure 6A, the ability of splenocytes harvested from Dec-treated tumor-bearing mice to expand was similar to that observed from control mice after the six day procedure. Furthermore, the frequencies of CD4+ and CD8+ T cells as well as the frequencies of CD25+ NK and NKT cells remained comparable on the final day of *ex vivo* procedure, day 6 (Figures 6B and 6D). Activated CD25+ NK cells and CD25+ NKT cells were also established one day after stimulation with B/I and IL-2 (Figure 6D). Importantly, however, significant reductions in CD4+ (40% vs. 70%; p=0.009) and CD8+ (25% vs. 50%; p=0.037) central memory T cells (CD44+ CD62L^{high}) with a reciprocal increase in CD4+ (50% vs. 12%; p=0.004) and CD8+ (60% vs. 35%; p=0.02) effector memory T cells (CD44+ CD62L^{int}) was observed in mice treated with Dec, as seen in Figure 6C. Thus, while splenocytes harvested from Dec-treated mice can be successfully reprogrammed and expanded, the use of such cells for ACT *in vivo* may limit the anti-tumor efficacy due to the reduced frequency of central memory T cells.

Next, we sought to determine the ability of such CTA-sensitized and reprogrammed splenocytes to produce an anti-tumor response in the presence of MMC cells and CTA-expressing MMC cells. As demonstrated in Figure 7, CTA-expressing MMC cells induced a 2-fold greater release of IFN- γ compared to control (p=0.0001). This suggests that reprogrammed CTA-sensitized

splenocytes for ACT against CTA-expressing primary tumors may generate an enhanced anti-tumor immune response.

ACT fails to reject CTA positive established primary tumors in FVBN202 mice.

We next studied the effect of utilizing reprogrammed CTA-sensitized splenocytes for ACT of FVBN202 mice bearing established primary cancer which had been pretreated with Dec to induce CTA expression. Dec injections began once tumors became palpable and ACT was performed when tumor had reached 50-70mm³. As seen in Figure 8, ACT used in this manner did not induce regression of the tumor; the growth kinetics were similar to the control group. However, the combination of Dec with ACT does appear to extend survival compared with ACT alone, demonstrated in Figure 1.

Altogether these data suggest that targeting established primary breast cancer using reprogrammed cellular immunotherapy, even with improved immunogenicity of the tumor and overcoming MDSCs, cannot produce an objective response. However, targeting CTA-expressing tumor cells using ACT in a setting of residual disease to prevent distant relapse may be an effective strategy.

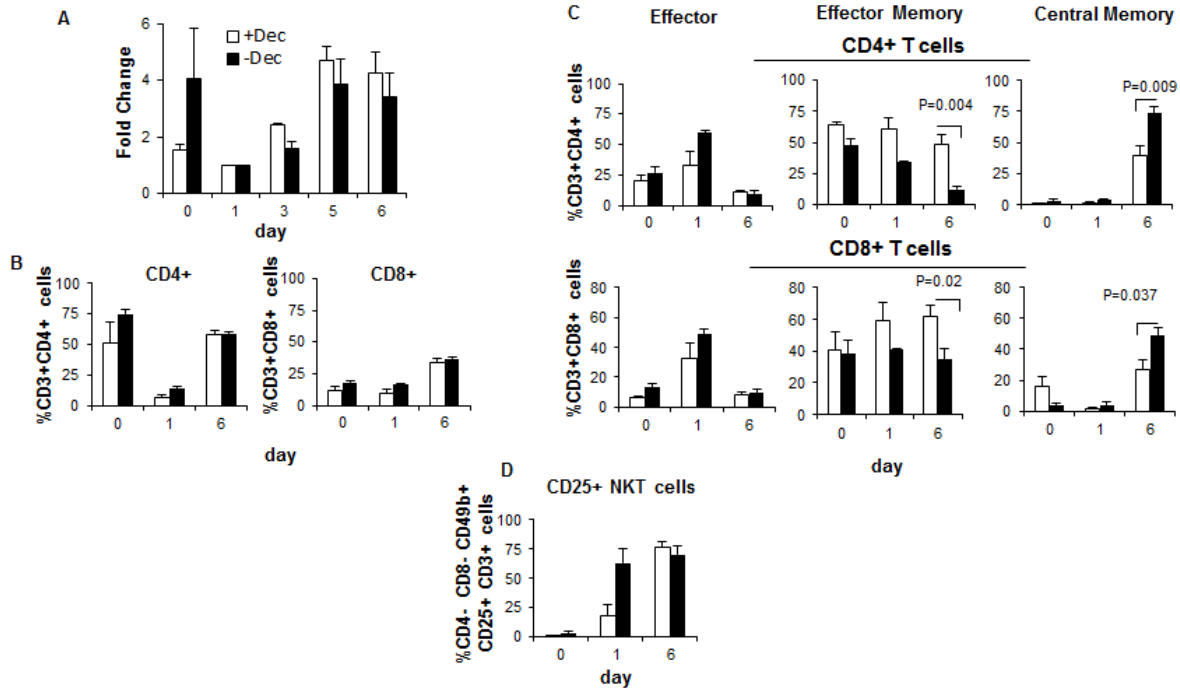


Figure 6. Expansion and phenotypic reprogramming of tumor-reactive splenocytes is similar between animals bearing primary cancer with and without Decitabine preconditioning. FVBN202 mice were challenged with 3×10^6 MMC cells intradermally. A portion of the mice went on to receive five sequential injections of Decitabine (2.5mg/kg) once tumors reached 1000mm^3 (+Dec), while the remaining mice were untreated (-Dec). Mice were euthanized and spleens were harvested 7 days after the final injection of Decitabine, and were then treated with B/I and γ -c cytokines *ex vivo*. A) Cell counts of viable tumor-reactive immune cells was determined by trypan blue exclusion; fold change was calculated by normalizing the cell count of each day to the number of cells present on day 1. Flow cytometry was used to determine the frequency of total CD4+ and CD8+ T cells (B), phenotype of CD4+ and CD8+ T cells (C), and the frequency of CD25+ NKT cells (D). Data represent four biological repeats for each group and mean \pm SEM.

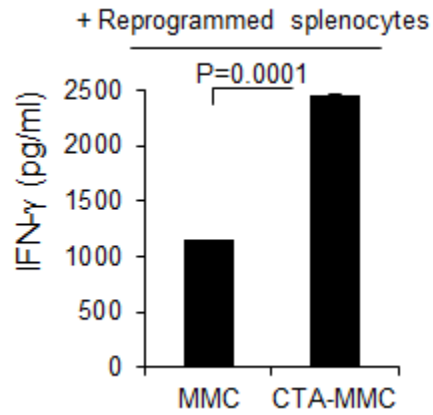


Figure 7. CTA-expressing MMC cells promote enhanced IFN-g release from CTA-sensitized reprogrammed splenocytes. MMC cells remained untreated (MMC) or were treated with Decitabine (3 μ M; 72hrs) to induce CTA expression (CTA-MMC). Tumor cells were then cocultured with reprogrammed splenocytes (1:10) for 20hours. IFN- γ was detected in the supernatant by ELISA. Data represent mean \pm SEM of duplicate wells.

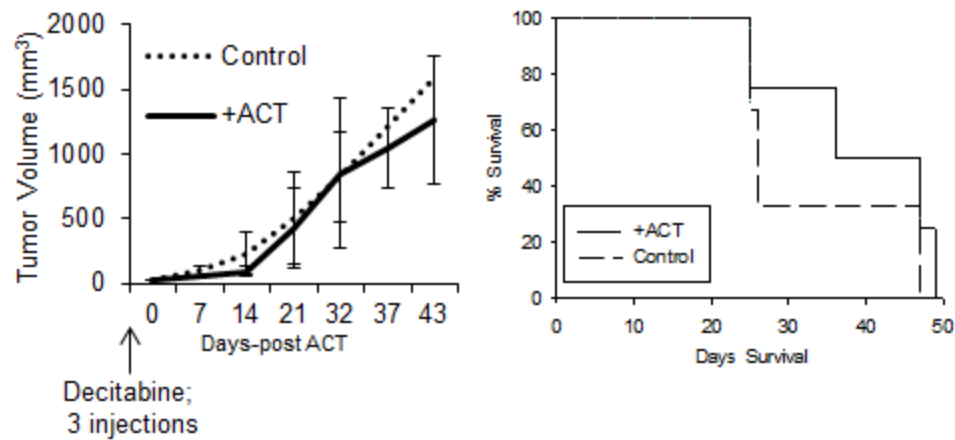


Figure 8. ACT fails to reject CTA-positive established primary tumors in FVBN202 mice. Animals were challenged intradermally with MMC (3×10^6) in the mammary gland region; after tumors reached 50-70mm³ all animals were treated with Decitabine (Every other day for 3 total injections; 2.5mg/kg; i.p.). Two days later, animals were conditioned with CYP (100 mg/kg; i.p.). The following day mice remained untreated (Control; n=3) or received ACT (+ACT; n=4) derived from a CTA⁺-tumor bearing donor. Data represent mean \pm SEM.

DISCUSSION

Development of cancer in immunocompetent individuals suggests that the host's unmodified immune response fails to protect the host from cancer due to the weak immunogenicity of many tumor cells. In addition, tumor-derived factors increase MDSCs which in turn dismantle anti-tumor immune responses, as previously reported by our group and others (15, 27, 28). In order to overcome such immunosuppressive mechanisms, we have developed a novel method to reprogram tumor-associated antigen (TAA)-sensitized immune cells, *ex vivo*, by pharmacologic activation using Bryostatins 1 and Ionomycin (B/I) and culture with the common gamma chain (γ -c) cytokines IL-7, IL-15 and IL-2. Reprogrammed immune cells display superior IFN- γ secretion, an enhanced frequency of highly efficacious central memory T cells (T_{cm}), as well as CD25⁺ NK and CD25⁺ NKT cells (16). Importantly, these cells establish a cellular crosstalk to render MDSCs immunostimulatory, rather than immunosuppressive and prevent murine breast cancer when used in ACT prophylactically (16). We also showed the clinical application of this reprogramming protocol using PBMC of patients with early stage breast cancer (54). Here, we sought to determine whether administration of ACT in a therapeutic setting against established primary tumors could induce tumor regression due to the ability of reprogrammed immune cells to overcome MDSCs. Contrary to our hypothesis, utilizing reprogrammed immune cells for ACT did not slow the rate of tumor growth or improve overall survival in FVBN202 mice bearing established primary MMC. These data suggest that tumors utilize multiple mechanisms which need to be tackled simultaneously. Therefore, we decided to combine reprogramming of tumor-sensitized immune cells with modulation of tumor cells, *in situ*, in order to improve tumor/immune cell crosstalk. The former was expected to overcome MDSCs and the latter was

expected to enhance immunogenicity of tumor cells by inducing the expression of highly immunogenic CTAs.

To induce the expression of CTAs in MMC, we used a demethylating drug, Dec, in order to hypomethylate the promoter of these genes and induce their expression (132-136). Therefore, Dec is expected to enhance immunogenicity of tumor cells. In fact, we showed that Dec was able to induce CTA expression in MMC as well as in human breast tumor cell lines, *in vitro*, as determined by qRT-PCR. The future implementation of additional techniques, such as immunohistochemistry or fluorescence *in situ* hybridization would also yield information as to the ability of Dec to homogenously induce CTA expression throughout the tumor. Treatment of tumor-bearing mice with Dec also resulted in the induction of CTAs, as determined by qRT-PCR, in the tumor as well as the elimination of MDSCs, *in vivo*. Such CTA expression was associated with the induction of CTA reactive immune response which produce higher levels of IFN- γ against CTA expressing MMC compared with wild type MMC. However, the combined use of Dec and ACT utilizing reprogrammed immune cells failed to produce objective responses against established primary MMC. These data suggest that tackling MDSC and enhancing the immunogenicity of tumor cells simultaneously may not be sufficient to induce objective responses against well-established breast cancer.

This failure could be associated with downregulation of neu expression in the tumor cells, *in vivo*. In addition, cessation of Dec administration could result in the reversion of the methylation patterns within the CTAs promoter region, consequentially resulting in the reduction or complete loss of CTAs within the tumor, as we demonstrate in Chapter Three. These data suggest that overcoming MDSCs and improving tumor cell immunogenicity is not sufficient to enhance the therapeutic efficacy of ACT, perhaps due to unsustained expression of CTAs and/or tumor

antigen loss or downregulation under immune pressure. In fact, the effect of anti-tumor immune responses in editing the tumor has been reported by several groups. For example, IFN- γ was shown to promote immunoediting and subsequent tumor escape in the CT26 colon carcinoma by down-regulation of the expression of gp70 immunogenic tumor antigen (137). Tumor-specific IFN- γ was also shown to be essential for successful initial immunotherapy, but it also impaired subsequent secondary or durable anti-tumor immune responses (138). Farrar et al. (139) demonstrated that IFN- γ producing T cells can establish and maintain cancer dormancy; however the progression of cancer from indolent to aggressive tumors occurred in the presence of increased expression of IFN- γ -inducible genes (140). Romieu-Mourez et al. (141) also showed that treatment of HER-2/neu positive cells with IFN- γ resulted in the suppression of protective anti-tumor immune responses. We also reported that immunotherapy of rat neu overexpressing mouse mammary carcinoma (MMC) elicited neu-specific IFN- γ producing CD8⁺ T cells that in turn facilitated breast cancer recurrence of neu antigen negative variant tumors following initial rejection of MMC tumor cells in immunocompetent mice (142, 143). The tumor antigen loss was due to hypermethylation of the neu promoter and loss of neu both at mRNA and protein levels (142, 144), resulting in escape of the tumor from further neu-specific immune responses. It is likely that DNA methylation may also impact HER-2/neu gene amplification in humans, as suggested by others (145). Subsequent studies showed that the tumor editing functions of CD8⁺ T cells induced epithelial to mesenchymal transition (EMT) of MMC, and as a result induced neu antigen loss and generated breast cancer stem cells (BCSC)-like tumor clones (144).

In clinical studies, a prospective randomized trial showed that tumor relapse in melanoma patients who were immunized with NY-ESO-1 vaccine was associated with downregulation of NY-ESO-1 and HLA class I in their tumor. This suggests immunoediting and escape of the

tumor under pressure from NY-ESO-1-specific immune responses (146). In patients with ductal carcinoma *in situ* (DCIS), HER-2/neu-targeted vaccination induced HER-2/neu-specific IFN- γ producing T cell responses and resulted in HER-2/neu antigen loss (147). Although the authors considered this HER-2/neu loss a positive outcome of the immune response, no follow-up studies have been performed to determine whether patients with HER-2/neu loss in their tumors might end up with recurrence of more invasive tumors. We reported that patients with HER-2/neu negative breast cancer had HER-2/neu-specific IFN- γ producing T cell responses which were associated with nuclear translocation of IFN- γ receptor α in the tumor site (86). This suggests that patients with HER-2/neu negative breast cancer might have had undetectable HER-2/neu positive premalignant tumors in the past that had lost HER-2/neu expression and progressed to invasive carcinoma under immune pressure. The fact that 55–75% of patients with premalignant DCIS overexpress HER-2/neu in their tumor lesions and 75% of breast cancers are HER-2/neu negative may suggest the progression of HER-2/neu positive DCIS to HER-2/neu negative breast cancer only in the tumor clones that express variable levels IFN- γ receptor α (148). This possibility is also supported by the observation that overexpression of HER-2/neu in DCIS lesions is usually accompanied by invasive foci (149). In fact, the low frequency of HER-2/neu expression (20–25%) in invasive breast cancer implies that HER-2/neu loss is an epiphenomenon of disease progression (150). Evaluation of HER-2/neu expression in tumor lesions of patients with breast cancer revealed overexpression of HER-2/neu in primary tumors and its loss in synchronous metastasis (151) as well as gain of HER-2/neu overexpression in metastatic tumors (152). Such discordance between primary and metastatic tumors in the expression of HER-2/neu suggests that HER-2/neu loss *in vivo* may be associated with distant recurrence or metastasis only in certain tumor clones. In prostate cancer, HER-2/neu positive tumors, DU145 and PC-3,

that responded to IFN- γ (likely due to sufficient expression of IFN- γ receptor α), showed down-regulation of HER-2/neu expression whereas another prostate tumor line, LNCaP, that failed to respond to IFN- γ did not show any change in the expression of HER-2/neu (153). Such failure of LNCaP was later shown to be due to the lack of JAK1 gene expression (154).

Despite the presence of pre-existing tumor-specific immune responses, breast cancer patients often do not benefit from immunotherapy (e.g. vaccines, antibodies). For example, although Trastuzumab (in combination with chemotherapy) prolongs the survival of women with advanced HER-2/neu⁺ breast cancer, the vast majority of women will develop resistance within one year of treatment initiation and 15% of patients are *de novo* resistant (155). Similarly, in a Phase II study by Peoples and colleagues, the benefit of a HER-2/neu peptide vaccine (i.e. prevention of recurrence) was minimal and non-significant despite the generation of high level HER-2/neu-specific CD8⁺ T cell responses (156). Although the reason for the resistance or lack of benefit is unclear, it could be related to the recent findings of Reim et al. (157), who showed that Trastuzumab associated with IFN- γ producing NK cells expanded tumorigenic CD44^{high}CD24^{low}HER-2/neu^{low} BCSC *in vitro*. We have observed such epigenetic effects of IFN- γ during neu antigen loss and tumor relapse in our mouse model of mammary carcinoma (142). Therefore, levels of IFN- γ production or levels of the expression of IFN- γ receptor α on tumor cells may determine whether a tumor inhibitory or a relapse promoting effect of IFN- γ may prevail. High levels of IFN- γ and/or IFN- γ receptor α expression or the lack of the expression can induce a robust tumor rejection via IFN- γ -dependent or -independent mechanisms whereas intermediate levels of IFN- γ and/or IFN- γ receptor α expression may facilitate tumor escape and relapse (158). This hypothetical model, which is suggested by our recent observations, may also predict the direction of discordance between primary and metastatic

breast cancers in the expression of HER-2/neu such that tumor clones with low levels of IFN- γ receptor α that escaped anti-tumor immune responses may retain HER-2/neu expression during metastasis.

In conclusion, the application of ACT combined with the blockade of a number of tumor escape mechanisms could not offer an effective therapeutic strategy against breast cancer because proliferating tumor cells undergo continuous change during cell division which could dismantle ACT. In the next chapter we tested anti-tumor efficacy of ACT combined with a demethylating drug, Aza, against MRD in patients with multiple myeloma after conventional chemotherapy and autologous stem cell transplantation (ASCT).

Chapter Two

INTRODUCTION

The results of Chapter One indicate that ACT using reprogrammed immune cells against established primary breast cancer was not effective, even when combined with Dec chemotherapy for rendering tumor cells highly immunogenic by the induction of CTA expression in tumor cells. In retrospect, this was not surprising. Human vaccines and immunotherapy against infectious diseases are only effective in a prophylactic setting either prior to exposure to the infectious agents, including pathogen-associated cancers, or during the incubation period or dormancy after the exposure. Therefore, we sought to determine the efficacy of immunotherapy against MRD when combined with Aza for the induction of CTA expression in residual tumor cells in order to prevent disease progression and relapse. In a phase II randomized clinical trial, patients with multiple myeloma (MM) received autologous lymphocyte infusion (ALI) in a setting of ASCT, generated to react with CTA-expressing myeloma cells using Aza and stimulated with the immune-modulatory agent Lenalidomide or Rev.

Stem cell transplantation following the modulation of tumor-immune crosstalk

Allogeneic stem cell transplantation (allo-SCT) is associated with a reduction in the relapse rate in patients with multiple myeloma (MM) on the basis of an allo-immune graft vs. myeloma effect, mediated by donor immune cells targeting tumor (myeloma)-specific antigens, resulting in prolonged remission. Allografting is, however, complicated by graft-versus-host disease and

unacceptable treatment-related mortality. On the other hand, patients undergoing high dose therapy with autologous stem cell transplantation (ASCT) remain at risk for relapse, despite maintenance and consolidation regimens (159). An alternative strategy is needed to relieve the burden of treatment toxicity observed in patients with myeloma while also maintaining and prolonging current treatment efficacy. Immunotherapeutic interventions mimicking graft-versus-myeloma effect in the ASCT setting may provide such an option. However efficacious, safe, and widely applicable strategies for immunotherapy remain elusive, limiting this option only to a select number of participants in clinical trials at tertiary cancer centers (160).

Cancer testis antigens (CTA) represent potential targets for immunotherapy in myeloma. These proteins are highly immunogenic with no natural self-tolerance because, under normal circumstances, they are only expressed in ‘immunologically privileged’ germ cells, and in the placenta (110). Aberrant CTA expression has been observed in both solid tumors and in hematological malignancies, particularly in MM (113). This often elicits a broad range of cellular and humoral immune responses. In myeloma, several reports have described sporadic over-expression of CTA and accompanying CTA-specific T cell and B cell responses (161-165). Induced CTA alloreactivity has also been reported in MM patients undergoing allografting, possibly associated with relapse-free responses (166). It is noteworthy that CTA expression is regulated by methylation of CpG islands in the promoters of these genes, which are mostly located on the X chromosome. There is evidence suggesting that therapy with Aza, a potent DNA methyl-transferase inhibitor, increases the expression of various CTA in a variety of *in vitro* and *in vivo* tumor models (128, 167, 168).

We hypothesized that *in vivo* induction of CTA by Aza may induce a CTA-specific T cell response if it is sequentially administered with Rev, which is widely used in the therapy of MM.

Rev functions in part by increasing T cell and NK cell tumor cytotoxicity *in vitro* (169, 170). Additionally, Rev stimulates T cell proliferation and secretion of interleukin 2 (IL-2) and IFN- γ in T cell co-stimulation assays (171, 172), therefore resulting in similar effect rendered by B/I during the reprogramming procedure described in chapter 1. Further, CTA-specific T cells generated by this combination of Aza and Rev, when adoptively transferred to ASCT recipients, could expand *in vivo* and provide robust protection from disease progression. Additionally, the alkylating agent, melphalan, has found a distinctive role in autologous stem cell transplantation (ASCT) and allogeneic stem cell transplantation (allo-SCT), due to its broad antitumor activity, ability to ablate the bone marrow, and potent immunosuppressive effects (173), and remains the most common conditioning agent in transplantation for myeloma (174). Melphalan has been shown to significantly reduce tumor burden in MM patients. As early as 1983, high-dose melphalan (140 mg/m²) in combination with ASCT led to a complete remission (CR; no detectable myeloma protein in the bone marrow) in one patient with MM (175). Later, it was shown in a cohort of 23 patients with refractory myeloma receiving 80-140 mg/m² of melphalan that tumor mass was reduced by more than 75% in 14 patients (176). Selby et al. (177) reported on the use of high-dose melphalan (140 mg/m²) therapy for 58 previously untreated patients with myeloma. A CR in 27% of the patients was achieved and 51% of the patients entered a partial response (PR; more than 50% reduction in myeloma protein and improvement in all other features). Therefore, in the setting of this study, transplant conditioning and ASCT produce both a state of MRD, as well as lymphodepletion, promoting the preferential proliferation of adoptively transferred CTA-specific T cells, leading to the promotion of effective adaptive cellular immunotherapy (160).

A multi-step phase II study was conducted to determine the feasibility of generating CTA-specific T cells in MM patients and their application in post-transplant maintenance to control residual disease (NCT01050790). MM patients received sequential Aza and Rev (Aza-Rev) to induce the expression of immunogenic CTA on malignant plasma cells and elicit a CTA-specific cellular immune response. The patients had autologous lymphocytes collected and cryopreserved following the second and third cycle of this regimen. After completion of this investigational regimen patients underwent stem cell mobilization and eventually ASCT. The autologous lymphocytes were adoptively transferred to the patients in the second month after transplant. Here, we demonstrate the feasibility of collecting and reinfusing autologous lymphocytes following ASCT in a setting of MRD. Further, we show induction of the expression of CTA in bone marrow of MM patients and an increase in CTA reactive T cells.

MATERIALS AND METHODS

Patients

Patients were enrolled on a prospective phase II clinical trial approved by the Virginia Commonwealth University (VCU) Institutional Review Board (MCC12430). MM patients referred to VCU's bone marrow transplant program had to meet the following eligibility criteria; presence of residual disease, with either quantifiable serum or urinary M protein or free light chains, in the presence of a positive immunofixation or clonal bone marrow plasma cells; age between 18 and 70 years; able to undergo high dose therapy and SCT; adequate performance status, marrow (absolute neutrophil count of $>1.5 \times 10^9/l$, platelet count $>100 \times 10^9/l$) and end organ function. Patients refractory to or progressing on therapy with lenalidomide were excluded. Patients with high $\beta 2$ -microglobulin (≥ 0.055 g/l) and adverse cytogenetic changes were offered tandem SCT, whereas those with standard risk disease underwent a single autograft.

Investigational regimen

Patients with MM who were in a partial remission or plateau phase underwent 3 cycles of therapy with Aza 75 mg/m² given subcutaneously from day 1-5 (Vidaza; Celgene Corporation, Summit, NJ, USA), and Rev 10 mg daily given orally, from day 6-21 (Celgene Corporation). The 3 cycles of therapy were administered at 4-week intervals prior to blood stem cell mobilization. No planned corticosteroids were administered during this therapy. Following 3 weeks of therapy in the second and third cycles of Aza-Rev, autologous lymphocytes were collected by a single, 18-litre lymphapheresis procedure and cryopreserved (Figure 9).

After completion of the third cycle of Aza-Rev, peripheral blood stem cell mobilization was performed using granulocyte colony-stimulating factor (G-CSF) 10 µg/kg/day subcutaneously from day 1 until the end of apheresis, either with or without plerixafor (0.24 mg/kg subcutaneously from day 4 until the end of apheresis). Patients then went on to receive high dose melphalan (either 140 or 200 mg/m²) on day -2, and underwent autologous SCT on day 0. GM-CSF (5 µg/kg/day) was administered from day 4 post-transplant for hematopoietic engraftment. Standard antimicrobial prophylaxis was administered.

Autologous lymphocyte infusion (ALI) was performed between day +30 to +60 of the SCT (following second transplant in tandem SCT recipients), after resolution of regimen-related toxicities and in the absence of active infections. Autologous lymphocytes collected following cycles 2 and 3 were infused together (Figure 9). Diphenhydramine and acetaminophen were administered for infusion reaction prophylaxis. Corticosteroid administration was avoided as much as possible following SCT and ALI. Patients were not given any routine maintenance therapy for myeloma following ALI, except for bisphosphonates when indicated. Periodic myeloma restaging was performed to monitor disease status.

qRT-PCR for the detection of CTA expression

Patient underwent bone marrow aspiration and biopsy before and after investigational therapy for standard histological studies. qRT-PCR was performed as described above. Both pre- and post-Aza-Rev treatment bone marrow samples were used to determine the expression of 10 human CTA transcripts [*MAGEA3*, *MAGEA4*, *MAGEA5*, *MAGEA6*, *MAGEC1*, *CTAG1B (NY-ESO-1)*, *SPACA3 (SLLP1)*, *AKAP4*, *SPA17*, *SPANXB1* & *SPANXB2*], using CTA-specific primers and human *GAPDH* (Table 4). Initially, qRT-PCR was performed using RNA isolated from

unfractionated Ficoll-Hypaque separated marrow mononuclear cells. Subsequent patients had CD138⁺ cells isolated from marrow mononuclear cells using an EasySep human CD138 positive selection kit, as instructed by the manufacturer (STEMCELL Technologies, Tukwila, WA, USA) followed by qRT-PCR analysis of CD138⁺ plasma cells and CD138⁻ fractions.

Figure 9

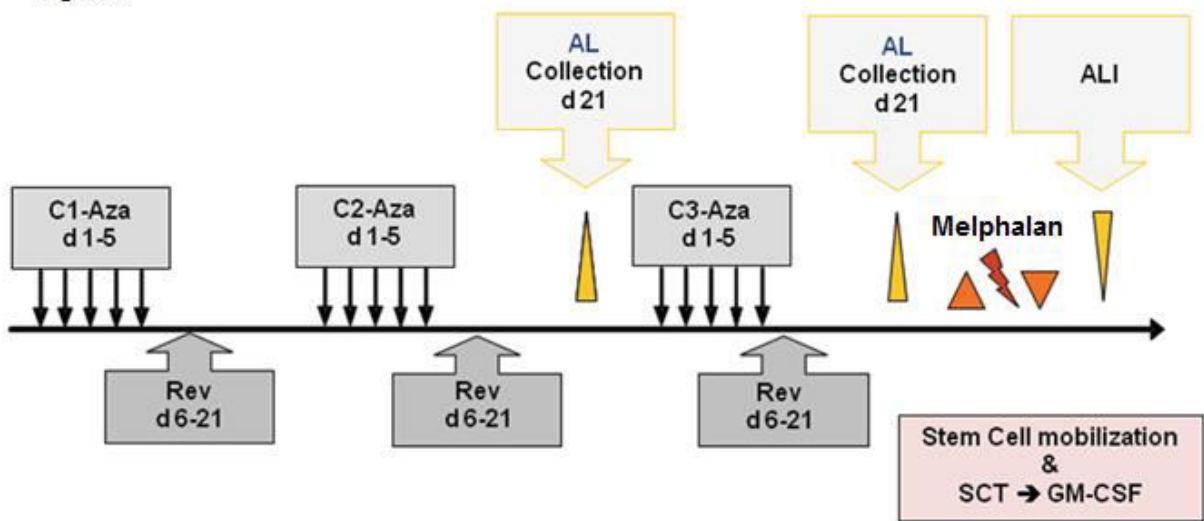


Table 4

Primers	Sense or Antisense Strand	Gene	Size (base pairs)
CTGAGGGGCCTGCCCAGTCT	Sense	<i>MAGEC1</i>	346
CGCGCCAACCTCGTCCACCTT	Antisense		
TGGAGGACCAGAGGCCCCC	Sense	<i>MAGEA3</i>	343
GGACGATTATCAGGAGGCCTGC	Antisense		
GAGCAGACAGGCCAACCG	Sense	<i>MAGEA4</i>	446
AAGGACTCTGCGTCAGGC	Antisense		
CCGGGCTCTGTGAGGAGGCA	Sense	<i>MAGEA5</i>	400
CGATGGCAGTGGGGATGGCG	Antisense		
TCGGTGAGGAGGCAAGTCCTG	Sense	<i>MAGEA6</i>	204
CTGGTCAGGGCAACAGGCGG	Antisense		
CAGGGCTGAATGGATGCTGCAGA	Sense	<i>CTAG1B</i>	332
GCGCCTCTGCCCTGAGGGAGG	Antisense		
CAGAAGGCGGTGGTGCCAG	Sense	<i>SPACA3</i>	302
CGTTGGGGACGTTCTGGGGTG	Antisense		
TGAAGGGCTGACACGCGAGA	Sense	<i>SPA17</i>	322
TCCCCGGAAGGCAGCTTGGAT	Antisense		
AGAGCTCTGGGCCACTGCGA	Sense	<i>SPANXB1 & B2</i>	184
CCCAGTTGGGGTCTCCGGCA	Antisense		
GCACTCAAGGCTGTAGGAGGGC	Sense	<i>AKAP4</i>	190
TGCACACACCCTGTGGCTG	Antisense		
ATTGCCCTCAACGACCACTTTG	Sense	<i>GAPDH</i>	264
TTGATGGTACATGACAAGGTGCGG	Antisense		

IFN- γ ELISA for the detection of CTA-specific T cell responses

Blood samples, autologous lymphocytes and stem cells from the patients were evaluated for the presence of T and NK cells, as well as for CTA (NY-ESO-1)-specific T cells, before and after Aza-Rev therapy and after ASCT. NY-ESO-1 was selected as the antigenic target to be studied due to its frequent up-regulation in the majority of patients (4/6) following Aza therapy and the availability of recombinant protein for performing IFN- γ ELISA, as previously described (16, 86). Briefly, cellular co-cultures were developed in which autologous lymphocytes were cultured with autologous monocyte-derived DCs (2:1) in the presence or absence of recombinant NY-ESO-1 (8 μ g/ml; 3H Biomedical; Uppsala, Sweden). Supernatants were collected after 20 h and subjected to IFN- γ ELISA (BD Biosciences, Franklin Lakes, NJ, USA).

Immuno-phenotypic analysis of the blood and stem cell apheresis product for measuring cellular immune parameters was performed, using a dual-platform technique on a Cytomics™ FC500 flow cytometer (Beckman Coulter Inc., Miami, FL, USA). Antibodies to CD3, CD4, CD8, CD34, and CD56 (Beckman Coulter, Inc.) were employed to quantify T cell subsets, haematopoietic stem cells, and NK cells.

Study design

This was a phase II study designed to test the feasibility of safely giving Aza-Rev to patients with MM, followed by the collection and administration of ALI. The study was designed with the expectation that $\geq 70\%$ of the patients would be able to mobilize a cell dose of 10^7 mononuclear cells/kg and that $\geq 80\%$ of the patients would be able to receive the ALI following SCT. The required sample size, $n = 19$, was based on an independent sample χ^2 test with a 0.05 one-sided significance level and 80% power to detect that 70% of the patients would be able to

mobilize the minimal cell dose against the null hypothesis of 40%, and also to detect that 80% of the patients will be able to get the infusions post-transplant against the null hypothesis of 50%. Drop-out was assumed to be 30%. Early stopping criteria for hematopoietic toxicity, were defined as grade 4 neutropenia or thrombocytopenia lasting beyond 7 days, and for non-hematopoietic toxicity, as any unexpected grade 4 toxicity attributable to the Aza-Rev regimen or ALI, with >30% incidence deemed unacceptable. Disease progression, while on investigational therapy, with a progression rate of >30% prior to SCT was also unacceptable as was the inability of >30% of the patients to proceed onto SCT. Disease response and progression were as defined by the International Myeloma Working Group (178).

Fourteen subjects have been enrolled to date. Temporal changes in CD3⁺, CD4⁺, CD8⁺ and CD56⁺ cell subsets at different time points, as well as differences in CTA expressions before and after Aza-Rev therapy were tested using the Wilcoxon signed-rank test. All tests were one-sided, and CTA expression increases were deemed significant for *P*-values < 0.05. The likelihoods for observing significant outcomes for study aims 1 and 2 are estimated using Bayes methods (179). We assigned conservative and pessimistic prior beta distributions for each success rate where the modes matched the null hypothesized success rates for each aim (40% for aim 1; 50% for aim 2), and assumed that the accumulated success rates were binomial processes based on *n* observed subjects. The predictive probability that we would observe efficacious outcomes for both aims (>70% mobilization for ALI and stem cells; >80% receive infusion) after observing the remaining (19-*n*) patients into the study would then be estimated using beta-binomial distributions.

RESULTS

Patient demographics

Between February 2010 and February 2012, 14 patients with a median age of 60 years (range 40–69) were enrolled (Table 5). Nine patients were African American, and seven were female. International Myeloma Working Group stage at diagnosis was I (n = 2), II (n = 6) and III (n = 6). Six had chromosomal abnormalities, consistent with high-risk disease. A median of two prior regimens had been administered (range 1–2) and eight had prior therapy with Rev. A median of 10.6 months had elapsed from diagnosis to start of Aza-Rev therapy and 10 cycles of therapy administered before study therapy was initiated (Table 5).

Response to Aza-Rev and autologous lymphocyte collection

All 14 patients completed 3 cycles of Aza-Rev and underwent two autologous lymphocyte (AL) collections. In order to determine if Aza-Rev therapy had a deleterious effect on the number of autologous T cells, a blood differential was performed. Circulating T cell counts were well preserved following Aza-Rev therapy (P=0.06) resulting in comparable yield of AL at a median 21 d following cycles 2 and 3 of Aza-Rev, with 0.87 ± 0.38 and $0.82 \pm 0.29 \times 10^8$ CD3+ cells/kg (n=14, P=0.20) respectively, with the first and second procedures (Table 6). There was no significant difference in the circulating T cell subset and NK cell counts between the two cycles of Aza-Rev therapy (Figure 10A). The circulating CD3+ cell count was significantly increased 2 weeks post-ALI compared with the pre-ALI value (P=0.04), as was the CD8+ cell count (P=0.02) (Figure 10B). CD4+ and CD56+ cell counts displayed an elevated trend when

comparing pre-ALI with 2 weeks-post ALI, however these values did not reach statistical significance ($P=0.06$).

***In vivo* induction of CTA expression with Aza-Rev in MM**

Quantitative RT-PCR evaluating a panel of 10 human CTAs in unfractionated bone marrow specimens collected before and after Aza-Rev from four patients demonstrated the induction of 6-8 CTAs in each patient (Figure 11A). In order to identify the cells which were being modulated to induce expression of CTAs, bone marrow cells from two patients were fractionated into CD138⁺ plasma cells (presumably residual tumor cells) and CD138⁻ cells. As shown in Figure 11B, CD138⁺ cells were the main source of Aza-induced CTA expression. *MAGE A4* and *MAGE A6* were expressed in bone marrow cells of all patients after investigational therapy ($n = 6$). Expression of other CTAs included *MAGE A3* (3/6), *MAGE A5* (4/6), *MAGE C1* (3/6), *NY-ESO-1* (4/6), *SLLP1* (3/6), *SP17* (5/6), *AKAP4* (5/6), and *SPANXB* (1/6). When compared to before and after therapy, CTA expression was significantly increased in 4 of 10 CTA as follows: *MAGEA4* ($P = 0.02$), *MAGE A6* ($P = 0.02$), *SP17* ($P = 0.03$) and *AKAP4* ($P = 0.02$). The increase in *MAGE A3* ($P = 0.09$), *MAGE A5* ($P = 0.08$), *MAGE C1* ($P = 0.13$), *NY-ESO-1* ($P = 0.07$), *SLLP1* ($P = 0.13$) and *SPANXB* ($P = 0.50$) expression, however, did not reach statistical significance.

Table 5 Patient characteristics.

UPN	Age (years)	MM subtype	Stage at diagnosis	Cytogenetics	Prior therapy	Number of cycles prior Rx	Response to prior Rx
12430-01	60	IgG λ	III*	N	TD	11	VGPR
12430-02	64	IgG κ	II	N	TD \times 1; RD	12	PR
12430-03	63	IgG κ	II	A ¹	TD; VD	8	PR
12430-04	68	IgG κ	II	N	RD	6	PR
12430-05	55	IgG κ	II	N	RD	10	VGPR
12430-06	53	IgG κ	III*	A ²	VDoxD	7	VGPR
12430-07	40	IgG κ	II	A ³	D; VD (4)	5	VGPR
12430-08	61	κ	III	A ⁴	VD	10	PR
12430-09	69	IgA λ	I*	N	VD	9	PR
12430-10	59	κ	III	N	VDox; RD	12	PR
12430-11	60	κ	I	N	V; RD	11	VGPR
12430-12	54	κ	II	A ⁵	RD	7	SD
12430-13	65	κ	II	A ⁶	RD; VD	12	VGPR
12430-14	52	IgG κ	III	A ⁷	RD	3	PR

UPN, unique patient number; MM, multiple myeloma; Rx, therapy; N, Normal; A, abnormal; TD, Thalidomide + dexamethasone; RD, Revlimid (lenalidomide) + dexamethasone; VD, Velcade (bortezomib) + dexamethasone; D, dexamethasone; VDox, Velcade + doxorubicin; SD, stable disease; PR, Partial remission; VGPR, Very good partial remission; REL, Relapse. Abnormal Cytogenetic/Fluorescent *in situ* hybridization results- 1: (+3, +7, +11, -13, +15, +17), 2: (-13, -17, *FGFR3:IGH@* translocation), 3: (-13, 14q32 translocation), 4: (46-51, X, -X, del 6q13, +11, -13, del 16q24, +19; +11q23/*ATM*, -13, *IGH@* translocation), 5: (-6q21/*MYB*, -13), 6: [-13q, t(11:14) *CCND1: IGH@*], 7: [t(11:14) *CCND1: IGH@*].

*Durie-Salmon staging, diagnostic β 2m not available. All other diagnosis staging used the International Myeloma Working Group criteria.

Table 6 Cell collection and clinical outcome following SCT and ALI.

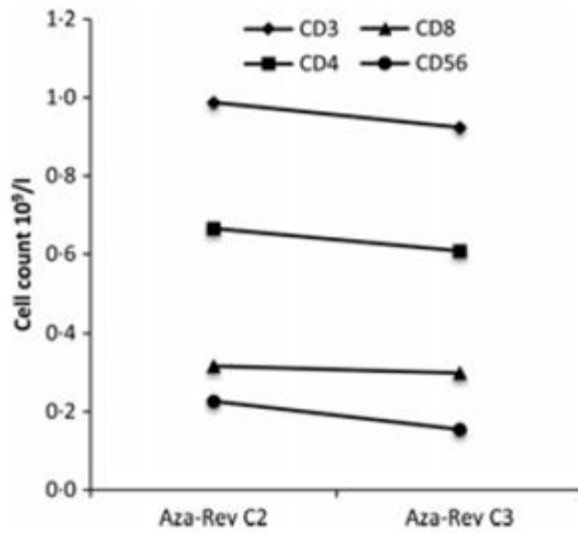
UPN	CD3 ⁺ cell yield		PBSC (CD34 ⁺)	PBSC (CD3 ⁺)	Time SCT to ALI (days)	Follow-up post -SCT (month)	Last disease status
	Apheresis 1	Apheresis 2					
12430-01*	0.58	0.26	8.65	3.08	39	19	VGPR
12430-02	1.03	0.99	8.72	5.5	52	17	CR
12430-03	0.95	0.82	10.71	6.22	46	15	VGPR
12430-04	0.51	0.48	10.28	5.26	49	12	CR
12430-05*	0.76	0.82	8.64	3.07	31	7	CR
12430-06*	0.94	1.01	14.57	4.57	32	7	REL
12430-07	1.57	1.44	16.9	6.48	49	6	SD
12430-08*	0.31	0.63	7.34	6.54	39	5	VGPR
12430-09	0.77	0.86	8.73	3.82	32	5	CR
12430-10	1.55	1.06	8.93	12.79	45	5	VGPR
12430-11	0.71	1.03	11.29	8.31	51	2	VGPR
12430-12	0.75	0.59	1.1	–	–	–	SD
12430-13†	1.46	0.84	6.44	9.07	–	<1	–
12430-14†	0.63	0.65	8.9	2.84	–	<1	–

SCT, autologous stem cell transplantation; PBSC, Peripheral blood stem cells; Cell dose reported: CD3⁺ cells- $\times 10^8$ cells/kg; CD34⁺ cells- $\times 10^6$ cells/kg; ALI, autologous lymphocyte infusion; SD, stable disease; CR, complete remission; VGPR, Very good partial remission; REL, Relapse. (–) Not evaluable.

*Tandem transplantation prior to ALI.

†ALI not infused to date.

A



B

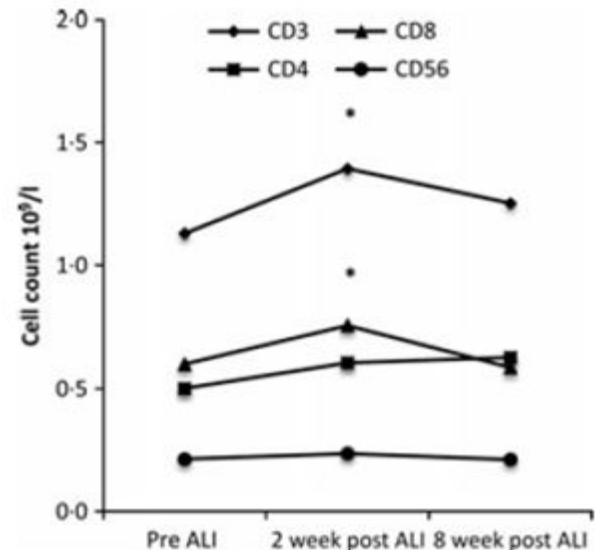


Figure 10. Aza-Rev does not modulate numbers of circulating lymphocytes, but ALI results in a transient increase CD3+ and CD8+ cells. A) Blood T cell subset and NK cell counts (mean) following investigational therapy. Counts prior to lymphaphereses 1 and 2, following cycles 2 (C2) and 3 (C3) of Aza-Rev. B) Blood T cell subset and NK cell counts (mean) following SCT. Counts pre-ALI, 2 and 8 weeks following ALI. * Signifies statically significant increase over earlier time point.

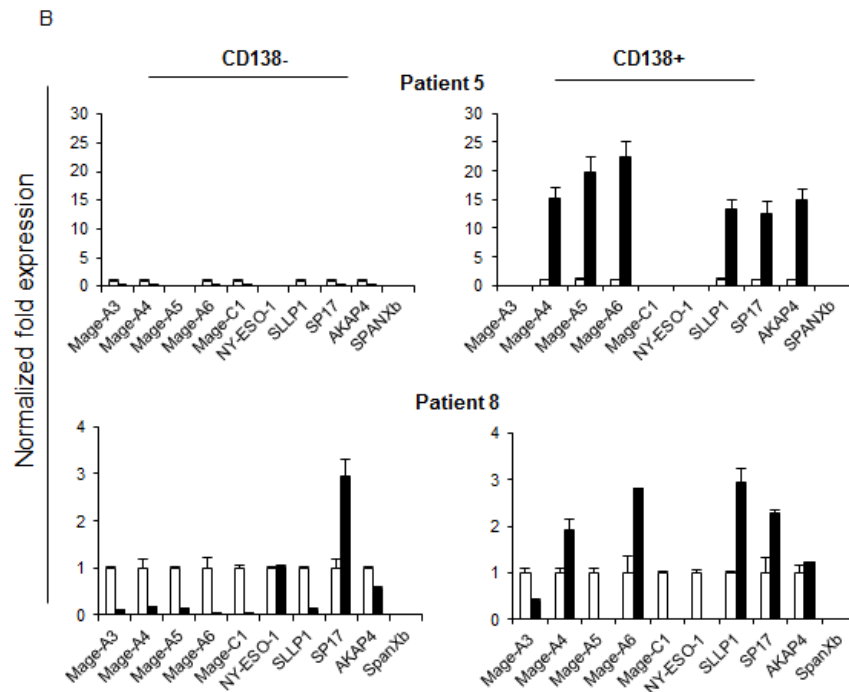
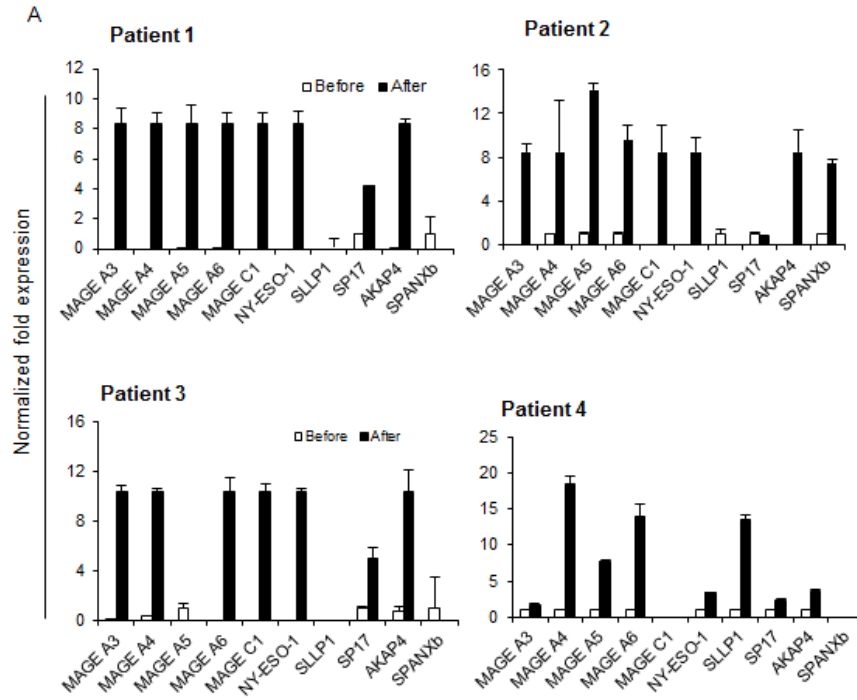


Figure 11. Induction of a panel of CTA expression in bone marrow of patients with MM following a 3-cycle administration of Aza-Rev. A) Fold expression of CTA prior to (before) and following 3 cycles of Aza-Rev in unfractionated bone marrow cells. B) Fold expression of CTA prior to and following 3 cycles of Aza-Rev in fractionated CD138- and CD138+ bone marrow cells of two patients. Data were normalized to human GAPDH. Error bars represent mean \pm SEM of duplicate wells.

CTA-specific T cell response following Aza-Rev therapy

In order to determine whether induction of CTA expression in the bone marrow cells was immunogenic by inducing CTA-specific T cell responses, peripheral blood mononuclear cells (PBMCs) of patients were used as source of T cells as well as monocyte-derived DCs. T cells were then cultured with autologous monocyte-derived DCs in the presence or absence of recombinant NY-ESO-1, and an antigen-specific IFN- γ release was determined using IFN- γ ELISA. As shown in Figure 12, for Patients 1 and 4, NY-ESO-1-reactive T cells appeared after the administration of the 1st cycle Aza-Rev and peaked following the 3rd cycle; Patient 2 had a preexisting NY-ESO-1 specific response which persisted after the 3rd cycle of Aza-Rev. However, the preexisting NY-ESO-1 specific response observed in Patient 3 markedly diminished following Aza-Rev therapy. As neither Patient 2 nor Patient 3 demonstrated expression of NY-ESO-1 before Aza-Rev therapy (Figure 11A), these data suggest aberrant expression of NY-ESO-1 in the tumor in the past, which established an adaptive memory response, and was then lost. Similarly, Patient 5 demonstrated an early response to NY-ESO-1, which declined following the 3rd cycle of Aza-Rev and at 8 weeks post-ALI before increasing at 11 months post-ALI. Interestingly, Patient 4, who displayed a low level of NY-ESO-1 induction in bone marrow (Figure 11A) also showed a low level of NY-ESO-1-reactive T cell responses, suggesting that the magnitude of CTA expression is directly correlated with the robustness of the T cell response. The release of IFN- γ 11months following ALI, was minimal (Patient 2) or was undetectable (Patient 4) in two patients who were in CR 1 year after transplantation. Significantly, these patients recorded further improvement in their response from very good partial response (VGPR; 90% or greater reduction in serum myeloma protein) to CR at 8 and 10 months following ALI. Patients 1 and 5, however, had a persistent weak NY-ESO-1-reactive

T cell response 1-year post transplant, in the presence of stable MRD. This response became undetectable by 15 months post-transplantation (data not shown) and both patients entered CR.

Loss of CTA expression following immunotherapy was associated with disease relapse

To identify potential escape mechanisms from ALI, CTA-expressing bone marrow cells were monitored pre- and post- Rev/Aza in one patient (Patient 14) whose tumor relapsed at 12-weeks post ALI. After the 3rd cycle of Aza-Rev, CTA-induction was observed specifically in CD138+ plasma cells (Figure 13A). CTA-expression within CD138+ plasma cells was completely lost at the time of disease progression (Figure 13B), suggesting transient expression of these antigens may facilitate escape from immune-mediated elimination and result in disease progression. However, we were not able to monitor the persistence of CTA expression in the majority of the patients because they obtained favorable outcomes to date, 8/14 currently in CR or VGPR with a mean follow up of 30 months (Table 7).

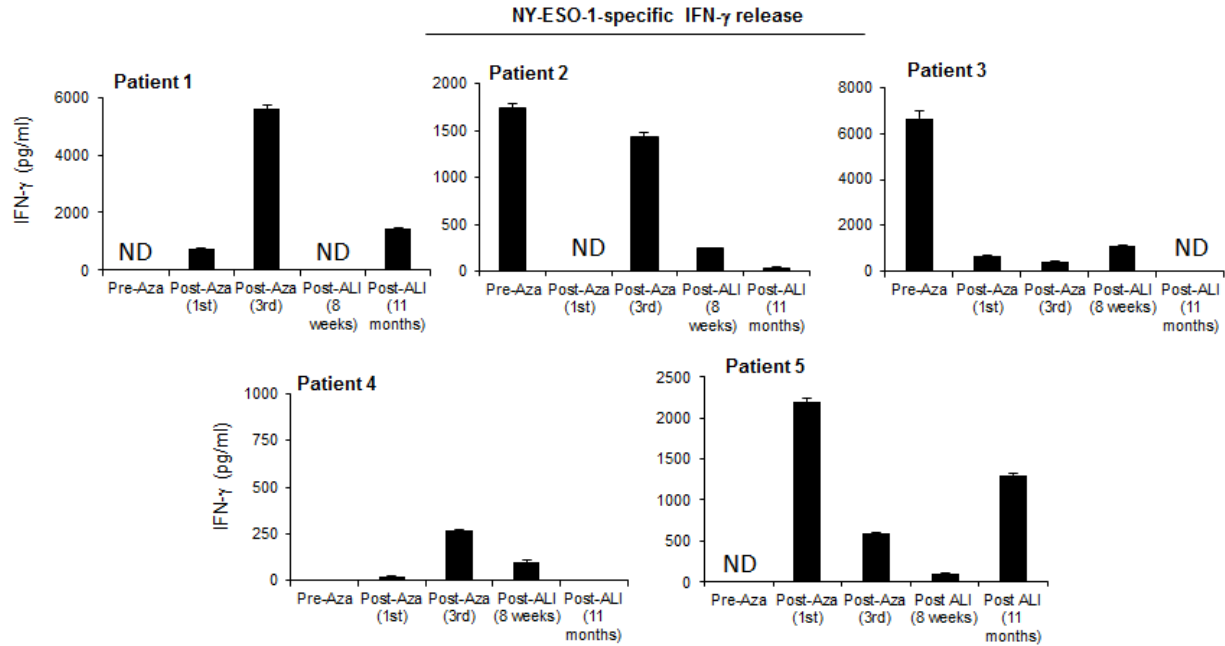


Figure 12. Induction of NY-ESO-1-reactive T cell responses in patients with MM. Lymphocytes were prepared from blood samples prior to, after 1st cycle or 3rd cycles Aza- Rev administration as well as 8 weeks or 11 months after autologous lymphocyte infusion (ALI). Lymphocytes were co-cultured with autologous dendritic cells (DCs) in 2:1 ratios in the presence or absence of recombinant NY-ESO-1 for 20 h. Interferon-gamma (IFN- γ) production was detected in the supernatant. Data are presented after subtracting background IFN- γ production by T cells plus DCs in the absence of the antigen. ND: not done. Error bars represent mean \pm SEM of duplicate wells.

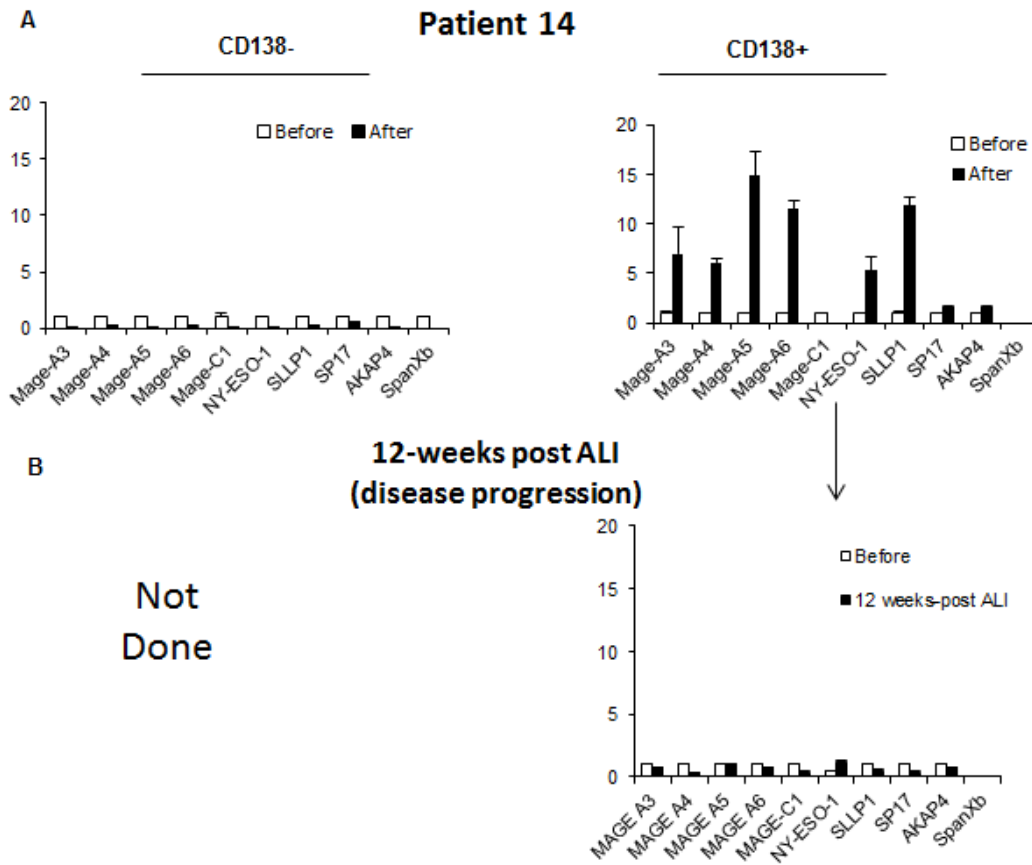


Figure 13. Induced expression of CTAs in bone marrow of patients is transient in one patient experiencing disease progression. A) Fold expression of CTA prior to (before) and following 3 cycles of Aza-Rev (after) in CD138- (left panel) and CD138+ (right panel) bone marrow cells. B) Fold expression of CTA prior to and 12 week after ALI in fractionated CD138- and CD138+ bone marrow cells (right panel) of one patient with disease progression. Data were normalized to human GAPDH. Error bars represent mean \pm SEM of duplicate wells.

Table 7

Patient #	follow up months	current disease status post transplant	Relapse/ Progressive Disease
1	36.3	CR	Yes
2	32.3	CR	
3		CR	
4	27.4	CR	
5	23.0	CR	
6	16.3	deceased	Yes
7	21.9	PD	Yes
9	20.5	CR	
10	22.0	VGPR	
11		progression	Yes
14	17.1	VGPR	Yes

DISCUSSION

This clinical trial demonstrated the *in vivo* epigenetic induction of highly immunogenic CTAs in patients with MM. Induction of CTAs was associated with a subsequent cell-mediated immune response; such CTA-sensitized lymphocytes were cryopreserved and used later as ALI. This was accomplished with the administration of a well-tolerated regimen of chemo-immunotherapy. In order to adequately control disease progression, this immunotherapy was designed in a stem cell transplant scheme, using ALI to target MRD following ASCT, rather than in a setting of large tumor-burden.

Presently, the only known curative therapy for patients with MM is allo-SCT. The graft-versus-myeloma effect observed following allografting has been linked to the emergence of tumor antigen-specific cellular and humoral immune response, particularly following donor lymphocyte infusion (180, 181). Similarly, antibodies against HY antigens in male recipients of female donor stem cells have been correlated with relapse-free responses following allo-SCT (182). A guiding principle in understanding such graft-versus-tumor and graft-versus-host responses is the allo-reactivity of donor T cells to ‘non-self’ minor histocompatibility antigens, oligopeptides that differ between human leucocyte antigen (HLA)-matched donors and recipients (183). Unfortunately, the recognition of such non-self-antigens also triggers graft-versus-host disease, which erodes the benefit observed in terms of relapse protection following allografting. Therefore, if the paradigm of immune recognition of ‘non-self’ can be extended to the autologous setting combined with an epigenetic modulator for the induction of CTA expression in tumor cells, one may then observe the graft-versus-tumor benefit without the risk of graft-versus-host disease.

Targeting CTAs hold promise in promoting a graft-versus-tumor-like response in an ASCT setting. In patients with malignant melanoma, NY-ESO-1 reactive T cells have been isolated and expanded *ex vivo* and re-infused into autologous recipients with dramatic responses recorded (112). Recently, *in vitro* evidence of CTA overexpression by epigenetic modification and an adaptive T cell response has been demonstrated against MAGEA4 in patients with Hodgkin lymphoma treated with Dec (184). Similar findings have been reported with acute myeloid leukemia and myelodysplasia, where therapy with Aza and valproic acid, has led to the emergence of cytotoxic T cells reactive to MAGEA1, MAGEA2, MAGEC2 and RAGEA1 peptides over the course of treatment (136). Our finding of CTA up-regulation in bone marrow and plasma cells from myeloma patients with residual disease treated with Aza-Rev *in vivo* corroborates these findings. Evaluation of the expression of a limited panel of CTA showed over-expression of multiple CTA in each patient tested following therapy with Aza. Interestingly, two patients (2&3) did not show expression of NY-ESO-1 before Aza-Rev therapy (Figure 11) but their lymphocytes demonstrated substantial release of IFN- γ in the presence of autologous DCs pulsed with recombinant NY-ESO-1 (Figure 12). This suggests that a memory response had been previously established against this CTA at a time when it was expressed, the expression of which was later lost. When we tested the efficacy of Aza-Rev to upregulate CTA expression in fractionated marrow cell populations, CTA induction in these patients appeared to be limited to CD138⁺ plasma cells, as opposed to normal hematopoietic cells. This may be due to abnormal regulation or activity of epigenetic modifiers, such as DNA methyltransferase, in cancer cells. Recently, altered histone methylation with a more open chromatin structure has been demonstrated in patients with chromosomal translocation (4:14) in myeloma, related to aberrant multiple myeloma SET domain (MMSET) activity (185). This suggests that malignant

myeloma cells may be more sensitive to epigenetic modulation in comparison with normal hematopoietic cells.

CTA reactivity has been invoked as a possible mechanism for graft-versus-leukemia responses in allo-SCT recipients (186). We tested NY-ESO-1 reactivity by incubating peripheral blood lymphocytes with recombinant NY-ESO-1 pulsed autologous DCs. NY-ESO-1-reactive T cells were observed in 5 patients tested, with reactivity correlating with level of NY-ESO-1 expression observed post Aza-Rev therapy, and being maintained for 2–11 months following SCT. The post-transplant maintenance of NY-ESO-1-reactivity was prolonged in one patient (Patient 1) with high levels of NY-ESO-1 expression and MRD before eventually improving to a CR. Additionally five other patients (Patients 2, 3, 4, 5 & 9) improved their response from VGPR to CR in the months following ALI. This suggests ongoing anti-tumor activity in the patient with prolonged MRD and possible maintenance of NY-ESO-1 (and other CTA) expression in malignant plasma cells. In the future, phenotypic characterization of patient's T cells at various time-points would also aid in determining if CTA-specific responses derived from effector or memory T cells, which would indirectly indicate the status of the patient's disease. Although we only tested the reactivity of autologous lymphocytes against NY-ESO-1, the CTA-specific T cell response elicited may be polyclonal; targeting several different CTA simultaneously providing enhanced protective capacity. Likewise, antigen loss as a mechanism of tumor escape will also need to be monitored by evaluating post-transplant persistence of CTA expression on plasma cells in bone marrow to determine the durability of this epigenetic modification. We observed loss of CTA expression which was associated with disease progression in one patient.

Thus, due to our observation in Chapter One that immunotherapy is ineffective in reducing tumor burden of bulky primary CTA-expressing cancer, the data in this chapter suggest that

targeting CTA-expressing tumor cells in a setting of MRD may be the best time to utilize cellular immunotherapy for other cancers, including breast cancer.

Chapter Three

INTRODUCTION

MDSCs are key cellular suppressors of anti-tumor immune responses in breast cancer patients. Tumor-derived factors drive the accumulation of MDSCs in the bone marrow, secondary lymphoid organs and at the site of the tumor, thereby inhibiting the efficacy of cellular immunotherapy against established tumors. A number of strategies have been used to enhance immunotherapy of cancer by overcoming MDSCs. These strategies fall into three major categories which include MDSC deactivation, depletion of MDSCs, or conversion of MDSCs to APCs. The latter approach identified NKT cells as a key facilitator in promoting MDSC maturation into mature myeloid cells with anti-tumor immune stimulatory function. Previous work has demonstrated that a function of invariant NKT cells is to promote the maturation of cells of myeloid lineage, particularly DCs, as discussed above. Likewise, it was reported very recently in an animal model of breast cancer metastasis that activated NKT cells decrease the frequency and immunosuppressive activity of MDSCs in tumor-resected mice (187). Additional studies have demonstrated that activated NKT cells convert MDSCs into immune-stimulatory APCs (52, 53). Using peripheral blood mononuclear cells (PBMC) of patients with early stage breast cancer, we also demonstrated that an optimal frequency of CD25+ NKT cells within unfractionated reprogrammed immune cells, cultured in the presence of MDSCs, induced the MDSCs to lose/downregulate CD11b which was associated with HLA-DR upregulation. Such phenotypic modulation was shown to promote anti-Her-2/neu immune responses, *in vitro* (54). Therefore, we suggest that inclusion of CD25+ NKT cells in adoptive cellular therapy will enhance the anti-tumor efficacy of adoptively transferred T cells by modulating MDSCs to become immunostimulatory.

Another barrier to a successful cancer immunotherapy is tumor immunoediting and escape from immunotherapy, which likely occurs in the event of robust anti-tumor immune responses. Despite the remarkable recent advances in cancer immunotherapy in prolonging patient survival, the ability of immunotherapy to treat common carcinomas, which account for majority of all cancer deaths, remains limited. This raises the question: is reduction of tumor burden and prolonging patient survival weeks to months an acceptable goal for 21st century cancer therapeutics, or should we further seek to understand the dynamic interplay between cancer cell and immune cell in order to offer a cure for cancer patients? The status of the tumor cells themselves when immunotherapy is employed likely determines the effectiveness of the therapy. The application of immunotherapy to highly proliferative tumors renders the tumors prone to immunoediting and subsequent immunological escape during cell division (188). An important point to consider is that human vaccines against infectious diseases are not effective in a setting of established disease. The rabies vaccine is an exception; however, it is ineffective as a single agent or at the onset of clinical illness. A successful history of human vaccines against infectious diseases suggests that cancer immunotherapy can be effective in a prophylactic setting either prior to exposure to infectious agents including pathogen-associated cancers, during the incubation period, or during dormancy after the exposure. For instance, the rabies vaccine can be used as post-exposure prophylaxis because the incubation period or dormancy for rabies is 1-3 months which provides a window for vaccination. However, it should be combined with anti-rabies immunoglobulin injections into the wound in order to control the infection and allow the vaccine to work. Prophylactic cancer vaccines have also been successful. The FDA has approved two vaccines, Gardasil® and Cervarix®, that protect against HPV infection which is the leading cause of cervical cancer worldwide (189). HPV infection is also responsible for some vaginal,

vulvar, anal, penile, and oropharyngeal cancers (190). The FDA has also approved a prophylactic cancer vaccine against HBV infection, which is a cause of liver cancer. Today, most children in the United States are vaccinated against HBV shortly after birth (191).

In general, conventional cancer therapies including chemotherapy and radiation therapy (RT), while inducing cell death in the majority of tumor cells, also promote tumor dormancy (192). Although these treatment-induced dormant cells become resistant to higher doses of nominal therapies, they may remain sensitive to immunotherapy during the dormant stage due to their inability to undergo immunoediting. Additionally, it was reported that the anti-tumor efficacy of many chemotherapeutic drugs is due to the induction of immunogenic cell death (ICD). ICD in turn induces anti-tumor immune responses, and may subject dormant tumor cells to persistent immune surveillance, resulting in the prevention of tumor recurrence (193-196).

In the previous chapter, we demonstrated that the combined use of ACT and a demethylating drug enhanced immunogenicity of tumor cells by inducing CTA expression, and resulted in objective responses when administered in a setting of MRD. In this chapter, we sought to evaluate the efficacy of ACT using reprogrammed T cells and NKT cells against experimental metastatic mammary carcinoma. First, we tested combined use of ACT and Dec against experimental metastatic breast cancer in FVBN202 mice, as well as the impact of Dec in tumor immunoediting and escape. Then, we performed mechanistic studies by using ACT alone without Dec in order to determine anti-tumor efficacy of ACT as well as its role in modulating MDSCs to become immune stimulatory APCs, *in vivo*. Finally, we tested our hypothesis that targeting dormant, but not highly proliferating, mammary tumor cells might overcome tumor immunoediting and escape. To this end, we conducted studies *in vitro* in order to determine sensitivity of different types of dormant tumor cells, indolent dormancy versus quiescent

dormancy, to immunoediting and escape. We demonstrate that quiescent, but not indolent, dormant tumor cells are resistant to immunoediting; thus, they could be the best target for immunotherapy.

MATERIALS AND METHODS

Mouse model

FVBN202 transgenic female mice (The Jackson Laboratory; Bar Harbor, ME) were used between 8 and 12 weeks of age throughout these experiments. These mice overexpress non-mutated, non-activated rat neu transgene under the regulation of the mouse mammary tumor virus promoter (130). These mice develop premalignant mammary hyperplasia similar to ductal carcinoma *in situ* prior to the development of spontaneous carcinoma (44). Premalignant events in FVBN202 mice include the accumulation of endogenous MDSCs (44). These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Tumor cell lines

The neu overexpressing mouse mammary carcinoma (MMC) cell line was established from a spontaneous mammary tumor harvested from FVBN202 mice. Tumor cells were maintained in RPMI 1640 supplemented with 10% FBS.

Ex vivo reprogramming and expansion of splenocytes

FVBN202 transgenic mice were inoculated in the mammary fat pad with 3×10^6 MMC cells. Tumor growth was monitored by digital caliper, and tumor volumes were calculated by volume (v) = $(L$ [length] \times W [width] 2)/2. As described previously (16, 54), and above, splenocytes were harvested 21–25 days after tumor challenge, when the tumor had reached $\geq 1000\text{mm}^3$.

Adoptive cellular therapy

Twenty-four hours prior to ACT, FVBN202 mice were injected i.p. with CYP (100 mg/kg) to induce lymphopenia. Individual groups of mice were challenged intravenously (i.v) with serial dilutions of MMC cells (3.5×10^5 , 1×10^5 , or 3.5×10^4). Mice then received reprogrammed splenocytes i.v. at a dose of 70×10^6 /mouse later the same day (+ACT), or remained untreated (No ACT). The study end-point and euthanasia occurred when the animals were considered moribund upon losing 10-20% of their initial body weight due to disease progression.

Characterization of splenocytes and tumor-infiltrating leukocytes

Spleens and metastatic tumor lesions of FVBN202 mice were harvested when the animals became moribund, and were then homogenized into a single cell suspension as described previously (16) and below; splenocytes were then characterized using flow cytometry. Reagents used for flow cytometry: anti-CD16/32 Ab (93), FITC-CD3 (17A2); FITC-CD11b (M1/70); FITC-anti mouse IgG (Poly4053); PE-GR-1 (RB6-8C5); PE-CD11c (N418); PE-F4/80 (BM8); PE-PD-1 (RMP1-30); PE-CD25 (3C7); PE-Ki-67 (16A8); Allophycocyanin-CD49b (DX5); Allophycocyanin-CD62L (MEL-14); Allophycocyanin-Annexin V; PercP/CY5.5-CD4 (GK1.5); Alexa Fluor 647-I-Aq (KH116); PercP/CY5.5-CD86 (GL-1); PercP/CY5.5-Rat IgG2a, k Isotype Control (RTK2758); PE-Dazzle-CD80 (16-10A1); PE-Dazzle-Armenian Hamster IgG Isotype Control (HTK888); PE/CY7-CD8 α (53-6.7); PE/CY7-CD40 (3/23); PE/CY7-Rat IgG2a, k Isotype Control (RTK2758); Brilliant Violet 421-PD-L1 (10F.9G2); Brilliant Violet 605-CD45 (30-F11); propidium iodide (PI), all of which were purchased from Biolegend (San Diego, CA). BD Horizon V450-Annexin V and FITC-Fixable Viability Stain (FVS); were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-rat neu antibody (anti-c-Erb2/c-Neu; 7.16.4), was

purchased from Calbiochem. All reagents were used at the manufacturer's recommended concentration. Cellular staining was performed as previously described by our group (16, 54), or as recommended by the manufacturer (Ki-67, FVS). Multicolor data acquisition was performed using a LSRFortessa X-20 (BD Biosciences). Data were analyzed using FCS Express v4.07 (De Novo Software; Glendale, CA).

Sorting of myeloid cells by FACS

Splenocytes were stained for surface expression of CD11b and Gr-1 as described above. Isolated cells were gated on the myeloid cell population based on their light scattering properties, thereby excluding cells of lymphoid origin. CD11b⁺ Gr1⁺ MDSCs and CD11b-/lo Gr1⁻ myeloid cells from the 'No ACT' and '+ACT' groups were then sorted into independent populations using a FACSAria (BD Biosciences) as previously described (54). Purity of sorted cells was consistently greater than 90%.

IFN- γ ELISA

Splenocytes from the 'No ACT' and '+ACT' groups were independently cultured in serum-free RPMI 1640 in order to enrich for non-adherent cells of lymphoid origin from adherent cells of myeloid origin. After 2 hours, non-adherent splenocytes were cultured in complete medium with irradiated MMC cells (140Gy) at a 10:1 ratio, and with or without sorted MDSCs or CD11b-/lo Gr1⁻ myeloid cells at a 2:1 ratio, for 20 hours. Supernatants were then collected and stored at -80°C until assayed. IFN- γ was detected in the supernatant using a Mouse IFN- γ ELISA kit (BD Biosciences), according to the manufacturer's protocol.

Isolation and characterization of lung metastases

Lungs were harvested from the 'No ACT' and '+ACT' groups after animals became moribund. Metastatic lesions were individually excised from the residual lung tissue, and were then digested in Trypsin-EDTA (0.25%; Life Technologies) overnight at 4°C. The following day, the suspension was incubated at 37°C for 30 minutes, followed by tissue homogenization to create a cellular suspension. The cell suspension was then washed twice with RPMI supplemented with 10% FBS. Residual red blood cells were then lysed using ACK lysing buffer, followed by an additional wash with RPMI 10% FBS. The cell suspension was then placed in cell culture and cultured with RPMI 10% FBS. Adherent metastatic tumor cells were then allowed to establish and proliferate for 10-14 days; they were then characterized for the expression of rat neu and PD-L1 using flow cytometry.

Characterization of metastatic tumor-infiltrating leukocytes

Lungs from each group were harvested and metastatic lesions were isolated as described above. After tissue digestion of the metastatic lesions and red blood cell lysis, 10^6 cells of the suspension were placed in flow tubes and stained for surface molecules as described above. All analysis was performed by gating on viable leukocytes (Annexin V⁻ CD45⁺), thereby discriminating out apoptotic cells and tumor cells.

Establishment of *in vitro* tumor cell dormancy

MMC cells were treated with three daily doses of Adriamycin (Doxorubicin Hydrochloride; Sigma-Aldrich; St. Louis, MO) (1µM/day for 2 hours). Residual, dormant MMC cells remained

adherent to tissue culture flasks, while the MMC cells susceptible to ADR-therapy became non-adherent and were removed from the culture periodically. Assessment of viability, Ki-67 expression and IFN- γ -induced PD-L1 upregulation by flow cytometry occurred 3 weeks after the final treatment. Similarly, three daily doses of RT (2Gy/day) were also used to establish dormant MMC cells. ADR and RT-induced dormant MMC cells were used in the cytotoxicity assay 8 days after the final treatment.

Cytotoxicity assay

Reprogrammed splenocytes were cultured with MMC cells or *in vitro* established dormant MMC cells at a 10:1 E:T ratio in complete medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 10% FBS, 10 mM L-glutamine) with 20 U/ml IL-2 (PeproTech). To determine resistance of dormant tumor cells to conventional therapies, ADR-induced dormant MMC cells were treated with a high dose of ADR (1 μ M for 24hrs), and RT-induced dormant MMC cells were treated with a high dose of RT (18Gy). After 48 hours, MMC cells were harvested and stained for rat neu (anti-c-Erb2/c-Neu; Calbiochem), Annexin V, and PI as previously described (16). Flow cytometry was used to analyze the viability of neu⁺ MMC cells.

Statistical analysis

Outcomes are summarized by basic descriptive statistics such as mean and standard error of the mean (SEM); differences between groups are illustrated using graphical data presented as mean \pm SEM. Statistical comparisons between groups were made using one-tailed and two-tailed

Student t test per the specific hypothesis. Time to death in the *in vivo* survival studies was calculated from baseline to the date of death (due to weight loss $\geq 10\%$). Kaplan-Meier curves and log-rank tests are used to illustrate time to death and to test the difference between each group. A p -value ≤ 0.05 was considered statistically significant.

RESULTS

Immunotherapy of metastatic tumor can prolong animal survival but fails to cure cancer

We have previously reported that ACT utilizing reprogrammed T cells/NKT cells in a prophylactic setting protected animals against primary tumors and recall tumor challenge. This protection was associated with the presence of memory T cells, and CD25+ NKT cells that rendered T cells resistant to the suppression by MDSC (197). Similar observations were made using PBMCs from patients with early stage breast cancer such that the presence of CD25+ NKT cells resulted in the modulation of CD33+CD11b+HLA-DR- MDSCs toward CD33+CD11b-/loHLADR+ myeloid cells which in turn overcome the suppressive function of MDSCs (198). Here, we sought to determine if ACT utilizing reprogrammed immune cells can protect animals against experimental metastasis. Due to the promising results obtained from the combined use of ALI and Aza-Rev in patients with MM, we sought to determine whether the combined use of ACT and Dec could eliminate experimental metastatic MMC. First, we demonstrated that ACT + Dec resulted in prolonging the survival of animals compared to the control groups, No-ACT or Dec alone (Figure 14; $p=0.0001$ and $p=0.037$, respectively). The evaluation of metastatic tumor cells showed that Dec alone facilitated downregulation of the neu antigen on metastatic MMC in the lung (Figure 15A, MFI: 442 vs. 202) as well as total loss of neu antigen in 36% of tumor cells compared with control MMC cell line containing a residual 5% of neu negative cells (Figure 15B). Since Dec induces the expression of CTAs and, therefore, functions as an *in situ* vaccination by eliciting endogenous T cell responses, neu loss or downregulation in animals who received Dec could be due to contribution of endogenous T cell response and Dec. To determine the contribution of Dec in neu antigen loss or downregulation, we performed *in vitro* studies by treatment of MMC with Dec alone where the endogenous immune response did not have any

contribution. We show that Dec treatment resulted in the downregulation of neu expression, but did not induce total neu loss (Figure 16, $p=0.008$). In addition, induction of CTAs in human tumor cell line by treatment with Dec was found to be transient. The expression of CTAs induced three days after treatment began was found to be severely diminished after the removal of Dec from the culture medium and then continuing the culture until day seven (Figure 17).

Therefore, due to the induction of tumor escape mechanisms in highly proliferating MMC by Dec, such as neu loss/downregulation and inability to stably express CTAs, we moved forward to test the efficacy of ACT against experimental metastases without the inclusion of Dec. Splenocytes of animals harboring primary mammary tumor or metastatic MMC in the lung can be reprogrammed, *ex vivo*, to generate memory T cells and CD25+ NKT cells (Figure 18). Recipients of ACT were conditioned by the injection of cyclophosphamide (CYP), and challenged i.v. with MMC cells. Animals served either as control (No ACT), or received ACT when proliferating MMC cells were present in the circulation (+ACT). As shown in Figure 19, ACT prolonged survival of animals regardless of whether the source of donor splenocytes was from primary tumor-bearing mice or from metastatic tumor-bearing mice. Therefore, we determined that using donor cells from primary tumor-bearing mice had the potential for more clinical application, in that lymphocytes from early stage breast cancer patients could be isolated and cryopreserved before the administration of conventional therapies. Then, in a setting in which the tumor sufficiently responds to initial therapy, cryopreserved lymphocytes could be thawed, reprogrammed and infused as ACT in order to target residual tumor cells and prevent relapse or prolong time-to-relapse. We have previously shown that cryopreserved PBMCs can be successfully reprogrammed (54).

We then challenged mice with a serial dilution of MMC cells to induce experimental metastasis in order to determine if tumor burden may impact the efficacy of ACT. We demonstrated that ACT prolonged survival of animals regardless of the dose of tumor challenge, but all mice succumbed to metastatic tumor in the lung (Figure 20A-C).

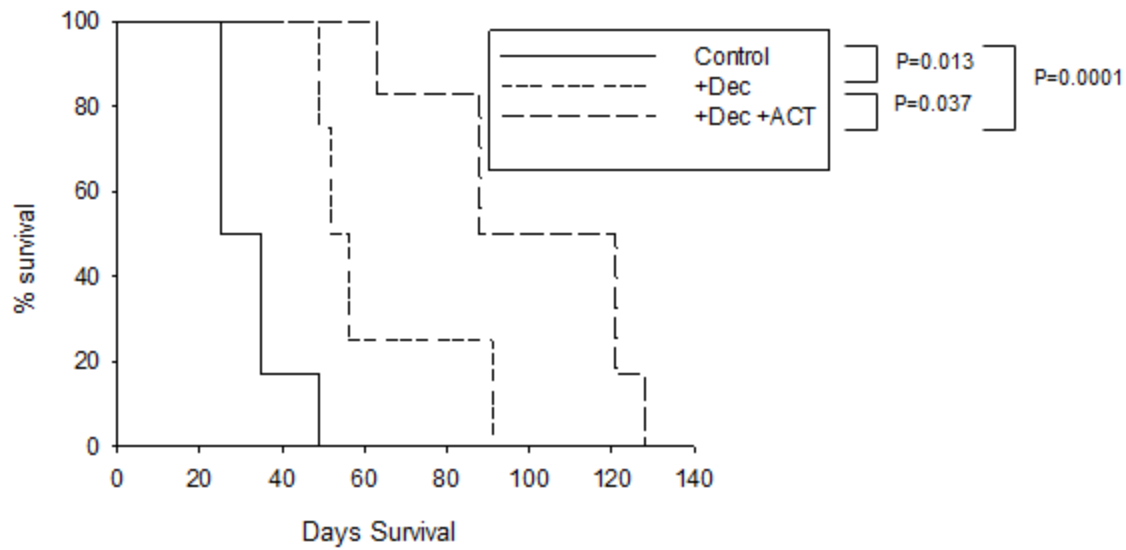


Figure 14. Combined ACT and Decitabine therapy prolongs survival in mice with circulating tumor cells. FVBN202 mice were challenged with 1×10^6 MMC cells i.v. Mice then either remained untreated (Control; n=4), received Decitabine (+Dec; n=4; 5 daily doses beginning on 3 days after tumor challenge), or received Decitabine and ACT (+Dec +ACT; n=6).

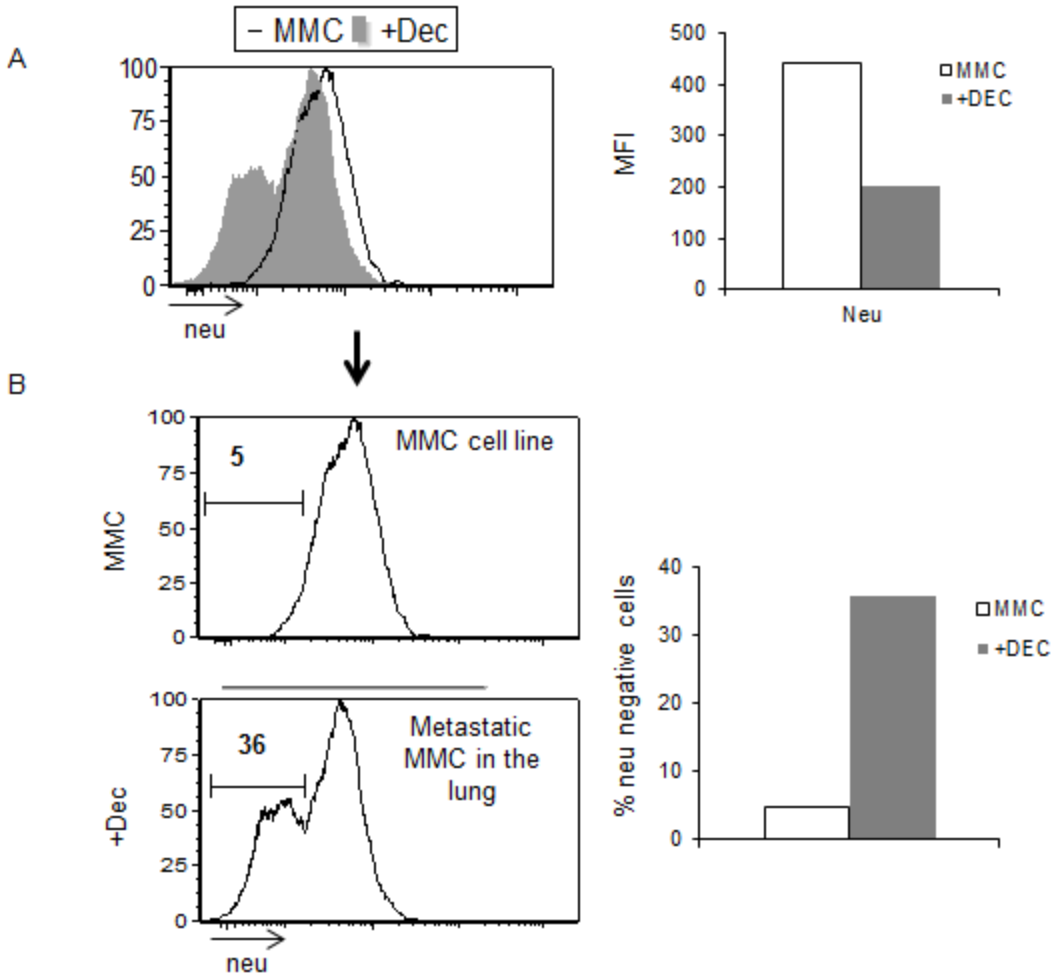


Figure 15. Decitabine induces downregulation and loss of neu expression on MMC cells, *in vivo*. FVBN202 were challenged i.v. with MMC cells (1×10^6); 3 days later they were injected with Decitabine (2.5mg/kg) once daily for 5 days (n=1) or remained untreated (MMC) (n=1). After the mice became moribund, metastases were excised from the lung and established *in vitro*. Neu median fluorescence intensity (MFI) (A) and percentage of neu negative cells (B) was quantified using flow cytometry 10-14 days after the animals had been euthanized.

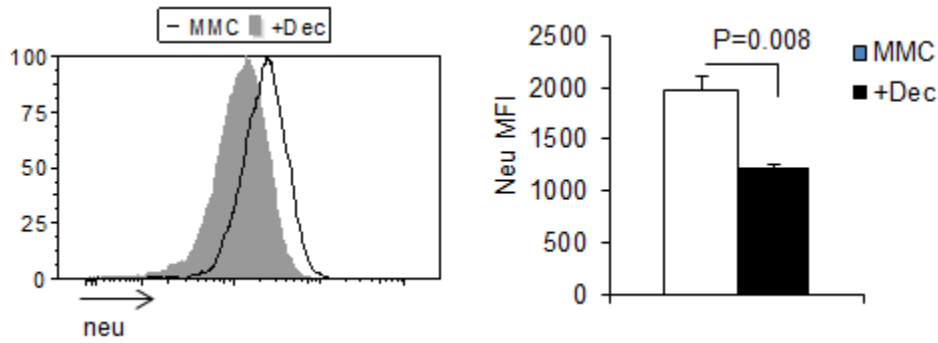


Figure 16. Decitabine downregulates neu expression on MMC cells, *in vitro*. MMC cells were treated with Decitabine (+Dec; 3uM) (n=3) or remained untreated (MMC) (n=3), *in vitro*. After 10 days of culture, neu expression was quantified using flow cytometry. Data represent mean MFI ± SEM.

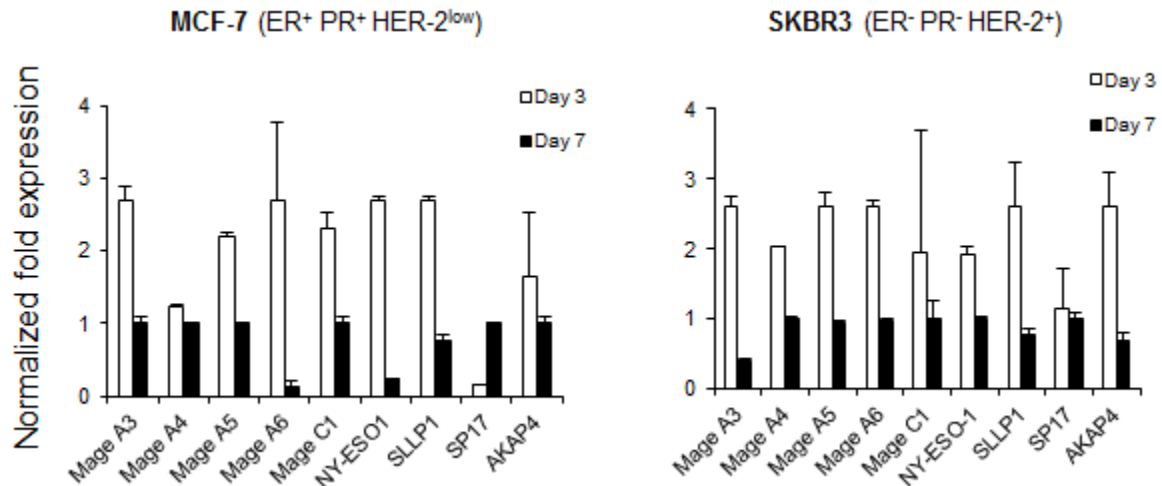


Figure 17. Induction of CTA expression is transient in human breast cancer cells . MCF-7 and SKBR3 cells were cultured with Decitabine (3 μ M) for 3days. RNA was then extracted from a portion of the cells (Day 3); Decitabine was removed from the remainder of the cells, which were then returned to culture for an additional four days (Day 7) before RNA was isolated. RNA was then converted to cDNA followed by qRT-PCR using primers specific for nine human CTAs. Expression was normalized to GAPDH. Data represent mean \pm SEM of duplicate wells.

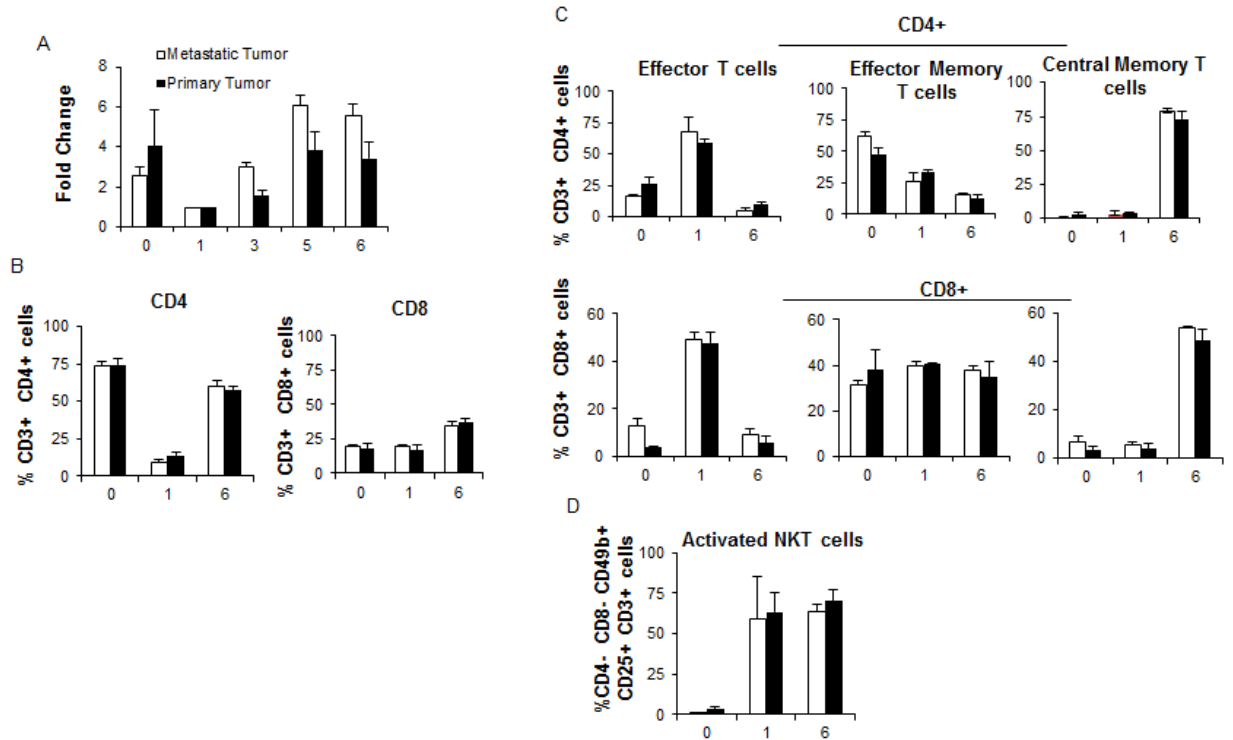


Figure 18. Expansion and phenotypic reprogramming of tumor-reactive splenocytes is similar between animals bearing primary and metastatic cancer. FVBN202 mice were challenged with 1×10^6 MMC cells i.v. or 3×10^6 MMC cells intradermally, spleens were harvested when mice became moribund or when tumor volume was $\geq 1000\text{mm}^3$, respectively, and were then treated with B/I and γ -c cytokines *ex vivo*. A) Cell counts of viable splenocytes were determined by trypan blue exclusion; fold change was calculated by normalizing the cell count of each day to the number of cells present on day 1. Flow cytometry was used to determine the frequency of total CD4+ and CD8+ T cells (B), phenotype of CD4+ and CD8+ T cells (C), and the frequency of CD25+ NKT cells (D). Data represent four biological repeats for each group and mean \pm SEM.

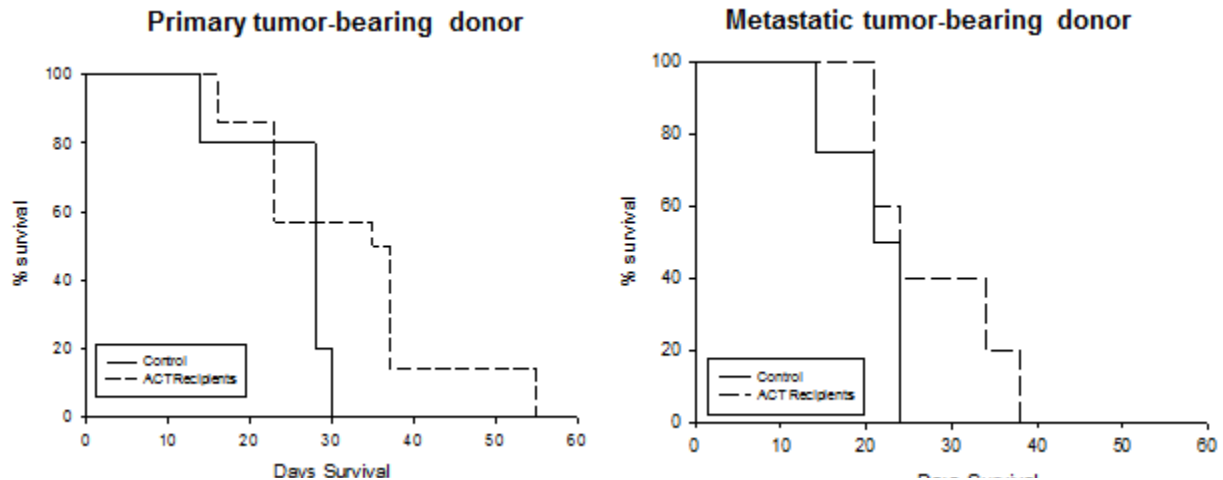


Figure 19. Reprogrammed immune cells derived from animals bearing primary cancer or experimental metastasis and used for ACT demonstrate similar functional efficacy, in vivo. FVBN202 mice were challenged with 1×10^6 MMC cells i.v. Mice then either remained untreated, or received ACT utilizing reprogrammed immune cells derived from primary tumor-bearing donors (left panel; Control n=4, +ACT n=7) or from metastatic tumor-bearing donors (right panel; Control n=3, +ACT n=5).

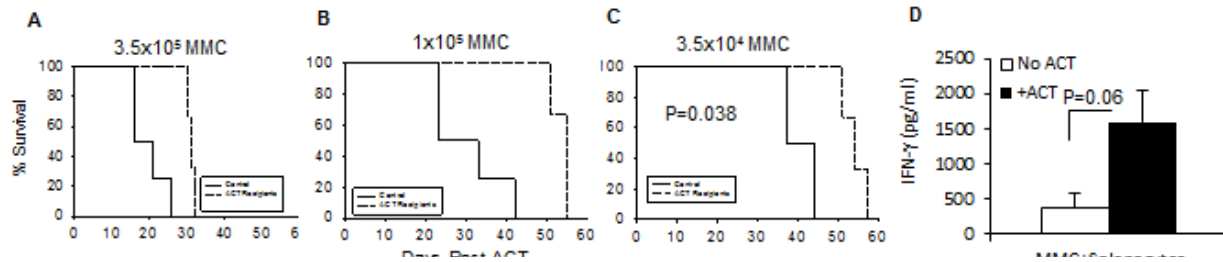


Figure 20. The efficacy of ACT is limited against circulating MMC despite reductions in tumor challenge. FVBN202 mice were challenged with serial reductions of proliferating MMC cells (A) 3.5×10^5 , B) 1×10^5 , and C) 3.5×10^4 , i.v., after having received ACT (ACT Recipients; n=3), or remaining untreated (Control; n=2). B) Splenocytes were harvested from untreated mice (No ACT; n=2) and ACT recipients (+ACT; n=3) and cultured in the presence of MMC cells (10:1) for 20 hours. Supernatants were collected and subjected to IFN- γ ELISA. Data represent mean \pm SEM.

ACT utilizing reprogrammed T cells and CD25+ NKT cells sustains an anti-tumor memory response, *in vivo*

In order to determine whether reprogrammed memory T cells were maintained *in vivo*, splenocytes of ACT recipients were collected when mice become moribund, and cultured with MMC tumor cells. As shown in Figure 20D, tumor-reactive IFN- γ production by endogenous splenic T cells from the +ACT group was greater than that produced by T cells from the No-ACT control group. These results suggest that ACT promotes the retention of long-lived anti-tumor immune responses, and that the lack of full protection against experimental metastases was not due to the loss of tumor-reactive immune cells.

ACT shifts splenic myeloid cells from Gr1+CD11b+ MDSCs to Gr1-CD11b-/lo myeloid cells

We next sought to determine whether the failure of ACT to result in complete elimination of the tumor was due to the inability of reprogrammed immune cells to modulate and overcome the suppressive function of MDSCs *in vivo*. Experimental animals (Figure 20) were sacrificed at the end of the trial when the ACT and No-ACT groups showed similar tumor burden in the lung (Figure 21A). ACT resulted in a significant reduction in trafficking of myeloid cells to the spleen in tumor-bearing animals ($p=0.002$), but the frequency remained higher than that observed in naïve mice ($p=0.001$). Importantly, ACT also reduced the frequency of MDSCs in the splenic myeloid cell compartment (Figure 21B, $p=0.005$), but not to the level observed in naïve mice ($p=0.008$). A similar trend was observed on total MDSCs (Figure 21B, $p=0.0001$ and $p=0.0009$). Conversely, ACT increased the proportion of Gr1-CD11b-/lo cells in the splenic myeloid cell compartment (Figure 21B, $p=0.004$), but remained lower than those in naïve mice ($p=0.002$).

Similarly, ACT increased percent total of Gr1-CD11b-/lo cells to a frequency similar to that of naïve mice, when compared with the No-ACT group (Figure 21B, $p=0.056$).

ACT modulates the phenotype of Gr1+CD11b+ MDSCs in the spleen

As the frequency of MDSCs appears to be altered upon ACT administration, we sought to further characterize these cells after ACT. Since the expression of MHC class II (MHCII) on MDSCs is associated with a contact-dependent mechanism of suppression (199), we sought to determine the frequency of MHCII expression on MDSCs, *in vivo*. Interestingly, splenic MDSCs of the +ACT group displayed elevated expression of MHCII in comparison to the No-ACT group (Figure 22A, 50% vs. 25%); and was similar to the frequency displayed by naïve mice. Conversely, splenic MDSCs of the No-ACT group were mainly MHCII- compared with the +ACT group or naïve mice (Figure 22B, 75% vs. 50%). The percentage of total MHCII+ MDSCs in the spleen of metastatic tumor-bearing mice was higher than that in naïve mice, and it did not change following ACT (Figure 23A). On the other hand, ACT reduced the total percentage of MHCII- MDSCs in the spleen (Figure 23B, $p=0.029$), though they remained higher than those in naïve mice ($p=0.039$). Since the total percentage of MHCII+ MDSCs and MHCII- MDSCs in the spleen remained increased compared with those in naïve mice, we hypothesized that the ratio of MDSCs to T cells in the spleen may have been altered. Therefore, we calculated the ratio of MDSCs to T cells because it is known that MDSCs suppress T cells function at high ratios to T cells (1:1 to 1:3). We found a higher proportion of MDSCs to T cells in the no-ACT group (1:3) compared to the ACT group (1:9) (Figure 23C, $p=0.029$), suggesting the MDSCs remaining after ACT have a reduced capacity to suppress the pool of anti-tumor T cell responses. The proportion of Gr1-CD11b-/lo myeloid cells to T cells did not change

following ACT (Figure 23D). Since the total percentage of MHCII+ MDSCs in the spleen did not change following ACT, we also looked at the expression of co-stimulatory molecules on these cells. As shown in Figure 23E-G, ACT resulted in a significant downregulation of the expression of CD80 (Figure 23E, $p=0.049$) and CD86 (Figure 23F, $p=0.033$), implying MDSCs in ACT recipients also may also have a reduced suppressive potential per cell (43, 200). Expression of CD40 did not change in the ACT group compared with the No-ACT group (Figure 23G).

ACT generated splenic Gr1-CD11b-/lo myeloid cells boost anti-tumor immune responses

We have previously reported that the presence of CD25+ NKT cells in reprogrammed immune cells induced the conversion of a fraction of MDSCs to CD11b-/lo MHC class II+ myeloid stimulatory cells, *in vitro* (54). Here, our *in vivo* studies also showed that only a fraction of MDSCs were converted to Gr1-CD11b-/lo myeloid cells; MDSCs were still present in ACT group, though at a lower frequency compared to the No-ACT group (Figure 21). In order to determine the immune stimulatory function of newly converted Gr1-CD11b-/lo myeloid cells, as well as the immune suppressive function of the remaining Gr1+CD11b+ MDSCs in the ACT group, splenic myeloid cells from ACT recipients were sorted into Gr1+CD11b+ and Gr1-CD11b-/lo cellular populations, and were then cultured with their own endogenous splenic-derived lymphocytes in the presence or absence of MMC cells (Figure 24A). Endogenous splenic-derived lymphocytes and sorted myeloid cells from the No-ACT group were used as controls (Figure 24B). ACT generated splenic Gr1-CD11b-/lo myeloid cells boosted the release of tumor-induced IFN- γ from endogenous lymphocytes (Figure 24A, $p=0.03$), whereas Gr1+CD11b+ MDSCs maintained suppressive function when used at a 1:2 ratio to lymphocytes

(Figure 24A, $p=0.008$). Cells derived from the No-ACT control group did not demonstrate tumor-induced IFN- γ release in the presence or absence of sorted splenic Gr1-CD11b-/lo myeloid cells (Figure 24B). Similar to our *in vitro* observations from animal studies (16), or patients with early-stage breast cancer (54), the increased tumor-specific IFN- γ production by lymphocytes derived from the +ACT group was associated with a higher frequency of splenic CD25+ NKT cells in these recipient mice (Figure 24C). The frequency of CD25+ NK cells did not increase following ACT (data not shown). These results suggest that Gr1-CD11b-/lo myeloid cells generated by ACT were functionally different from those of the No-ACT group, and that the activity of CD25+ NKT cells *in vivo* may render them immunostimulatory. Our previous studies *in vitro* showed that Gr1-CD11b-/lo myeloid stimulatory cells were converted from a fraction of Gr1+CD11b+ MDSCs by CD25+ NKT cells (54).

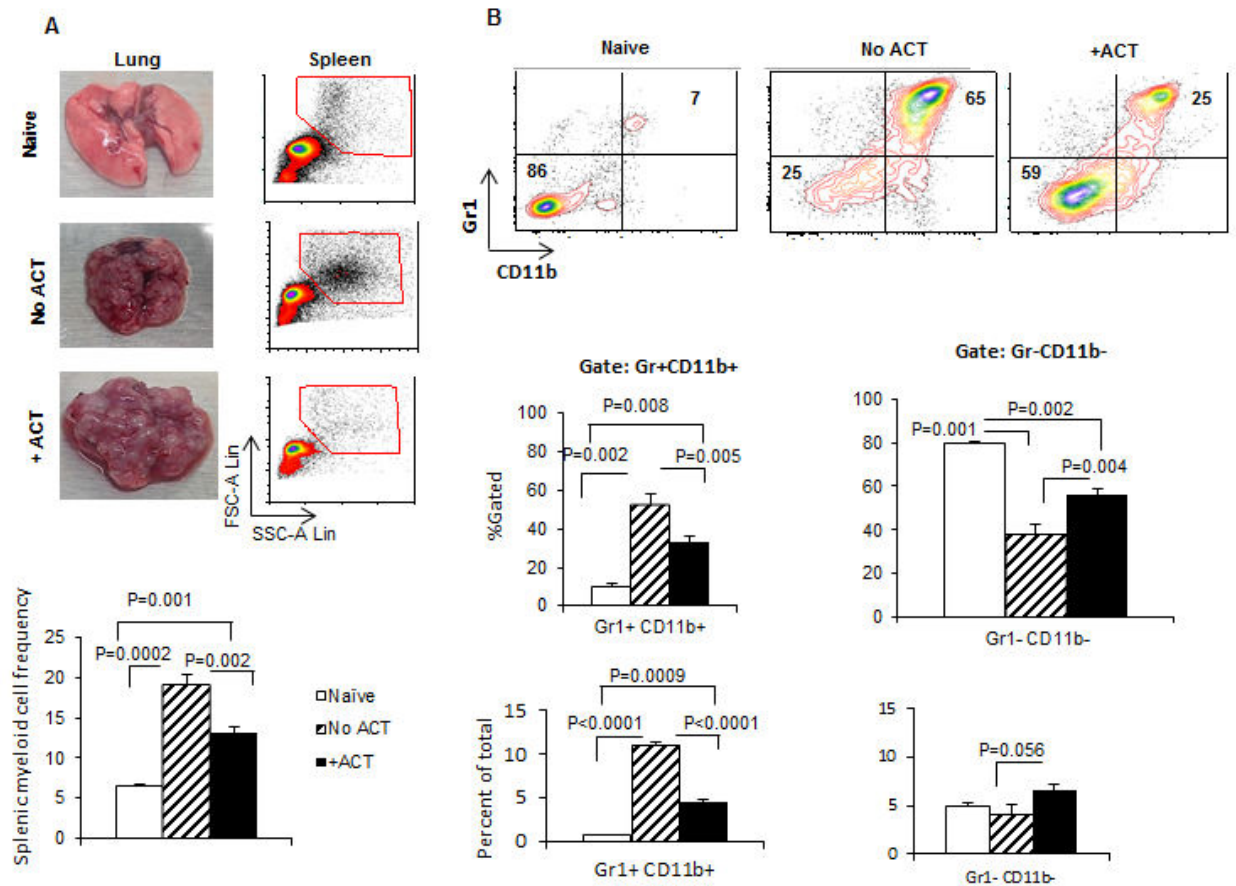


Figure 21. ACT modulates the splenic myeloid cell compartment. FVBN202 mice receiving ACT (n=12), or untreated mice (n=9) were intravenously challenged with MMC. Animals were euthanized after bulky metastatic lesions had developed and the mice became moribund, ~30-40days post-ACT. A) Tumor burden in the lungs of control mice, or mice receiving ACT is qualitatively similar (left panel), however ACT reduces the accumulation of myeloid cells in the spleen (right panel). B) Relative frequencies of splenic myeloid Gr1- CD11b- cells, and Gr1+ CD11b+ cells (upper and middle panels) and total frequency of splenic myeloid Gr1- CD11b- cells, and Gr1+ CD11b+ cells (lower panel) assessed using flow cytometry in naive mice (n=3), untreated mice (No ACT) or mice receiving ACT (+ACT). Myeloid cell discrimination was determined by the light scattering properties of splenocytes. Data represent mean \pm SEM.

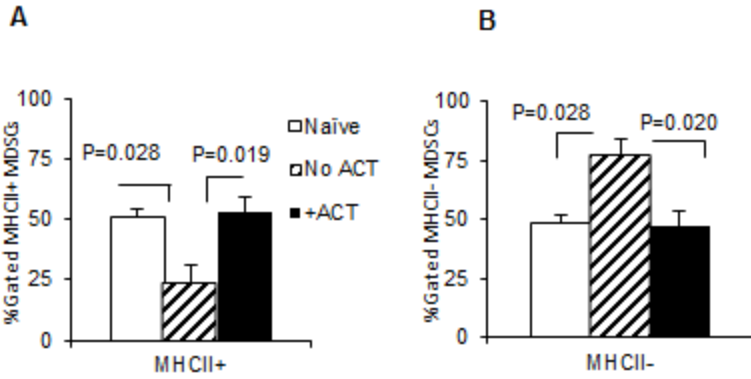


Figure 22. ACT modulates the phenotype of splenic MDSCs. FVBN202 mice that had received ACT (n=8), or remained untreated (n=4) and were intravenously challenged with MMC, and naïve mice (n=3) were analyzed for the expression of MHCII on MDSCs using flow cytometry after tumor challenged mice became moribund. A) Relative frequencies of splenic MDSCs expressing MHCII, and (B) relative frequency of MDSCs negative for MHCII. Data represent mean \pm SEM.

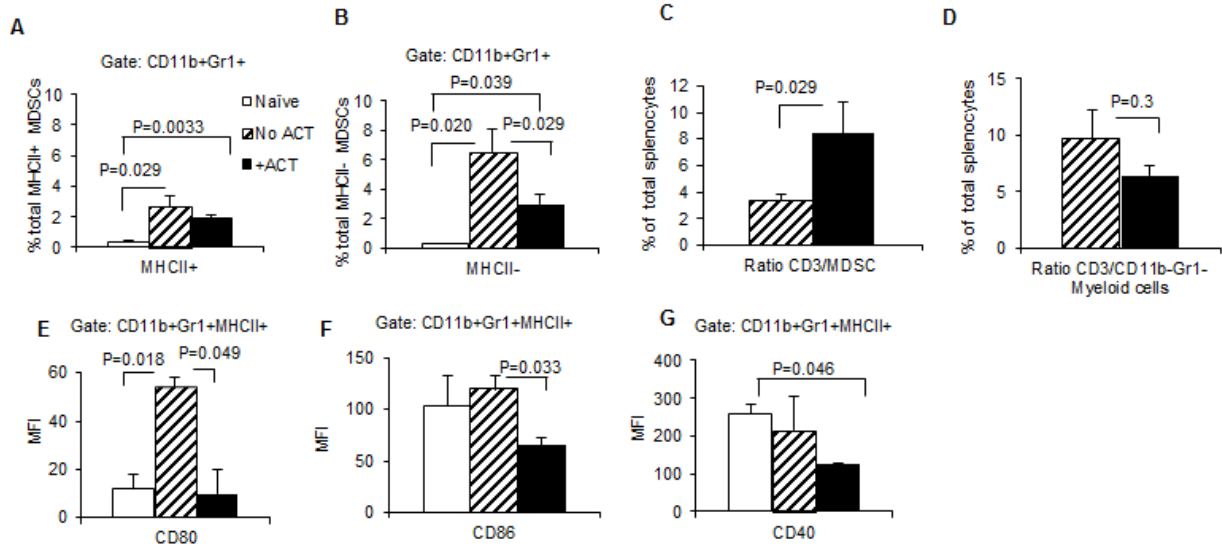


Figure 23. ACT modulates the phenotype of splenic MDSCs. Splens of FVB/N202 mice that had received ACT (n=8), or remained untreated (n=4) and were intravenously challenged with MMC, and naive mice (n=3) were analyzed by flow cytometry after tumor-bearing mice became moribund. (A&B) Total frequency of splenic MHCII+ MDSCs. The frequency of total splenic CD3+ T cells was determined, and was then divided by the total splenic frequency of MDSCs (C), and the total splenic frequency of CD11b-Gr1- myeloid cells (D). Median fluorescence intensity (MFI) of CD80 (E), CD86 (F), and CD40 (G) was quantified on the surface of MHCII+ MDSCs. Data were collected using flow cytometry. Data represent mean \pm SEM.

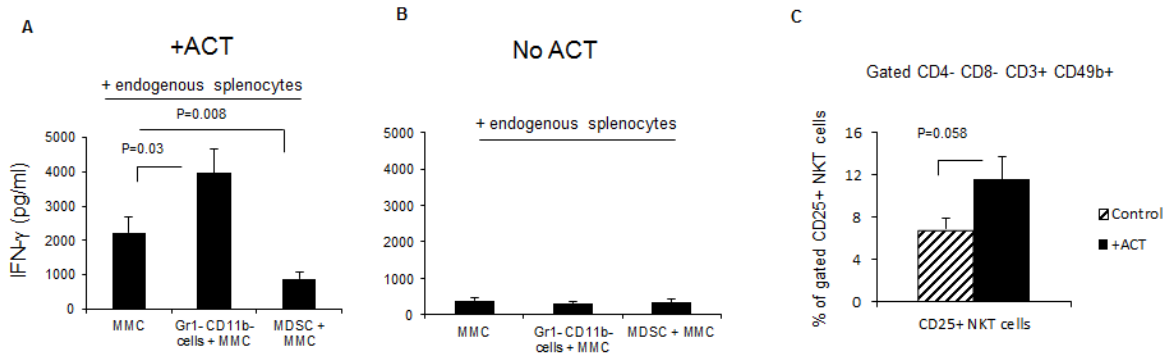


Figure 24. ACT generated Gr1- CD11b- cells promote tumor cell-mediated IFN- γ release by endogenous splenocytes. FACS sorted CD11b-Gr1- or CD11b+ Gr1+ myeloid cells from (A) ACT recipients (n=3) or from (B) No-ACT control mice (n=2) were co-cultured without or with MMC (5:1) and without or with endogenous splenocytes (1:2) for 20 hours; supernatant IFN- γ concentration was determined by ELISA. (n=1). C) Splenocytes were harvested from No-ACT (n=3) and +ACT groups (n=3); the frequency of CD25+ NKT cells was quantified in each group by flow cytometry. Data represent mean \pm SEM.

ACT generated splenic Gr1-CD11b-/lo myeloid cells are unique immune stimulatory cells, which differ from conventional APCs

Since Gr1-CD11b-/lo myeloid cells generated by ACT showed immune stimulatory function (Figure 24), and had a higher frequency in contrast with the No-ACT group (Figure 21B), we next sought to characterize these cells phenotypically. As shown in Figure 25A, frequency of MHCII expressed by CD11b-/loGr1- myeloid cells in the +ACT group and in naïve mice was higher than that in the no-ACT group. Gr1-CD11b-/loMHCII+ myeloid cells in the +ACT group and naïve mice also showed an increased MFI of the co-stimulatory molecule, CD86, compared with the No-ACT group (Figure 25B). In addition, the +ACT group downregulated the co-stimulatory molecules, CD40 and CD80, on Gr1-CD11b-/loMHCII+ myeloid cells compared with naïve mice (Figure 25C, $p=0.0002$ and $p=0.012$, respectively) and the no-ACT group (Figure 25D, $p=0.053$ and $p=0.016$, respectively).

In order to determine whether Gr1-CD11b-/loMHCII+ myeloid cells responded to toll-like receptor (TLR) stimulation and resulted in their maturation, as has been described for conventional APCs (201-203), these cells were stimulated with the TLR-4 agonist, lipopolysaccharide (LPS), *in vitro*. We used splenocytes from the No ACT group as source of Gr1-CD11b-/loMHCII+ myeloid cells because they expressed lower levels of MHCII and CD86 expression at baseline. As can be seen in Figure 26, LPS induced the maturation of Gr1-CD11b-/loMHCII+ myeloid cells by upregulating the expression of MHCII (MFI: 2000 vs. 5500; $p=0.007$), as well as the co-stimulatory molecules CD86 (MFI: 37 vs. 150; $p=0.002$), CD40 (MFI: 580 vs. 925; $p=0.009$), and CD80 (MFI: 50 vs. 100; $p=0.03$).

Since ACT generated Gr1-CD11b-/lo immune stimulatory myeloid cells displayed characteristics of APCs by expressing signal I (MHCII) and signal II (CD86), and these cells

demonstrated maturation patterns generally observed in APCs upon LPS stimulation, we sought to determine whether these cells were conventional APCs. As shown in Figure 27, nearly all Gr1-CD11b-/loMHCII+ myeloid cells lacked expression of the DC marker CD11c (Figure 27, 94-97%). The frequency of these myeloid stimulatory cells did not change during tumor challenge or ACT, accounting for 3-4% of total splenocytes (Figure 27). In addition, these myeloid stimulatory cells did not express the pan marker of macrophages, F4/80 (Figure 28, 95-98%).

Immunotherapy induces tumor escape in proliferating tumor cells and indolent dormant cells, but not in quiescent dormant cells

Since tumor-reactive lymphocytes persisted following ACT, and modulation of myeloid cells occurred by reducing MDSCs and increasing myeloid stimulatory cells, we sought to determine whether tumor immunoediting resulted in the escape of tumor cells from elimination by ACT, and ultimately death of the animal. Metastatic tumor lesions were isolated from the lung at the end of the trial and analyzed for the expression of the tumor antigen, neu, and programmed death ligand 1 (PD-L1). The tumor lesions isolated from +ACT group showed downregulation of the neu antigen on the tumor cells compared with control MMC tumor cell line and the lesions isolated from the no-ACT group (Figure 29A, left panel; $p=0.00003$ and $p=0.0008$, respectively).

Additionally, 25% of MMC cells isolated from metastatic tumor lesions demonstrated total loss of neu expression compared with control MMC tumor cell line and the lesions isolated from the No ACT group (Figure 29A, right panel; $p=0.002$ and $p=0.01$, respectively). This suggests that metastatic MMC cells may escape detection from neu-specific cellular immunity. Additionally, we detected an upregulation of PD-L1 on the lung metastatic tumor cells compared with control

MMC tumor cell line (Figure 29B; MFI 390 vs. 78, $p=0.00002$). Interestingly, the No ACT group had higher expression of PD-L1 in the tumor compared with the ACT group (MFI 1360 vs. 390, $p=0.011$). The immune suppressive function of PD-L1 requires engagement with programmed death receptor 1 (PD-1), which renders immune cells tolerogenic (204, 205). Importantly, 40-50% of reprogrammed T cells and NKT cells that were used for ACT expressed PD-1 (Figure 30), but only CD8+ T cells were observed to upregulate PD-1 as a result of reprogramming (Figure 30; $p=0.01$). Therefore, we also analyzed tumor-infiltrating T cells for PD-1 expression, to determine the potential for the PD-1/PD-L1 axis to mediate T cell tolerance within the tumor site. Interestingly, as seen in Figure 31A, tumor infiltration of CD8+ T cells was greater in mice receiving ACT compared to untreated mice (14% vs. 3% respectively; $p=0.02$). However, expression of PD-1 remained intact on tumor infiltrating CD8 + T (Figure 31B). We did not observe CD4+ T cell infiltration into the tumor lesions (data not shown). Splenic T cells and NKT cells that were isolated from the +ACT and No-ACT group when animals became moribund also expressed PD-1, though there was no statistical difference between the groups (Figure 32, 10% of T cells and 50% of NKT cells). All together, these data suggest that although ACT promotes the infiltration of CD8+ T cells, the highly proliferative nature of the metastatic tumors may evade such anti-tumor immune responses by emerging with reduced expression of the tumor antigen, neu, and upregulating the co-inhibitory molecule PD-L1. Thus, we then began to question if residual tumor cells that remain after conventional cytotoxic therapy, which are generally dormant, also employ similar escape mechanisms or if they were perhaps more sensitive to immune-mediated elimination.

To determine whether dormant tumor cells were resistant to escape and immunoediting, MMC tumor cells were treated with ADR in order to establish tumor dormancy. A clinically relevant

proliferation marker, Ki67, along with a viability dye (FVS), were used to detect viable, indolent tumor cells (Ki67+/FVS-) and quiescent tumor cells (Ki67-/FVS-). As shown in Figure 33A, ADR induced apoptosis in the majority of MMC cells; 3 weeks after treatment the number of FVS- viable MMC cells was reduced from 77% to 31% ($p=0.005$). The remaining residual viable tumor cells that escaped chemotherapy-induced apoptosis entered a dormant state, as there was no significant increase in the number of tumor cells between one week and three weeks after the completion of ADR chemotherapy (Figure 33B). To determine if dormant tumor cells could exploit immune escape mechanisms, we treated dormant MMC cells with IFN- γ , a cytokine produced and secreted by inflammatory cells in the tumor microenvironment, three weeks after the completion of ADR treatment in order to provoke PD-L1 expression (206, 207). We evaluated the expression of PD-L1 on viable proliferating control MMC cells, without (Untreated) and with IFN- γ treatment (Untreated \rightarrow IFN- γ), as well as on viable dormant tumor cells without (+ADR) and with IFN- γ treatment (+ADR \rightarrow IFN- γ). ADR or ADR \rightarrow IFN- γ treatment increased Ki67- quiescent tumor dormancy (Figure 34A, left panel, $p=0.02$ and $p=0.001$, respectively), and reduced the frequency of Ki67+ indolent MMC cells (Figure 34A, right panel, $p=0.02$ and $p=0.001$, respectively). IFN- γ treatment induced upregulation of PD-L1 on Ki67+ proliferating MMC (Figure 34B, left panel, $p=0.002$) and Ki67+ indolent tumor cells (Figure 34B, left panel, $p=0.01$). Interestingly, Ki67- control MMC cells and Ki67- quiescent MMC cells did not upregulate PD-L1 (Figure 34B, right panel). As Adriamycin significantly increases the population of quiescent MMC cells which are less responsive to an inflammatory environment which may upregulate the expression of PD-L1 compared with indolent dormant cells or proliferating cells, this suggests that immunotherapy applied after conventional cytotoxic therapy may be more effective in eliminating Ki-67- tumor cells generated by chemotherapy.

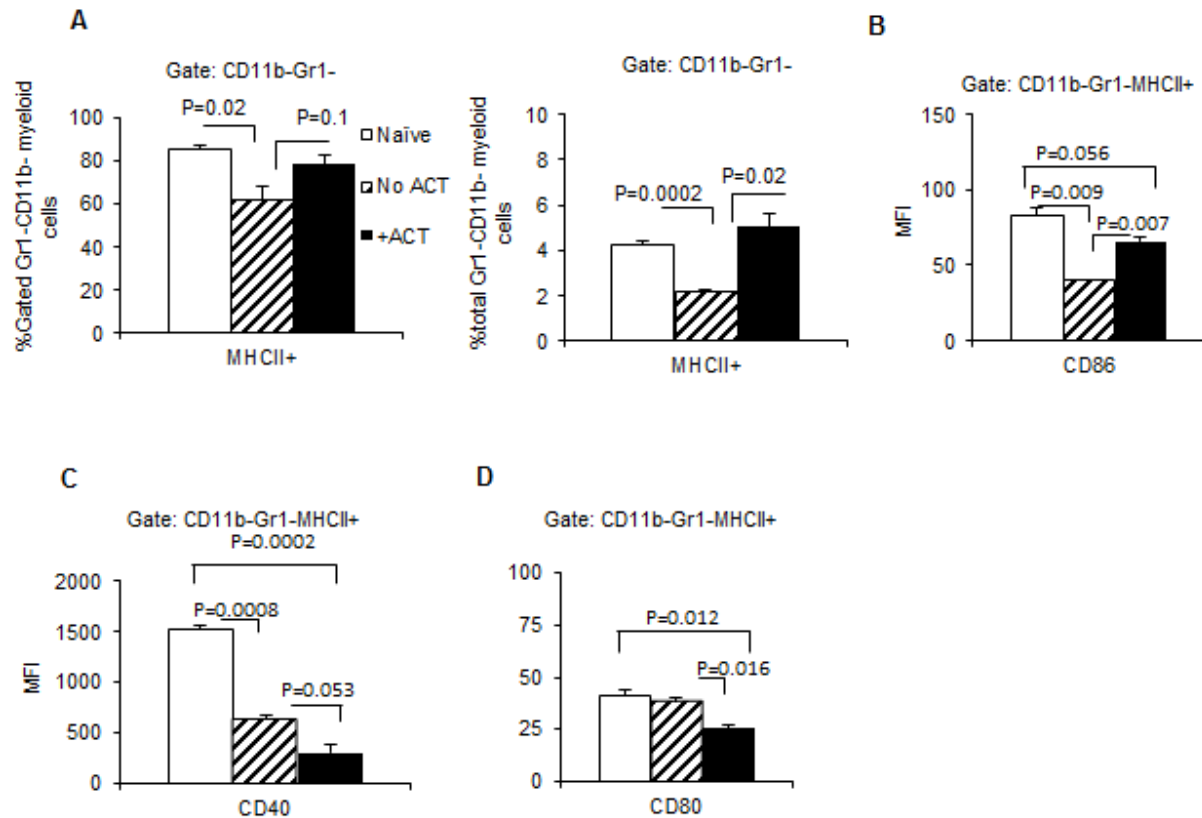


Figure 25. ACT modulates the phenotype of splenic CD11b-Gr1- myeloid cells. Splensens of FVBN202 mice that had received ACT (n=8), or remained untreated (n=4) and were intravenously challenged with MMC, and naïve mice (n=3) were analyzed by flow cytometry after tumor-bearing mice became moribund. (A) Gated frequency and total frequency of MHCII on splenic CD11b-Gr1- myeloid cells. Median fluorescence intensity (MFI) of CD86 (B), CD40 (C), and CD80 (D) was quantified on the surface of MHCII+ CD11b-Gr1- myeloid cells. Myeloid cell discrimination was determined by the light scattering properties of splenocytes. Data were collected using flow cytometry. Data represent mean \pm SEM.

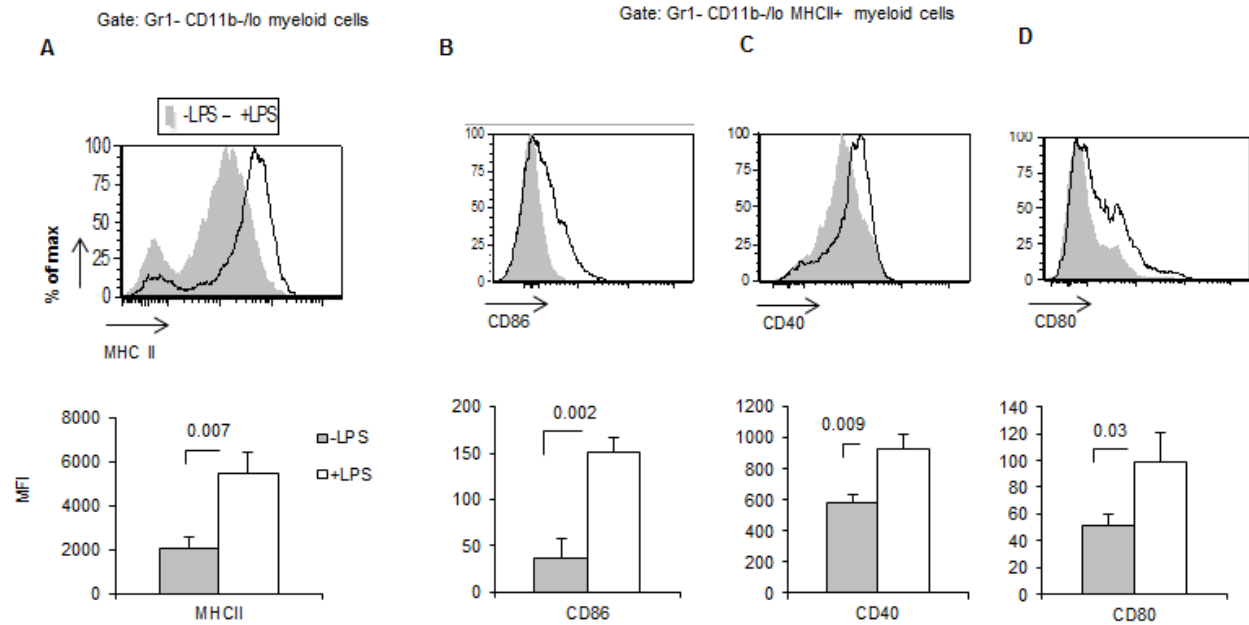


Figure 26. Splenic Gr1-CD11b-/- myeloid cells demonstrate characteristics of APC maturation upon stimulation with LPS. Spleens of FVBN202 mice were harvested from moribund mice that had not received ACT (n=3) after i.v. challenge with MMC. Gr1- CD11b-/- cells were sorted from the spleen and were analyzed by flow cytometry after stimulation with LPS (1µg/ml; 22hrs). (A) MFI of MHCII on splenic Gr1-CD11b-/- myeloid cells. Splenic Gr1- CD11b-/- MHCII+ myeloid cells were also analyzed for the surface expression of (B) CD86, (C) CD40, and (D) CD80. Myeloid cell discrimination was determined by the light scattering properties of splenocytes. Data represent mean ± SEM.

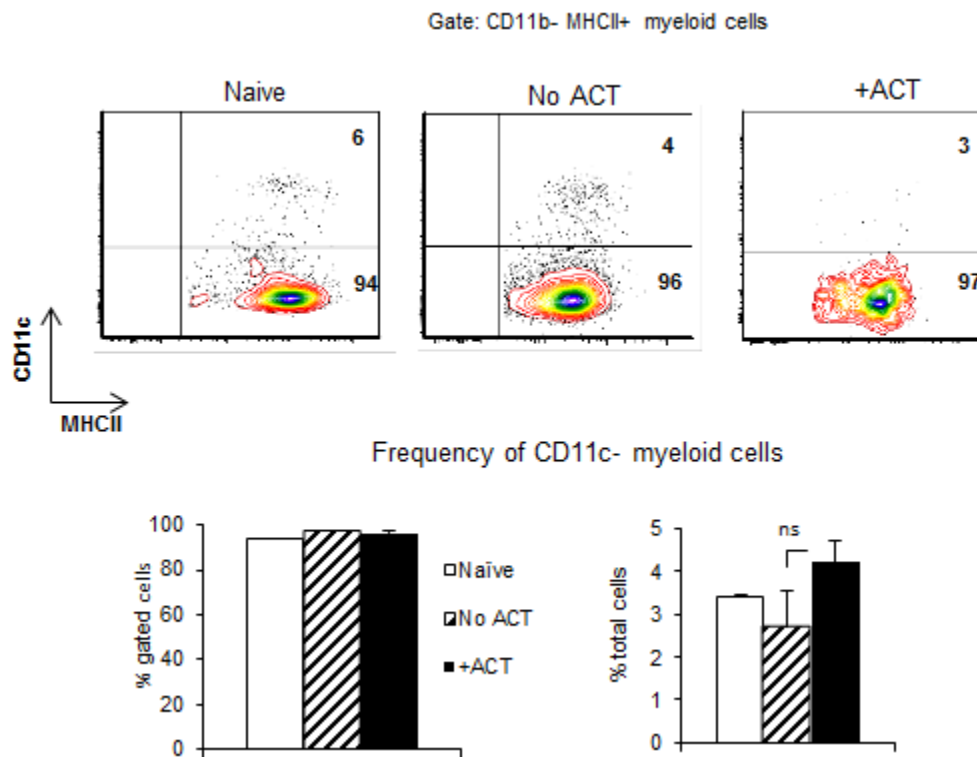


Figure 27. CD11b negative myeloid cells are not enriched with CD11c+ DCs upon ACT. Splens of FVBN202 mice that had received ACT (n=2), or remained untreated (n=2) and were intravenously challenged with MMC, and naïve mice (n=3) were analyzed by flow cytometry after tumor-bearing mice became moribund. A) Relative frequencies (left panel) and total frequency (right panel) of splenic CD11c-CD11b-myeloid cells. Myeloid cell discrimination was determined by the light scattering properties of splenocytes. Data represent mean \pm SEM.

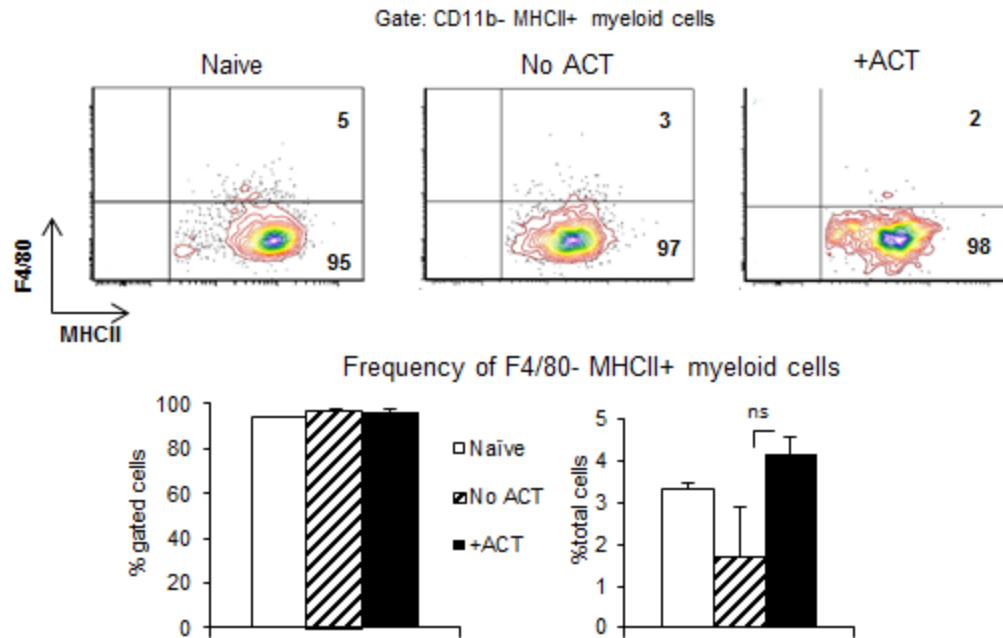


Figure 28. CD11b negative myeloid cells are not enriched with F4/80+ macrophages upon ACT. Splensens of FVBN202 mice that had received ACT (n=2), or remained untreated (n=2) and were intravenously challenged with MMC, and naïve mice (n=3) were analyzed by flow cytometry after tumor-bearing mice became moribund. A) Relative frequencies (left panel) and total frequency (right panel) of splenic F4/80-CD11b- myeloid cells. Myeloid cell discrimination was determined by the light scattering properties of splenocytes. Data represent mean \pm SEM.

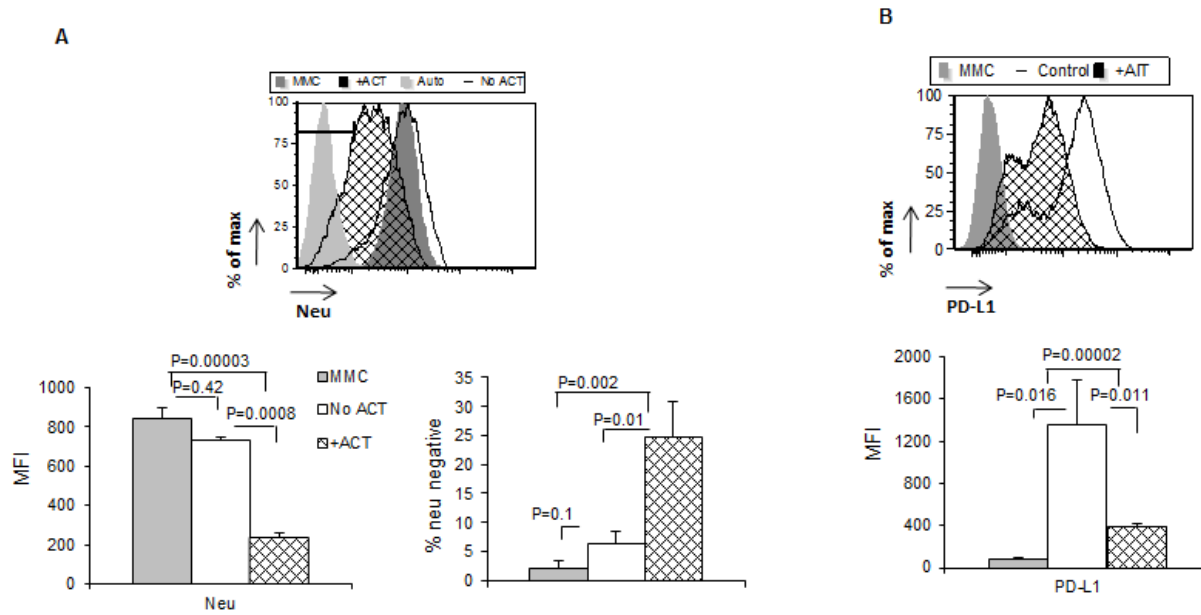


Figure 29. ACT promotes immunoediting of lung metastatic lesions. Metastatic lesions in the lung of FVBN202 mice that remained untreated (No ACT; n=3) and ACT recipients (+ACT; n=6) were harvested when mice became moribund. Tumor lesions were digested and were placed into cell culture for 10-14 days. MFI of neu and frequency of neu loss (A) and MFI of PD-L1 (B) were then quantified using flow cytometry. Data represent mean \pm SEM.

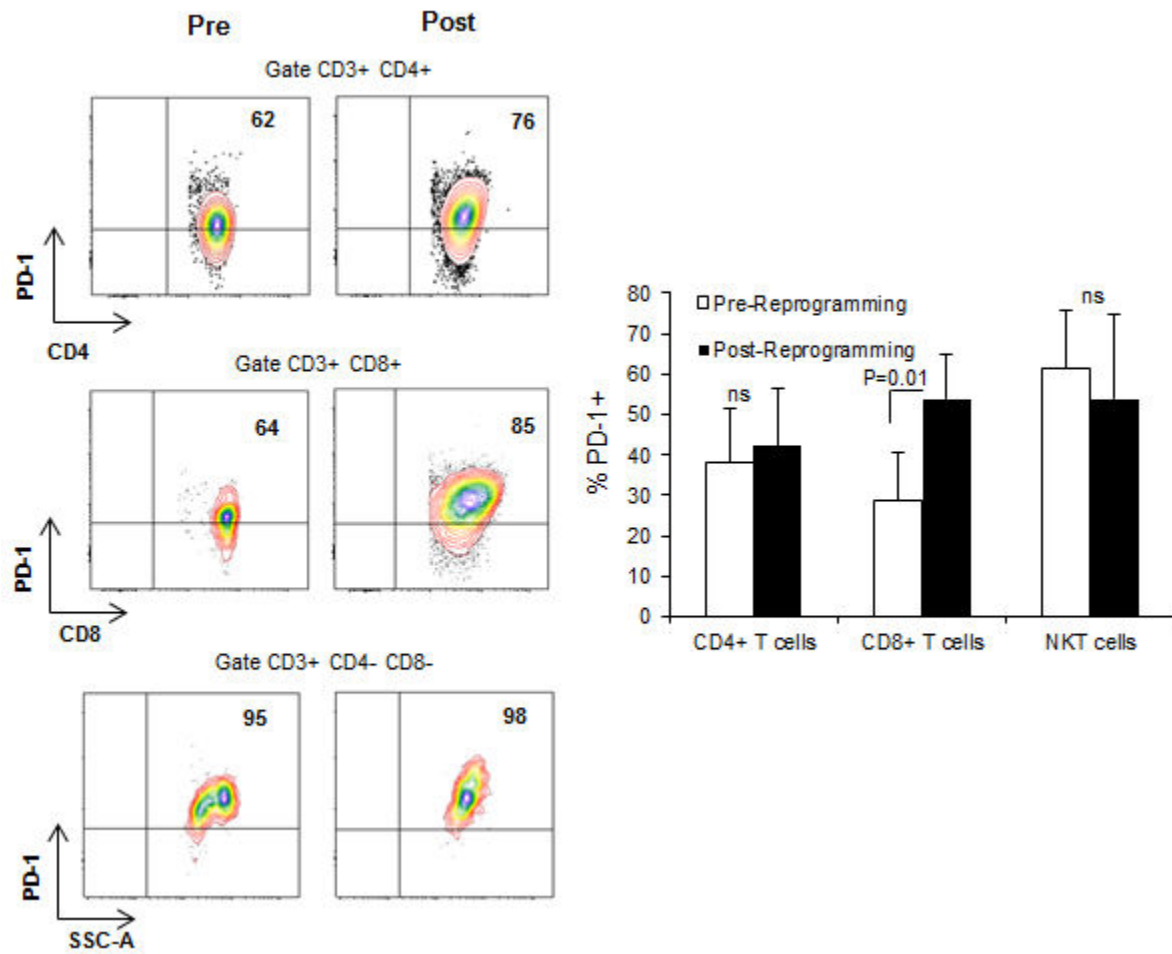


Figure 30. Reprogrammed splenocytes express PD-1. Spleens of FVBN202 mice bearing primary mammary carcinoma (n=4) were harvested after tumors were $\geq 1000\text{mm}^3$. PD-1 expression was then quantified on the splenocytes pre- and post-reprogramming. Data represent mean \pm SEM.

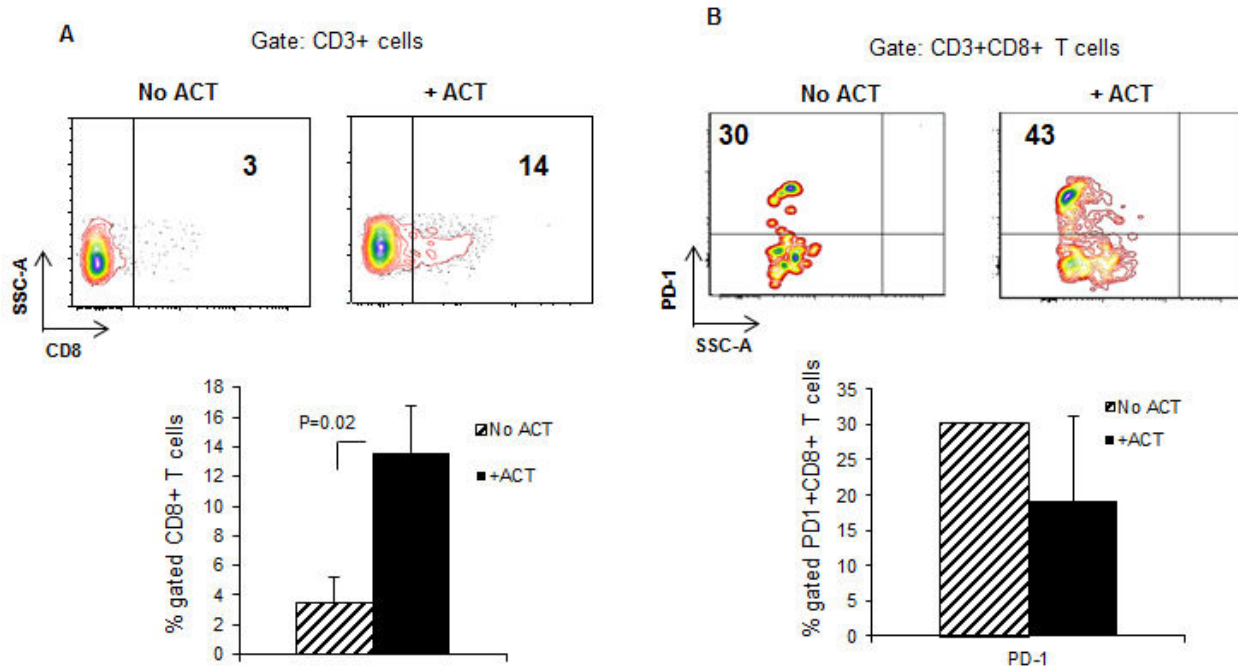


Figure 31. The frequency of tumor-infiltrating CD8+ T cells increases upon ACT, yet retain PD-1 expression. Metastatic lesions in the lung of FVBN202 mice that remained untreated (No ACT) and ACT recipients (+ACT) were harvested when mice became moribund. Tumor lesions were digested and (A) the frequency of CD8+ T cell infiltration (No ACT, n=3; +ACT, n=3), and (B) expression of PD-1 (No ACT, n=1; +ACT, n=3) were then quantified relative to CD45+ AnnexinV- leukocytes. Data represent mean \pm SEM.

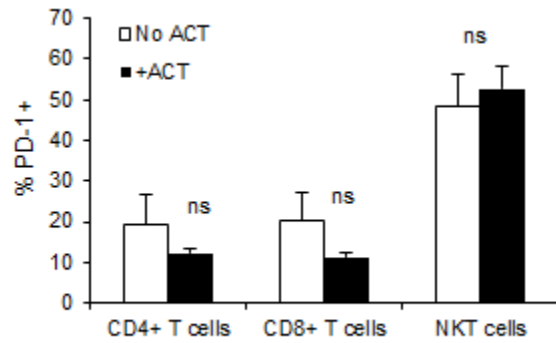


Figure 32. Splenic lymphocytes retain PD-1 expression after ACT. Spleens of FVBN202 mice that had received ACT (n=4), or remained untreated (n=3) and were intravenously challenged with MMC were analyzed by flow cytometry after tumor-bearing mice became moribund in order to quantify PD-1 expression. Data represent mean \pm SEM.

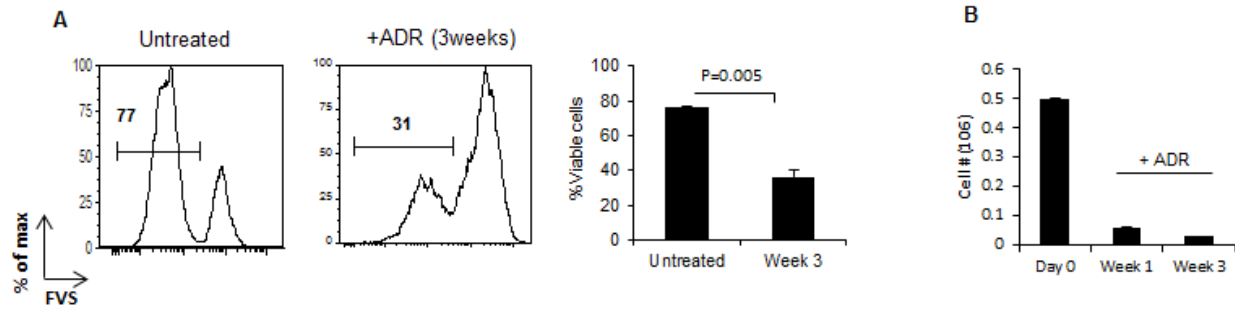


Figure 33. ADR-treatment results in the emergence of a viable, dormant MMC cells. A) MMC tumor cells were treated with 3 daily doses of ADR (1 μ M for 2 hrs), then remained untreated for 3 weeks. A) The frequency of viable MMC cells was determined by quantifying FVS- cells using flow cytometry. B) At weeks 1 and 3 post-treatment, adherent and viable tumor cells were counted by trypan blue exclusion. Representative data from triplicate experiments Data represent mean \pm SEM.

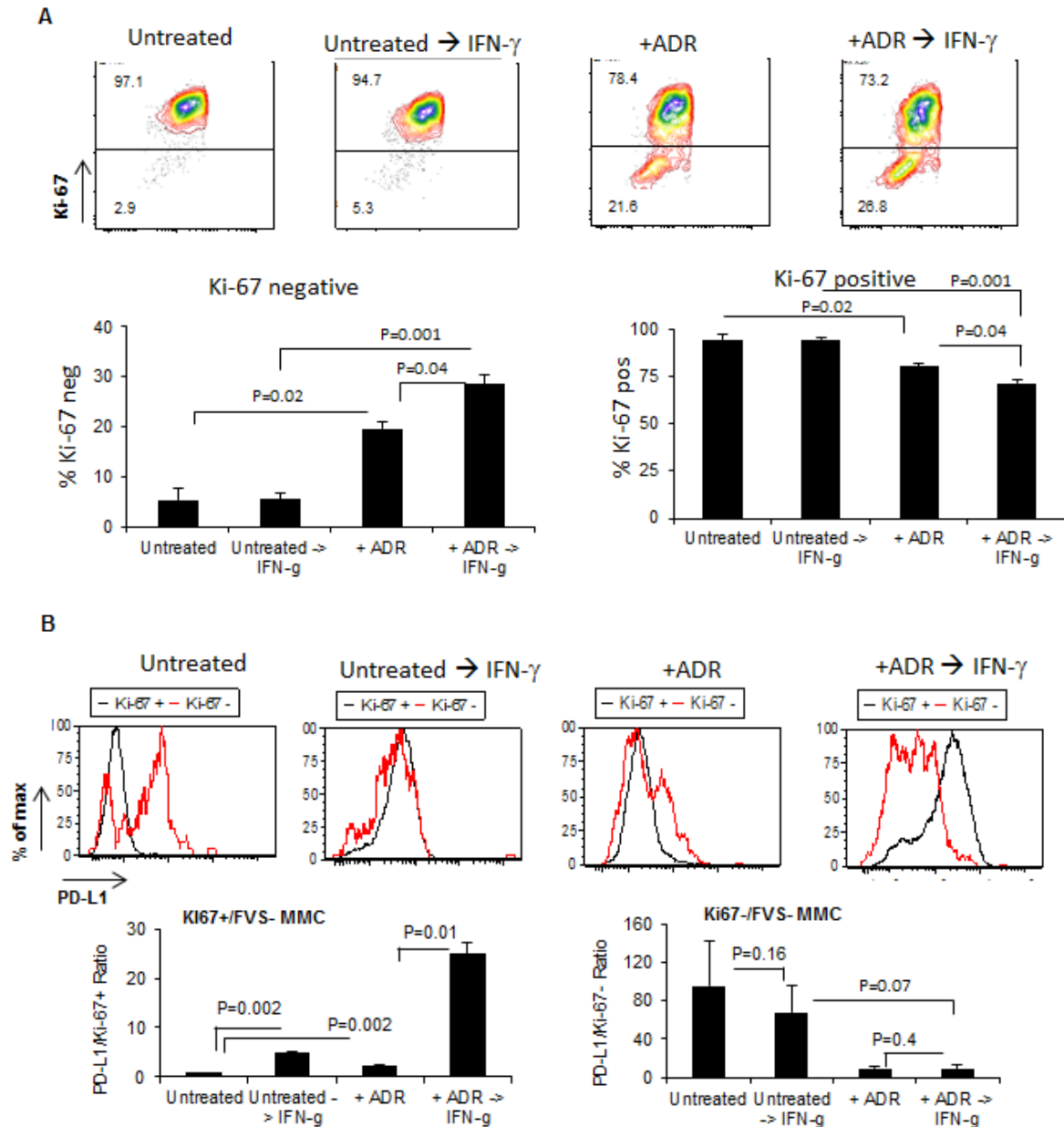


Figure 34. IFN- γ induces upregulation of PD-L1 on Ki67+ but not on Ki-67- tumor cells. MMC cells were treated for 3 consecutive days with Doxorubicin (1 μ M, 2hrs) or left untreated. Three weeks later Doxorubicin-treated and untreated MMC cells were stimulated with IFN- γ (50ng/ml) for 12-16 hours to induce the expression of PD-L1. A) Expression of Ki-67 was determined in control MMC cells (untreated) as well as ADR treated cells (+ADR), +/- IFN- γ stimulation. B) The Expression of PD-L1/cell was calculated by dividing PD-L1 MFI by the frequency of Ki-67 negative cells in Doxorubicin-treated and untreated MMC cells, +/- IFN- γ stimulation. Data represent 3 independent experiments and mean \pm SEM.

Dormant MMC cells established by ADR become resistant to chemotherapy, but remain sensitive to immunotherapy

In order to determine whether dormant MMC cells established by ADR chemotherapy remain sensitive to immunotherapy, dormancy was established by treating cells with three daily doses of ADR treatment (1 μ M/day for 2 hrs) (Figure 35A); eight days after the final treatment, MMC cells received a high dose of ADR (1 μ M for 24 hrs), or were cultured with reprogrammed immune cells for 48 hrs. ADR treatment induced apoptosis in MMC cells (Figure 35B, $p=0.01$). Tumor cells that survived apoptosis became chemo-refractory; additional ADR treatment at a higher dose (1 μ M for 24 hrs) did not induce cell death (Figure 35B, average 40% vs. 54%). However, they remained sensitive to tumor-reactive lymphocytes. In the presence of tumor-reactive immune cells, the frequency of viable ADR-treated dormant MMC dropped from 40% to 8% (Figure 35B, $p=0.003$). In fact, immunotherapy was more effective than a high dose of chemotherapy in inducing apoptosis in dormant MMC (Figure 35B, $p=0.02$). We also established dormant MMC by three daily doses of RT (2Gy/day); again surviving dormant cells became refractory to RT. An additional RT at a higher dose (18 Gy) did not markedly decrease the frequency of viable tumor cells (Figure 36, 53% vs. 52%). However, RT-refractory MMC cells remained sensitive to tumor-reactive lymphocytes as the viability dropped from 53% to 8% (Figure 36, $p=0.002$). In fact, immunotherapy was more effective than a high dose RT on inducing apoptosis in dormant MMC (Figure 36, $p=0.01$).

In order to determine the efficacy of targeting dormant tumor cell *in vivo*, we performed a preliminary study in which we treated experimental animals bearing primary MMC with ADR, followed by ACT once the tumor growth plateaued, which represents tumor dormancy. As seen in Figure 37, both animals treated with ADR exhibited suppression of tumor growth.

Importantly, the animal which also received ACT exhibited demonstrable tumor regression by day 24. This data suggests that MMC cells which are in a dormant state can be targeted more effectively than highly proliferative tumor cells, *in vivo*.

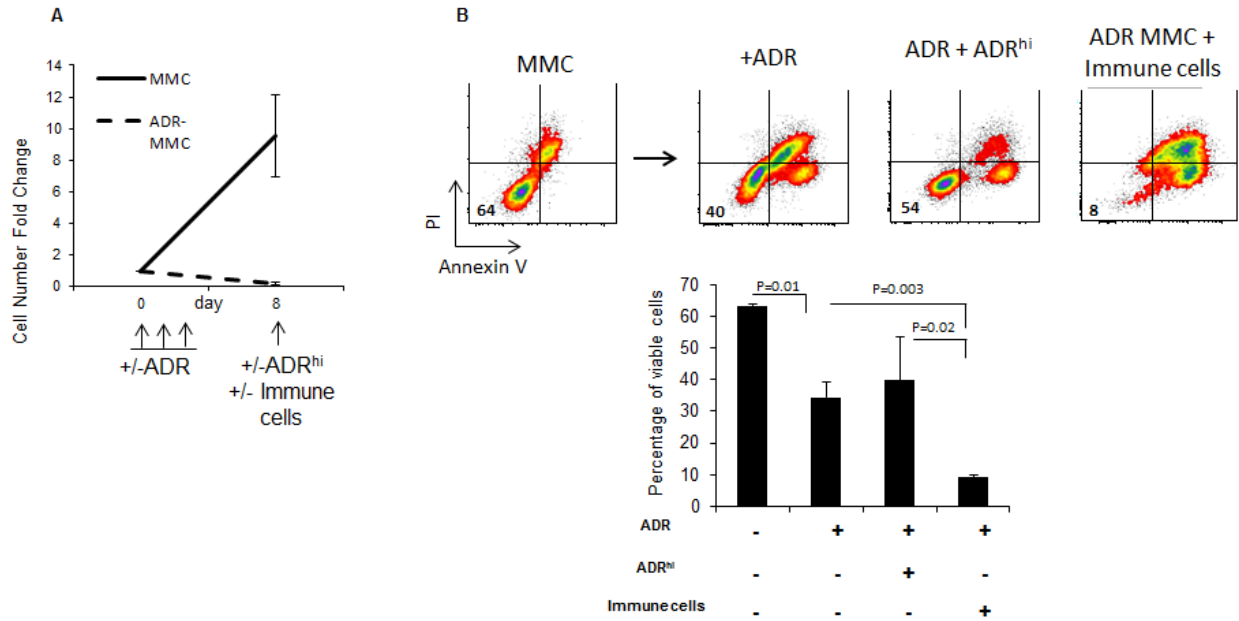


Figure 35. Immunotherapy displays cytotoxic function against treatment-refractory dormant tumor cells, *in vitro*. MMC cells (n=3) treated with Doxorubicin (1 μ M, 2hrs) for 3 consecutive days and remained in culture for 8 days total, in order to establish tumor cell dormancy *in vitro* (A). On day 8, these dormant tumor cells were treated with ADR (1 μ M, 24hrs) (ADR-treated MMC + ADR) or reprogrammed immune cells (ADR-treated MMC + Immune cells; ADR-treated MMC + Immune cells). Two days later, cells were stained with Annexin V/PI and analyzed by flow cytometry (B). Data represent three biological repeats and mean \pm SEM.

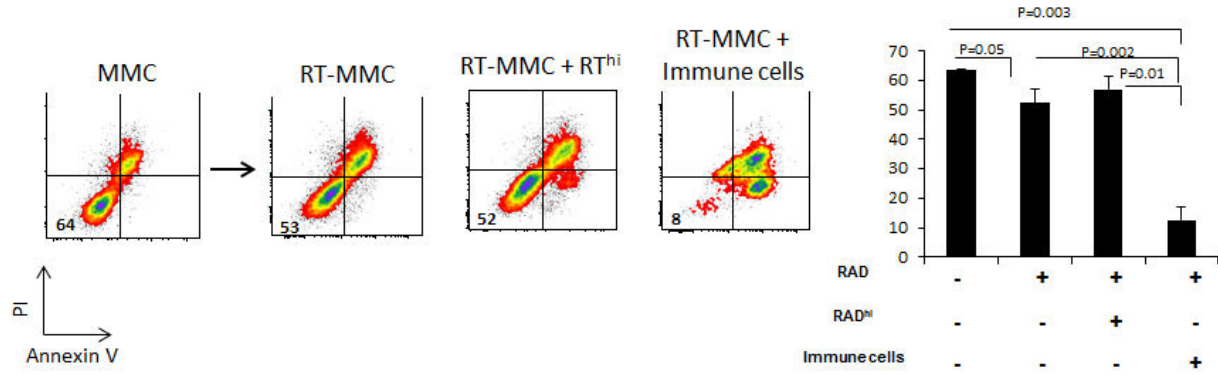


Figure 36. Immunotherapy displays cytotoxic function against treatment- refractory dormant tumor cells, *in vitro*. MMC tumor cells were treated with 3-daily doses of 2Gy RT (RT-treated MMC) in order to establish tumor cell dormancy *in vitro*. On day 8, these dormant tumor cells were treated with RT (RT-treated MMC + 18Gy RT) or reprogrammed immune cells (RT-treated MMC + Immune cells). Two days later, cells were stained with Annexin V/PI and analyzed by flow cytometry. Data represent two biological repeats and mean \pm SEM.

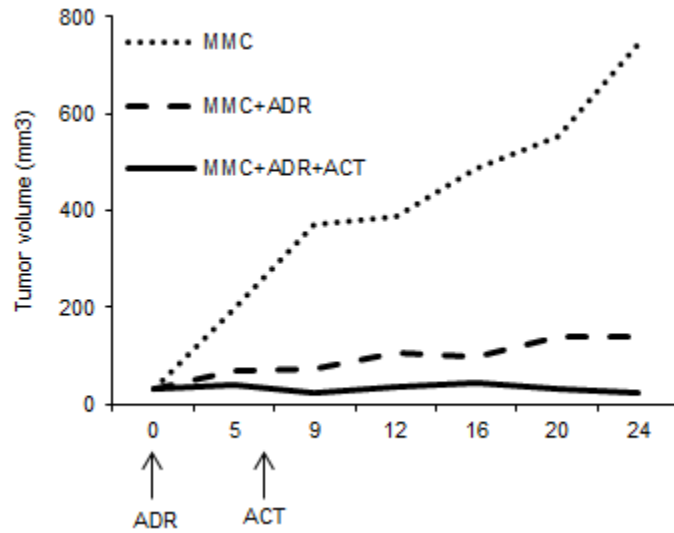


Figure 37. ACT induces regression of Adriamycin (ADR)-induced established indolent primary breast cancer. Animals were challenged with MMC (3×10^6) intradermally in the mammary gland region; after tumors reached 30-50mm³ animals were treated with Adriamycin (MMC+ADR; 20 mg/kg; i.v.), or remained untreated (MMC). Six days later, one mouse received ACT (MMC+ADR+ACT).

DISCUSSION

We developed an experimental metastatic mouse model by i.v. injection of highly proliferative MMC cells to FVBN202 mice. Animals in this model became moribund within 20-40 days, and presented with lung metastases upon macroscopic inspection. This model represents the onset of advanced stage disease. We demonstrated that concurrent use of Dec with ACT using reprogrammed NKT and T cells prolonged survival of the experimental animals, but failed to eliminate the tumor as all mice eventually succumbed to metastatic disease in the lung. Failure in tumor elimination was associated with downregulation of the tumor antigen, neu, on metastatic tumor cells. Neu downregulation occurred in the presence of Dec, *in vitro*, whereas total neu loss and downregulation were evident in the presence of Dec treatment, *in vivo*. Additionally, *in vitro* studies demonstrated that Dec-induced CTA expression was transient; tumor cells quickly downregulated expression of CTA transcripts upon the cessation of Dec treatment. These data suggest that Dec may function as an *in situ* vaccination by inducing immune responses against the tumor which in turn facilitate total antigen loss. Additional studies involving ACT without Dec treatment confirmed that total neu antigen loss was mediated by anti-tumor immune responses, *in vivo*, whereas our *in vitro* studies demonstrated that Dec alone only had the capacity to induce downregulation of neu antigen.

ACT without Dec also prolonged animal survival but, again, it failed to eliminate the tumor as animals eventually succumbed to metastatic disease in the lung. The data suggest that this was due to the induction of tumor escape mechanisms by these highly proliferative tumor cells, including upregulation of PD-L1 and loss of neu antigen by the tumor, which may result from immunoediting due to the increased infiltration of CD8+ T cells in these tumors. Very importantly, we identified and characterized novel APCs that were established from a portion of

MDSCs resulting from the administration of ACT containing CD25+ NKT cells, *in vivo*. These novel Gr1-CD11b-/lo immunostimulatory cells displayed a phenotype distinct from conventional APCs, specifically DCs and macrophages. Finally, promising results in patients with MM (Chapter Two) were obtained when ACT was administered during a setting of MRD, likely when indolent and/or quiescent tumor cells were present. Therefore, we hypothesized that targeting indolent, but not highly proliferating, mammary tumor cells might overcome tumor immunoediting and escape. Therefore, we conducted studies in order to determine the sensitivity of different types of dormant cells, indolent dormancy versus quiescent dormancy, to immunoediting and escape. Our *in vitro* studies demonstrated that quiescent, but not indolent, dormant tumor cells are resistant to immunoediting; thus, they may represent the ideal target for immunotherapy.

We have previously reported that PBMCs collected from patients with early stage breast cancer can be reprogrammed and expanded *ex vivo* (54). Here, we compared two different sources of tumor-sensitized immune cells, including animals harboring early stage primary tumor or those harboring metastatic tumor in the lung. We demonstrated that immune cells from both sources can be reprogrammed and expanded *ex vivo*. ACT utilizing these reprogrammed immune cells produced similar objective responses. We demonstrated that failure of ACT in complete elimination of the tumor was not due to the status of metastatic tumor burden, loss of tumor-reactive T cells, or the presence of MDSCs. In fact, ACT facilitated modulation of myeloid cells by inducing a shift from MDSCs toward immune-stimulatory myeloid cells, resulting in a ratio of MDSCs to T cells below the optimal suppressive level. However, highly proliferative tumor cells were sensitive to immunoediting due to the activity of ACT. The escape mechanisms induced by ACT included the induction of PD-L1 and loss of neu antigen on metastatic tumor

cells. A higher expression of PD-L1 in the no-ACT group compared with the ACT group suggests that endogenous innate immune response may be involved in the upregulation of PD-L1 on tumor cells. It was reported that innate IFN- γ is essential for upregulation of PD-L1 expression (207). Intriguingly, an adaptive immune response following ACT reduced PD-L1 expression on tumor cells compared with the no-ACT group, though it was still significantly higher than MMC tumor prior to challenge. This is important because reprogrammed T cells and NKT cells that were used for ACT expressed PD-1, and PD-1 expression was sustained after ACT. However, reprogrammed T cells also produce perforin and granzyme B (16) allowing them to induce apoptosis in tumor cells before they begin to upregulate PD-L1 mediated by IFN- γ . IFN- γ produced by reprogrammed T cells increases the level of PD-L1 expression compared with control MMC. Therefore, prolonged survival in the ACT group could be associated with lower expression of PD-L1 in MMC compared with no-ACT group, though animals succumbed to metastatic tumor, as their tumors begin to undergo antigen loss by downregulating the expression of neu. Therefore, our data suggest that tumors utilize numerous mechanisms to change during cell division and escape from immunotherapy. These mechanisms were shown to overcome tumor immune surveillance and reduce the efficacy of immunotherapy (142, 144, 148, 208). On the other hand, dormant tumor cells which were established by chemotherapy or RT became chemo-resistant or RT-resistant but remained sensitive to immunotherapy. Our findings are consistent with the reports on the efficacy of ACT in patients with metastatic melanoma utilizing tumor-infiltrating lymphocytes (TIL) grown in IL-2. ACT utilizing IL-2 expanded TIL resulted in tumor regression in 49% of patients (209). When ACT was combined with total body irradiation (TBI) objective responses increased to 72%. Among treated groups, 20% had

complete tumor regression and over 10 years relapse-free survival (76). Thus far, of the 34 complete responders in the NCI trials, one has recurred (210).

Dormant tumor cells contain Ki67+ indolent cells and Ki67- quiescent cells. Indolent tumor cells are still capable of proliferating, but represent balanced cellular proliferation and death; therefore they generally remain dormant. Thus, due to their proliferative nature, they remained sensitive to immunoediting and escape. On the contrary, Ki67- quiescent dormant cells displayed a reduced potential for immunoediting; they failed to upregulate PD-L1 in the presence of IFN- γ stimulation. Our data suggest that administration of immunotherapy after the completion of conventional cancer therapies, when tumor dormancy is established, could effectively target dormant tumor cells. The challenge, however, is to develop a combinatorial chemotherapeutic strategy which predominantly establishes quiescent dormancy so that tumors are incapable of escape from subsequent immunotherapy.

Recently, there have been dramatic advances in the field of cancer immunotherapy. However, these advances have been limited to increasing patients' survival for a limited period of time rather than offering a cure for cancer patients. For instance, Sipuleucel-T (Provenge) has extended survival of patients with metastatic prostate cancer by a median of 4.1 months (211). Blockade of immune checkpoint molecules has also prolonged survival in patients with advanced cancer. For instance, anti-CTLA-4 antibody (Ipilimumab) therapy resulted in a 3.5-month gain in overall survival in patients with stage III or IV metastatic cutaneous melanoma (212). Cumulative response rates for anti-PD-1 antibody therapy among patients with non-small-cell lung cancer, melanoma, and renal-cell cancer were 18%, 28%, and 27%, respectively. Responses were durable such that 20 of 31 responses lasted 1 year or more in patients with 1 year or more of follow-up (213). On the other hand, administration of immunotherapy in prophylactic settings

has been successful against many infectious diseases, as well as against HPV-associated cervical cancer. In addition, the application of stem cell transplantation and donor-derived lymphocyte infusion is successful only against MRD rather than against active and advanced stage disease. Therefore, it is reasonable to expect that administration of immunotherapy during MRD or tumor dormancy could prevent distant recurrence of breast cancer, thereby eliminating mortality associated with the advanced stages of the disease.

We also demonstrated that ACT utilizing reprogrammed T cells and CD25+ NKT cells resulted in the modulation of Gr1+CD11b+ MDSCs toward Gr1-CD11b-/lo immune stimulatory myeloid cells. These findings support our previous *in vitro* observations as well as the observations by other groups showing that NKT cells can convert MDSCs to APCs (16, 52-54, 187). We have previously reported that Gr1+CD11b+ MDSCs became immune stimulatory cells in the presence of NKT cells; fractionated reprogrammed T cells alone resulted in the failure of ACT in protecting animals from MMC tumor challenge (16). Here, we showed that conversion of MDSCs to Gr1-CD11b-/lo immune stimulatory myeloid cells takes place only in a fraction all of MDSCs. The remaining MDSCs persisted in their suppressive function, *in vitro*. Importantly, however, the proportion of T cells to MDSCs, *in vivo*, was found to be below the optimal suppressive ratio. It is yet to be determined if Gr1-CD11b-/lo immune stimulatory myeloid cells are predominantly converted from granulocytic or monocytic MDSCs. We have reported that MDSCs in MMC-bearing animals are mainly composed of the granulocytic phenotype, though only monocytic MDSCs were found to be able to suppress T cell responses (27). Therefore, if granulocytic MDSCs are converted to Gr1-CD11b-/lo immune stimulatory cells, they could dominate monocytic MDSCs. This could explain why the remaining MDSCs from the ACT group retained their immune suppressive function in the absence of newly converted Gr1-

CD11b-/lo immune stimulatory cells. However, the presence of newly converted immune stimulatory myeloid cells results in the failure of MDSCs to sufficiently suppress anti-tumor T cells, *in vitro* (16, 54). This could also explain the multifaceted function of monocytic versus granulocytic MDSCs in the exacerbation and amelioration of different diseases associated with the suppression or induction of specific types of the immune response. We have suggested that the term myeloid regulatory cells (Mregs) can better explain the functions of MDSCs which contain both monocytic and granulocytic phenotypes (214). In fact, we propose that MDSCs are a phenotype of Mregs which cannot be converted to immune stimulatory myeloid cells in a setting of cancer. In addition, controversial reports on the role of these cells in autoimmune diseases can be consolidated and understood in the context of their regulatory function under certain conditions, which is not merely limited to immune suppressive function of Gr1+CD11b+ Mregs. The newly converted immune stimulatory myeloid cells of the ACT group showed CD11b characteristics of APCs by expressing MHC class II as well as co-stimulatory molecules. However, they differed from conventional DCs or macrophages. The Gr1-CD11b-/loMHCII+ immune stimulatory cells that were established following ACT were also phenotypically different from those in tumor-bearing control mice, i.e., they differed in the expression of MHC class II and co-stimulatory molecules. The newly converted Gr1-CD11b-/lo myeloid cells were also functionally different from those in tumor-bearing control mice, as only those from the ACT group showed immune stimulatory function. These data suggest that ACT by using CD25+ NKT cells and T cells could act as *in situ* vaccine by converting a fraction of MDSCs to a unique APC which require further characterization.

In conclusion, we demonstrate that ACT can prolong survival of animals harboring an active disease. Our data suggest that ACT could cure breast cancer if it is administered during tumor

dormancy rather than during the active state of the disease. We also identified a new type of Gr1-CD11b-/lo immune stimulatory myeloid cell that were converted from Gr1+CD11b+ MDSCs, and which differed from conventional APCs. Future work to characterize the phenotype, function, and development of these cells could lead to strategies to harness the natural accumulation of MDSCs in cancer patients in order to promote their conversion to myeloid stimulatory cells which could lead to a more effective generation of endogenous anti-tumor immune responses.

Conclusions

The overall results of this work suggest that currently available clinically translatable immunotherapeutic strategies for the treatment of breast cancer may be best implemented to function as a relapse prophylaxis, and provides insights into the potential to reprogram the tumor microenvironment to support anti-tumor immune function.

In **Chapter One**, we evaluated the efficacy of treating established primary mammary carcinoma in the FVBN202 mouse with ACT utilizing reprogrammed immune cells, as developed by our group. We found this strategy to be ineffective in eliminating or inducing regression of primary mammary carcinoma, even after inducing the expression of highly immunogenic CTAs within the tumor. This suggests that established breast cancer may not represent the optimal target to which our immunotherapeutic strategy of ACT may be effective. In **Chapter Two**, we participated in a Phase II randomized clinical trial using Aza combined with an immunomodulator Rev and ACT in patients with multiple myeloma who harbored MRD. We demonstrated that Aza-Rev induced the expression of a panel of CTAs specifically within plasma cells harbored in the bone marrow. Such induction of CTA expression generated NY-ESO-1-specific lymphocytes which were collected and later reinfused into patients as a cellular therapy after chemotherapy and ASCT, in order to target residual malignant cells and prevent disease recurrence. This strategy was effective in generating objective responses for patients with residual multiple myeloma. Therefore, in **Chapter Three** we demonstrated that ACT using reprogrammed NKT cells and T cells combined with modulation of tumor using Dec is effective in prolonging survival of animals harboring early advanced experimental metastatic mammary carcinoma. However, this treatment failed to eliminate the tumor because of the induction of tumor immunoediting and escape which is characterized by neu antigen loss/downregulation and

upregulation of PD-L1 in tumor cells. Importantly, dividing tumor cells were sensitive to immunoediting whereas quiescent dormant cells were resistant to immunoediting and escape. We also demonstrated the striking effect of ACT utilizing reprogrammed T and NKT cells to modulate the splenic myeloid cell compartment, resulting in a reduction in the frequency of splenic MDSCs while also stimulating the generation of a novel phenotype of immune stimulatory cell, which functioned to enhance endogenous anti-tumor function *in vitro*.

Future Perspective

The results of this work suggest that utilizing ACT containing reprogrammed T cells and NKT cells may yield the most effective responses when used in a setting of minimal residual disease, during a period of dormancy after the implementation of conventional therapies. Future studies should investigate the ability of conventional therapeutics to induce quiescent dormancy, which is resistant to immunoediting and escape, followed by the administration of immunotherapy in order to prevent advanced stage disease.

Furthermore, our work suggests the ACT actively functions to modulate splenic myeloid cells to generate cells capable of promoting anti-tumor immune responses. Additional studies are required to determine if such ACT-generated Gr1-CD11b-/lo myeloid stimulatory cells possess antigen presentation capacity, as well as if they can be categorized into a cell lineage by characterization of the expression of common granulocytic or monocytic proteins, such as Ly6G and Ly6C, respectively. Also, it will be prudent to determine if such immune stimulatory cells are directly generated from MDSCs, or arise from an independent progenitor. Such information may prove to be essential in the development of our knowledge of the methods by which developing tumors usurp myelopoiesis, and may therefore generate novel strategies by which one may reestablish the 'normal' myelopoietic program to generate myeloid cells with immune stimulatory properties to further enhance the immune response against breast, and perhaps other solid tumors.

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209. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002;298(5594):850-4.
210. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* 2015;348(6230):62-8.
211. Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010;363(5):411-22.
212. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010;363(8):711-23.
213. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012;366(26):2443-54.
214. Manjili MH, Wang XY, Abrams S. Evolution of our understanding of myeloid regulatory cells: From MDSCs to mregs. *Front Immunol* 2014;5:303.

VITA

Personal Summary:

Kyle Kristopher Payne was born on the 11th of December, 1981 in Bedford, Indiana, and is an American citizen. He graduated with an honors diploma from Bedford North Lawrence High School in 2000 and went on to attend Purdue University from 2000-2001. He then attended Indiana University from 2001-2005 where he received a Bachelor of Science in Biology, with distinction, while also studying Chemistry and History among many other disciplines. After several contemplative years, Kyle entered Virginia Commonwealth University in the autumn of 2009 as a premedical certificate student, and then rediscovered his love of research under the mentorship of Dr. Masoud H. Manjili, who supported Kyle's transition to the MS program in May 2010 and Kyle's subsequent transfer to the PhD program in May 2011.

Education

- July 2015 Doctor of Philosophy; Virginia Commonwealth University, Richmond, VA
Dissertation project: Immunotherapy of Cancer: Reprogramming Tumor/Immune Cellular Crosstalk to Improve Anti-Tumor Efficacy
Director: Masoud H. Manjili, DVM, PhD
- May 2005 Bachelor of Science- Biology, *with distinction*; Indiana University
Minor Concentrations: Chemistry & History

Patents

Manjili MH, Kmiecik M, Toor AA, Idowu MO, Bear HD, **Payne KK**, Marincola FM, Wang E. Gene signatures associated with rejection or recurrence of cancer (PCT/US2012/030312, September 28, 2012).

Provisional Patents

Manjili MH, Toor A, Kmiecik M, **Payne KK**. Cancer testis antigens are prognostic biomarkers for breast cancer patients (Serial No: 61/537130; 09/21/2011)

Fellowships

1. The American Association of Immunologists Careers in Immunology Fellowship
Project Title: Cancer Immunotherapy: Reprogramming tumor-immune cellular crosstalk
Funded Years: 2014-2015

Awards/Honors

1. The American Association of Immunologists - 2015 AAI Trainee Abstract Award
Abstract Title: Adoptive Cellular Therapy containing T cells and CD25+ NKT cells modulates myeloid cells and stimulates endogenous anti-tumor immune function
2. St. Jude Children's Research Hospital – Postdoctoral Recruitment-2014
3. The Ohio State University – Selected to attend the 2014 Postdoctoral Recruitment Event
4. Virginia Commonwealth University - Nominated to attend the 64th Lindau Nobel Laureate Meeting - 2014

5. Adaptive Biotechnologies Corporation - Young Investigator Award

Project Title: Molecular signatures of T cell-mediated tumor rejection: the application of high throughput sequencing for immunomonitoring of cancer therapy

Awarded: August 2012

6. Virginia Commonwealth University Graduate Student Travel Award

Project Title: Immunotherapy of cancer: re-programming tumor-immune crosstalk

Awarded: October 2012

7. Virginia Commonwealth University Graduate Student Travel Award

Project Title: Sequential common gamma-chain cytokines facilitate differentiation of tumor-reactive T cells that are resistant to MDSC and can induce rejection of breast tumors. Awarded: April 2011

Professional Society Membership & Service

- Society for Immunotherapy of Cancer –Student Member (2011-present)
 - Early Career Scientist Committee Member (2014-present)
 - Co-chair 2014 Annual Meeting Professional Development Session
- The American Association of Immunologists – Trainee Member (2014-present)
- American Association for Cancer Research – Associate Member (2010-present)
- International Society for Translational Medicine – Member (2011-present)
- American Association of University Professors – Member (2014-present)

Manuscript Reviews

- Pathology – Research and Practice (2015)

Teaching Experience

1. Virginia Commonwealth University – School of Medicine - Richmond, Virginia

- Invited Instructor - MICR 686; Advanced Immunology – Spring Semester, 2015

Topic: Crosstalk between NK/NKT cells and MDSC can rescue T cells from suppression

2. Virginia Commonwealth University – School of Medicine - Richmond, Virginia

- Invited Instructor - MICR 686; Advanced Immunology – Spring Semester, 2014

Topic: *Ex vivo* reprogramming of human T cells for adoptive immunotherapy of cancer

3. Virginia Commonwealth University – School of Medicine - Richmond, Virginia

- Invited Instructor - MICR 686; Advanced Immunology – Spring Semester, 2013

Topic: Crosstalk between NK/NKT cells and MDSC can rescue T cells from suppression

4. Virginia Commonwealth University – School of Medicine - Richmond, Virginia

- Invited Instructor - MICR 686; Advanced Immunology – Spring Semester, 2012

Topic: Epigenetic modulation as an immunotherapeutic approach in cancer

5. Virginia Commonwealth University – School of Dentistry - Richmond, Virginia

- Graduate Teaching Assistant – MICR 513; Infection and Immunity – Fall Semester, 2011

6. Indiana University – Department of Biology - Bloomington, Indiana

- Undergraduate Teaching Instructor – Biological Mechanisms – Spring and Fall Semester, 2004

Community Service

- Higher Achievement – Richmond, Virginia; 2013-2015

Peer-Reviewed Publications

1. Manjili MH, **Payne KK**. (2015). Prospects in cancer immunotherapy: treating advanced stage disease or preventing tumor recurrence? *Discovery Medicine*. 19(107):427-31. [Review].
2. **Payne KK**, Bear HD, Manjili MH. (2014). Adoptive cellular therapy of cancer: exploring innate and adaptive cellular crosstalk to improve anti-tumor efficacy. *Future Oncol*. 10(10): 1779-94. [Review].
3. Kmiecik M, **Payne KK**, Wang XY, Manjili MH. (2013). IFN- γ R α is a key determinant of CD8+ T cell-mediated tumor elimination or tumor escape and relapse in the FVB mouse. *PLoS One*. 8:12, e82544
4. Gowda M, **Payne KK**, Godder K, Manjili MH. (2013). HLA-DR expression on myeloid cells can be a potential prognostic factor in patients with high risk Neuroblastoma. *Oncoimmunology*. 2:10, e26616

5. **Payne KK**, Zoon CK, Wan W, Marlar K, Borelli CA, Keim RC, Kenari MN, Kazim AL, Bear HD, Manjili MH. (2013). Peripheral blood mononuclear cells of patients with breast cancer can be reprogrammed to enhance anti-HER-2/neu reactivity and overcome myeloid-derived suppressor cells. *Breast Cancer Res Treat.* 142(1):45-57
6. Ascierto ML, Idowu MO, Zhao Y, Khalak H, **Payne KK**, Wang XY, Dumur CI, Bedognetti D, Tomei S, Ascierto PA, Shanker A, Bear HD, Wang E, Marincola FM, De Maria A, Manjili MH. (2013). Molecular signatures mostly associated with NK cells are predictive of relapse free survival in breast cancer patients. *J Transl Med.* 11:145.
7. Meier JM, Roberts C, Avent K, Hazlett AF, Berrie J, **Payne KK**, Hamm D, Chung HM, Desmarais C, Hogan KT, Archer KJ, Manjili MH, Toor AA. (2013). Fractal Organization of the Human T Cell Repertoire in Health and Following Stem Cell Transplantation. *Biol Blood Marrow Transplant.* 19(3):366-77
8. **Payne KK**, Toor AA, Wang XY, Manjili MH. (2012). Immunotherapy of cancer: re-programming tumor-immune crosstalk. *Clin Dev Immunol.* 2012:760965 [Review]
9. **Payne KK**, Manjili MH. (2012). Adaptive immune responses associated with breast cancer relapse. *Arch. Immunol. Ther. Exp.* 60:345–350 [Review]
10. Toor AA, **Payne KK**, Chung HM, Hazlett AF, Sabo RT, Clark WB, Kmiecik M, Anderson J, Buskey A, Roseff S, Williams DC, Roberts C, McCarty J, Manjili MH (2012). Epigenetic Induction of Adaptive Immune Response in Multiple Myeloma: Sequential Aza and Lenalidomide to Generate Cancer Testis Antigen Specific Cellular Immunity. *Br J Haematol.* 158(6):700-11

11. Manjili MH, **Payne KK**. (2012). Cancer immunotherapy: re-programming cells of the innate and adaptive immune systems. *Oncoimmunology*, 1:2, 201-204 [Review]
12. Kmiecik M*, Basu D*, **Payne KK***, Toor A, Yacoub A, Graham L, Sale L, Bear HD, Manjili MH. (2011). Activated NK T cells and NK cells render T cells resistant to MDSC and result in an effective adoptive cellular therapy against breast cancer in the FVBN202 transgenic mouse. *J Immunol*, 187; 708-717 ***Authors Contributed Equally**
13. Kmiecik M, **Payne KK**, Idowu MO, Grimes MM, Graham L, Ascierto ML, Wang E, Bear HD, Manjili MH. (2011). Tumor escape and progression of HER-2/neu negative breast cancer under immune pressure. *J Transl Med*, 9:35
14. Sumpter TL, **Payne KK**, Wilkes DS. (2008). Regulation of the NFAT pathway discriminates CD4+CD25+ regulatory T cells from CD4+CD25- helper T cells. *J Leukoc Bio*, 83: 708-717.
15. Smith GN, Mickler EA, **Payne KK**, Lee J, Duncan M, Reynolds J, Foresman B, Wilkes DS. (2007). Lung transplant metalloproteinase levels are elevated prior to bronchiolitis obliterans syndrome. *Am J Transplant*, 7(7):1856-61.

Extramural Presentations

1. **Payne KK**, Graham L, Bear HD, Manjili MH. (2015). Adoptive Cellular Therapy containing T cells and CD25+ NKT cells modulates myeloid cells and stimulates endogenous anti-tumor immune function. [Oral Presentation]. Presented at: *IMMUNOLOGY 2015™, Annual Meeting of The American Association of Immunologists, May 8-12, The American Association of Immunologists, Inc., New Orleans, Abstract VAC7P.1038*

2. **Payne KK**. (2014). Cancer immunotherapy: Reprogramming tumor/immune cellular crosstalk to improve anti-tumor efficacy. [Oral Presentation] Presented at St. Jude Children's Research Hospital; Postdoctoral Candidate Seminar, Memphis Tennessee; December 16, 2014.
3. **Payne KK**. (2014). Reprogramming tumor-immune crosstalk for the immunotherapy of cancer. [Oral Presentation] Presented at The Ohio State University; 2014 Postdoctoral Recruitment Event, Columbus Ohio; October 2, 2014.
4. **Payne KK**, Graham L, Bear HD, Manjili MH. (2014). Cancer immunotherapy: treatment of established breast cancer or prevention of tumor relapse and metastasis? [abstract] Presented at: *IMMUNOLOGY 2014™, Annual Meeting of The American Association of Immunologists, May 2-6, The American Association of Immunologists, Inc., Pittsburgh, Abstract 71.27*
5. **Payne KK**, Zoon CK, Wan W, Marlar K, Borelli CA, Keim RC, Kenari MN, Kazim AL, Bear HD, Manjili MH. (2013) Peripheral blood mononuclear cells of patients with breast cancer can be reprogrammed to enhance HER-2/neu reactivity and overcome myeloid-derived suppressor cells. [abstract] SITC 28th Annual Meeting, National Harbor, Maryland; November 7-10. Abstract number 191.
6. Meier J, Scalora A, Avent K, Berrie J, **Payne KK**, Hamm D, Desmarais C, Sanders C, Hogan KT, Grant S, Archer K, Manjili MH, Roberts CH, Toor AA (2012). Fractal Organization of the Human T Cell Repertoire in Health and Following Stem Cell Transplantation. [abstract] 54th ASH Annual Meeting and Exposition, Atlanta, Georgia; December 8-11.

7. **Payne KK**, Roberts C, Sabo R, Chung H, Clark W, McCarty J, Manjili MH, Toor AA. (2012). Aza for induction of adaptive immunity in multiple myeloma. [abstract] 2nd International Workshop on The Biology, Prevention, and Treatment of Relapse After Hematopoietic Stem Cell Transplantation. National Institutes of Health, Bethesda, Maryland; November 5-6.
8. **Payne KK**, Hall CE, Toor AA, Bear HD, Wang XY, Manjili MH. (2012). Immunotherapy of cancer: re-programming tumor-immune crosstalk. [abstract] SITC 27th Annual Meeting, Bethesda, Maryland; October 24-28. Abstract number 18.
9. Toor AA, **Payne KK**, Chung HM, Roberts CH, Sabo RT, Kmiecik M, Clark W, McLaughlin C, Buskey A, Anderson J, Manjili RH, Roseff SD, McCarty JH, Manjili MH. (2011). Adoptive Immunotherapy In Multiple Myeloma: Epigenetic Modification and Immunomodulation by Sequential Aza and Lenalidomide to Generate Cancer Testis Antigen Specific Cellular Immune Responses. [abstract] 53rd ASH Annual Meeting and Exposition, San Diego, California; December 10-13.
10. **Payne KK**, Toor AA, Manjili MH. (2011) Cancer Testis Antigens as prognostic biomarkers for breast cancer patients. [abstract] SITC 26th Annual Meeting, Bethesda, Maryland; November 4-6.
11. Ascierto ML, **Payne KK**, Kmiecik M, Idowu MO, Dumur C, Bedognetti D, De Maria A, Wang E, Marincola FM, Manjili, MH. (2011). Role of NK and NKT cells in Breast Cancer Patients: association with favorable prognosis. [abstract] SITC 26th Annual Meeting, Bethesda, Maryland; November 4-6.
12. **Payne KK**, Kmiecik M, Basu D, Ramakrishnan V, Bear H, Manjili M (2011). Sequential

common gamma-chain cytokines facilitate differentiation of tumor-reactive T cells that are resistant to MDSC and can induce rejection of breast tumors. [abstract] In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract number 775.

Intramural Presentations

1. **Payne KK**. Cancer Immunotherapy: Reprogramming Tumor/Immune Cellular Crosstalk to Improve Anti-Tumor Efficacy. [Oral Presentation] Final Defense. VCU Department of Microbiology and Immunology, School of Medicine; July 9, 2015
2. **Payne KK**, Graham L, Bear HD, Manjili MH. (2015). Adoptive Cellular Therapy containing T cells and CD25+ NKT cells modulates myeloid cells and stimulates endogenous anti-tumor immune function. [abstract] VCU Massey Cancer Center Research Retreat, May 22, 2015
3. **Payne KK**, Graham L, Bear HD, Manjili MH. (2015). Adoptive Cellular Therapy containing T cells and CD25+ NKT cells modulates myeloid cells and stimulates endogenous anti-tumor immune function. [Abstract] 11th Annual VCU Women's Health Research Day, April 2, 2015
4. Sappal S, **Payne KK**, Wan W, Idowu M, Dumur CI, Manjili MH. Cancer Testis Antigen Expression Effect on Breast Cancer Recurrence. [abstract] Medical Student Honors Day, VCU School of Medicine, May 1, 2015
5. Wallace MM, **Payne KK**, Keim RC, Dumur CI, Manjili MH. Molecular pathways of breast cancer dormancy and relapse. [abstract] Medical Student Honors Day, VCU School of Medicine, May 1, 2015

6. **Payne KK**. Re-programming of tumor-immune crosstalk for the immunotherapy of breast cancer. [Seminar Speaker] VCU Department of Microbiology and Immunology Seminar, November 21, 2013
7. **Payne KK**, Zoon CK, Wan W, Marlar K, Borelli CA, Keim RC, Kenari MN, Kazim AL, Bear HD, Manjili MH. Peripheral blood mononuclear cells of patients with breast cancer can be reprogrammed to enhance HER-2/neu reactivity and overcome myeloid-derived suppressor cells. [abstract] VCU Massey Cancer Center Research Retreat, June 13, 2013
8. **Payne KK**, Kmiecik M, Wang XY, Manjili MH. IFN- γ R α is a key determinant of CD8+ T cell-mediated tumor elimination or tumor escape and relapse. [abstract] VCU Massey Cancer Center Research Retreat, June 13, 2013
9. Saleem SJ, Martin RK, Zellner HB, Dean LM, Ngyen K, **Payne KK**, Bear HD, Manjili MH, Irani AM, Conrad DH. Mast Cell-histamine release promotes (is required) for the activity of monocytic myeloid derived suppressor cells. [abstract] VCU Massey Cancer Center Research Retreat, June 13, 2013
10. **Payne KK**. Epigenetic modulation of tumor and immune cell re-programming as a therapeutic approach against cancer. [Seminar Speaker] VCU Department of Microbiology and Immunology Seminar, November 29, 2012
11. **Payne KK**, Hall CE, Toor AA, Bear HD, Wang XY, Manjili MH. (2012). Immunotherapy of cancer: re-programming tumor-immune crosstalk. [abstract] VCU Massey Cancer Center Research Retreat, October 18, 2012

12. **Payne KK**, Zoon C, Graham L, Borrelli C, Detwiler M, Wan W, Kazim L, Bear HD, Manjili MH. Reprogramming of tumor-sensitized immune cells for immunotherapy of breast cancer. [Seminar Speaker] VCU Breast Cancer Research Interest Group, August 2, 2012.
13. **Payne KK**. Epigenetic modulation of tumor and immune cell re-programming as a therapeutic approach against cancer. [Seminar Speaker] VCU Department of Microbiology and Immunology Seminar, January 19, 2012
14. **Payne KK**. *In situ* vaccination to overcome tumor escape and relapse. [Seminar Speaker] VCU Breast Cancer Research Interest Group, July 7, 2011
15. Reed T, **Payne KK**, Kmiecik M, Knutson KL, Dumur CI, Manjili MH, Hartman, MCT. (2011) Discovery of a cell surface marker for early detection of tumor escape and relapse of breast cancer. [abstract] VCU Massey Cancer Center Research Retreat, November 7, 2011
16. **Payne KK**, Toor AA, Manjili MH. (2011) Cancer Testis Antigens as prognostic biomarkers for breast cancer patients. [abstract] VCU Massey Cancer Center Research Retreat, November 7, 2011
17. **Payne KK**, Roberts CH, Hazlett A, Alesi E, Kmiecik M, Sabo RT, Clark W, Manjili RH, McCarty JM, Chung HM, Toor AA, Manjili MH. (2011) Epigenetic Modifications Induce Adaptive Immunity: Generation of Cancer Testis Antigen specific cellular immune responses in multiple myeloma patients. [abstract] VCU Massey Cancer Center Research Retreat, November 7, 2011
18. **Payne KK**, Toor AA, Manjili MH. (2011) Cancer Testis Antigens as prognostic biomarkers for breast cancer patients. [abstract] Virginia Commonwealth University; Watts Research Poster Symposium, October 18-19, 2011

19. **Payne KK**, Kmiecik M, Basu D, Ramakrishnan V, Bear H, Manjili M (2011). Sequential common gamma chain cytokines can expand cells of the adaptive and innate immune systems that can overcome myeloid-derived suppressor cells and provide long-term memory against breast cancer upon adoptive cellular therapy. [Abstract] 7th Annual VCU Women's Health Research Day, April 28, 2011
20. **Payne KK**, Kmiecik M, Basu D, Ramakrishnan V, Bear H, Manjili M (2010). Sequential common gamma-chain cytokines facilitate differentiation of tumor-reactive T cells that are resistant to MDSC and can induce rejection of breast tumors. [abstract] Virginia Commonwealth University Massey Cancer Center Research Retreat, October 15, 2010