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Preconditioning of the tumor microenvironment by means of low dose chemotherapies for an effective immunotherapy of breast cancer

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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

First, I will dedicate this study to my Almighty God,

Who gave me strength and knowledge every day of my life.

To my parents "(deceased)" who inspired me to be strong despite of many obstacles in life.

To my brothers and sisters for their eternal love.

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List of Abbreviations

ADR – Adriamycin

AIT - Adoptive immunotherapy

AO – Acridine orange

$ATG5^{KD}$ - Stable knockdown of ATG5

B/I - Bryostatin 1/ionomycin

CHAPS - (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate)]

CISD2 - CDGSH iron sulfur domain 2

CLL - chronic lymphocytic leukemia

CQ – Chloroquine

CT – Untreated MMC

CTC - Circulating tumor cells

CYP – Cyclophosphamide

DCs - Dendritic cells

DNA - Deoxyribonucleic acid

DT - FAC-treated dormant MMC

DTC - disseminated tumor cells

DTT - dithiothreitol

ECE – ET-1 converting enzyme

EDTA - Ethylenediaminetetracetic acid

ER - Endoplasmic reticulum

ETR_A – endothelin receptor A

ETR_B – endothelin receptor B

ET-1 – endothelin ligand

FAC – 5-Fluorouracile, Adriamycin, and Cyclophosphamide

FACS - Fluorescence-activated cell sorting

FBS - Fetal bovine serum

FVS - Fixable viability stain

GBM - glioblastoma multiform

H&E – Hematoxylin and Eosin

HC - health control tissues

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

ICD - Immunogenic cell death

IFN-γ - Interferon gamma

IHC – Immunohistochemistry

InDels – Deletions and insertions

LAMP2A - lysosomal membrane associated protein 2 A

Maci - Macitentan

MAPK - mitogen activated protein kinase

MDSC - Myeloid-derived suppressor cells

MHC - Major histocompatibility complex

MMC – neu overexpressing mouse mammary carcinoma

P13K - phosphatidylinositol 3-kinase

PD-1 - Programmed cell death protein 1

PDL-1 - Programmed death ligand 1

PI - Propidium iodide

SCLC - Small cell lung cancer

SEM - Standard error of the mean

SNP - Single nucleotide polymorphism

TC - Untreated MMC control

TNBC – Triple negative breast cancer

TSLPR – Thymic stromal lymphopoietin receptor

γ-c - Gamma-chain

5-FU – 5-Fluorouracile

Abstract

PRECONDITIONING OF THE TUMOR MICROENVIRONMENT BY MEANS OF LOW DOSE CHEMOTHERAPIES FOR AN EFFECTIVE IMMUNOTHERAPY OF BREAST CANCER

By

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

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Breast cancer mortality is mainly due to distant recurrence of the disease arising from dormant tumor cells established by cancer therapies. Patients who initially respond to cancer therapies often succumb to distant recurrence of the disease. It is not clear why people with the same type of breast cancer respond to treatments differently; some escape from dormancy and relapse earlier than others. In addition, some tumor clones respond to immunotherapy while others do not. We investigated how autophagy plays a role in accelerating or delaying recurrence of neu overexpressing mouse mammary carcinoma (MMC) following adriamycin (ADR) treatment, and in affecting response to immunotherapy. We explored two strategies: 1) transient blockade of autophagy with chloroquine (CQ), which blocks fusion of autophagosomes and lysosomes during ADR treatment, and 2) permanent inhibition of autophagy by a stable knockdown of ATG5 ($ATG5^{KD}$), which inhibits the formation of autophagosomes in MMC during and after ADR treatment. We found that while CQ prolonged tumor dormancy, but that stable knockdown of autophagy resulted in early escape from dormancy and recurrence. Interestingly, $ATG5^{KD}$ MMC contained an increased frequency of ADR-induced polyploid-like cells and rendered MMC resistant to immunotherapy. On the other hand, a transient blockade of autophagy did not affect the sensitivity of MMC to immunotherapy. Our observations suggest that while chemotherapy-induced autophagy may facilitate tumor relapse, cell-intrinsic autophagy delays tumor relapse, in part, by inhibiting the formation of polyploid-like tumor dormancy.

Although immunotherapy of breast cancer by means of anti-HER2 antibodies prolongs survival of breast cancer patients, disease recurrence remains a major challenge. On the other hand administration of human vaccines against infectious disease in a preventive setting or during latency/dormancy has been successful in offering a cure. Here, we sought to use adoptive immunotherapy (AIT) at the time of tumor dormancy in order to prevent progression of breast cancer.

We used a low dose immunogenic chemotherapy by means of 5-FU, Adriamycin, and Cyclophosphamide (FAC) in order to stabilize tumor progression prior to AIT using autologous tumor-reactive lymphocytes. Low dose FAC established local tumor dormancy, inhibited distant tumor dormancy occurring long before distant metastasis, and induced predominate a Ki67-quiescent type of tumor dormancy, which is less susceptible to tumor immunoediting. Dormant tumor cells expressed the cell survival pathways, including the endothelin receptor/ligand (ETR_A, ETR_B and ET-1) and PD-L1, thereby protecting them from elimination by AIT. In addition, tumor-reactive CD8+ T cells also produced ET-1 as a survival ligand for ETR_A positive tumor cells. A combination of AIT with the blockade of tumor cell survival pathways resulted in a significant improvement of AIT against tumor dormancy. We also showed that the inhibition Bcl-xL downstream of the tumor cell survival pathways is specifically effective against dormant tumor cells, suggesting a combination of AIT with small molecules inhibitors of Bcl-xL. Altogether, we showed that distant tumor dormancy is established long before distant recurrence of breast cancer, and that the expression of several tumor cell survival pathways in dormant cells protects them from immunotherapy. Our results suggest that immunotherapeutic targeting of tumor dormancy combined with the blockade of a common downstream cell survival pathway could prevent tumor progression and recurrence of the disease.

Chapter 1. Introduction

1.1. Breast cancer, tumor dormancy and tumor relapse

1.1.1. Naturally-occurring tumor dormancy prior to or during the detection of primary cancer

A recent review on tumor dormancy highlighted clinical evidence in support of the existence of natural tumor dormancy in healthy individuals (1). This phenomenon is called “cancer without disease” (2). Post-mortem autopsy specimens obtained from individuals who did not have cancer-related disease in their life-time revealed that 30% of healthy women had in situ malignant lesions in their breast, though only 1% of women in this age range get breast cancer. Similar observations were reported for prostate lesions in cancer free men. Interestingly, all autopsied individuals aged 50 to 70 had in situ carcinomas in the thyroid gland, whereas the incidence of thyroid cancer in this age group is only 0.1% (2). Pancreatic intraepithelial neoplasia being in dormant state is remarkably common, particularly in cancer free elderly (3). These data suggest that tumor dormancy precedes primary cancer development, and that tumor cells could remain dormant for the life-time of an individual without ever causing cancer. Recent evidence from patients with solid malignancies also indicates that metastasis is a very early event such that even small tumors (<5 mm) can establish metastasis long before they become detectable at the primary site. This phenomenon is defined as early dissemination but late metastasis, because metastatic cells could lie dormant for even a decade and then re-emerge as metastatic disease. More recently, the observations made in two groups of cancer patients have further challenged the classical view of tumor metastasis. The first group of patients are those with metastatic lesions either before the primary tumor became clinically detectable, or when harboring primary cancer at very early stage without local invasion. For instance, patients with stage M0 breast cancer could relapse after

complete resection of their primary tumor, and their metastatic tumor had significantly fewer genetic abnormalities than the primary tumor (4). Studies in melanoma model demonstrated that tumor cells were disseminated throughout the body even before primary tumor became clinically detectable (5). Mechanistic studies revealed that in early lesions prior to establishment of breast cancer there was a sub-population of early cancer cells that spread to distant organs. Further studies demonstrated that progesterone-induced signaling induces dissemination of malignant cells from early lesions shortly after Human epidermal growth factor receptor-2 (HER2) activation and prior to breast cancer development (6).

1.1.2. Breast cancer therapies, tumor dormancy and recurrence

In general, patients with cancer are often at risk of developing distant recurrence of the disease years or decades after successful treatment of their primary cancer. This phenomenon can be explained by cancer dormancy, a stage that residual disease is present but remains asymptomatic, and most often, undetectable. Tumor dormancy is present in almost all cancers, particularly breast cancer. Up to 30% of early stage breast cancers with no evidence of metastasis will relapse in distant organs less than a decade after the treatment of primary cancer (7). Dormant breast cancer cells have been detected as adherent disseminated tumor cells (DTC) that reside in metastatic-free distant organs (8), as well as in the bone marrow of breast cancer patients (9,10). Adherent DTC has also been reported in a mouse model of breast cancer (11). Another form of tumor dormancy is present in the bloodstream as circulating tumor cells (CTCs) (12). CellSearch® System has been applied to noninvasive monitoring of CTCs in breast cancer patients as well as the isolation of single cells for genomic analysis of tumor dormancy (13). Detection of non-adherent CTC in cancer patients is not limited to those with metastatic disease, as patients with non-metastatic cancer or early stage breast cancer also show CTC (14,15). Detection of CTC in breast cancer

patients even 22 years after the completion of conventional cancer therapies (14) suggest that cancer survivors are at risk of tumor recurrence at any time as soon as CTC become adherent and re-grow in a distant organ.

Immunotherapeutic targeting of active stage breast cancer has been able to prolong patients survival, but like other cancer therapies has not been able to overcome tumor dormancy and recurrence. Trastuzumab has been the most promising immunotherapeutic for HER2 overexpressing breast cancer either as a single agent or in combination with chemotherapy by improving the progression free survival up to 4.9 months (16) or 7.4 months (17), respectively. However, around 70% of HER2 positive breast cancers do not respond to Trastuzumab (18), and 31% of patients who respond will experience a recurrence within one year (19,20). The addition of Pertuzumab to Trastuzumab and chemotherapy has improved 4-year disease-free survival only by 3.2% in patients with early stage breast cancer (21).

1.2. A paradoxical role of autophagy in tumor dormancy and relapse

Cell-intrinsic autophagy is a housekeeping mechanism in the cells to get rid of misfolded or aggregated proteins, which otherwise become toxic to the cells. Cancer therapies such as chemotherapy and radiation therapy, which cause Deoxyribonucleic acid (DNA) breaks and Endoplasmic reticulum (ER) stress, can increase autophagy beyond homeostatic autophagy which is termed treatment-induced autophagy. Autophagy preserves cellular integrity by degrading p62 which otherwise could either go to the nuclei and inhibit DNA repair (22) or accumulate in the cytosol and increase genomic instability (23). Efficient autophagy could sensitize tumor cells to treatment-induced apoptosis (22,24) or facilitate tumor dormancy (25–27). On the one hand, inhibition of autophagy by chloroquine (CQ) may sensitize tumor cells to chemotherapy (28,29); on the other hand, induction of autophagy by metformin suppresses several types of cancers

(30,31). The state and localization of p53 are important factors which impact the efficiency and outcome of autophagy such that nuclear localization of mutant p53 ($p53^{mu}$) participates in drug resistance whereas its cytosolic localization inhibits autophagy; on the other hand, nuclear localization of wild type p53 ($p53^{wt}$) results in drug-induced apoptosis or dormancy, and its cytosolic localization inhibits autophagy (32,33). Autophagic flux represents the dynamic process of autophagosome generation, their fusion with lysosomes, and the degradation of autophagic substrates in autolysosomes. Mammalian autophagy can occur via at least two different pathways, which include ATG5/ATG7/LC3B-dependent conventional pathway and an ATG5/ATG7/LC3B-independent alternative pathway (34). LC3 is currently the most widely used autophagosome marker because the amount of LC3B reflects the number of autophagosomes and autophagy-related structures. However, the LC3B amount at a given time point does not necessarily estimate the autophagic activity, because not only autophagy activation but also inhibition of autophagosome degradation greatly increases the amount of LC3B. Degradation of p62 is another widely used marker to monitor autophagic activity because p62 directly binds to LC3 and is selectively degraded by autophagy. Accumulation of p62 protein is believed to result from impaired autophagy, a condition that has been described in many tumor types, including cancers of the lung, breast, colon, and prostate where p62 accumulation has been linked to poor prognosis (35–38).

1.3. Low dose chemotherapy and the induction of anti-tumor immune responses

Low-dose metronomic chemotherapy, which utilizes lower, less toxic, doses given on a close regular or daily basis over prolonged periods, has been shown to control tumor progression in patients with early stage as well as those with advanced-stage cancers (39). While a standard dose of chemotherapy is highly toxic and unsuitable for being simultaneously combined with

immunotherapy, low dose metronomic chemotherapy does not harm lymphocytes, and also induces anti-tumor immune responses (40–43). Low dose chemotherapy is a promising combination partner for immunotherapy. For instance, breast cancer patients who were treated with low dose cyclophosphamide (CYP) showed decreased Tregs and increased effector T cells as well as NK-dependent anti-tumor immunity (40,41). Similar results were reported for low dose 5-Fluorouracile (5-FU) in depleting Myeloid-derived suppressor cells (MDSCs) (42). CYP was also reported to induce Immunogenic cell death (ICD) (44). Low dose metronomic chemotherapy was shown to be a suitable preparative regimen for vaccination approach in order to boost anti-tumor immune responses against dormant cells (43).

1.4. Tumor dormancy and tumor survival pathways

Although dormant tumor cells might contain cancer stem cells that did not respond to chemotherapies, dormant cells do not always show markers of cancer stem cells (1,45,46). Several reports have shown that the upregulation of various stress-induced UPR-associated genes like heat shock proteins and cyclophilin B regulate metastatic tumor dormancy (47–49). In addition, tumor cells can survive cancer therapeutics through the expression of survival receptors that transmit signals for the induction of anti-apoptotic genes in tumor cells. For instance, in humans, endothelin receptor A (ETR_A) acts as a survival receptor by inducing the expression of anti-apoptotic genes in prostate cancer (50). Its ligand, ET-1 is produced by the prostate epithelia (50). The presence of the ETR_A/ET-1 pathway at the tumor site makes tumor infiltrating T cells less effective in patients with prostate cancer. A higher responsiveness of melanoma patients to immunotherapy compared with patients with prostate cancer or ovarian cancer is associated with the upregulation of ETR_A in prostate and ovarian cancers but not in melanoma (51). Human Dendritic cells (DCs) also produce ET-1 upon activation (52), which in turn supports survival of T cells during activation as

well as survival of tumor cells that express ETR_A. ET-1 produced by T cells or tumor cells could also act as a paracrine or autocrine loop, respectively, for tumor cell survival.

ET-1 is a vasoconstrictor, which forms the endothelin system together with ET-1 converting enzyme (ECE) and ETR_A and ET receptor B (ETR_B). ECE is a membrane-bound neutral metalloprotease that cleaves the 38-aa inactive intermediate big ET-1 into a 21-aa active ET-1 on the cell membrane (53). As shown in Figure 1, major pathways and effectors downstream of ET receptors include mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways, adenylyl cyclase and phospholipases (PLCβ and PLA2) (54). ET-1 through ETR_A promotes tumor cell survival and proliferation in colorectal cancer (55), and in breast cancer where a statistically significant correlation between ETR_A expression and reduced disease-free survival time has been reported (56–58). This is associated with the induction of Bcl-2/Bcl-xL anti-apoptotic molecules downstream of ETR_A signaling (Figure 1). The affinity ETR_A for ET-1 is greater than that for ET-2 and the lowest affinity for ET-3, whereas the ETR_B shows equal affinity for ET-1, ET-2 or ET-3. There are cancers expressing predominantly ETR_A such as ovarian, nasopharyngeal, thyroid, prostate, colon, pancreatic, gastric, renal, and breast cancers, and others predominately expressing ETR_B such as melanoma, glioblastoma multiform (GBM), and astrocytoma, and those expressing both ETR_A and ETR_B such as oral, lung, bladder, vulvar cancers, and Kaposi's sarcoma (59). However, the role of ETR_A in facilitating tumor escape from breast cancer therapies, immunotherapy in particular, is unknown.

Expression of programmed death ligand 1 (PD-L1) is another mechanism of tumor cell survival through the engagement with PD-1, which in turn results in the induction of an anti-apoptotic gene, Bcl-xL in PD-L1 positive tumor cells (52) (Figure 2). An altered expression of PD-L1 correlates with not only cancer progression but also autoimmune diseases. For instance, PD-L1 loss has been

reported in children with systemic lupus erythematosus, and expression of PD-L1 is restored only during disease remission (60). The expression of PD-L1 on activated T cells support their survival such that PD-L1-deficient T cells express lower Bcl-xL, which is an anti-apoptosis gene, than wild-type cells and are more sensitive to apoptosis *in vivo* (61) (Figure 2). Tumor cells exploit this pathway by the expression of PD-L1 in order to survive immune surveillance. We have reported that tumor escape from immunotherapy was associated with the expression of PD-L1 on metastatic mammary tumor cells (62). These receptors support tumor escape and progression in the presence of cancer therapies.

When cells undergo death triggered by chemotherapeutic agents, the Bcl-2-family-dependent mitochondrial apoptotic pathway is activated. The Bcl-2 family consists of three main groups: pro-survival (e.g., Bcl-2, Mcl-1, Bcl-xL), multi-domain pro-apoptotic (e.g., BAX, BAK), and BH3-only pro-apoptotic (e.g., BAD, BID, BIM, Noxa) (63–65). It has been reported that monotherapy with navitoclax (ABT-263, targeted to inhibit Bcl-2 and Bcl-xL) or venetoclax (ABT-199, targeted to inhibit Bcl-2) is effective in chronic lymphocytic leukemia (CLL) and small cell lung cancer (SCLC) in clinical trials (66–68). ABT-199 is a first-in-class oral Bcl-2 inhibitor for the treatment of CLL, and is also in a phase 1b clinical trial for Bcl-2 positive metastatic breast cancer (69), phase I/II trial for non-Hodgkin lymphomas (including diffuse large B-cell lymphoma, mantle cell lymphoma, follicular lymphoma) and acute myeloid leukaemia, and phase I development for multiple myeloma, systemic lupus erythematosus (70). Due to the increasing complexity of developing Mcl-1 specific inhibitors, these drugs have been published only in the past year. A Sevier/Novartis first in-class Mcl-1 inhibitor, S63845, has shown monotherapeutic activity in diverse hematological cancers (71). Another Mcl-1 inhibitor, AMG 176 (Amgen), has also demonstrated to have monotherapeutic activity as well (72). AMG 176 is already in clinical trials

(NCT02675452) as is a Servier Mcl-1 inhibitor with a closely-related structure to S-63845 (NCT02979366). When combined with chemotherapy, a selective inhibitor of Bcl-xL (A-1331852) is highly effective against solid tumors without causing neutropenia observed with ABT-199 (73).

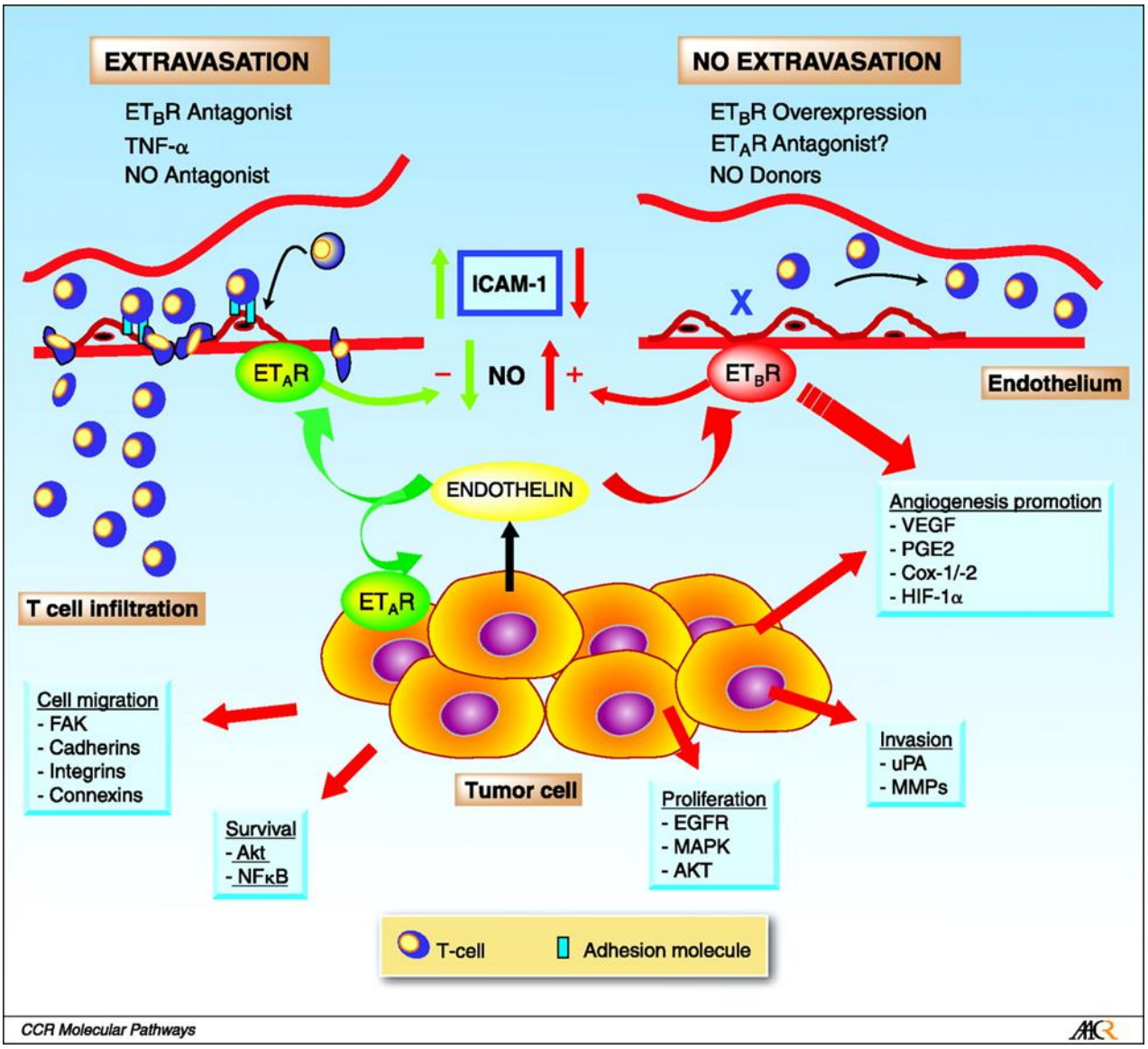


Figure 1. Tumor survival pathway: Endothelin receptor A

Adapted from Clin Cancer Res. 2009; 15:4521-8

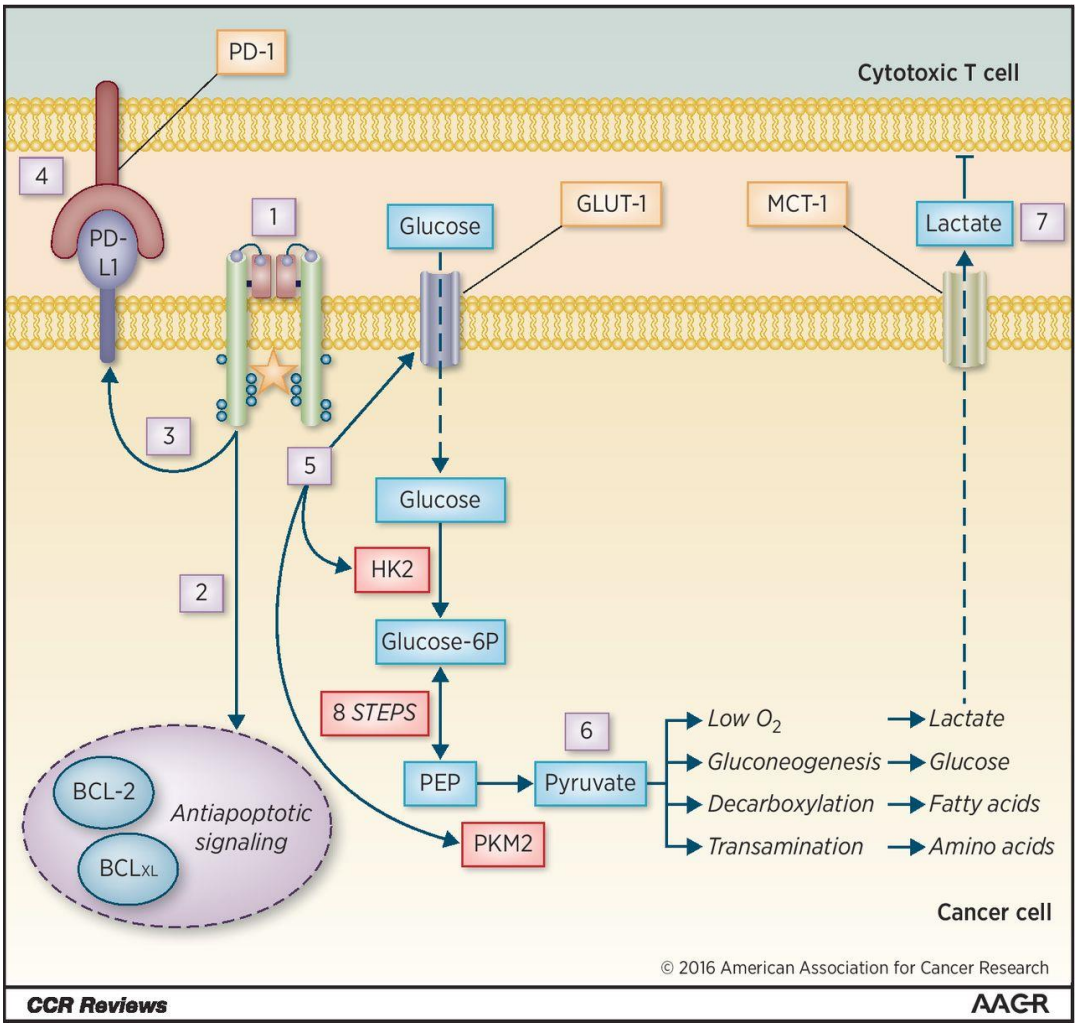


Figure 2. Tumor survival pathway: PD-L1

Clin Cancer Res. 2016; 22:4005-13

Chapter 2. Materials and Methods

2.1. Mouse model

FVBN202 transgenic female mice (The Jackson Laboratory; Bar Harbor, ME) were used. These mice overexpress non-mutated, non-activated rat neu transgene under the regulation of the mouse mammary tumor virus promoter (74). These mice develop premalignant mammary hyperplasia similar to ductal carcinoma *in situ* prior to the development of spontaneous carcinoma (75). These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

2.2. Tumor cell line

The neu overexpressing mouse mammary carcinoma (MMC) cell line was established from spontaneous mammary tumors harvested from FVBN202 mice (76). Tumor cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and cells were kept at 37ºC with 5% CO2. Cells were routinely passaged when needed with 0.25% Trypsin or 10mM Ethylenediaminetetracetic acid (EDTA) solution (Quality Biologicals Inc., Gaithersburg, MD).

2.3. Genetic silencing of ATG5 in MMC

Mission shRNA bacterial stocks for ATG5 and scrambled Control were purchased from Sigma Aldrich. Lentiviruses were produced in HEK 293TN cells co-transfected using Endo F ectinTM Lenti Transfection Reagent (GeneCopoeia, 1001-01) with a packaging mixture of psPAX2 and pMD2.G constructs (Addgene). Media containing the viruses was used to infect MMC cells; puromycin (1 μg/ml) was used as a selection marker to enrich for infected cells.

2.4. Antibodies

All antibodies were purchase from Biolegend (San Diego, CA) unless otherwise stated. Antibodies were used as instructed by the supplier. Antibodies include: anti-CD16/32 (clone 93), APC-anti-mouse IgG (Poly4053), PE-Ki67 (16A8), Alexa flour 488-Ki67 (11F6), Brilliant Violet 605-CD45 (30-F11), FITC-Annexin V, APC-Annexin V, 7-AAD viability staining solution and Propidium Iodide solution (PI), mouse anti-rat neu (anti–c-Erb2/c-Neu; 7.16.4, Calbiochem, Billerica, MA), FITC- Fixable viability stain (FVS) (BD Biosciences), anti-CD16/32 antibody (clone 93), FITC-CD3 (17A2); BUV395-CD3; FITC-CD11b (M1/70); APC-CD11b; FITC-anti-mouse IgG (Poly4053); PE-GR-1 (RB6-8C5); PE-PD-1 (RMP1-30); PE-Ki67 (16A8); allophycocyanin-CD62 ligand (MEL-14); Brilliant Violet 421-CD44; FITC-Annexin V; FITC-CD4 (GK1.5); Brilliant Violet 737-CD4; APC-CD8α (53-6.7); Brilliant Violet 711-CD8α; Brilliant Violet 421-PD-L1 (10F.9G2); PE-PD-L1; Brilliant Violet 786-CD45; (30-F11); Brilliant Violet 421 anti-IgG secondary or APC Streptavidin and PI, all of which were purchased from BioLegend (San Diego, CA, USA). APC-CY7-FVS were purchased from BD Horizon. Anti-mouse Endothelin 1 monoclonal antibody (TR.ET.48.5), Anti-rabbit Endothelin A receptor polyclonal antibody, and Anti-rabbit Endothelin B receptor polyclonal antibody was purchased from Thermofisher. Anti-rat neu antibody (anti–c-Erb2/c-Neu; 7.16.4), was purchased from Calbiochem. Biotin mouse anti-mouse H-2D[q]/H-2L[q] (MHCI) was purchased from BD pharmingen. All reagents were used at the manufacturer's recommended concentration.

2.5. Experimental tumor dormancy

In vitro tumor dormancy was established by the treatment of MMC or ATG5^{KD} MMC tumor cells with 3 daily doses of ADR (Sigma-Aldrich, 1uM for 2 hrs). During ADR treatment, MMC tumor cells were cultured without or with CQ (Sigma-Aldrich, 10uM, 3 hrs prior to and during ADR

treatment). By 2 weeks after the treatment, all groups did not show any increases in the number of adherent cells, which is the characteristic of tumor dormancy. For in vivo induction of tumor dormancy, FVBN202 mice were challenged with ADR-treated dormant MMC or $ATG5^{KD}$ MMC (i.v. injection of 1 million viable cells), or untreated MMC followed by 3 weekly treatments of ADR (i.v., 9 mg/kg) or with 3 weekly treatment of ADR + 60 mg/kg CQ (i.p.).

For a low dose chemotherapy, in vitro, MMC were seeded at 5 x 10^6 cells/flask for 24 hours prior to experiment. On the day of treatment, cells were seeded at 3 x 10^6 cells/flask and incubated at 37°C with 5% CO2 for 2 hours. Once adherent, cells were treated with a combination of low dose metronomic FAC (5uM 5-FU + 0.1uM ADR; Sigma-Aldrich, St. Louis, MO; plus 2uM CYP; Baxter healthcare corporation, Deerfield, IL) (77–79)**.** Cells were washed with sterile 1X PBS, and fresh medium (RPMI-1640, 10% FBS) was added to the culture. Treatment was repeated for five consecutive days. Cells were cultured for 10 days after the last treatment. Medium was changed as needed.

2.6. Cytotoxicity assay

Freshly isolated tumor-primed splenic T cells or *ex vivo* expanded splenic T cells were cultured with MMC or FAC treated MMC at a 10:1 E:T ratio in 3 ml complete medium (RPMI-1640 supplemented with 100 U/ml of penicillin, 100 µg/ml streptomycin, 10% FBS, 10mM L-glutamine and 5x10^{-5}M 2-mercaptoethanol) with 20U/ml of IL-2 (Peprotech) in 6 well culture dishes. After 48 hs cells were harvested and with 10 mM EDTA, and washed twice with 1X Annexin buffer (BD Biosciences, San Jose. CA) at 1200 rpm for 8 minutes at 4ºC in round bottom polystyrene tubes. Cells were blocked with anti-mouse CD16/32 antibody for 20 minutes on ice, followed by two washes, and staining for CD45, c-Erb2/c–Neu, and Annexin V for 20, 20, 15 minutes respectively. PI (Sigma, Saint Louis, MO) and 400µL of 1X Annexin buffer was added

immediately prior to data acquisition according to the manufacturer's protocol (BD Pharmingen). The gated $CD45^-$ or neu+ tumor cells were analyzed for tumor cell apoptosis (62).

2.7. Ex vivo reprograming of splenic lymphocytes for AIT

Reprogramming of tumor-sensitized immune cells was performed as previously described by our group (62). FVBN202 mice were inoculated with 3 million MMC in the mammary fat pad and growth was monitored by digital caliper. Spleens were harvested when tumor had reached ≥ $1000mm^3$. 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) (B/I), and 80 U/ml of IL-2 (Peprotech) for 16–18 hours. Lymphocytes were then washed three times and cultured in complete medium with recombinant murine IL-7 and IL-15 (20ng/mL of each, Peprotech). After 24 hours, 20 U/mL of IL-2 was added to the culture and the following day cells were washed and cultured with 40 U/mL of IL-2. After 48 hours, cells were washed again and 40 U/mL of fresh IL-2 was added. 24 hours later, lymphocytes were washed again and cultured with 40 U/mL of fresh IL-2. Lymphocytes were harvested 24 hours later on the 6^{th} day and were used for *in vitro* studies as cytotoxicity experiments or *in vivo* studies for AIT. FVBN202 mice were injected i.p. with CYP (100 mg/kg) to induce lymphopenia one day prior to AIT. AIT was performed i.v. at a dose of 70×10^6/mouse three days after tumor challenge or once the tumor became palpable ($50\text{-}70mm^3$).

2.8. Tumor challenge studies

Primary tumor model: FVBN202 mice were inoculated with 3 million MMC in sterile 1X PBS subcutaneously in the mammary region. Mice were weighed and tumor size was monitored by digital caliper twice weekly. Tumor size was calculated by V[volume] = (L[length] $_x$ W[width]^2)/2.

Three days after tumor challenge, Mice were injected with a low dose metronomic FAC (10 mg/kg 5-FU+3 mg/kg ADR+10 mg/kg CYP every day for nine days i.p.) (80–82). As soon as the tumor reached 1000mm^3 in the control group, we sacrificed the experimental animals and their tumors were collected in complete medium for further analysis.

Experimental metastatic tumor model: FVBN202 mice were inoculated with 1 million MMC in sterile 1X PBS intravenously. Mice were weighed twice weekly. Three days after tumor challenge, Mice were injected with a low dose metronomic FAC (10 mg/kg 5-FU + 3 mg/kg ADR + 10 mg/kg CYP every day for nine days, i.p.). The animals were euthanized when they had a weight loss of ≥10% in the control group, we sacrificed the experimental animals and the liver and lungs were collected in a complete medium for further analysis.

2.9. Blockade of tumor survival pathways by using anti-PD-1 antibody or macitentan

FVBN202 mice were inoculated with 3 million MMC in sterile 1X PBS subcutaneously in the mammary region. Mice were weighed and tumor size was monitored by digital caliper twice weekly. Tumor size was calculated by V [volume] = (L[length] $_x$ W[width]^2)/2. Three days after tumor challenge, mice were injected with a low dose metronomic FAC. Mice then received reprogrammed splenocytes i.v. at a dose of 70 × 10^6/mouse or remained untreated. Mice received i.p. injections with 100 μg of anti–Programmed cell death protein 1 (anti-PD-1) antibody (Bioxcell) in a total volume of 300 ul of dilution buffer (5 times every 3 days) (83), or oral gavage of 0.3 ml macitentan (Biovision) (30 mg/kg, every day for six days) (84). As soon as the tumor reached 1000mm^3 in the control group, we sacrificed the experimental animals and their tumor, liver, and lungs were collected in a complete medium for further analysis.

2.10. Establishment of dormant tumor cell line from the lungs

Under aseptic conditions liver and lungs were minced and 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 gentle tissue homogenization to create a cellular suspension. The cell suspension was then washed twice with RPMI-1640 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 cultured with RPMI-16 20 % FBS for 2 weeks followed by culture in RMPI-1640 supplemented with 10% FBS.

2.11. Flow cytometry analysis

A multi-color staining and flow cytometry analyses of the tumor, lungs or liver were performed as previously described by our group (62), with some modifications. Infiltrating lymphocytes or neu positive tumor cells were analyzed by multi-color staining of resected tissues. The tissues were minced into smaller pieces using scissors and forceps. A plunger from a disposable 5mL syringe was used to mechanically disaggregate the tissues against a secured filter. Complete medium was used to wash any remaining cells through the filter.

For the detection of distant dormant tumor cells, liver and lungs were harvested and homogenized into a single cell suspension as previously described (62). Ki67 expression was determined as previously described by our group (25,62). Cells were fixed with 70% ethanol and stained with anti-Ki67 for 30 minutes at room temperature. Prior to fixation, cells were stained with Fixable Viability Stain (FVS) for 20 minutes at room temperature, washed and stained with anti-neu antibody for 20 minutes on ice. Cells were washed twice with FACS buffer (1X PBS, 10% FBS, 0.1% sodium azide) prior to data acquisition.

Reagents used for flow cytometry include the following: anti-CD16/32 antibody (clone 93), FITC-CD3 (17A2); BUV395-CD3; FITC-CD11b (M1/70); APC-CD11b; FITC-anti-mouse IgG (Poly4053); PE-GR-1 (RB6-8C5); PE-PD-1 (RMP1-30); PE-Ki67 (16A8); allophycocyanin-CD62 ligand (MEL-14); Brilliant Violet 421-CD44; FITC-Annexin V; FITC-CD4 (GK1.5); Brilliant Violet 737-CD4; APC-CD8α (53-6.7); Brilliant Violet 711-CD8α; Brilliant Violet 421-PD-L1 (10F.9G2); PE-PD-L1; Brilliant Violet 786-CD45; (30-F11); Brilliant Violet 421 anti-IgG secondary or APC Streptavidin and PI, all of which were purchased from BioLegend (San Diego, CA, USA). APC-CY7-FVS were purchased from BD Horizon. Anti-mouse Endothelin 1 monoclonal antibody (TR.ET.48.5), Anti-rabbit Endothelin A receptor polyclonal antibody, and Anti-rabbit Endothelin B receptor polyclonal antibody was purchased from Thermofisher. Anti-rat neu antibody (anti–c-Erb2/c-Neu; 7.16.4), was purchased from Calbiochem. Biotin mouse anti-mouse H-2D[q]/H-2L[q] (MHCI) was purchased from BD pharmingen. All reagents were used at the manufacturer's recommended concentration. Data acquisition was performed on a Beckon-Dickenson FACS Fortessa X-20 flow cytometer (BD Biosciences). All data was analyzed using FCS express software 5 (De Novo Software, Loa Angeles, CA).

2.12. Inhibition of Bcl-2, Mcl-1 and Bcl-xL

For T cell induced cytotoxicity, MMC or FAC-treated dormant MMC were cultured with or without the inhibitor of Bcl-2 (ABT-199, 1 μM), Mcl-1 (S-63845, 1 μM) or Bcl-xL (A-1331852, 1 μM) for 3 hours, washed and cultured with T cells for 24 hours. For Western blot analysis, MMC or FAC-treated dormant MMC were cultured with the inhibitors for 24 hours, and then lysed for Western blot analysis.

2.13. Western blot

Whole cell lysates were prepared with CHAPS lysis buffer [20 mM Tris (pH 7.4), 137 mM NaCl, 1 mM dithiothreitol (DTT), 1% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate)], 1:200 ratio of protease inhibitor cocktail, and 1:100 ratio of phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich)]. Equal amounts of proteins were loaded on a SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and analyzed by immunoblotting. Antibodies for BIM, BCL-X_L, Survivin, Cleaved-Caspase3 (5A1E), GAPDH (D16H11), HRP-linked anti-rabbit IgG, and HRP-linked anti-mouse IgG antibodies were from Cell Signaling Technology (Beverly, CA); MCL-1 (ADI-AAP-240-F) was from Enzo Life Sciences (Farmingdale, NY); BCL-2 (100) was from Sigma-Aldrich (St. Louis, MO); p53 (DO-1) was from Santa Cruz Biotechnology (Santa Cruz, CA). ECL2 Western blotting substrate was purchased from Thermo Fisher Scientific (Rockland, IL).

2.14. Statistical analysis

Data are summarized as means and standard errors of the mean (SEM) with differences between groups being illustrated with graphical data presented as mean ± SEM. Statistical comparisons were made using a one-tailed or two-tailed Student *t* test and a p-value <0.05 was considered significant (*: <0.05, **: <0.005. ***: <0.0005)

Chapter 3. Autophagy, tumor dormancy and recurrence

3.1. Rationale, hypothesis and experimental design

Autophagy plays a paradoxical role in the promotion and inhibition of cancer. On the one hand, autophagy has a cancer-promoting role by protecting tumor cells from chemotherapy or providing a source of energy for tumor cells to survive under hypoxic and acidic conditions despite the lack of mature vessels (85). On the other hand, inhibition of autophagy by disruption of *Beclin 1* or deletion of *ATG5* increases the frequency of spontaneous malignancies (86) or liver tumor (87), respectively. Recently, four different mechanisms have been proposed to describe paradoxical functions of autophagy in cancer, which include cytotoxic, cytostatic, cytoprotective and non-protective autophagy (88). There are also three major types of autophagy which include micro-autophagy involving the direct engulfment of cytosolic material by lysosomes through invagination, chaperone-mediated autophagy involving HSP70 and the lysosomal membrane associated protein 2 A (LAMP2A), and macro-autophagy which is a highly conserved pathway involving the formation of autophagosomes, which fuse with lysosomes. To this end, ATG5 is involved in the elongation of autophagosomes to engulf toxic material for degradation. A stable knockdown of ATG5 results in the inhibition of the formation of autophagosomes and progression of macro-autophagy (89). CQ, on the other hand, does not have any effects on autophagosomes but it blocks the fusion of autophagosomes and lysosomes, thereby preventing the completion of macro-autophagy. In order to investigate the role of macro-autophagy in tumor dormancy and relapse, we performed a transient inhibition of macro-autophagy by means of CQ during chemotherapy, which mainly inhibits chemotherapy-induced autophagy while cell-intrinsic autophagy will be restored after the completion of chemotherapy. We also performed a permanent inhibition of cell-intrinsic macro-autophagy by a stable knockdown of ATG5 in tumor cells. We

demonstrated that cell-intrinsic, but not chemotherapy-induced, autophagy can inhibit tumor relapse.

3.2. Results

3.2.1. Adriamycin induces autophagy in MMC

In order to determine whether ADR induces autophagy and in turn establishes tumor dormancy, MMC cells were treated with ADR in the presence or absence of CQ, a pharmacological agent used to block the final stages of autophagy, specifically the fusion of autophagosomes with lysosomes that is necessary for digestion of the cargo in the autophagosomes (frequently termed "autophagic flux"). CQ blocked this autophagic flux as evidenced by the enhanced accumulation of acidic vesicles (red signals) (Figure 3A, ADR and ADR+CQ). We further monitored degradation of the p62/SQSTM1 protein as a marker of autophagic flux, and LC.3B expression as a marker of autophagosomes formation (since LC3 is a component of the autophagosomes). As shown in Figure 3B, ADR did not induce degradation of p62/SQSTM1 although it elevated LC.3B, suggesting that ADR induces autophagy but fails to drive autophagy to completion and p62/SQSTM1 degradation.

3.2.2. A transient blockade of autophagy by CQ during ADR treatment delays tumor relapse in vitro but not in vivo

Since CQ is being used to sensitize tumor cells susceptible to chemotherapy (90), we sought to determine whether blockade of autophagy by CQ during ADR treatment affects tumor dormancy and relapse. We showed that the presence of CQ during ADR treatment, in vitro, resulted in prolonging tumor dormancy such that, while ADR treated MMC resumed cell proliferation 6 weeks after the treatment, ADR+CQ treated MMC remained dormant (Figure 4A). In order to

confirm tumor cell relapse after 6 weeks, flow cytometry analysis of ADR-treated MMC was performed, and indicated a shift of Ki67- non-proliferating cells to Ki67+ proliferating cells with a greater viability (Figure 4B). In fact, MMC cells remained apoptotic by producing floater dead cells following ADR treatment (Figure 5A) which compensated for cell proliferation and maintained tumor dormancy for 3 weeks after the completion of ADR treatment. Follow up studies on floater cells showed they were all apoptotic (Figure 5B). A transient blockade of autophagy by CQ did not affect susceptibility of tumor cells to ADR-induced apoptosis (Figure 6). On the other hand, a transient blockade of autophagy during ADR chemotherapy, in vivo, did not prolong tumor dormancy in FVBN202 mice (Figure 7).

3.2.3. A transient blockade of autophagy by CQ during ADR treatment does not change susceptibility of tumor cell to immunotherapy

In order to determine whether a transient blockade of autophagy during ADR treatment affects susceptibility of dormant MMC to immunotherapy, dormant MMC were cultured with either Interferon gamma (IFN-γ) or MMC-reactive T cells three weeks after treatment with ADR or ADR+CQ. As shown in Figure 8, untreated MMC or dormant MMC treated with ADR or ADR+CQ all remained susceptible to IFN-γ treatment or T cells.

3.2.4. A stable knockdown of autophagy reduces susceptibility of MMC to ADR treatment

CQ only transiently blocks fusion of autophagosomes and lysosomes during ADR treatment such that after removal of CQ, accumulated autophagosomes could eventually be fused with lysosomes to complete autophagy. In order to determine the role of autophagy in tumor dormancy or relapse, we used shRNA for a stable knockdown of ATG5 ($ATG5^{KD}$) which inhibits formation of autophagosomes in MMC. Scrambled shRNA was used as control (Figure 9A). The $ATG5^{KD}$ MMC and scrambled control MMC were irradiated to confirm that $ATG5^{KD}$ MMC cells were

deficient in autophagy, using p62 and LC.3B as read outs (Figure 9B). Tumor cells remained intact for the expression of neu antigen, as well as cell proliferation in vitro and in vivo following knockdown of autophagy (Figure 9C-E). Flow cytometry analysis determined a lower level of viability in MMC compared with $ATG5^{KD}$ MMC following ADR treatment (Figure 10).

3.2.5. A stable knockdown of autophagy results in earlier tumor relapse associated with increased frequency of polyploid-like cells and resistance to immunotherapy

In order to determine whether a higher viability of $ATG5^{KD}$ MMC following ADR treatment (Figure 8) facilitates an earlier tumor relapse compared with wild type MMC, follow up studies were performed for three weeks after ADR treatment. As shown in Figure 11A, $ATG5^{KD}$ MMC survived better than autophagy-competent MMC following ADR treatment showing a significantly higher number of cells by 3 weeks after the treatment. Flow cytometry analysis of tumor cells showed greater levels of apoptosis in wild type MMC compared with $ATG5^{KD}$ MMC (Figure 11B, $p<0.001$). Interestingly, $ATG5^{KD}$ MMC cells contained a higher number of polyploid-like cells following ADR treatment compared with autophagy-competent MMC (Figure 11B, $p<0.03$).

In order to determine the in vivo relevance of our in vitro findings, FVBN202 mice were used. Tumor dormancy was first established by ADR treatment in vitro; FVBN202 mice (n=7/group) were then challenged i.v. with one million viable dormant tumor cells. Animals were then sacrificed when they became moribund (lost 10% weight) as a result of massive lung metastasis. As can be seen in Figure 12A, animals that were challenged with ADR-treated $ATG5^{KD}$ MMC developed lung metastasis significantly sooner than those that were challenged with ADR-treated MMC. Hematoxylin/eosin and immunohistochemistry analyses of tumor lesions determined a higher frequency of polyploid-like and Ki67+ tumor cells in animals that were challenged with

ADR-treated ATG5^{KD} MMC (Figure 12B). Finally, ATG5^{KD} MMC were found to be resistant to T cell-induced apoptosis compared with autophagy-competent MMC (Figure 13).

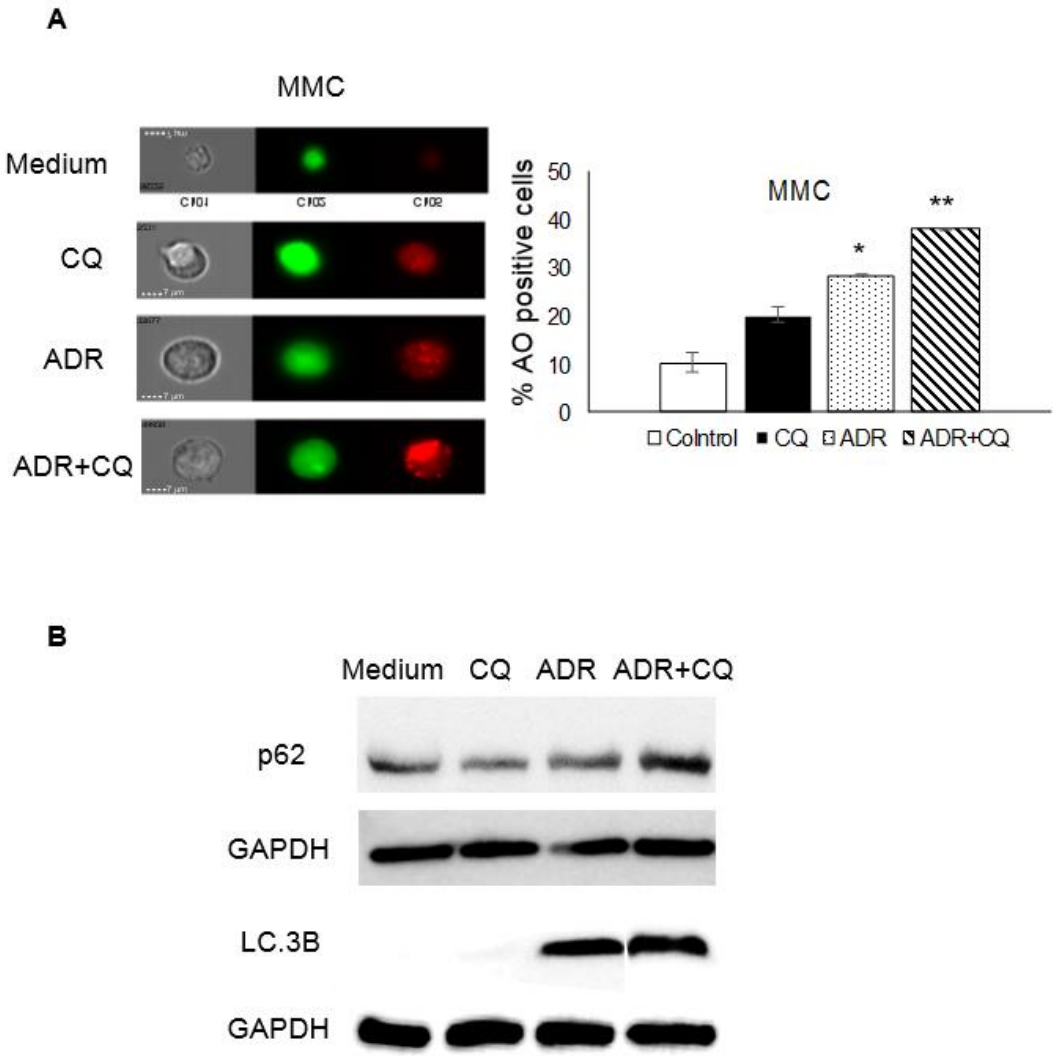


Figure 3. CQ blocks ADR-induced autophagy. MMC tumor cells received three daily doses of ADR alone (1 µM ADR for 2 hrs) (ADR) or in the presence of CQ (10 µM 3 hrs before ADR and 2hrs during ADR treatment) (ADR+CQ), washed after each daily treatment and analyzed by acridine orange (AO) one day after the last treatment. Untreated MMC (Medium) or MMC treated with CQ (CQ) served as controls. A) Acridine orange (AO) staining was analyzed for acidic vesicles (red) using image cytometry. Data represent triplicate experiments. B) Levels of p62/SQSTM1 and LC.3B after treatment with ADR ± CQ indicative of autophagy induction in the absence of autophagic flux (B).

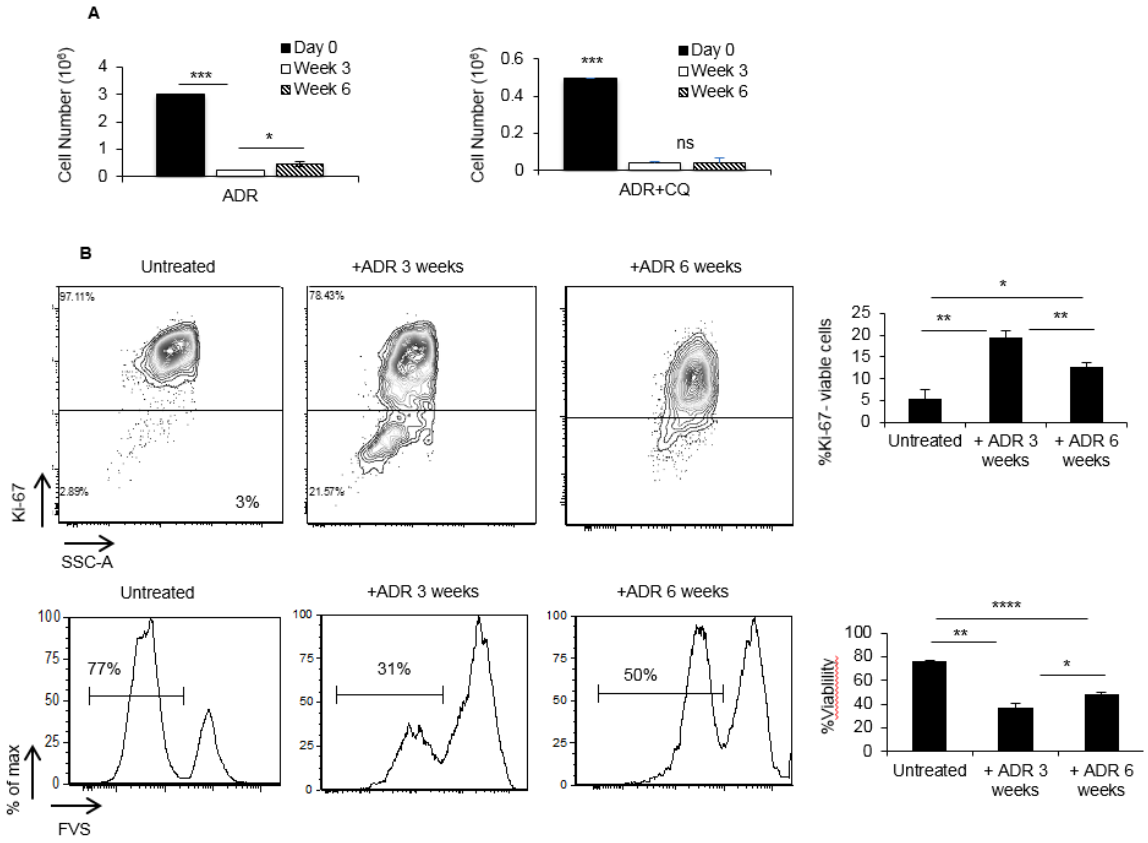


Figure 4. ADR-induced dormant tumor cells remain dormant in the presence of CQ. MMC tumor cells were treated with 3 daily doses of ADR (1uM for 2 hrs), with one group receiving CQ (10uM) 3 hrs prior to and during ADR treatment. Both groups remained untreated for 3 weeks and 6 weeks, *in vitro*. A) Adherent viable cells were counted using trypan blue exclusion at various time points. Data represent 3 replicates ± Standard error of the mean (SEM). B) At weeks 3 and 6 post-treatment, Ki-67 expression on gated FVS- viable cells (upper panel) and cell viability (lower panel) were quantified within the population of adherent tumor cells. Data represent 2-3 replicates ± SEM. Four independent experiments have been carried out which have shown similar results.

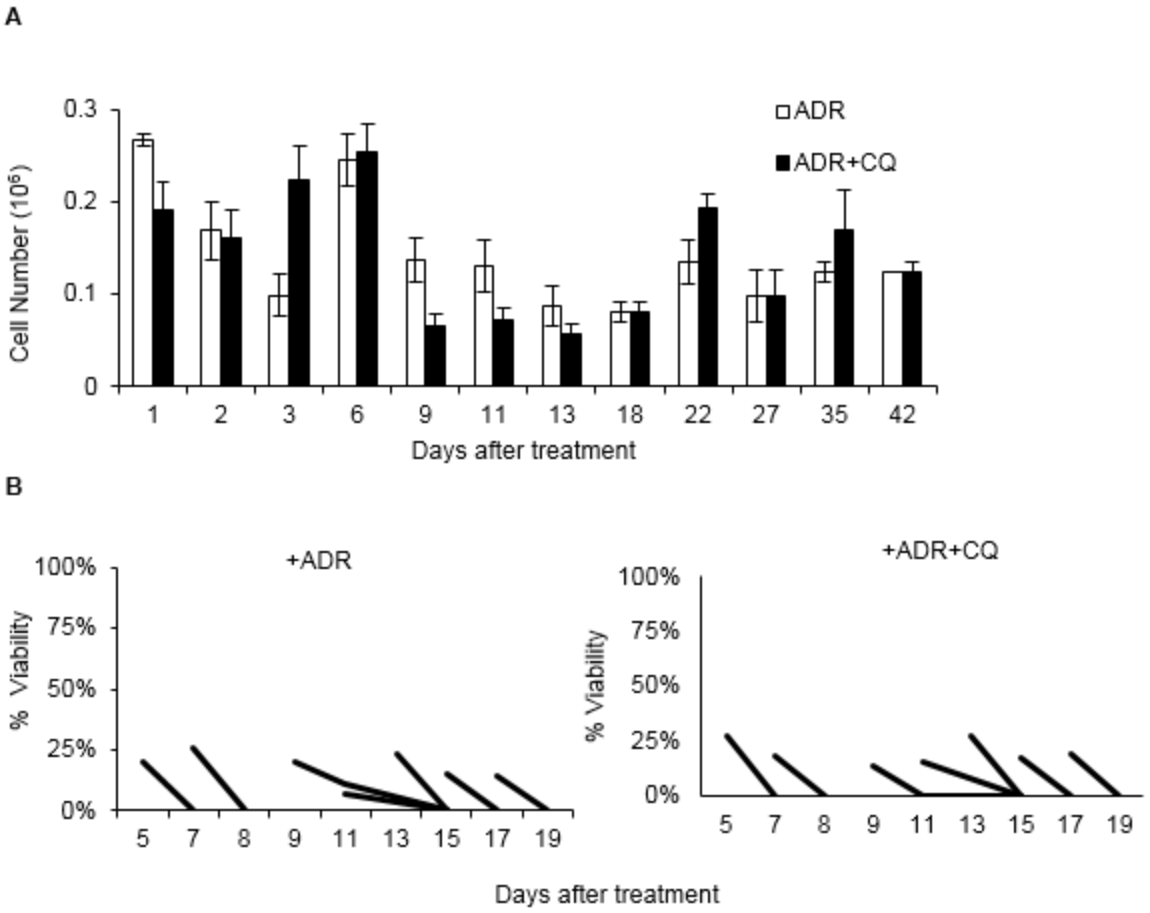


Figure 5. ADR-induced dormant tumor cells produce floater apoptotic cells, in vitro. MMC tumor cells (3 x 10^6 cells/flask) were treated with 3 daily doses of ADR (1uM for 2 hrs), with one group receiving CQ (10uM) 3 hrs prior to and during ADR treatment. Both groups remained untreated for 3 weeks and 6 weeks, *in vitro*. A) Floater cells were collected whenever culture medium was replaced and cell number and viability was assessed via trypan blue exclusion. Data represent 3 independent experiments and mean ± SEM. B) Floater cells were cultured separately for 2-3 days each time they were collected, and assessed for viability 2-4 days later by using trypan blue staining.

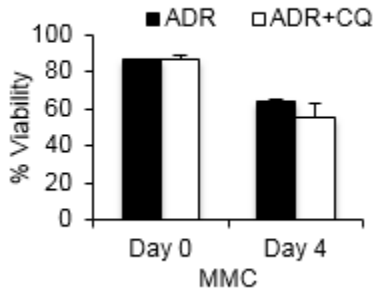


Figure 6. A transient blockade of autophagy by CQ did not change the susceptibility of MMC to ADR treatment. MMC tumor cells were treated with ADR alone (1 uM ADR for 2 hrs) (ADR) or in the presence of CQ (10 uM 3 hrs before ADR and 2hrs during ADR treatment) (ADR+CQ). Tumor cells were analyzed by Annexin v/PI staining prior to treatment (Day 0) or three days after the treatment (Day 4). Experiments were performed in triplicates.

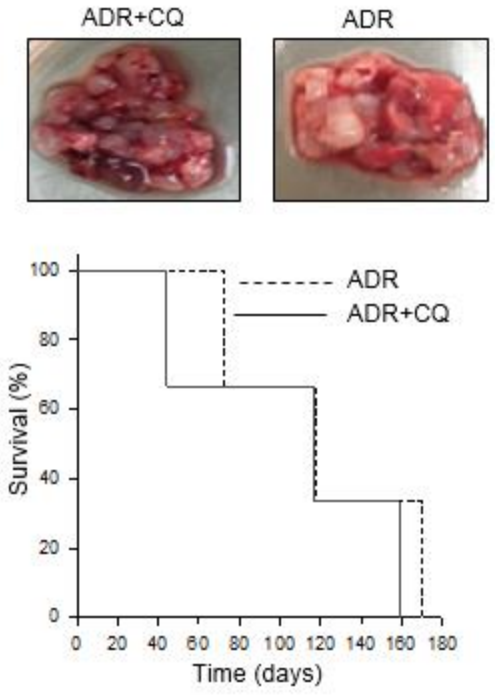


Figure 7. A transient blockade of autophagy by CQ during ADR treatment fails to maintain tumor dormancy, in vivo FVBN202 mice (n=3/group) were challenged with MMC (i.v. injection of 1 million viable cells), and three days after tumor challenge animals were split into two groups: one group received 3 weekly treatments of ADR (i.v., 9 mg/kg), and another group received 3 weekly treatment of ADR + 60 mg/kg CQ (i.p.). Animals were sacrificed when they became moribund. Figure shows Kaplan-Meier survival curve and tumors in the lungs.

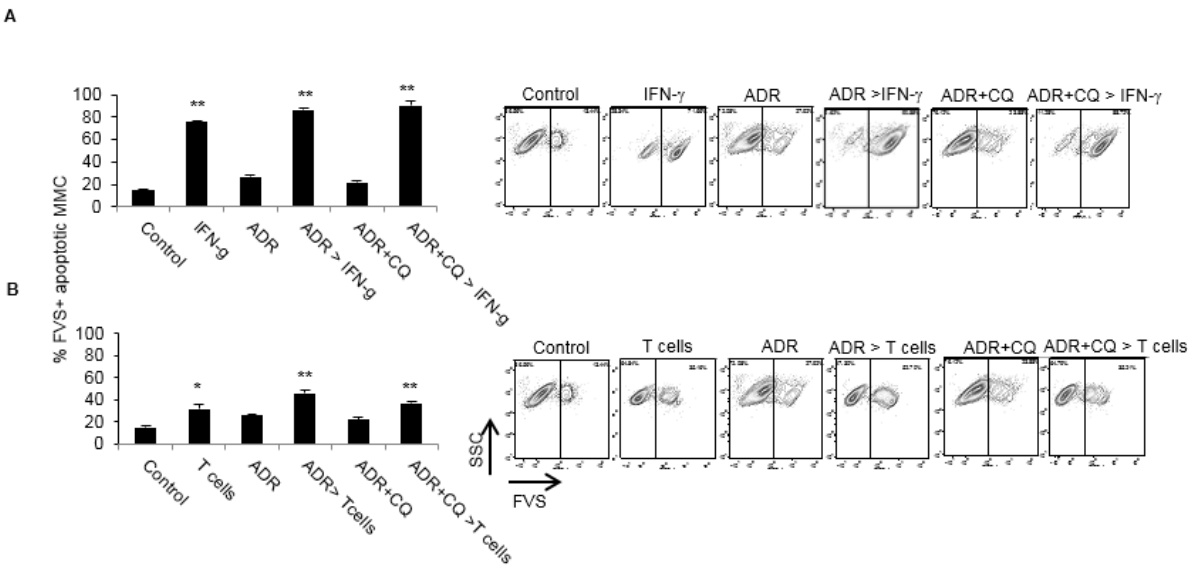


Figure 8. Dormant tumor cells established by ADR or ADR+CQ remain susceptible to immunotherapy. The in vitro tumor dormancy was established three weeks after three daily treatments of MMC with ADR or ADR+CQ. Untreated MMC cells were used as control. A) Apoptosis was determined by FVS viability staining in MMC (control), ADR-treated dormant MMC (ADR), ADR+CQ-treated dormant MMC (ADR+CQ), as well as control MMC cultured with three daily doses of IFN-γ and analyzed two days later (50 ng/ml) (IFN-γ), ADR-treated dormant MMC cultured with three daily doses of IFN-γ (50 ng/ml) and analyzed two days later (ADR > IFN-γ), or ADR+CQ-treated dormant MMC cultured with three daily doses of IFN-γ (50 ng/ml) and analyzed two days later (ADR+CQ > IFN-γ). B) Apoptosis was determined by FVS viability staining of MMC (control), MMC cultured with MMC-sensitized T cells for 48 hrs (T cells), ADR-treated dormant MMC (ADR), ADR-treated dormant MMC cultured with MMC-sensitized T cells for 48 hrs (ADR > T cells), ADR+CQ-treated dormant MMC (ADR+CQ), or ADR+CQ-treated dormant MMC cultured with MMC-sensitized T cells for 48 hrs (ADR+CQ > T cells). Samples from the co-culture of T cells and tumor cells were analyzed on gated CD45- tumor cells. Splenic T cells were collected from MMC tumor-bearing FVBN202 mice

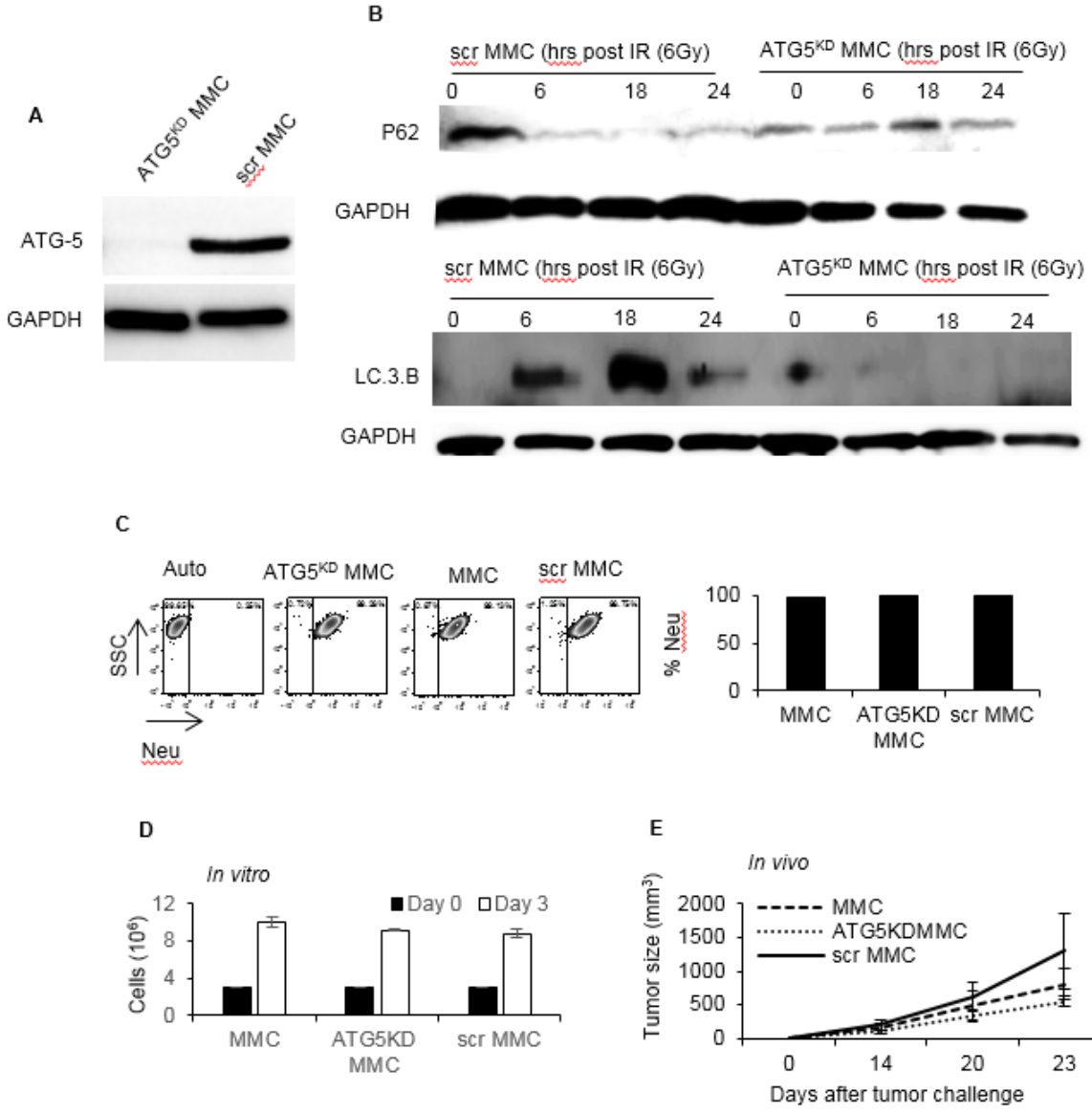

A
ATG5KD MMC
scr MMC
ATG-5
GAPDH
B
scr MMC (hrs post IR (6Gy)
ATG5KD MMC (hrs post IR (6Gy)
0 6 18 24 0 6 18 24
P62
GAPDH
scr MMC (hrs post IR (6Gy)
ATG5KD MMC (hrs post IR (6Gy)
0 6 18 24 0 6 18 24
LC.3.B
GAPDH
C
Auto
ATG5KD MMC
MMC
scr MMC
SSC
Neu
% Neu
100
50
0
MMC
ATG5KD MMC
scr MMC
D
In vitro
Day 0
Day 3
Cells (10^6)
12
8
4
0
MMC
ATG5KD MMC
scr MMC
E
In vivo
Tumor size (mm3)
2000
1500
1000
500
0
MMC
ATG5KDMMC
scr MMC
0
14
20
23
Days after tumor challenge

Figure 9. ATG5 knockdown tumor cells and wild type MMC show a similar pattern of growth. MMC cells were stably transfected with lentivirus expressing shRNA against ATG5 to establish autophagy-deficient cells ($ATG5^{KD}$ MMC). Control MMC (MMC) were stably transfected with scrambled control vector as autophagy-competent cells (scr MMC). A) Cell lysates were collected and used for immunoblotting against ATG5. B) $ATG5^{KD}$ MMC and scr MMC were treated with IR (6G) and cells lysates were collected at 6, 18, 24 hrs post treatment. Autophagy was determined by degradation of p62 and accumulation of LC.3.B C) Expression of Neu protein was determined on autophagy-competent control MMC (MMC or scr MMC) and autophagy-deficient MMC ($ATG5^{KD}$ MMC) using FACS analyses. D) Tumor cell proliferation was determined in a 3-day culture using trypan blue exclusion. E) FVBN202 mice (n=3) were inoculated with autophagy-competent MMC (MMC or scr MMC) or autophagy-deficient MMC ($ATG5^{KD}$ MMC) (3×10^6 cell/mouse, s.c. inoculation), and tumor growth was monitored by using a digital caliper. Data represents triplicate experiments.

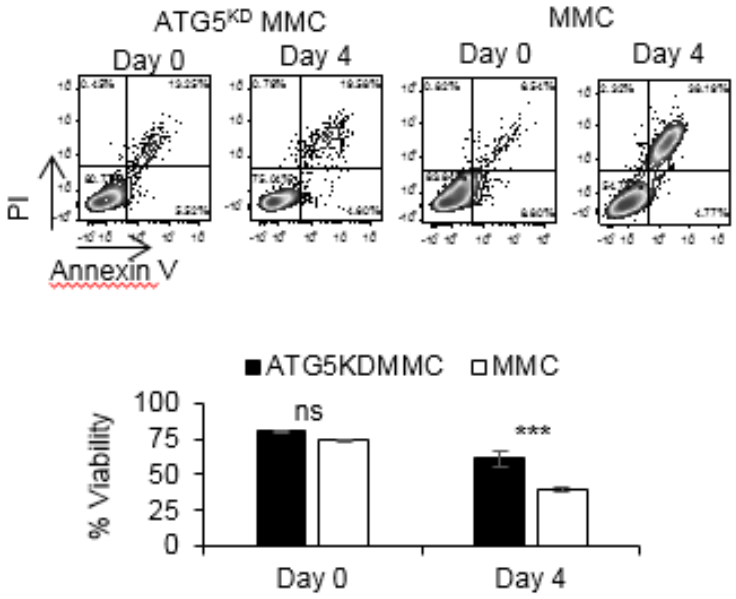


Figure 10. Autophagy knockdown tumor cells become less susceptible to ADR-induced apoptosis. Autophagy-deficient MMC ($ATG5^{KD}$ MMC) or autophagy-competent MMC (MMC) were treated with a single dose of ADR alone (1 uM ADR for 2 hrs). Tumor cells were analyzed by Annexin V/PI staining prior to treatment (Day 0) or three days after the treatment (Day 4). Experiments were performed in triplicates.

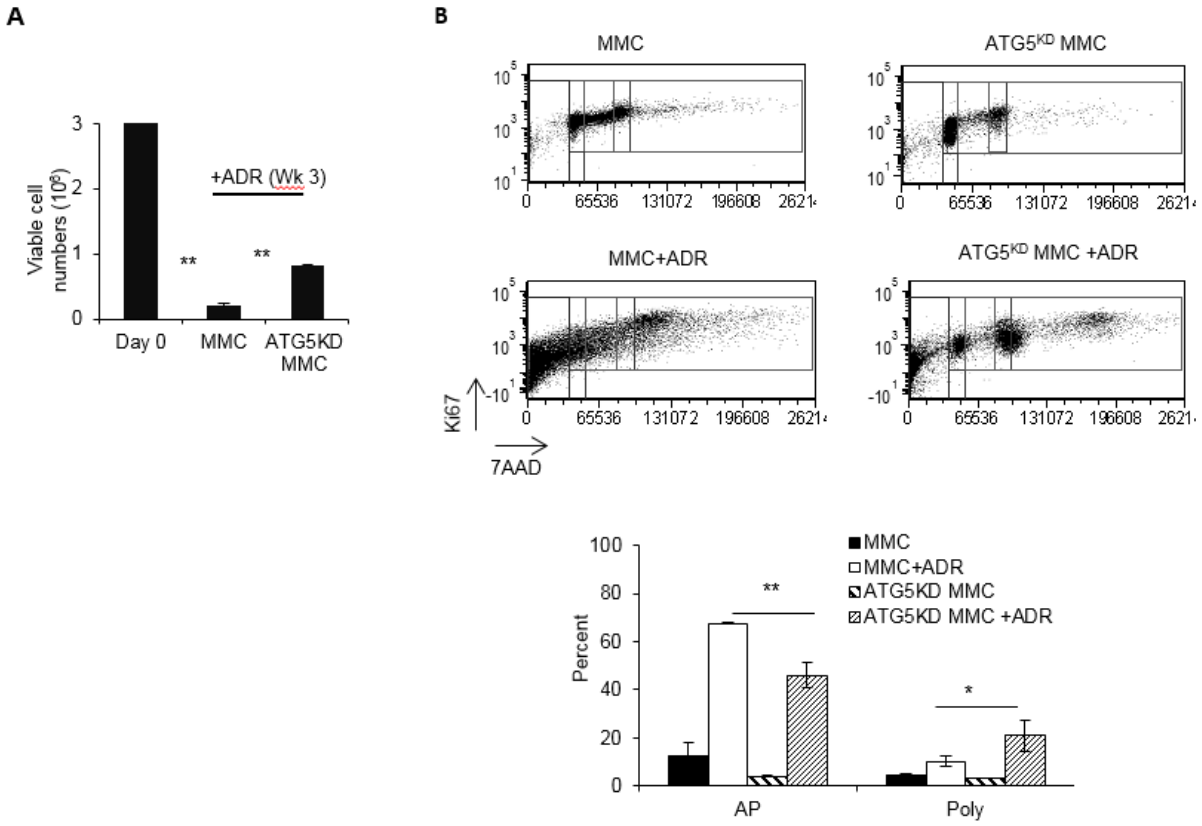


Figure 11. ADR-induced tumor dormancy in autophagy knockdown tumor cells with polyploid-like morphology compared with autophagy competent tumor cells, *in vitro*. MMC or ATG5^{KD} MMC tumor cells (3 million cells, Day 0) were treated with 3 daily doses of ADR (1uM for 2 hrs), and viable cells were counted at week 3 using trypan blue exclusion. Data represent triplicate experiments (A). Dot plots from each experimental group gated for cell cycle phase based upon DNA content (7-AAD) and Ki-67 expression. Events falling to the left of the G1/G0 gates are considered apoptotic cells (AP). Events falling to the far right of the G2/M gate are considered polyploid-like cells (Poly) (B). Three independent experiments have been performed and data represent 3 replicates ± SEM.

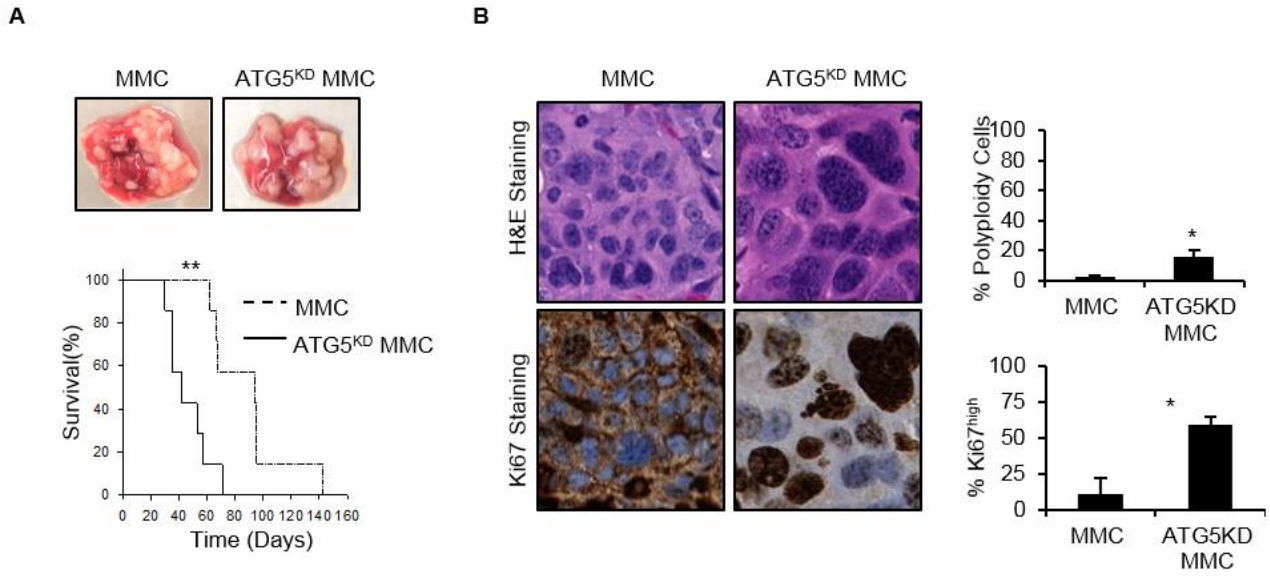


Figure 12. Earlier relapse of autophagy knockdown tumor cells with polyploidy-like morphology compared with autophagy competent tumor cells, in vivo. A) FVBN202 mice (n=7) were challenged i.v. with 10^6 cells ADR-treated dormant control MMC (MMC), or ADR-treated dormant ATG5^{KD} MMC (ATG5^{KD} MMC). Animals were euthanized as soon as they became moribund. Representative tumor relapse in the lung and survival curve are shown. B) Relapsed tumors were collected and immunohistochemistry slides were prepared by either staining samples with hematoxylin and eosin (H&E) or by Ki67 staining followed by subsequent digitization and analysis with NDP View software (Hamamatsu Photonics). At twenty-times magnification, three representative 0.02mm^2 areas were chosen from the H&E slides containing approximately 100 cells to measure nuclear envelope size. Cells containing a nuclear envelope equal to or greater than 16um with visible multi-nuclei were considered polyploid-like or high grade cells. The corresponding cell was then analyzed on the Ki67 stained slide to determine Ki67 expression levels. Data was collected from three biological samples. Significance is based on a two-tailed t-test of $p<0.05$.

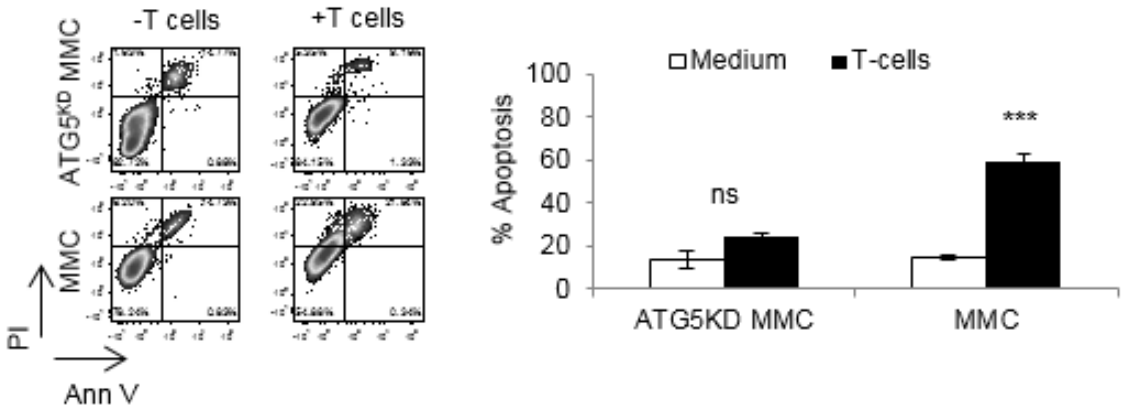


Figure 13. Autophagy knockdown tumor cells become resistant to T cell-induced apoptosis. Neu overexpressing autophagy-deficient MMC ($ATG5^{KD}$ MMC) or autophagy-competent MMC (MMC) were co-cultured with MMC-sensitized T cells and then gated CD45-Neu+ tumor cells were analyzed by Annexin V/PI staining. Data represents triplicate experiments.

3.3. Discussion

Cell-intrinsic autophagy is an ongoing process, which regulates cellular metabolism and homeostasis. Autophagy is also induced by insults such as chemotherapy. Here, we studied a paradoxical role of autophagy in tumor promotion and tumor inhibition by a transient inhibition of autophagy only during chemotherapy or a stable knockdown of autophagy in MMC tumor cells. While the former transiently blocked autophagy and cell-intrinsic autophagy was restored after the completion of chemotherapy, the latter permanently blocked chemotherapy-induced autophagy and cell-intrinsic autophagy. We demonstrated that inhibition of chemotherapy-induced autophagy by CQ did not increase susceptibility of tumor cells to chemotherapy-induced apoptosis. Nevertheless, chemotherapy-induced autophagy appeared to accelerate tumor relapse such that use of CQ during chemotherapy delayed tumor relapse in vitro. Our observation is consistent with other reports showing that increased autophagy in residual breast cancer after neoadjuvant chemotherapy was correlated with increased risk of tumor relapse (91). A transient blockade of autophagy during chemotherapy of tumor-bearing animals did not affect tumor relapse, perhaps, because tumor inhibitory effects of in vivo chemotherapy were not as effective as in vitro drug treatment. Also, chemotherapy-induced autophagy did not affect the sensitivity of tumor cells to apoptosis induced by IFN-γ or tumor-reactive T cells.

We also demonstrated that, unlike chemotherapy-induced autophagy, cell-intrinsic autophagy accelerated tumor relapse. A stable knockdown of cell-intrinsic autophagy by ATG5 shRNA resulted in a reduced sensitivity of tumor cells to chemotherapy- or T cell-induced apoptosis, and accelerated tumor relapse in vivo. These effects coincided with an increased frequency of multinuclear polyploid-like dormant cells. These observations suggest that chemotherapy-induced autophagy could have tumor-promoting effects and facilitate tumor relapse, whereas cell-intrinsic

autophagy could synergize with cancer therapeutics and delay tumor relapse. In fact, cell-intrinsic autophagy would seem to inhibit the formation of multinuclear cells following chemotherapy, and to prevent chemotherapy-induced genetic instability associated with resistance to cancer therapeutics. Similar observations have been made in other breast tumor models by showing that CQ but not knockdown of Beclin 1 or ATG12 sensitized the tumor to chemotherapy (28). Therefore, anti-tumor effects of autophagy inhibitors such as CQ is likely to be because of the inhibition of chemotherapy-induced autophagy while anti-tumor effects of autophagy inducers such as rapamycin may result from enhanced cell-intrinsic autophagy (92,93). It has been reported cancer stem cells play a role in tumor dormancy (94) and drug resistance (95), and that immunotherapeutic targeting of breast cancer stem cells inhibits growth of mammary carcinoma (96). However, we did not detect the enrichment of CD44+CD24- cancer stem cells following ADR-induced tumor dormancy (data not shown).

Anticancer drugs and ionizing radiation tend to induce autophagy in tumor cells (97). Treatment-induced autophagy could lead to apoptosis (98) and tumor cell dormancy (99). We have already reported that dormant tumor cells established by ADR treatment or radiation therapy, in vitro, developed resistance to these treatments but remained susceptible to immunotherapy (62). Therefore, evaluation of apoptosis or tumor growth inhibition as a single factor without evaluating tumor dormancy and relapse may not be sufficient for understanding anti-cancer efficacy of autophagy inhibitors such as CQ. Inhibition of autophagy by CQ during chemotherapy diminishes the expression of DNA repair proteins, resulting in tumor growth inhibition in carboplatin-resistant BRCA1 wild-type TNBC orthotopic xenografts (100). In triple negative breast cancer, CQ sensitizes tumor cells to paclitaxel chemotherapy (101). In several tumor models, CQ

synergistically augmented sunitinib cytotoxicity on tumor cells (90). However, the role of CQ in inhibiting tumor recurrence has yet to be determined.

Cells that are deficient in autophagy show increased levels of reactive oxygen species which result in the accumulation of DNA damage, increased double-strand breaks and polyploid-like nuclei (26,102). To this end, cell-intrinsic autophagy protects the cell from genomic instability induced by the accumulation of toxins within the cell (103). It has been reported that Beclin1 knockout mice fail to maintain genomic integrity by increasing DNA double stranded breaks and gene amplifications (102). A higher expression of Beclin 1 in healthy breast tissue than in breast cancer suggests a deficiency in cell-intrinsic autophagy in tumors (104), which could contribute to genomic instability during tumorigenesis. In breast cancer patients who received adjuvant chemotherapy, presence of tumor cell intrinsic autophagy contributed to reduced risk of tumor relapse (105). Expression of ATG5 in the tumor specimens is also associated with relapse-free survival in breast cancer patients (106). In glioma, reduced tumor cell progression and relapse by knockdown of CDGSH iron sulfur domain 2 (CISD2) was associated with the activation of Beclin 1-mediated autophagy (107).

Our observations suggest that any deficiency in tumor cell-intrinsic autophagy could result in a reduced sensitivity of breast cancer to chemotherapy or immunotherapy. Therefore, IHC analysis of tumor biopsies before and after neoadjuvant or adjuvant chemotherapy could determine cell-intrinsic and chemotherapy-induced autophagy, respectively, and in turn might predict the risk of distant recurrence of the diseases accordingly. In future studies, other murine and human breast tumor cell lines as well as other types of carcinoma cells should be used in order to determine whether our findings offer a general mechanism of autophagy-associated tumor dormancy and relapse, or it might be a cancer specific phenomenon.

Chapter 4. Immunotherapeutic targeting of tumor dormancy

4.1. Rationale, hypothesis and experimental design

Cancer immunotherapeutics often prolong survival of patients without offering a consistent cure or eliminating the disease relapse for cancer patients. On the other hand, immunotherapies have been able to eliminate many infectious diseases such as polio, measles etc. This is because immunotherapies work perfectly in a preventive setting before clinical symptoms of the disease are evident or during a latency/dormancy period. Therefore, the central hypothesis to be tested is that immunotherapeutic targeting of breast cancer during tumor dormancy can prevent disease progression and/or recurrence but cannot eliminate solid tumors. Currently, there is no therapeutic that can effectively target dormant tumor cells and prevent distant recurrence of the disease. Recently, we reported that dormant tumor cells that became resistant to chemotherapy or radiation therapy remained susceptible to immunotherapy (62). However, chemotherapy-induced dormant tumor cells could undergo immunoediting because they contain $Ki67^{-}$ quiescent cells and $Ki67^{low}$ indolent cells; while indolent cells show a balance between cell proliferation and cell death, quiescent cells are arrested in G0 and cannot undergo cell division. We have reported that unlike quiescent cells, $Ki67^{low}$ indolent cells are susceptible to immunoediting and could lead to tumor escape and relapse (62). Other groups also reported that 40% of CTCs undergo apoptosis (108), perhaps because of being in the state of indolent dormancy. To inhibit local tumor growth, we will use a low dose chemotherapy. A low dose immunogenic chemotherapy will include 5-Fluorouracile+Adriamycin+Cyclophosphamide (FAC), which leaves predominantly $Ki67^{-}$ quiescent tumor cells incapable of undergoing immunoediting. While a standard dose of chemotherapy is highly toxic for proliferating effector T cells and unsuitable for being simultaneously combined with immunotherapy, a low dose chemotherapy does not harm

lymphocytes, and also induces anti-tumor immune responses (40–43). For instance, breast cancer patients who were treated with low dose CYP showed decreased Tregs and increased effector T cells as well as NK-dependent anti-tumor immunity (40,41). Similar results were reported for low dose 5-FU in depleting MDSCs (42). CYP was also reported to induce immunogenic cell death (ICD) (44). Low dose chemotherapies were shown to be a suitable preparative regimen for a vaccination approach in order to boost anti-tumor immune responses against dormant cells (43). We will also use tumor-sensitized lymphocytes for adoptive immunotherapy of the locally established dormant tumor cells. It was reported that the ex vivo reprogramming of tumor sensitized lymphocytes by means of bryostatin 1/Ionamycin (B/I) and the common gamma chain cytokines IL2, IL7 and IL15 generates tumor-reactive T cells, NK cells and NKT cells that are resistant to MDSCs (62,109). AIT by means of such reprogrammed lymphocytes collected from the spleen or lymph nodes of tumor bearing mice protected animals from tumor progression and prolonged survival of animals bearing neu overexpressing mouse mammary carcinoma (MMC) or triple negative 4T1 tumors (62,110).

4.2. Results

4.2.1. A low dose FAC induce predominatly Ki67^{-} quiescent type of tumor dormancy, *in vitro* and *in vivo*

We have reported that a standard dose chemotherapy simultaneously induced tumor cell apoptosis and established tumor dormancy in MMC tumor cells (25). We also reported that standard dose chemotherapy induced a transient local tumor dormancy which contained two populations: Ki67^{low} indolent and Ki67^{-} quiescent dormant MMC cells (62). Here, we examined tumor biopsies and surgical excisions of breast cancer patients, who partially responded to standard neoadjuvant therapies, in order to determine the state of a transient local tumor dormancy. We detected Ki67^{low}

indolent cells (light brown) and Ki67$^-$ quiescent cells (blue) in the tumor excisions compared to the parallel biopsy specimens (Figure 14, left panel). Neoadjuvant therapies resulted in a significant increase in the percentage of Ki67$^-$ quiescent cells (p=0.003) as well as a significant decrease in the percentage of Ki67$^+$ cells (p=0.003) (Figure 14, right panel).

Previously, we also reported that Ki67^{low} indolent cells were more susceptible than Ki67$^-$ quiescent cells to IFN-γ-induced PD-L1 expression (25,62). Here, we wanted to determine whether using a low dose cytostatic chemotherapy could induce predominantly a quiescent type of tumor dormancy, thereby overcoming tumor immunoediting. To test our hypothesis, we established tumor dormancy, *in vitro*, by treating MMC cells with a low dose FAC for 5 consecutive days, and analyzed dormant cells 10 days after the completion of FAC treatment. We found that low dose FAC was capable of establishing tumor dormancy by reducing tumor cell numbers (Figure 15A), as well as increasing the proportion of Ki67$^-$ quiescent cells (Figure 15B). In order to determine the *in vivo* relevance of our findings, FVBN202 mice bearing MMC tumor cells in the mammary region received a low dose FAC to establish a transient local tumor dormancy. Low dose FAC inhibited tumor growth in the mammary region (Figure 16). Flow cytometry analysis of neu positive MMC showed a lower number of FVS- viable tumor cells in the mammary region, and a predominance of Ki67$^-$ quiescent tumor cells following FAC treatment (Figure 16).

4.2.2. A low dose FAC inhibits tumor immunoediting

Since low dose FAC induced predominantly a quiescent type of tumor dormancy, we wanted to see if dormant tumor cells become less susceptible to immunoediting. We examined two types of tumor immunoediting which included, those that are independent of cell division such as JAK-STAT signal transduction-dependent PD-L1 expression (111), and those that take place during cell division such as tumor antigen loss (112). We found that FAC-induced dormant MMC are less

susceptible to IFN-γ-induced PD-L1 expression compared with MMC control (Figure 17A). Analysis of the $Ki67^+$ and $Ki67^-$ fractions of tumor cells for the expression of PD-L1 showed that $Ki67^+$ cells were more susceptible to IFN-γ-induced PD-L1 expression compared with $Ki67^-$ quiescent cells (Figure 17B). On the contrary, IFN-γ-induced downregulation of the neu antigen was evident only in MMC control, specifically in the $Ki67^+$ fraction, whereas FAC-induced dormant MMC cells did not undergo immunoediting as IFN-γ did not induce neu antigen loss in them (Figure 17C-D).

4.2.3. T cells collected from early stage breast cancer are best source for AIT

For the sake of clinical translation of our findings, it was important to determine whether autologous lymphocytes for AIT should be collected from patients with early stage breast cancer or those harboring metastatic disease. So, splenocytes that were collected from animals with primary tumor in the mammary region or those with experimental metastasis in the lungs were re-programmed and then cultured with MMC tumor cells to evaluate their anti-tumor function ex vivo. As shown in Figure 18A, splenocytes from animals bearing primary tumor in the mammary region expanded better than those bearing lung metastasis, though with a similar cell viability. Either source induced apoptosis in MMC while those from animals harboring primary tumor in the mammary region were slightly more effective than those collected from animals harboring MMC in the lungs (Figure 18B).

4.2.4. Autologous T cells collected prior to chemotherapy expand better than T cells collected after chemotherapy

Lymphocytes were collected before or after a low dose FAC chemotherapy from FVBN202 mice bearing primary tumor in the mammary region, and re-programmed *ex vivo* as previously described

by our group (62). Lymphocytes from pre-chemotherapy were expanded better than those from post-chemotherapy while both sources showed a comparable viability (Figure 19).

4.2.5. The failure of AIT following a low dose FAC against primary tumor is associated with the induction of the PD-L1 and ETR_A survival pathways following AIT

In order to determine whether a low dose FAC that limits tumor growth but does not eliminate the tumor can be effectively targeted by immunotherapy, FVBN202 mice bearing primary MMC in the mammary region were used. For AIT, we used our established protocol, which included the collection of the spleen or lymph nodes from tumor-bearing animals and expanding the lymphocytes, ex vivo, in the presence of common gamma chain cytokines (γ-c - Gamma-chain) (IL2, IL7, IL15) for 6 days. The expanded lymphocytes contained CD8+ and CD4+ T cells as well as NK/NKT cells, and were resistant to MDSCs (62,109). We also reported a similar tumor-specific efficacy for the expanded autologous lymphocytes using PBMC of patients with early stage breast cancer (113). Here, we found that immunotherapeutic targeting of solid tumors following a low dose FAC chemotherapy promoted the progression of primary tumor in FVBN202 mice (Figure 20). Flow cytometry analysis of tumor microenvironment showed a significantly higher expression of ET-1, ETR_A and ETR_B and the retention of PD-L1 expression in FVS negative viable tumor cells following AIT (Figure 21A). Tumor infiltrating viable T cells also showed a significantly higher expression of ET-1 on CD8+ T cells and CD4+ T cells (Figure 21B). In addition, a higher frequency of viable CD8+PD-1+ T cells was detected following AIT (Figure 21C). Flow cytometry analysis of tumor infiltrating myeloid cells also showed a significantly higher expression of ET-1 in MDSCs, CD11b+ and Gr1+ cells in the AIT group (Figure 21D). These data suggest that tumor survival pathways became highly active following immunotherapeutic targeting of FAC-treated solid mammary tumor, as adoptively transferred T

cells and tumor infiltrating myeloid cells could provide ET-1 for inducing the survival of ETR_A positive tumor cells. In order to determine if the expression of ET-1 on T cells was due to the *ex vivo* expansion and/or an event that took place following AIT, splenic lymphocytes of donor mice were analyzed before and after the *ex vivo* expansion. We detected a higher level of the expression of ET-1, ETR_A and ETR_B in the *ex vivo* expanded CD8+ T cells but not CD4+ T cells (Figure 22

4.2.6. A direct interaction between T cells and FAC-induced dormant tumor cells fail to induce the expression of ETR_A and ETR_B on dormant cells

In order to determine whether the interaction between tumor cells and lymphocytes at the tumor site might increase the expression of endothelin pathway, an *ex vivo* co-culture study was performed using untreated MMC or FAC-treated dormant MMC as target cells. We showed that a direct interaction between T cells and dormant tumor cells did not induce the expression of ET-1, ETR_A or ETR_B on dormant cells (Figure 23).

4.2.7. A blockade of the ETR_A or PD-L1 survival pathways improves immunotherapy of local tumor dormancy

Since failure of AIT against established tumors containing dormant cells was associated with increases in the endothelin survival pathway and the retention of PD-L1 expression, we wanted to determine if a blockade of the survival pathways in dormant tumor cells might improve the efficacy of AIT. We used a dual endothelin receptor antagonist, Macitentan (Maci), or anti-PD1 antibody. While Macitentan or anti-PD1 did not inhibit the growth of the FAC-treated primary tumor (Figure 24A), they improved anti-tumor efficacy of AIT against FAC-treated primary tumors (Figure 24B).

4.2.8. A low dose FAC reduces distant tumor dormancy, which is maintained by immunotherapy

It was reported that FVBN202 mice bearing primary tumor cells show disseminated tumor dormancy in the bone marrow (114). However, the timing at which tumor cells disseminate from the primary site to other organs remains elusive due to lack of an experimental model. Here, we used FVBN202 transgenic mice that express rat neu oncogene in their mammary rumors allowing the detection of invisible dormant tumor cells at the distant organs by FACS analysis of the rat neu positive cells. FVBN202 mice bearing MMC in the mammary region or experimental metastatic MMC in the lungs were used. Tumor-bearing mice without any treatment served as tumor control. FVBN202 mice bearing primary tumor in the mammary region or experimental metastasis in the lungs showed no palpable tumor at the distant sites (Figure 25A-B, upper panels). Flow cytometry analysis of tumor control group showed the presence of neu positive dormant tumor cells in the lungs and in the liver of mice bearing primary tumor in the mammary region (Figure 25A) and in the liver of mice bearing metastatic tumor in the lungs (Figure 25B). Low dose FAC that inhibited tumor growth at the primary site, also decreased but not eliminated tumor dormancy at the distant sites (Figure 25A-B, middle and lower panels). All distant dormant tumor cells were dominated with $Ki67^-$ quiescent cells regardless of the treatments (Figure 25A). An improved efficacy of AIT in the presence of Macitentan or anti-PD1 antibody did not eliminate distant tumor dormancy, but retained dormant cells in a predominantly $Ki67^-$ quiescent state (Figure 26). Dormant tumor cells in the lungs and in the liver showed different patterns of the expression of PD-L1, ET-1, ETR_A or ETR_B depending on the type of treatments that animals received (Figure 27A-B).

In order to determine whether the most effective immunotherapy using AIT combined with anti-PD1 therapy was associated with the infiltration of effector T cells at the distant sites, T cells were

analyzed using a multi-color flow cytometry. In the lungs and liver, all groups showed a higher proportion of viable CD4+ T cells than CD8+ T cells (Figure 28A-B). In the lungs, a predominant CD4+ naïve T cells were present in all groups (Figure 28A). However, the proportion of CD8+ T effector cells increased in animals who received anti-PD1+AIT (Figure 28A). In the liver, all groups but the control showed a predominant CD4+ T effector cells (Figure 29B). CD8+ T effector cells were dominant only in animals who received anti-PD1 and AIT (Figure 28B). The *ex vivo* culture of the lungs and the liver showed a higher dormant tumor burden in the lungs than in the liver (Figure 29A). Flow cytometry analysis of dormant cells recovered from the lungs showed the expression of neu antigen and a dominant Ki67+ cells (Figure 29B).

Culture of dormant tumor cells isolated from the lungs of FVBN202 female mice who had received a low dose FAC for 9 consecutive days in the presence of autologous lymphocytes used for AIT showed the inhibition of tumor relapse *ex vivo*. As shown in Figure 29C, dormant tumor cells relapsed, *ex vivo*, in the absence of tumor-reactive lymphocytes while those in the presence of the lymphocytes did not grow.

4.2.9. The inhibitor of Bcl-xL increases apoptosis in FAC-induced dormant tumor cells

Since the cell survival receptors PD-L1 and ETR_A use the anti-apoptotic Bcl-2/Bcl-xL as a common downstream signaling pathway, we wanted to determine whether the inhibition of Bcl-2/Bcl-xL promotes apoptosis of dormant tumor cells. First, we showed that FAC-treated dormant MMC retained the expression of the anti-apoptotic Bcl-2/Bcl-xL/Mcl-1 while downregulating the expression of the cell proliferation promoting survivin (Figure 30A). The inhibition of Bcl-2, Mcl-1 or Bcl-xL for 24 hours showed an increased cleavage of caspase 3 in dormant tumor cells in the presence of the specific inhibitor of Bcl-xL inhibitor, A-1331852 (Figure 30B).

4.2.10. A low dose FAC induces mutations in dormant tumor cells

To determine whether a higher susceptibility of dormant MMC was associated with an increased rate of mutations, RNAseq analysis of FAC-treated dormant MMC (DT) as well as untreated MMC control (TC) or healthy control tissues (HC) collected from the tail of FVBN202 mice was performed. We detected a distinct pattern of gene expression in dormant tumor cells (Figure 31). Dormant MMC also showed an increased number of mutations as a single nucleotide polymorphism (SNP) (Figure 31). Dormant MMC also showed an increased number of InDels (Figure 31), referring to insertions or deletions of small fragments (one or more nucleotides) when compared to reference genome. In addition, dormant MMC showed an increased expression of MHC class I haplotypes, H-2D, H-2Q and H-2K compared with MMC control (Figure 31). These data suggest that low dose FAC could change not only the pattern of antigenic peptides but also the proportion of Major histocompatibility complex (MHC) haplotypes, thereby creating dormancy-associated antigens.

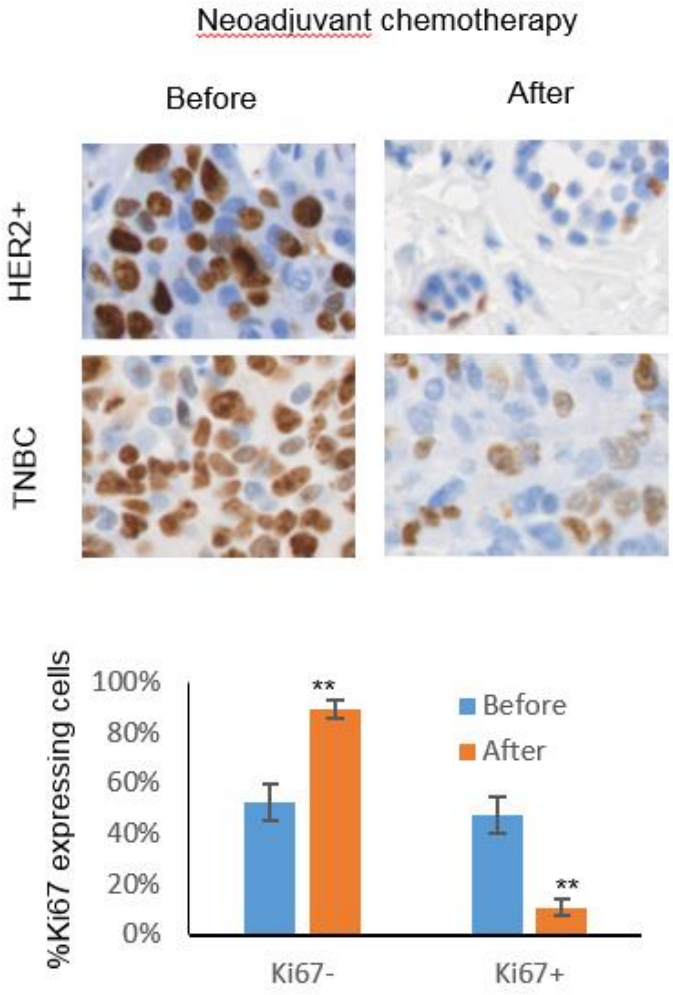


Figure 14. Neoadjuvant therapies induce local tumor dormancy. Tumor biopsies (before) and surgical excisions (after) of patients with triple negative breast cancer (TNBC, n=2) or HER2 positive breast cancer (HER2+, n=2) were subjected to Ki67 staining. Patient with HER2 positive breast cancer received neoadjuvant Pertuzumab, Trastuzumab, Docetaxel and Carboplatin, and those with TNBC received neoadjuvant Taxol, Carboplatin, Adriamycin and Cytoxan, followed by surgery. A) IHC analysis showing Ki67high (dark brown), Ki67low (light brown) and Ki67- (blue). B) Percent Ki67- or Ki67+ in tumor biopsies (before, n=4) or surgical excisions (after, n=4) were analyzed using the Vectra® Polaris™ Automated Quantitative Pathology Imaging System and InForm software (PerkinElmer) (1x1 field, 20x resolution).

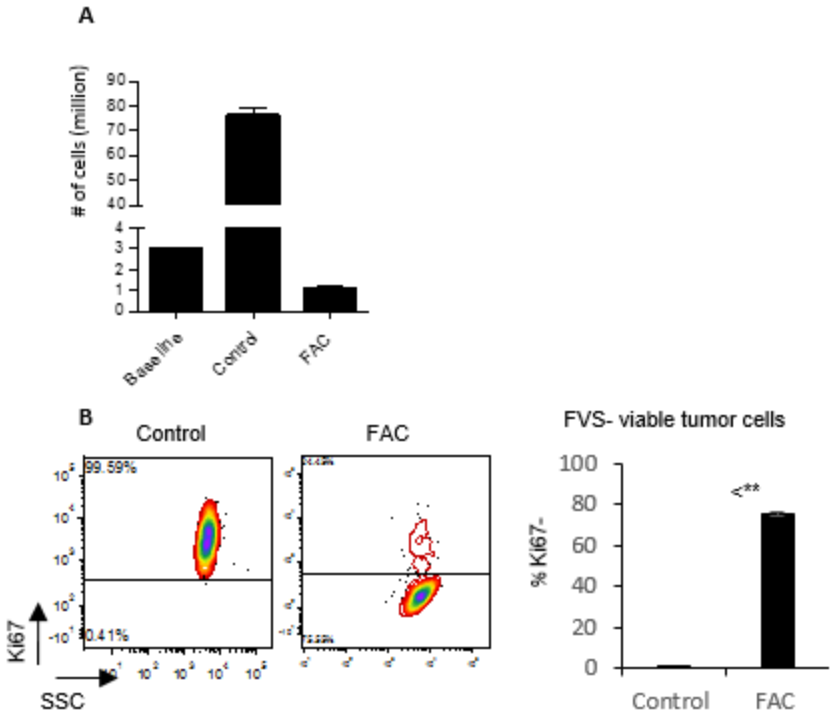


Figure 15. Low dose FAC induced predominantly Ki67- quiescent tumor dormancy, *in vitro*. MMC (Baseline, 3 million cells) were treated with FAC (5-FU (5uM) + ADR (0.1 uM) + CYP (2uM)) daily for 5 days (FAC). Untreated MMC served as control (Control). One day after the last treatment, cells were washed and cultured with medium for additional 10 days. Cells were then detached from the flasks with 10 uM EDTA, counted using trypan blue exclusion (A), and stained with FVS, anti-neu and anti-Ki67 Abs for flow cytometry analysis. Gated FVS- neu+ viable tumor cells were analyzed for the expression of Ki67 (B). Data represent triplicate experiments.

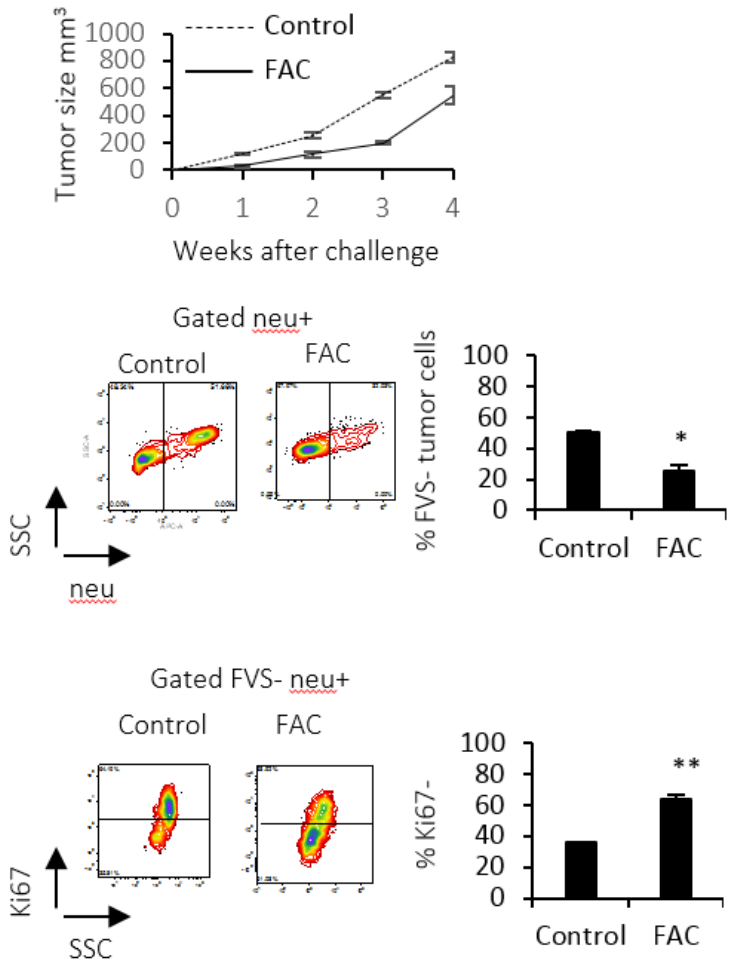
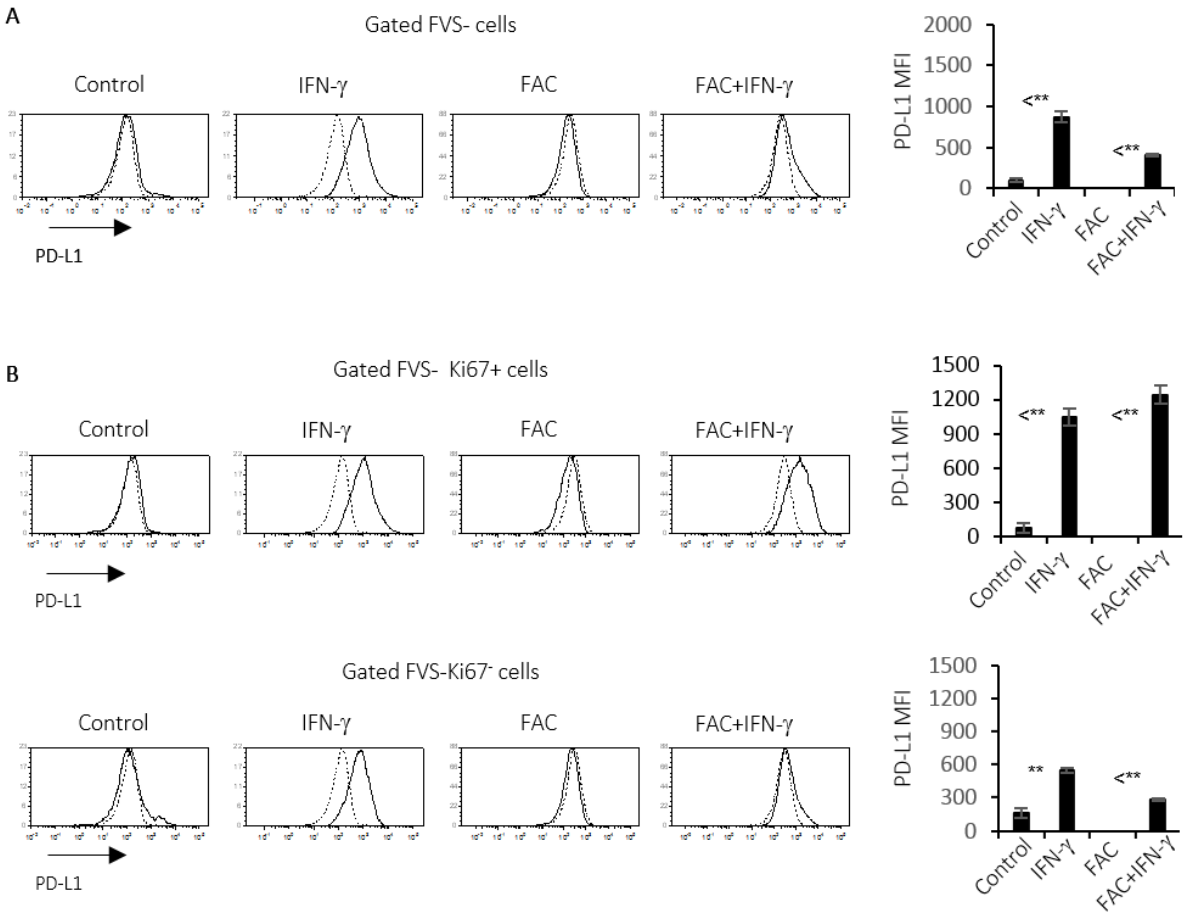


Figure 16. Low dose FAC induced predominantly Ki67- quiescent tumor dormancy, *in vivo*. Female FVBN202 mice (6-8 weeks old) were challenged with 3 million neu positive MMC in the mammary region. Mice were then split into two groups, who received no treatment (Control, n=2) or received 9 daily treatments with a low dose FAC ((10 mg/kg 5-FU+3 mg/kg ADR+10 mg/kg CYP, i.p., n=3). Animals were euthanized when tumor reached 1000 mm^3 in the control group. Tumors or lungs were removed, and subjected to flow cytometry analysis using APC-CY7-FVS, APC-anti-neu, and PE-anti-Ki-67 Abs. The upper panel shows palpable tumors, the middle panel shows gated FVS-neu+ viable tumor cells, and the lower panel shows the expression of Ki67 on FVS-neu+ viable tumor cells.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Gated FVS- cells
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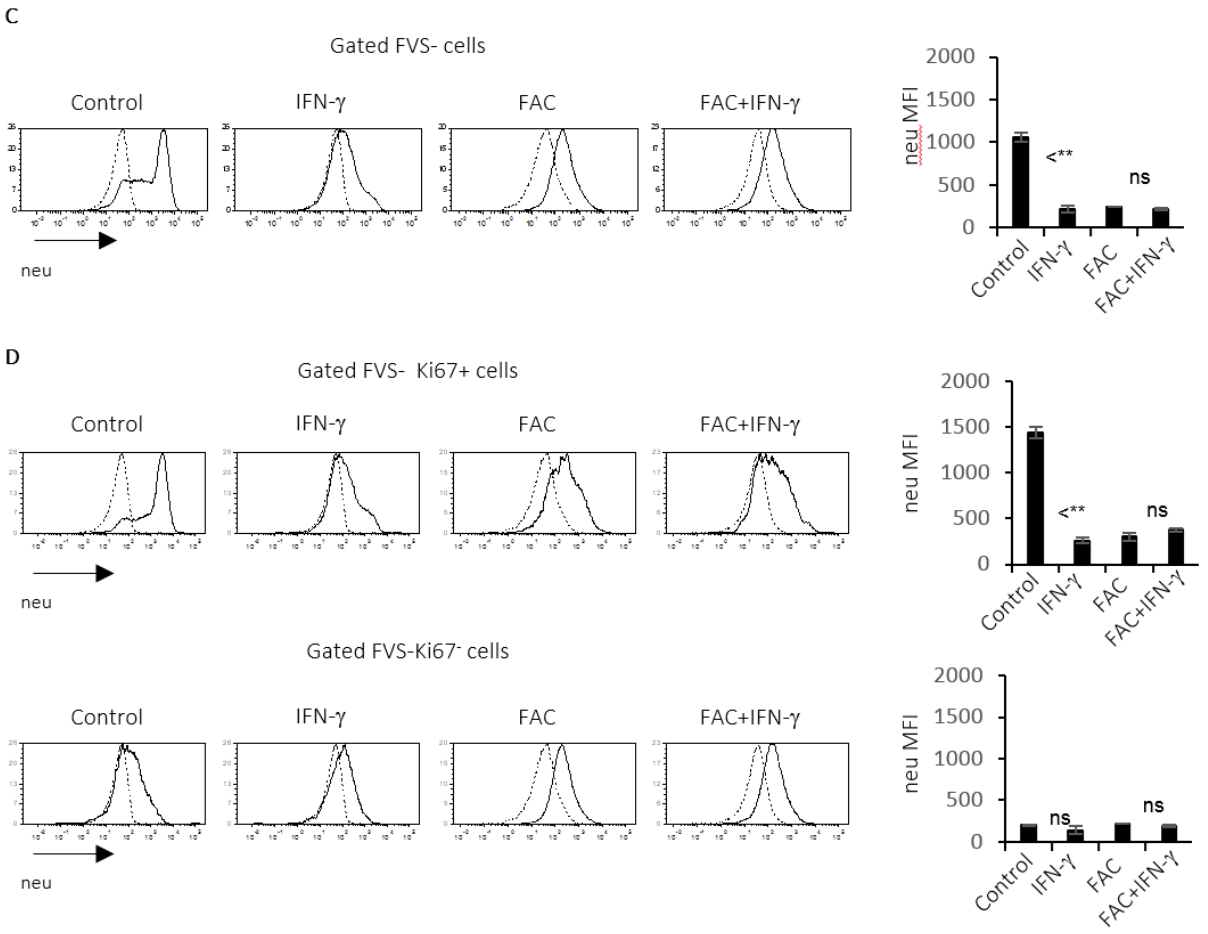


Figure 17. FAC-treated dormant cells are less susceptible to immunoediting because of the dominance of Ki67- dormancy. Untreated MMC (Control) or FAC-treated dormant MMC (FAC), 10 days after FAC, were cultured in the absence or presence of IFN-γ (50 ng/ml) for 12 hours (IFN-γ or FAC+ IFN-γ). A) Gated FVS- viable cells were analyzed for the expression of PD-L1. B) Gated FVS-Ki67+ or FVS-Ki67- cells were analyzed for the expression of PD-L1. C) Gated FVS- viable tumor cells were analyzed for the expression of neu antigen. D) Gated FVS-Ki67+ or FVS-Ki67- cells were analyzed for the expression of neu antigen. Data represents mean ± SEM of triplicate experiments.

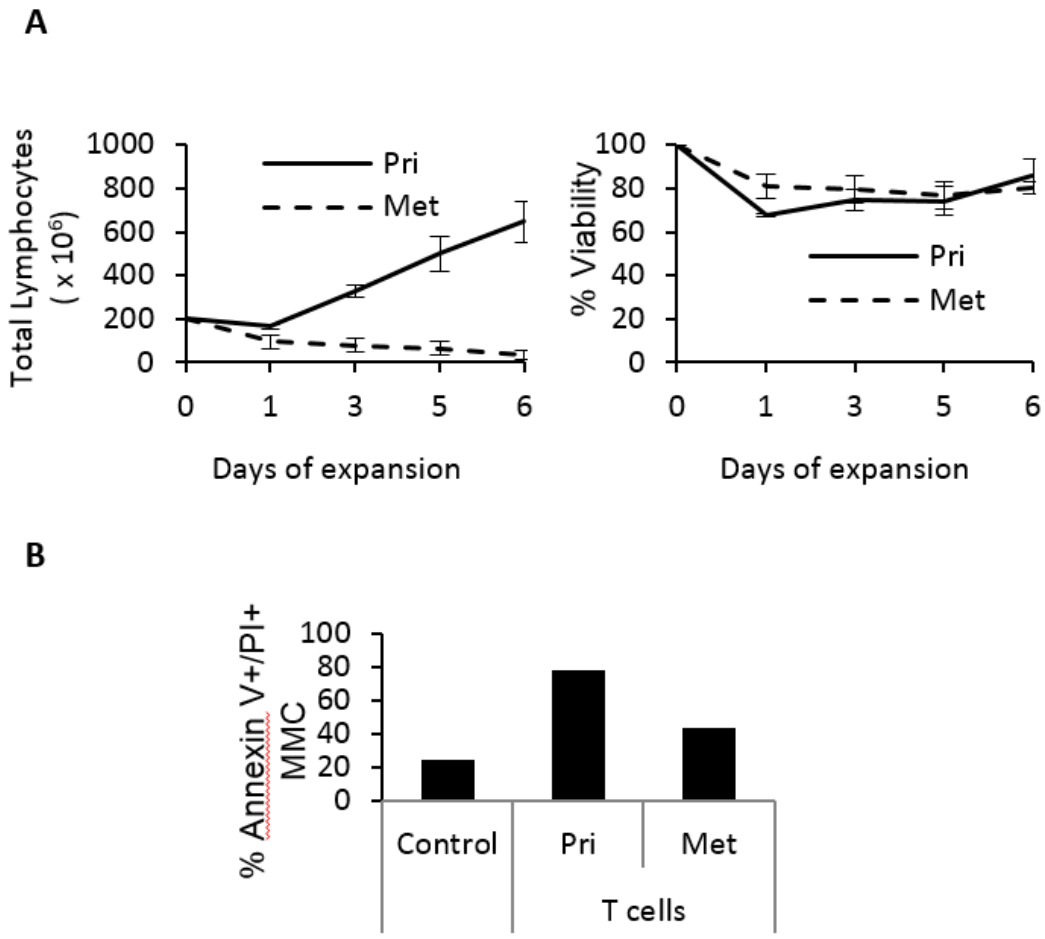


Figure 18. Primary tumor bearing mice are best donors of lymphocytes for autologous AIT. To establish donors for AIT, female FVBN202 mice (3 mice/group) were challenged with 3 million MMC in the mammary fat pads (Pri) or one million cells i.v. (Met). Animals were sacrificed 3-4 weeks after tumor challenge and their splenocytes were stimulated with bryostatin 1 (2 nM) and ionomycin (1 uM) (B/I) and 80 U/ml IL-2 for 16 hours followed by cultured with IL-7 (20 ng/ml) + IL-15 (20 ng/ml) and IL-2 (40 U/ml) for 6 days. A) lymphocyte counts (left panel) and percentage of viability (right panel) were performed during a 6-day expansion ex vivo using trypan blue exclusion. B) MMC cells were co-cultured with lymphocytes that were expanded from animals bearing primary MMC (Pri) or metastatic MMC (Met), and apoptosis in gated APC-neu+ cells was determined using FITC-Annexin V/PI staining.

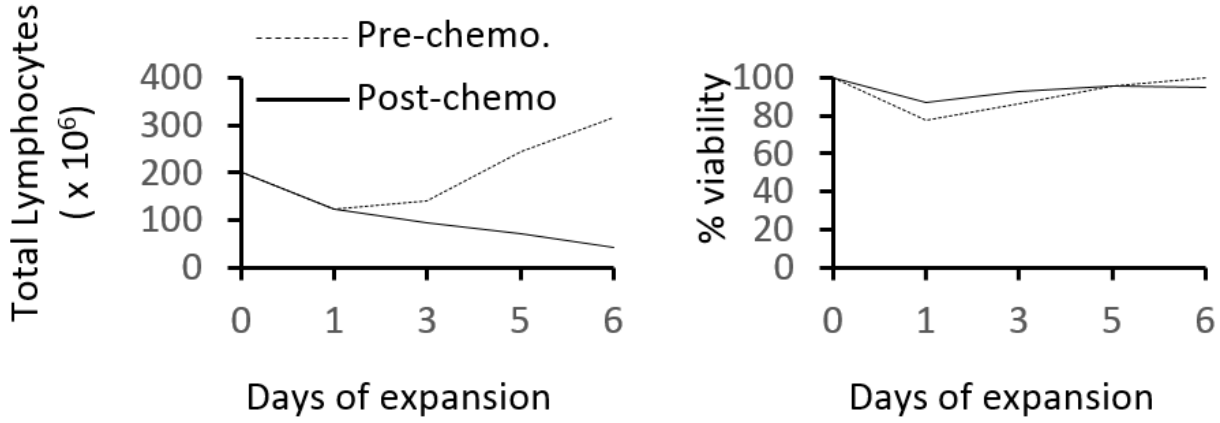


Figure 19. Autologous T cells collected prior to chemotherapy expand better than T cells collected after chemotherapy. To establish donors for AIT, female FVBN202 mice (3 mice/group) were challenged with 3 million MMC in the mammary fat pad. Mice were then split into two groups, who received no treatment (pre-chemo) or received 9 daily treatments with a low dose FAC (post-chemo). Animals were sacrificed 3-4 weeks after tumor challenge and their splenocytes were expanded using B/I and IL-2, IL-7, and IL-15 for 6 days. Lymphocyte counts were performed during a 6-day expansion ex vivo using trypan blue exclusion.

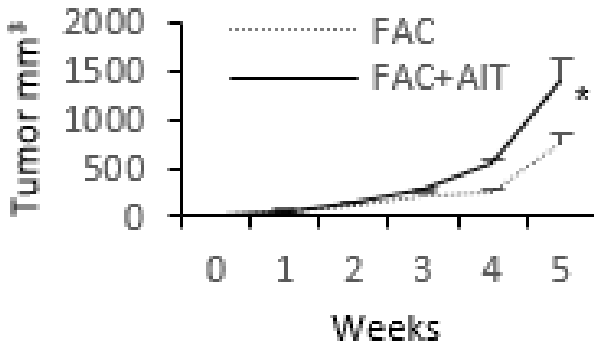


Figure 20. AIT following of a low dose FAC chemotherapy of primary breast cancer fails to protect animals from tumor growth. Female FVBN202 mice (4 mice per group) were challenged with 3 million MMC in the mammary region. Three days after tumor challenge, all groups received 9 daily doses of FAC ((10 mg/kg 5-FU+3 mg/kg ADR+10 mg/kg CYP (i.p.) followed by a single dose of CYP (100 mg/kg) before being split into two groups who did not receive AIT (FAC) or received AIT (FAC+AIT) (i.v. injection of 70 million lymphocytes/mouse). Tumor growth was monitored by using a digital caliper.

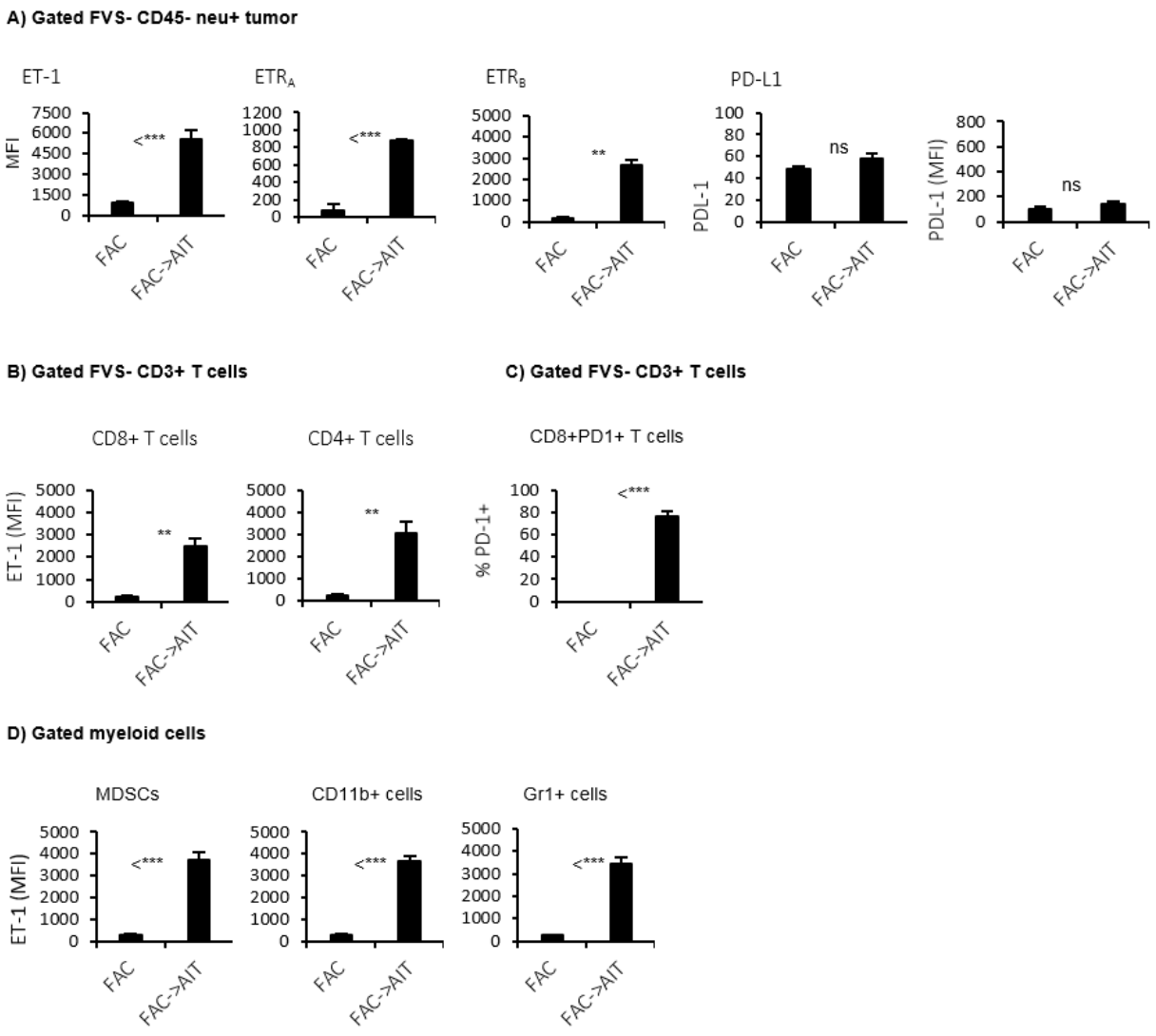


Figure 21. AIT following of a low dose FAC chemotherapy of primary breast cancer induces the expression of endothelin survival pathway. FVBN202 mice bearing FAC-induced dormant MMC that were established in Figure 20 were sacrificed and their tumor were analyzed by flow cytometry. A) Gated FVS- CD45- neu+ tumor cells were analyzed for the expression of ET-1, ETR_A, ETR_B or PD-L1. B) Gated FVS-CD3+CD8+ T cells or FVS-CD3+CD4+ T cells were analyzed for the expression of ET-1, ETR_A, ETR_B. C) Gated FVS-CD3+CD8+ T cells expressing PD-1. D) Gated FVS-CD11b+Gr1+ or CD11b+ or Gr1+ were analyzed for the expression of ET-1.

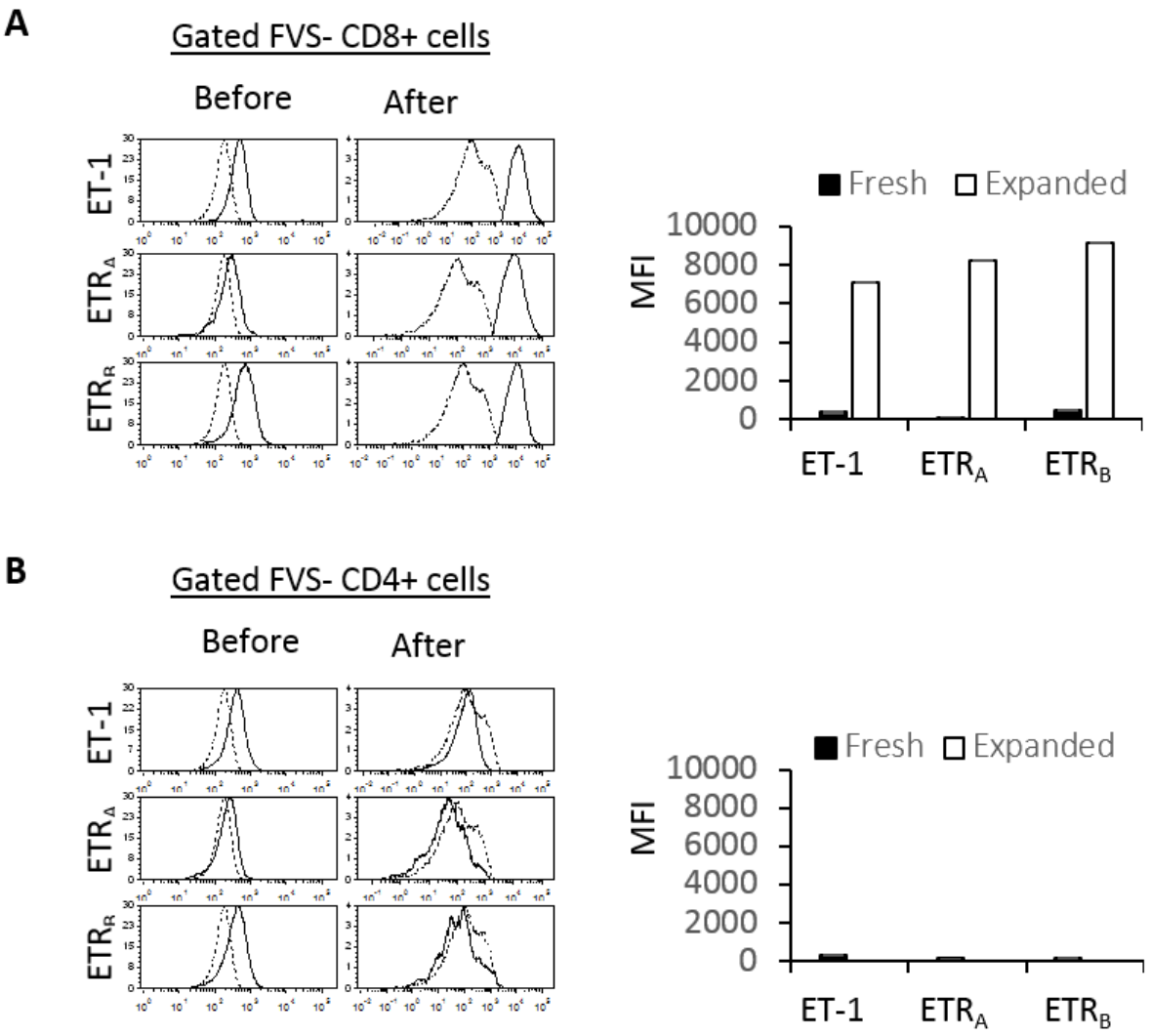


Figure 22. The CD8+ T cell fraction of tumor-reactive lymphocytes used for AIT show an increased expression of the endothelin receptor/ligand. Splenic lymphocytes collected from tumor-bearing FVBN202 mice prior to the ex vivo expansion (before) or after the expansion (After) were analyzed for the expression of endothelin ligand and receptors. Gated FVS- viable CD8+ T cells (A) or CD4+ T cells (B) were analyzed for the expression of ET1, ETR_A or ETR_B. Data represent triplicate experiments.

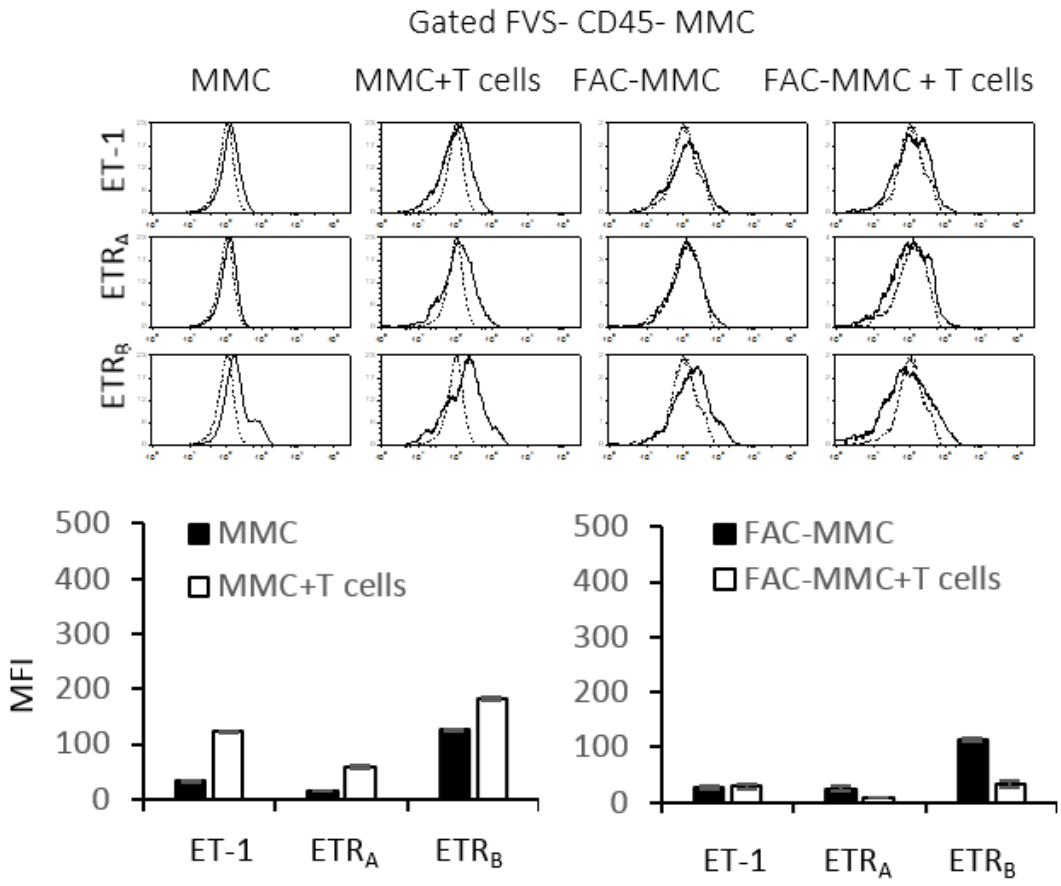


Figure 23. A direct interaction between T cells and FAC-induced dormant tumor cells fail to induce the expression of ETR_A and ETR_B on dormant cells. MMC or FAC-treated dormant MMC alone or co-cultured with tumor-reactive T cells in a 10:1 E:T ratio for 24 hours and subjected to a multi-color flow cytometry. Gated FVS-CD45-neu+ MMC were analyzed for the expression of ET-1, ETR_A or ETR_B. Data represent triplicate experiments.

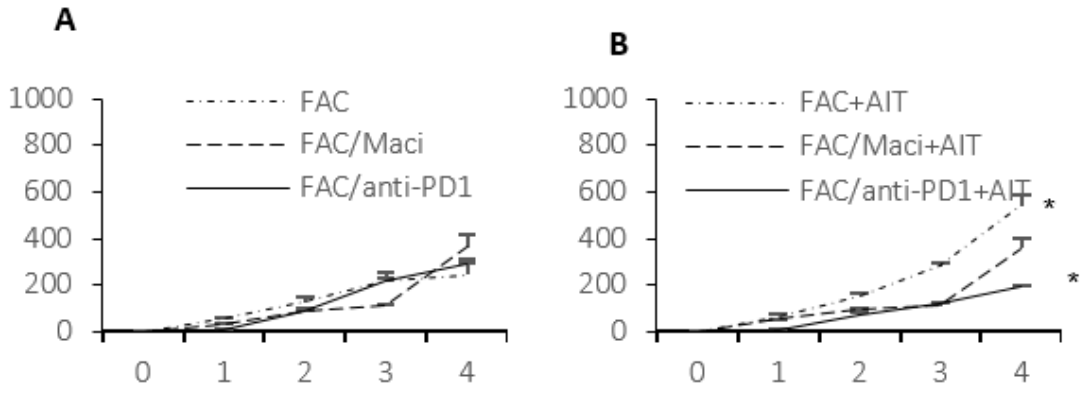
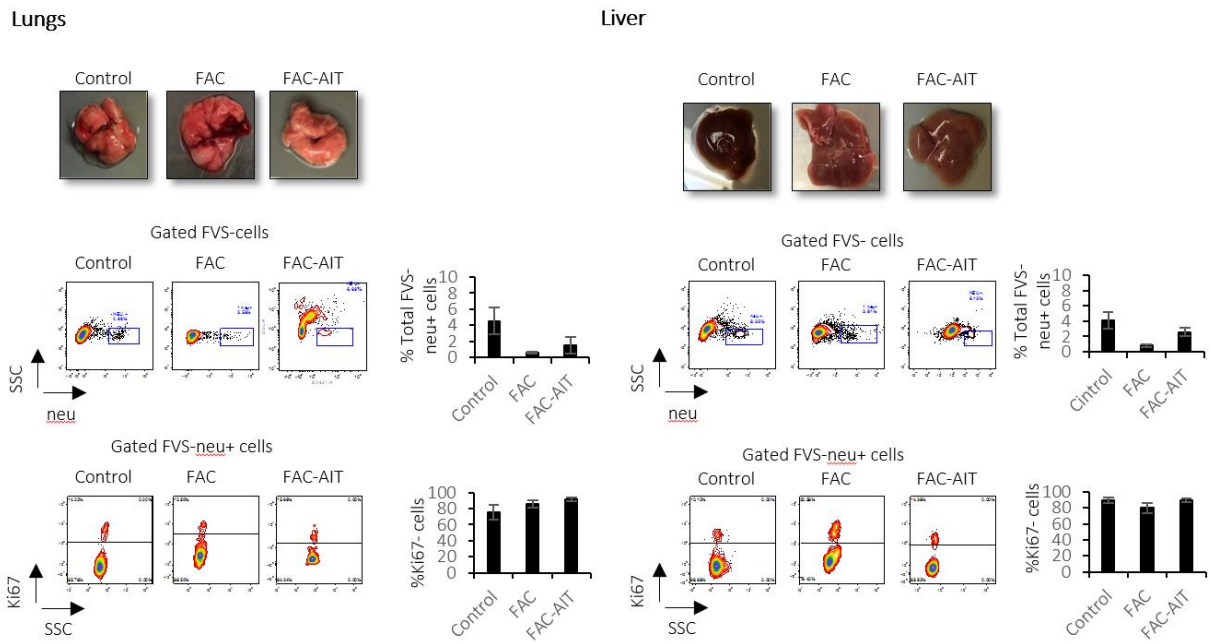


Figure 24. The blockade of the tumor survival pathways improves immunotherapy of tumor dormancy. Female FVBN202 mice (6-8 weeks old) were challenged with 3 million neu positive MMC in the mammary region, and 3 days after they received different treatments as follow. A) All groups received 9 daily treatments with low dose FAC ((10 mg/kg 5-FU+3 mg/kg ADR+10 mg/kg CYP, i.p. (FAC, n=4). One group did not receive any treatment (FAC), received 6 daily treatments with macitentan (30mg/kg orally) (FAC+Maci, n=3), or received 5 treatments with anti-PD1 antibody (100mg/kg anti-PD-1, i.p. every three days) (FAC/anti-PD1, n=3). B) Additional groups who received the three treatments above also received AIT (n=3 mice/group). Tumor size was monitored by a digital caliper twice weekly. Tumor size was calculated by V [volume] = (L[length] x W[width]2)/2.

A) Primary tumor in the mammary region



B) Experimental metastasis in the lungs

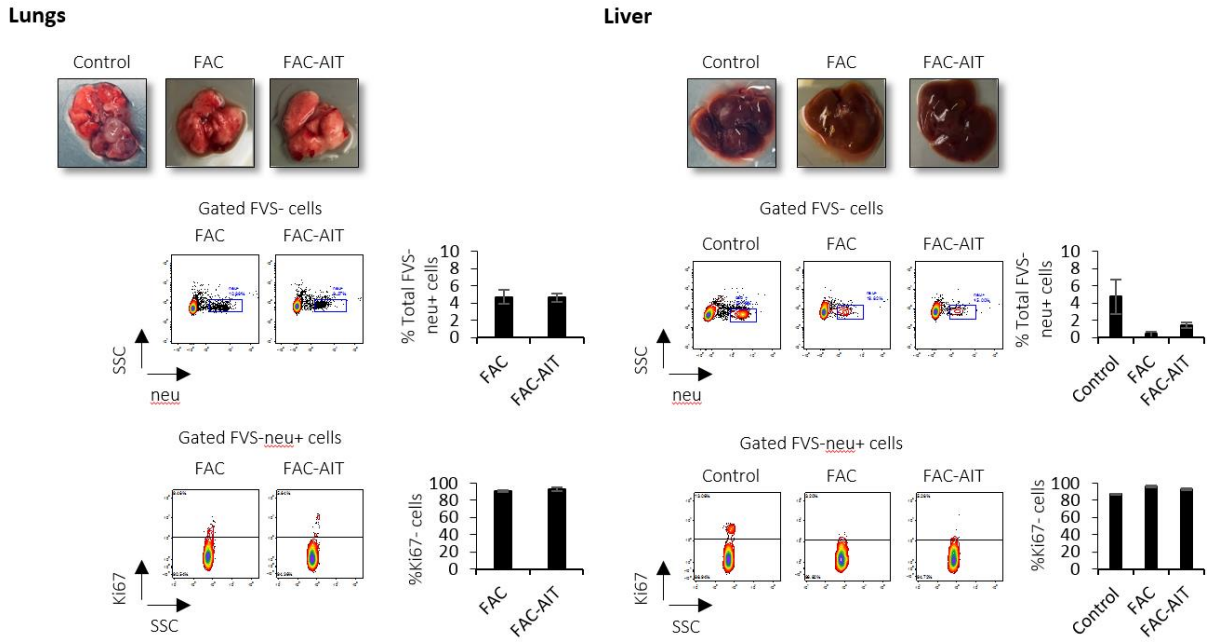


Figure 25. Low dose FAC reduces distant tumor dormancy. Female FVBN202 mice (6-8 weeks old) were challenged with 3 million neu positive MMC in the mammary region (A) or 1 million MMC i.v. for establishing experimental metastasis in the lungs (B). Mice were then split into three groups, who received no treatment (Control) or received 9 daily treatments with a low dose FAC (FAC, i.p.) followed by i.v. injection of 70 million lymphocytes/mouse (FAC-AIT). Animals were euthanized when tumor reached 1000 mm3 in the control group (A), or when control group became moribund by losing over 10% weight (B). Lungs and liver were removed and subjected to a multi-color flow cytometry analysis using APC-CY7-FVS, APC-anti-neu, and PE-anti-Ki-67 Abs. Data represent 3-4 mice per group.

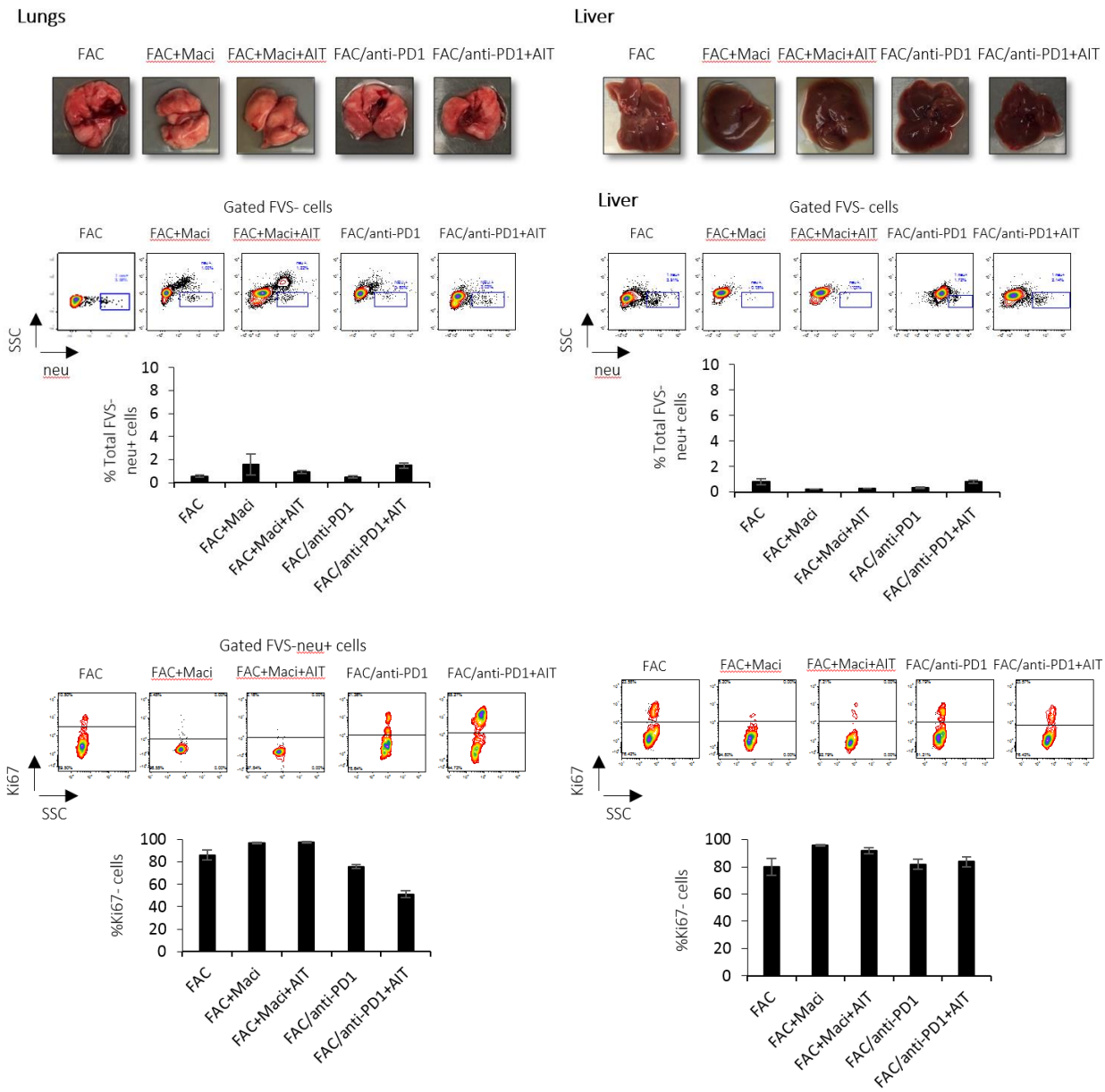
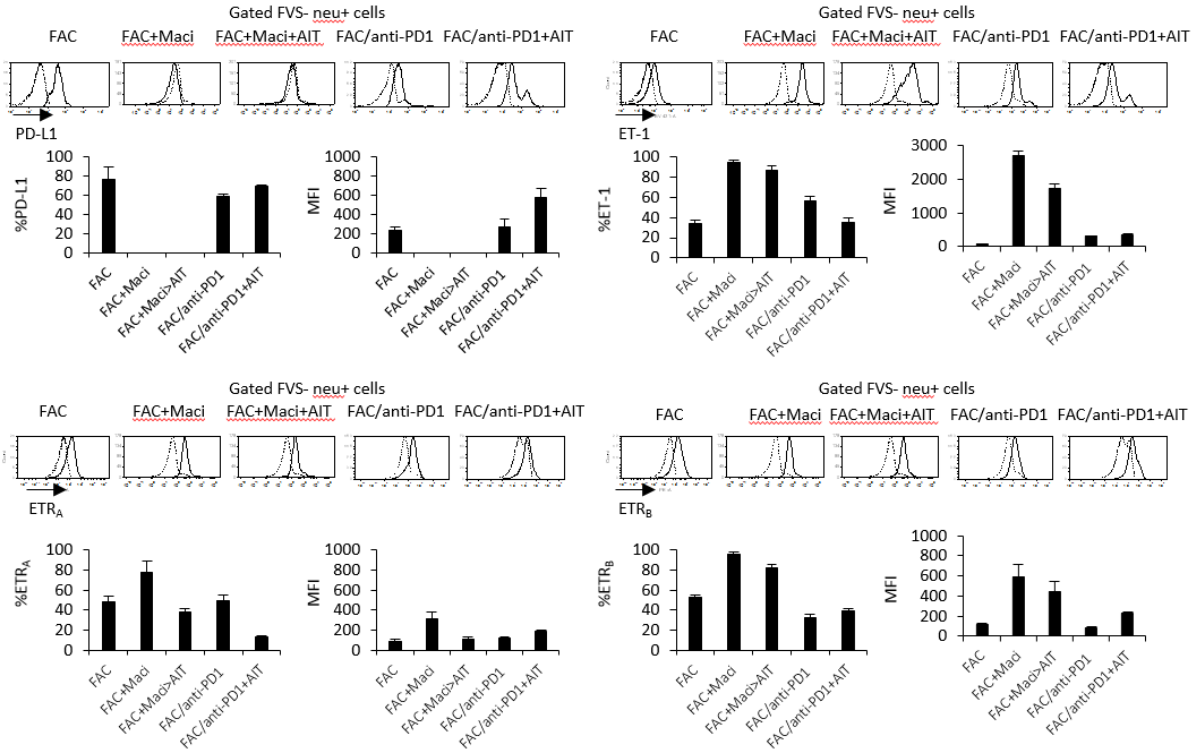

Lungs
FAC
FAC+Maci
FAC+Maci+AIT
FAC/anti-PD1
FAC/anti-PD1+AIT
Liver
Gated FVS- cells
SSC
neu
% Total FVS- neu+ cells
Gated FVS-neu+ cells
Ki67
%Ki67- cells

Figure 26. Immunotherapeutic targeting of FAC-induced dormant MMC combined with the blockade of tumor survival pathways do not eliminate distant dormant tumor cells but spared predominantly Ki67- quiescent type of dormancy. Female FVBN202 mice (6-8 weeks old) were challenged with 3 million neu positive MMC in the mammary region. Three days after tumor challenge, mice received 9 daily treatments with low dose FAC, and split into 5 groups: no additional treatment (FAC), 6 daily treatments with macitentan (30mg/kg orally) alone (FAC+Maci) or followed by AIT (FAC+Maci+AIT), 5 treatments with anti-PD1 antibody alone (100mg/kg anti-PD-1, i.p. every three days) (FAC/anti-PD1) or followed by AIT (Fac/anti-PD1+AIT). Animals were euthanized when tumor reached 1000 mm3 in the control group. Lungs and liver were removed and subjected to flow cytometry analysis for the detection of neu positive dormant tumor cells and expression of Ki67 on FVS-neu+ viable dormant tumor cells. Data represents 3-4 mice/group.

A) Lungs



B) Liver

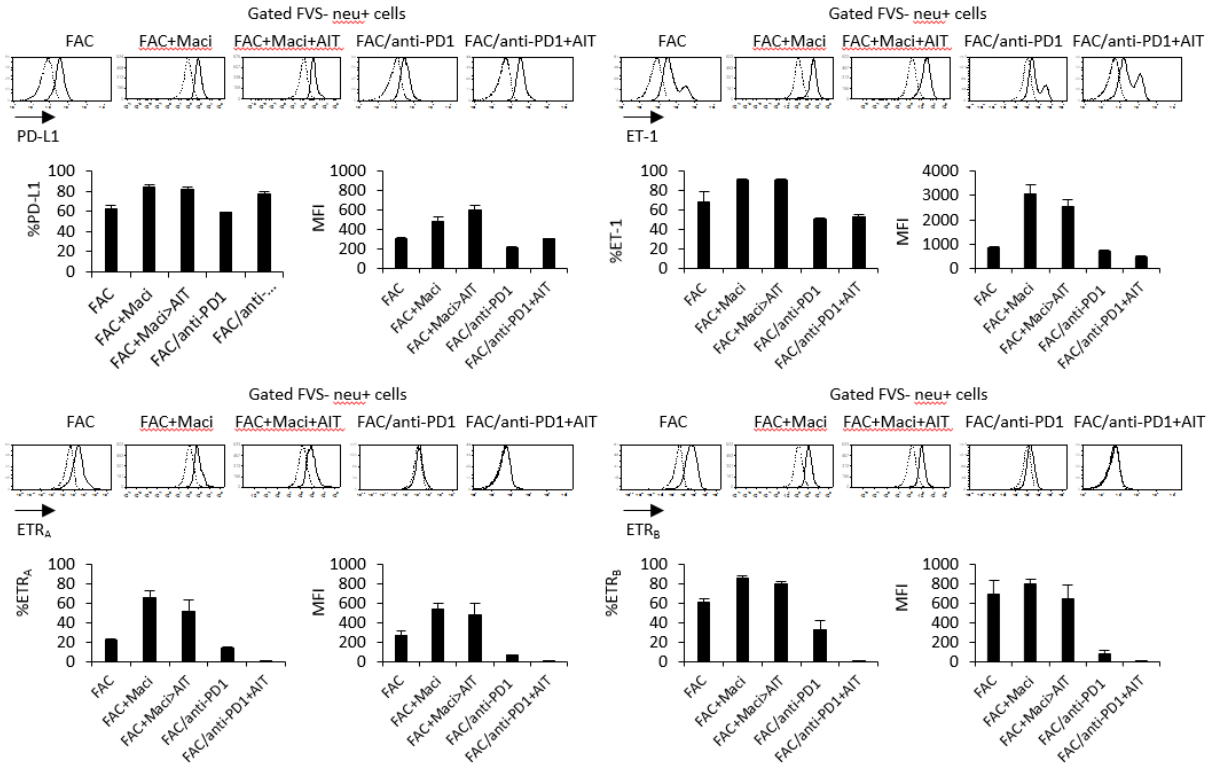
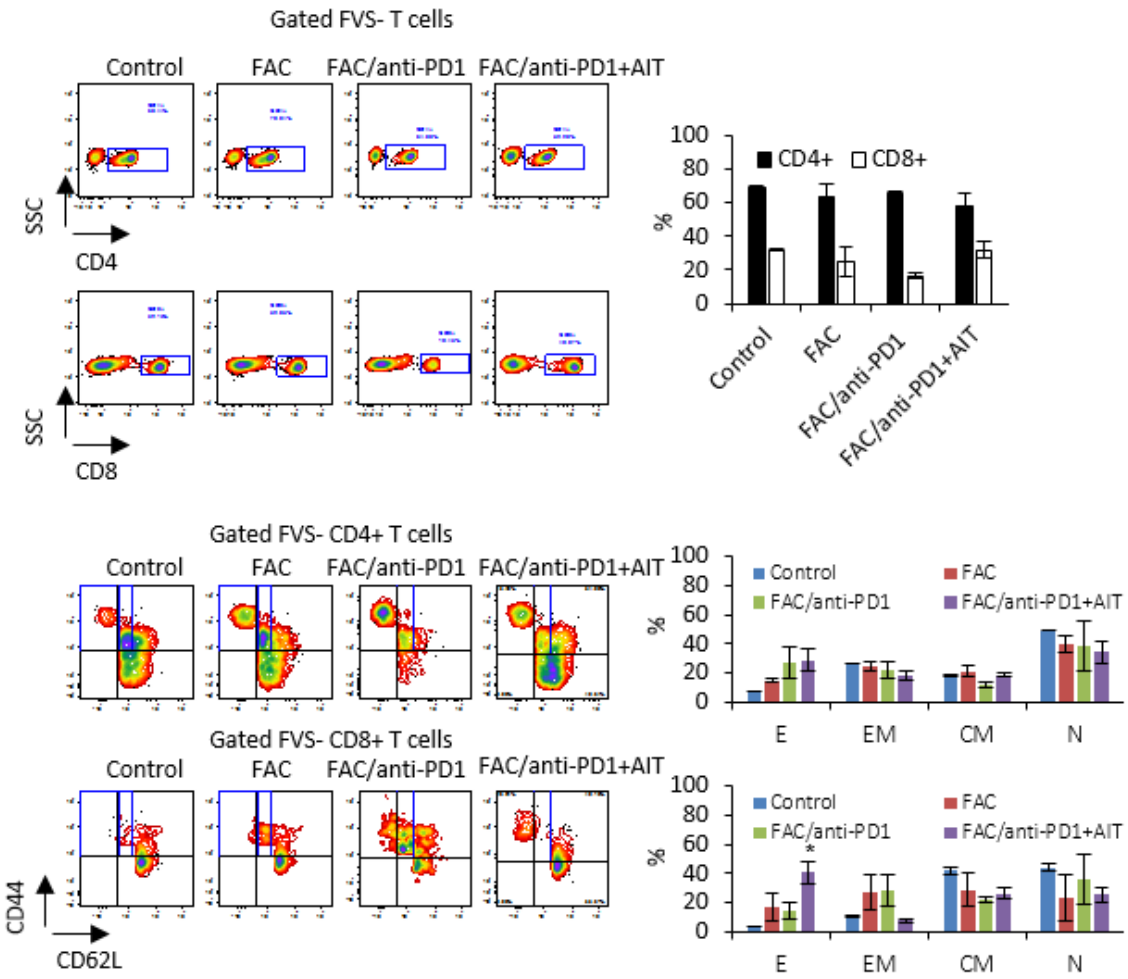


Figure 27. Dormant tumor cells express tumor survival pathways regardless of the treatment types. Female FVBN202 mice (6-8 weeks old) were challenged with 3 million neu positive MMC in the mammary region. Mice were then split into five groups. Three days after tumor challenge, mice received 9 daily treatments with low dose FAC, and split into 5 groups: no additional treatment (FAC), 6 daily treatments with macitentan (30mg/kg orally) alone (FAC+Maci) or followed by AIT (FAC+Maci+AIT), 5 treatments with anti-PD1 antibody alone (100mg/kg anti-PD-1, i.p. every three days) (FAC/anti-PD1) or followed by AIT (Fac/anti-PD1+AIT). Animals were euthanized when tumor reached 1000 mm3 in the control group. Lungs (A) and liver (B) were removed and subjected to flow cytometry analysis for the detection of PD-L1, ET-1, ETR_A and ETR_B on FVS-neu+ viable dormant tumor cells. Data represents 3-4 mice/group.

A) Lungs


Gated FVS- T cells
Control
FAC
FAC/anti-PD1
FAC/anti-PD1+AIT
SSC
CD4
SSC
CD8
CD4+
CD8+
%
100
80
60
40
20
0
Control
FAC
FAC/anti-PD1
FAC/anti-PD1+AIT
Gated FVS- CD4+ T cells
Gated FVS- CD8+ T cells
CD44
CD62L
E
EM
CM
N
*

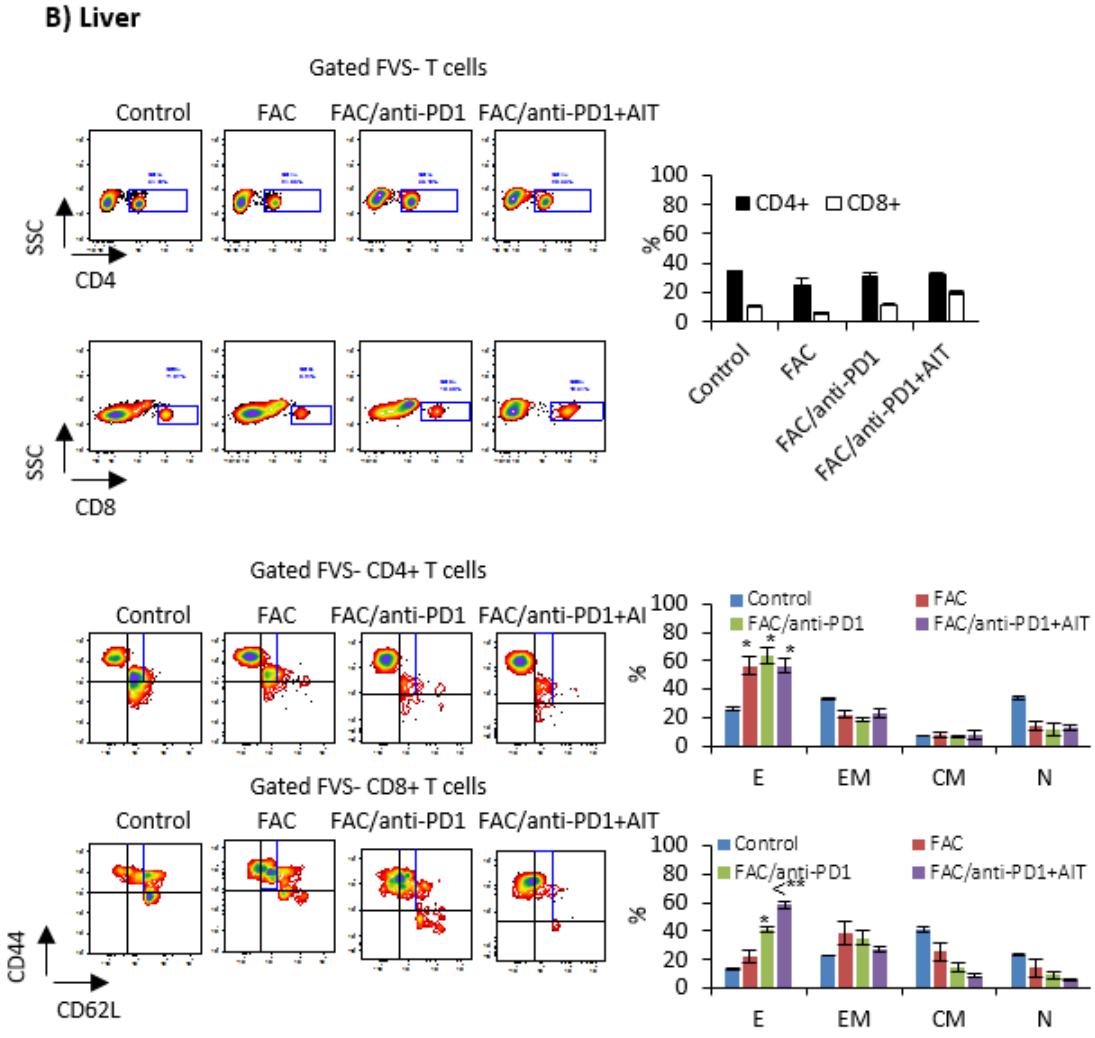
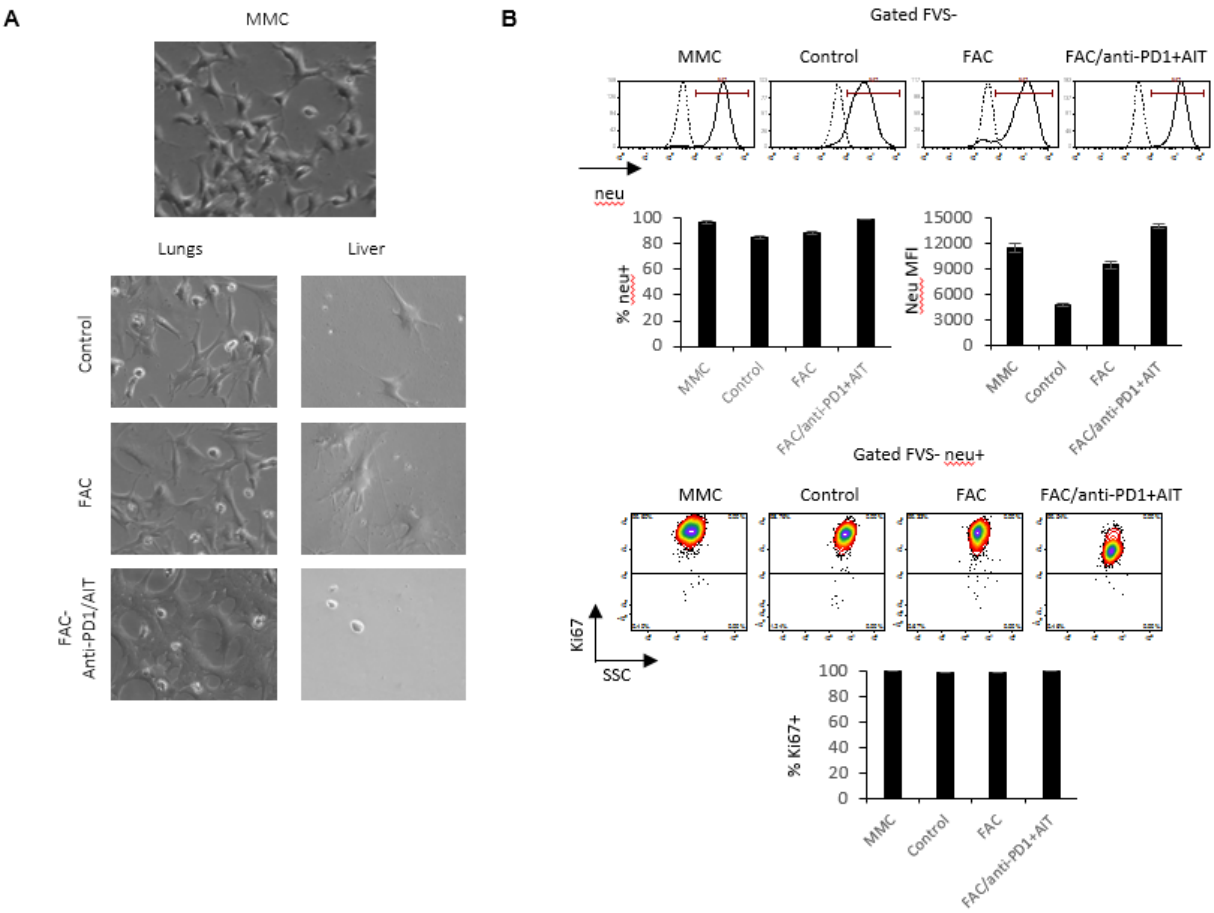


Figure 28. AIT increases the proportion of effector T cells in the distant sites Female FVBN202 mice (6-8 weeks old) were challenged with 3 million neu positive MMC in the mammary region. Mice were then split into four groups. Three days after tumor challenge, mice either remained untreated (Control) or received 9 daily treatments with low dose FAC and split into 3 groups: no additional treatment (FAC), 5 treatments with anti-PD1 antibody alone (100mg/kg anti-PD-1, i.p. every three days) (FAC/anti-PD1) or followed by AIT (Fac/anti-PD1+AIT). Animals were euthanized when tumor reached 1000 mm3 in the control group. Lungs (A) and liver (B) were removed and subjected to flow cytometry analysis for the detection of T cell phenotypes.



C

12 days co-culture with T cells

Days ex vivo: 18 18 14

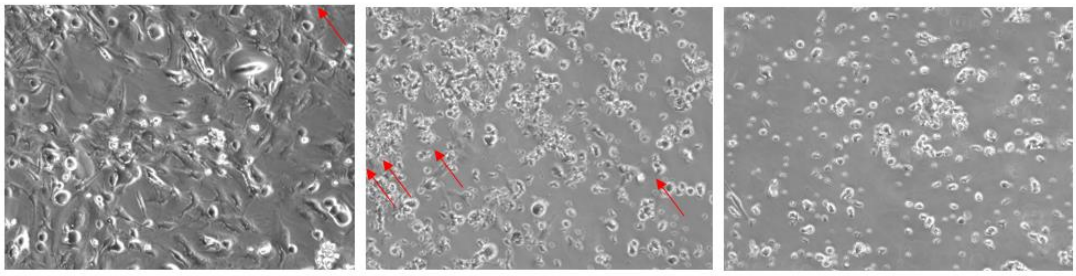


Figure 29. FAC-induced dormant tumor cells retain the expression of neu antigen. A) MMC tumor cell line and dormant tumor cells recovered from the lungs and the liver of FVBN202 mice bearing primary MMC in the mammary region who received no treatment (Control) or received 9-daily doses of FAC alone (FAC) or combined with anti-PD1 and AIT (FAC-Anti-PD1/AIT) were analyzed. B) FVS- viable cells were analyzed for the expression of neu or Ki67 using MMC tumor cell line or dormant tumor cells recovered from the lungs of animals bearing primary tumor in the mammary region who received no treatment (control), or received a low dose FAC chemotherapy without further treatment (FAC) or followed by anti-PD1 antibody and AIT (FAC/Anti-PD1+AIT). C) dormant tumor cells isolated from the lungs of FVBN202 female mice who had received a low dose FAC for 9 consecutive days were cultured alone (left panel) or with tumor-reactive autologous lymphocytes (4×10^6) either 6 days after the recovery of dormant cells from the lungs (middle panel) or two days after recovery (right panel) in the presence of 40 unit/ml IL-2 (3 times during the culture as needed) and 20 ng/ml IL-7 (one time on day 5 of the co-culture). The pictures were taken at 100X magnification on day 12 of the co-couture. Arrows show dormant tumor cells.

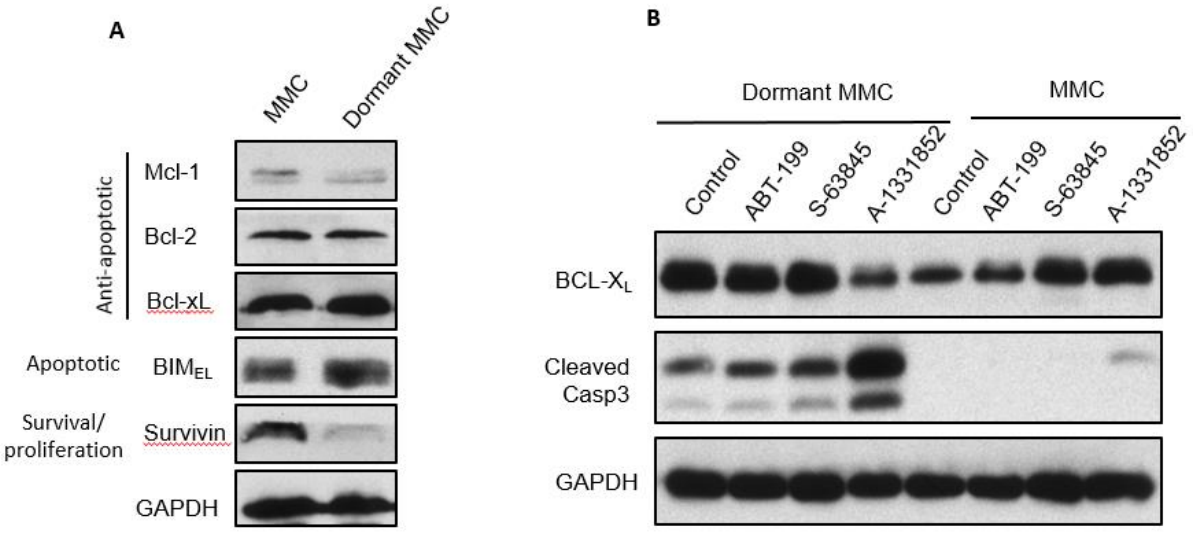


Figure 30. FAC-induced dormant tumor cells retain the expression of Bcl-2/Bcl-xL. A) Untreated tumor cells (MMC) or FAC-treated dormant cells (Dormant MMC) were analyzed for the expression of anti-apoptotic (Mcl-1, Bcl-2, Bcl-xL, survivin) or pro-apoptotic (Bim) proteins. B) Untreated tumor cells (MMC) or FAC-treated dormant cells (Dormant MMC) were treated with 10 µL DMSO (Control) or with the inhibitor of Bcl-2 (ABT-199, 1 uM), Mcl-1 (S63845, 1 uM) or Bcl-xL (A-1331852, 1 uM) for 24 hours. Cell lysates were analyzed for the expression of p53, Bcl-xL and cleavage of caspase 3.

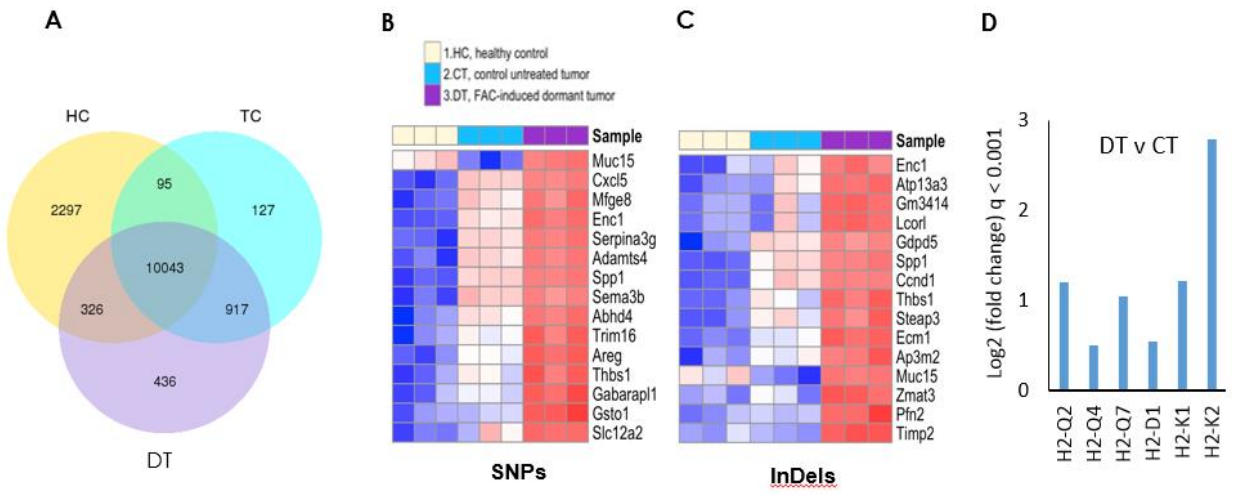


Figure 31. A low dose FAC increases SNPs, deletions and insertions (InDels), and increases the expression of MHC class I haplotypes in dormant tumor cells. Tumor dormancy was established ten days after the completion of 5 daily treatments of MMC with low dose FAC in vitro (DT). Untreated MMC (CT) or healthy tail tissue of FVBN202 mice (HC) were subjected to RNAseq and bioinformatic analysis for the detection of gene expression using Venn diagram (A), SNPs (B), InDels (C), or fold change in the expression of MHC class I (D). We collected the total number of mutations per gene in samples in the "HC" (healthy control), "CT" (control, untreated tumor), and "DT" (FAC-induced dormant tumor) groups. Genes with progressively increasing mutational load were prioritized using one-way ANOVA and comparison of fold changes between the groups. The top 15 genes showing the largest and most significant increase in mutational load were selected.

4.3. Discussion

We showed that FVBN202 transgenic mouse model of neu overexpressing breast cancer establishes distant tumor dormancy in the lungs and in the liver without any sign of distant metastasis very early during tumor progression at the primary site. The use of a low dose FAC resulted in the inhibition of the progression of primary tumor, inhibition of distant tumor dormancy, as well as the dominance of a $Ki67^-$ quiescent type of tumor dormancy. FAC-induced dormant tumor cells became less susceptible to IFN-γ–induced tumor immunoediting compared with non-dormant tumor cells. To this end, FAC-induced dormant tumor cells showed a lower expression of the neu antigen, but they did not downregulate the expression of the neu antigen in the presence of IFN-γ. On the other hand, non-dormant tumor cells remained highly susceptible to the IFN-γ–induced downregulation of the neu antigen, which was evident only in the $Ki67^{low}$ indolent cells. Furthermore, FAC-induced dormant tumor cells showed a lower expression of PD-L1 in the presence of IFN-γ compared with non-dormant tumor cells. IFN-γ-induced PD-L1 expression was more abundant in $Ki67^{low}$ indolent cells than in $Ki67^-$ quiescent cells. These data suggest that dormant tumor cells became resistant to tumor immunoediting mechanisms that take place during cell division, but they remain susceptible to the immunoediting mechanisms that are independent of cell division. It has been reported that IFN-γ-induced PD-L1 expression is the result of JAK-STAT signal transduction pathway (111), while neu expression in the FVBN202 transgenic mice is regulated in part by epigenetic modification of the MMTV promoter during cell division (112).

Immunotherapeutic targeting of primary breast cancer following a low dose FAC by means of an adoptive transfer of tumor-sensitized autologous lymphocytes failed to eliminate the tumor in FVBN202 mice. We found that such failure of AIT against FAC-treated tumor cells was associated

with the upregulation of tumor survival pathways including the expression of ET-1, ETR_A/ETR_B and PD-L1. Adoptively transferred CD8+ T cells, but not CD4+ T cells, used for AIT expressed ET-1. A direct interaction between dormant tumor cells and T cells, *ex vivo*, did not increase the expression of ETR_A on dormant tumor cells. Analysis of the tumor microenvironment showed that FAC-induced dormant cells, tumor-reactive immune cells as well as myeloid cells produced ET-1, which could act through autocrine and paracrine pathways to support the survival of tumor cells expressing ETR_A. To this end, we showed that blockade of the endothelin receptors by means of Macitentan increased apoptosis in FAC-induced dormant tumor cells, *ex vivo*. In addition, blockade of the tumor survival pathways by means of Macitentan or anti-PD1 antibody during AIT resulted in a significant improvement in the efficacy of AIT against primary tumors in FVBN202 mice.

We also demonstrated that distant tumor dormancy occur very early during tumor progression at the primary site, and they could be kept dormant under the immune surveillance. To this end, dominance of CD4+ and CD8+ T effector cells in the liver was associated with the inhibition of tumor relapse, *ex vivo*. On the other hand, dominance of CD4+ and CD8+ T naïve cells in the lungs was associated with the relapse of dormant tumor cells from the lungs, *ex vivo*. Finally, co-culture of tumor-reactive lymphocytes used for AIT with dormant tumor cells isolated from the lungs resulted in the inhibition of tumor relapse, *ex vivo*. These data suggest that administration of immunotherapy during actual tumor dormancy when no solid tumor is detectable, i.e., in cancer survivors, could prevent distant recurrence of breast cancer.

Although blockade of the endothelin and PD-L1 survival pathways improved immunotherapy of breast cancer, presence of yet to be identified redundant tumor survival pathways could inhibit a complete elimination of the FAC-treated primary tumor cells by immunotherapy. Therefore,

targeting a common downstream pathway of tumor survival molecules would be more feasible for an effective immunotherapy of breast cancer following a neoadjuvant low dose chemotherapy. For instance, the anti-apoptotic Bcl-2/Bcl-xL is induced downstream of ETR_A or PD-L1 signaling, as well as some other known cell survival pathways such as the cytokine thymic stromal lymphopoietin receptor (TSLPR) pathway which has been reported in breast cancer (115–117). To this end, we showed that inhibition of Bcl-xL by means of A-1331852 specifically induced apoptosis of FAC-induced dormant tumor cells.

Altogether, our results show that immunotherapeutic targeting of tumor dormancy, but not locally controlled solid tumors, could retain distant tumor cells in a dormant state and prevent their recurrence. In future studies, use of A-1331852 during immunotherapeutic targeting of primary tumors following a neoadjuvant chemotherapy is expected to augment anti-tumor efficacy of AIT. In addition, use of a low dose FAC in a neoadjuvant setting flowed by surgical resection of the primary tumor prior to immunotherapy is expected to improve the efficacy of AIT against a local and distant recurrence of the disease. Finally, Low dose FAC could induce mutations in dormant tumor cells, which could be used as a neoantigen personalized vaccine for immunotherapeutic targeting of tumor dormancy to achieve complete response.

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VITA

Personal summary:

Hussein Faraj Elewi Aqbi was born on the 24th of May, 1981 in Baghdad, Iraq, and is an Iraqi citizen. He graduated with an honors diploma from Al-Mustafa High School in 2000 and went on to attend Mustansiriyah University from 2000-2004 where he received a Bachelor of Science in Biology, with distinction. After several contemplative years, Hussein entered Baghdad University in the fall of 2007-2009 where he received a Master of Science in Microbiology, Hussein entered Virginia Commonwealth University in the fall of 2015 where he started his Ph.D. in school of medicine Microbiology and Immunology department, then rediscovered his love of research under the mentorship of Dr. Masoud H. Manjili.

EDUCATION:

2014-2018	Ph.D (Microbiology and Immunology) Virginia Commonwealth University Richmond, Virginia, USA **Thesis Title:** Preconditioning of the tumor microenvironment by means of low does chemotherapies for an effective immunotherapy of breast cancer
2006-2009	M.S (Microbiology) Baghdad University Baghdad, Iraq **Thesis Title:** Extraction of mannan from yeast Saccharomyces cerevisiae and study of its agglutination
2000-2004	B.S (Biotechnology) Mustansiriyah University Baghdad, Iraq

EMPLOYMENTS/POSITION:

08/2014 - Till date:	Doctoral Student, Dept. of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia, USA.
05/2005– 01/2014:	Lecturer for BS, Dept. of Biology College of Science, Mustansiriyah University, Baghdad, Iraq.
08/2004 - 04/2011:	Branch office Manager of the Independent High Electoral Commission, Baghdad, Iraq
11/2018 – 11/2019:	Laboratory specialist in the Massey Cancer Center, Virginia Commonwealth university, Richmond, Virginia, USA

RESEARCH INTERESTS

My current research Centre on Immunotherapy of tumor dormancy: The objective of this project is to identify molecular or cellular events that establish tumor dormancy after chemotherapy and those that lead to disease recurrence.

PAPERS PUBLISHED IN PEER REVIEWED JOURNALS:

1. Payne KK, **Aqbi HF**, Butler SE, Graham L, Keim RC, Wan W, Idowu MO, Bear HD, Wang XY, Manjili MH. $Gr1^{-/low}CD11b^{-/low}$MHCII+ myeloid cells boost T cell anti-tumor efficacy. *J Leukoc Biol* 2018 Jul 9. doi: 10.1002/JLB.5A0717-276RR. [Epub ahead of print] PMID: 29985529
2. **Aqbi HF**, Tyutyunyk-Massey L, Keim RC, Butler SE, Thekkudan T, Joshi S, Smith TM, Bandyopadhyay D, Idowu MO, Bear HD, Payne KK, Gewirtz DA, Manjili MH. *Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy. Oncotarget* 9(31): 22128-22137, 2018 PMID: 29774126

3. **Aqbi HF**, Wallace M, Sappal S, Payne KK, Manjili MH**.** IFN-γ orchestrates tumor elimination, tumor dormancy, tumor escape and progression. *J Leukoc Biol* 2018 Feb 22. doi: 10.1002/JLB.5MIR0917-351R. [Epub ahead of print] PMID: 29469956

PULISHED ABSTRACTS OR PROCEEDINGS:

1. **Aqbi HF**, Cara Coleman, Michael Idowu, Manjili MH. Low-dose neoadjuvant chemotherapy dominates Ki67- quiescent tumor dormancy for an effective immunotherapy of breast cancer, IMMUNOLOGY 2019™ AAI Annual Meeting, San Diego, California, May 9-13, 2019 [received 2018 AAI Laboratory Travel Grant Award]
2. **Aqbi HF**, Smith TM, Idowu MO, Butler SB, Payne KK, Manjili MH. Autophagy-deficient breast cancer shows early escape from dormancy and recurrence following chemotherapy, IMMUNOLOGY 2018™ AAI Annual Meeting, Austin, TX, May 4-8, 2018 [received 2018 AAI Laboratory Travel Grant Award]
3. **Aqbi HF**, Smith TJ, McKiver B, Joshi S, Keim R, Idowu MO, Guo C, Wang XY, Payne KK, Manjili MH. Autophagy and chemotherapy-induced tumor dormancy. Cancer Immunology & Immunotherapy: from conception to delivery, NIH, Washington D.C., October 12-13, 2017.
4. **Aqbi HF**, Butler SE, Keim R, Idowu MO, Manjili MH. Chemotherapy-induced tumor dormancy and relapse. IMMUNOLOGY 2017™ AAI Annual Meeting, Washington D.C., May 12-16, 2017.
5. Butler SE, Payne KK, Keim RC, **Aqbi HF**, Manjili MH. Chemotherapy-induced indolent tumor dormancy is maintained by a balance of cell proliferation and death. 2016 VCU Massey Cancer Center Research Retreat, Richmond VA, June 17, 2016
6. **Aqbi HF**, Wallace MT, Payne KK, Keim RC, Dumur CI, Manjili MH. Treatment-induced tumor dormancy. The inaugural Gordon Archer Research Day in Infectious Disease. Richmond VA, 11/23/2015

MEMBERSHIP AND PROFESSIONAL SCIENTIFIC SOCIETIES:

2016 - Till date - Member in the American Association of Immunologists.

WORKSHOPS/PRESENTATIONS:

1. Poster Presentation in the First annual Gordon archer research day in ID, Microbiology and immunology, Poster title: Treatment- induced tumor dormancy, Richmond, VA, November 23, 2015.
2. Department research seminar presentation in Microbiology and immunology department, Presentation title: Immunotherapy for breast cancer: treatment-induced indolent and quiescent types of tumor dormancy. Richmond, VA, May 02, 2016.
3. Department research seminar presentation in Microbiology and immunology department, Presentation title: Immunotherapeutic targeting of tumor dormancy. Richmond, VA, April 27, 2017.
4. Poster Presentation in 2017 AAI Annual Meeting. Poster title: Chemotherapy- induced tumor dormancy and relapse. Washington D.C. May 12-16, 2017.
5. Poster Presentation in Cancer Research Retreat. Poster title: Chemotherapy- induced tumor dormancy and relapse. Richmond, VA, June 16,2017.
6. Poster Presentation in Cancer Immunology and Immunotherapy meeting at the NIH. Poster title: Autophagy and chemotherapy-induced tumor dormancy. Washington D.C. October 12-13,2017.
7. Department research seminar presentation in Microbiology and immunology department, Presentation title: Chemo-immunotherapy of breast cancer to overcome tumor escape and relapse. Richmond, VA, November 16, 2017.
8. Poster Presentation in 2018 AAI Annual Meeting. Poster title: Autophagy-deficient breast cancer shows early escape from dormancy and recurrence following chemotherapy. Austin, Texas. May 04-08, 2017.
9. Poster presentation in 2019 AAI Annual Meeting. Poster title: Low-dose neoadjuvant chemotherapy dominates Ki67- quiescent tumor dormancy for an effective immunotherapy of breast cancer. San Diego, California, May 9-13, 2019
10. Oral presentation in 2019 AAI Annual Meeting. Poster title: Low-dose neoadjuvant chemotherapy dominates Ki67- quiescent tumor dormancy for an effective immunotherapy of breast cancer. San Diego, California, May 9-13, 2019