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TITLE: Treatment-Induced Autophagy Associated with Tumor Dormancy and Relapse

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Studies relating to the role autophagy in tumor dormancy revealed that transient inhibition of autophagy during ADR treatment resulted in prolonging tumor dormancy. However, complete knockdown of autophagy gene expedited tumor relapse. We also identified two distinct types of ADR-induced tumor dormancy including Ki67+/low indolent and Ki67- quiescent tumor dormancy. Whereas that former was sensitive to immunoediting, escape and relapse, the latter was found to be resistant to tumor immunoediting and escape. Adoptive immunotherapy (AIT) was also found to support regression of ADR-induced dormant tumor cells.

15. SUBJECT TERMS
Autophagy, tumor dormancy, tumor relapse, chemotherapy, immunotherapy

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1. **INTRODUCTION:**
   The objective of the proposal is to understand the role of autophagy in chemotherapy induced tumor dormancy and recurrence.

2. **KEYWORDS:**

   tumor dormancy, tumor relapse, immunotherapy, immunoediting, autophagy

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

1) Understand the role of autophagy in chemotherapy-induced tumor dormancy (Aim 1)
2) Understand the role of tumor IFN-gamma Ra in determining tumor recurrence under immune pressure (Aim 2)

What was accomplished under these goals?

Transient inhibition of ADR-induced autophagy by CQ prolongs tumor dormancy whereas a complete inhibition of autophagy expedites tumor growth and relapse following ADR treatment

In the previous progress report we showed that inhibition of autophagy by CQ during ADR treatment, in vitro, resulted in prolonging tumor dormancy such that ADR treated MMC resumed cell proliferation 6 weeks after the treatment when ADR+CQ treated MMC remained dormant (Figure 1). Flow cytometry analysis of ADR-treated MMC confirmed resumption of cell proliferation by shifting Ki67- non-proliferating cells to Ki67+ proliferating cells with a greater viability (Figure 2). CQ only transiently blocks fusion of autophagosome and lysosome during ADR treatment such that after removal of CQ, accumulated autophagosome could eventually be fused with lysosome to complete autophagy. Therefore, we used autophagy deficient MMC (ATG5-/- MMC) and scrambled control MMC (Scr MMC) to determine the role of autophagy in tumor dormancy or relapse, in vitro and in vivo. In vitro studies showed that ATG5-/- MMC resumed proliferation, indicative of relapse, sooner than MMC or Scr MMC by 3 weeks following ADR treatment (Figure 3A). In order to determine in vivo relevance of our in vitro findings, FVBN202 mice were used. Tumor dormancy was first established (n=7/group) were then challenged i.v. with one million viable 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 3B, animals that were challenged with ATG5-/- MMC developed lung metastasis significantly sooner than those that were challenged with MMC. Follow up studies in the animals who were challenged with Scr MMC are still in progress, which shows similar trend to MMC so far. These data suggest that autophagy supports ADR-induced tumor dormancy. On the other hand, a transient inhibition of autophagy by CQ only during ADR treatment even further prolonged tumor dormancy (Table 1). We are conducting gene array analysis to determine whether function of CQ in prolonging ADR-induced tumor dormancy is beyond a transient inhibition of autophagy.

Dormant MMC cells established by ADR or radiation therapy (RT) become resistant to higher doses of chemotherapy or RT, but remain sensitive to immunotherapy

In order to determine whether dormant MMC cells established by ADR treatment remain sensitive to tumor-reactive immune cells, dormancy was established by treating MMC with three daily doses of ADR (1μM/day for 2 hs); eight days after the final treatment, MMC cells received a high dose of ADR (1μM for 24 hs), or were cultured with tumor-reactive immune cells for 48 hs. ADR treatment induced apoptosis in MMC cells (Figure 4A-B, p=0.01). Tumor cells that survived apoptosis became chemo-refractory such that additional ADR treatment at a higher dose (1μM for 24 hs) did not induce cell death (Figure 4A-B, average 40% vs. 54%). However, they remained sensitive to tumor-reactive immune cells.

In the presence of tumor-reactive immune cells, the frequency of viable ADR-treated dormant MMC dropped from 40% to 8% (Figure 4A-B, p=0.003). In fact, lymphocytes were more effective than a high dose of chemotherapy in inducing apoptosis in dormant MMC (Figure 4A-B, p=0.02). We also established dormant MMC by three daily doses of RT (2 Gy/day); again surviving dormant cells became refractory to RT. An additional RT at a higher dose (18 Gy) did not markedly decrease the frequency of viable tumor cells (Figure 4B-C, 53% vs. 52%). However, RT-refractory MMC cells remained sensitive to tumor-reactive lymphocytes as the viability dropped from 53% to 8% (Figure 4B-C, p=0.002). Recapitulating our results with chemotherapy-induced tumor cell dormancy, tumor-reactive immune cells were more effective than high dose RT at inducing apoptosis in dormant MMC (Figure 4B-C, p=0.01). In order to determine whether higher levels of apoptosis in dormant tumor cells were due to their greater sensitivity to immune cells rather than a higher reactivity of the immune cells, IFN-γ ELISA was performed using re-programmed immune cells cultured with either MMC tumor cells or ADR-, RT-induced dormant MMC cells. As shown in Fig. 4D, tumor-reactive immune cells produced comparable levels of IFN-γ upon stimulation with MMC or dormant MMC (RT-MMC, ADR-MMC).

ADR induces two types of tumor dormancy: indolent and quiescent

In order to determine whether dormant MMC cells that were established by ADR were in the state of non-proliferative quiescent dormancy or were capable of sluggish proliferation (balanced proliferation and death), MMC cells were stained with a clinically relevant proliferation marker, Ki67. ADR treatment shifted Ki67 positive (Ki67+/high) highly proliferating MMC towards Ki67+/low indolent cells as shown by a significant drop in the Ki67's MFI (Figure 5, p= 0.026), as well as shifting towards Ki67 negative (Ki67-) quiescent cells (Fig. 5, 3% vs. 22%, p= 0.01). In order to determine whether indolent and quiescent types of tumor dormancy were present in vivo, FVBN202 mice were inoculated with MMC in the mammary gland ($3x10^6$ cells/mouse). Animals were either served as control (MMC) or received ADR chemotherapy when tumors became palpable (MMC+ADR). As shown in Figure 6, ADR chemotherapy inhibited tumor growth and established local dormancy. Animals were then euthanized and their tumors subjected to H & E staining and IHC for Ki-67. Whereas all tumors in control group (MMC) were Ki-67+ (dark brown), tumors in the treatment group (MMC+ADR) were mostly Ki-67+/low indolent cells (light brown) and some Ki-67- quiescent cells (background blue color).

Indolent but not quiescent dormant tumor cells are prone to immunoediting and escape from immunotherapy

In order to determine sensitivity of dormant tumor cells to immunoediting and escape from immune response (1-4) , expression of PD-L1, a suppressor of the immune response, was determined on dormant cells. Since IFN-γ was shown to be a major product of the immune response that induces tumor immunoediting, we wanted to determine if IFN-γ upregulates PD-L1 expression on Ki67+/low indolent dormant cells and/or Ki67- quiescent dormant tumor cells. First, ADR-induced tumor dormancy was established 3 weeks after the treatment cessation (Figure 7; + ADR). Dormant MMC were then treated with IFN-γ (+ ADR → IFN-γ) and analyzed for the expression of PD-L1 after 12 hrs. MMC cells (untreated) or MMC cells pulsed with IFN-γ (Untreated → IFN-γ) or ADR-treated dormant MMC (+ADR) served as controls. We detected the IFN-γ-induced upregulation of PDL-1 on Ki67+ untreated MMC and on Ki67+/low ADR-treated indolent dormant MMC (Fig. 7B); however, IFN-γ did not upregulate PD-L1 on Ki67- quiescent tumor cells (Figure 7C).

AIT of FVBN202 mice resulted in the regression of ADR-induced indolent tumor

In order to determine whether AIT following chemotherapy protects animals from tumor growth, tumor-bearing FVBN202 mice were injected with ADR (2X10 mg/Kg, i.v.) to establish predominant indolent dormancy in vivo (Figure 8). As soon as tumor growth was inhibited and plateaued (7 days after ADR treatment), animals remained untreated (MMC+ADR) or received AIT by reprogrammed immune cells (MMC+ADR+AIT). AIT was performed, as previously described by our group (5, 6) . Follow up studies showed further inhibition of indolent tumors only following AIT (Figure 8). In the proposed research, we

will perform long-term follow up studies to determine whether indolent tumors will eventually relapse. We will also use ADR+CQ to establish a predominant quiescent type of tumor dormancy before the administration of AIT.

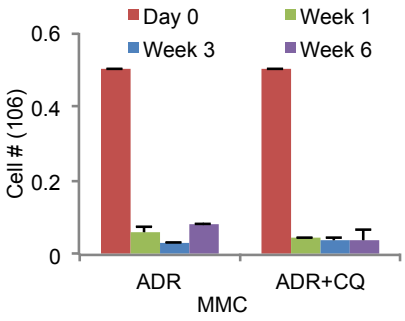


Fig. 1. Use of CQ prolongs ADR-induced tumor dormancy in MMC. MMC tumor cells were treated with 3 daily doses of ADR (1uM for 2 hrs) in the absence or presence of CQ (10nM for 5 hrs; 2hrs before ADR + 3hrs during ADR). At weeks 1, 3, and 6 post-treatments, adherent tumor cells were counted by trypan blue exclusion. Experiments were performed in duplicates.

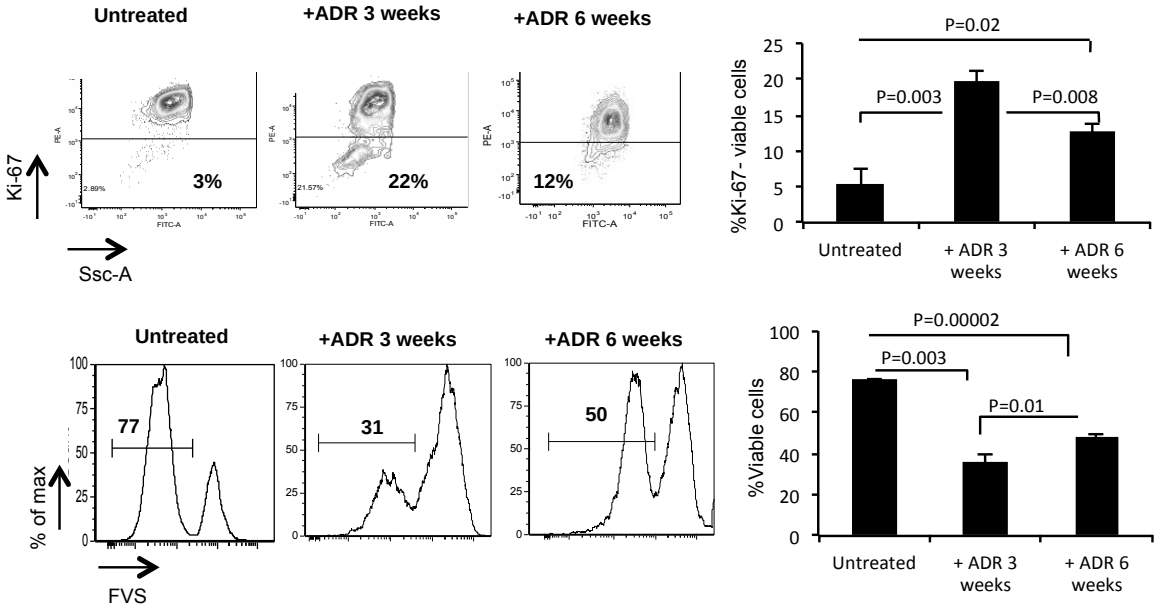


Figure 2. Dormant tumor cells recover proliferative capacity as a function of time. MMC tumor cells were treated with 3 daily doses of ADR (1uM for 2 hs), then remained untreated for 3 weeks and 6 weeks, *in vitro*. At weeks 3 and 6 post-treatment, Ki-67 expression and viability were quantified within the population of adherent tumor cells. Data represent 3 independent experiments and mean ± SEM.

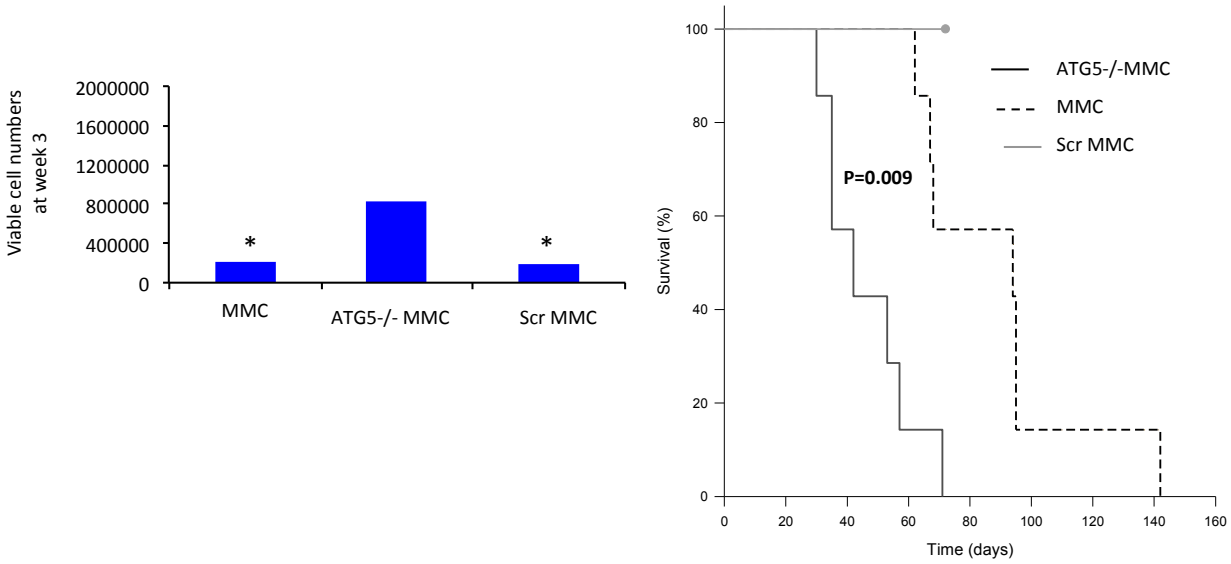


Figure 3. Autophagy deficient MMC relapse sooner than autophagy competent MMC. MMC, ATG5-/- MMC or Scr MMC tumor cells (3 million cells) were treated with 3 daily doses of ADR (1uM for 2 hrs), and were counted at week 3 using trypan blue exclusion (A). At week 3 of ADR treatment, ATG5-/- MMC, MMC or Scr MMC were injected i.v. into FVBN202 mice (n=7 mice/group, 1 million viable cells/ mouse). Overall survival was determined.

Table 1. Autophagy deficient MMC relapse sooner that autophagy competent MMC

ADR treatment (Days 1-3)	Recovery (Days 4-21)	Beginning of relapse
No autophagy	No autophagy	Week 3
Autophagy	Autophagy	Week 6
No autophagy	Autophagy	Relapse-free for 6 weeks

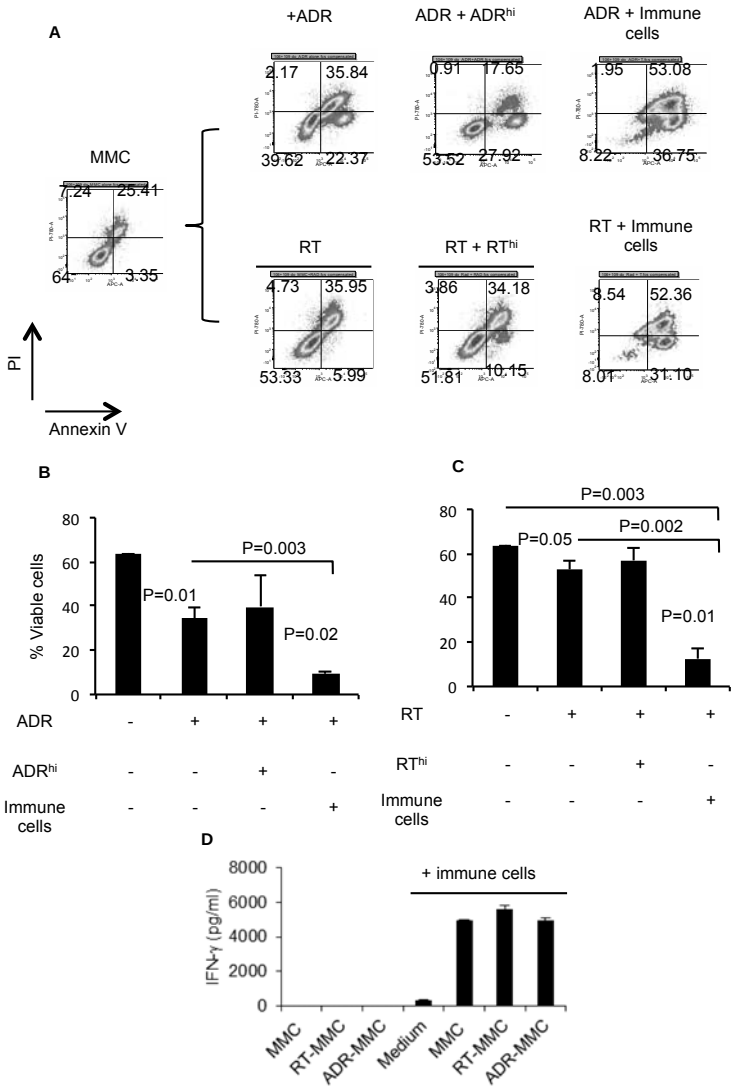


Figure 4. Immunotherapy displays cytotoxic function against treatment-refractory dormant tumor cells, *in vitro*. A) MMC cells (n=3) treated with ADR (1μM, 2 hs) or 2 Gy RT (RT-treated MMC) for 3 consecutive days and remained in culture for 8 days total, in order to establish tumor cell dormancy *in vitro*. B) On day 8, these dormant tumor cells were treated with a high dose ADR (1μM, 24 hs) (ADR-treated MMC + ADR^{hi}) or reprogrammed immune cells (ADR-treated MMC + Immune cells; ADR-treated MMC + Immune cells). Two days later, cells were stained with Annexin V/PI and analyzed by flow cytometry. Data represent three biological repeats and mean ± SEM. C) On day 8, these dormant tumor cells were treated with 18 Gy RT (RT-treated MMC + RT^{hi}) or reprogrammed immune cells (RT-treated MMC + Immune cells). Two days later, cells were stained with Annexin V/PI and analyzed by flow cytometry. D) MMC tumor cells (MMC) or dormant MMC cells (RT-MMC, ADR-MMC) were cultured in the absence or presence of the re-programmed immune cells in a 10-1 ratio for 24 hs. Control immune cells were cultured alone (Medium). IFN-γ release was detected in the supernatant using ELISA. Data represent two biological repeats and mean ± SEM.

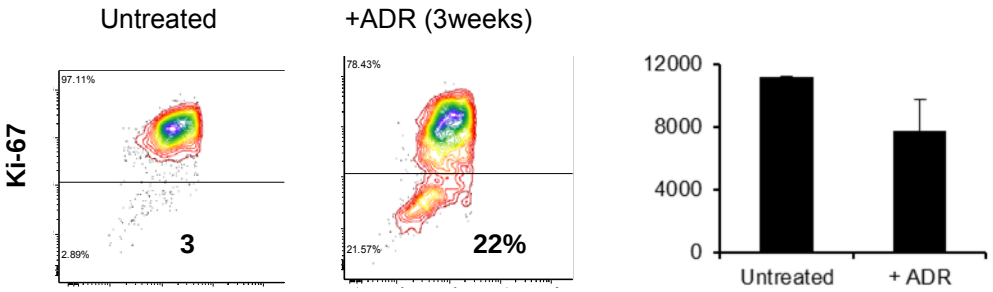


Figure 5. ADR induces indolent (Ki67+/low) and quiescent (Ki67-) types of tumor dormancy. MMC cells were treated with three daily doses of ADR (1 mM, 2hrs). Adherent MMC were analyzed for the expression of Ki67 before and 3 weeks after the treatment.

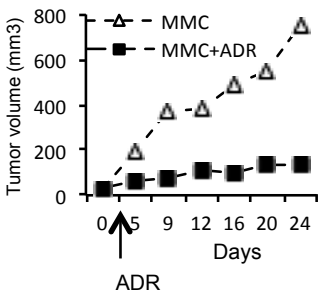
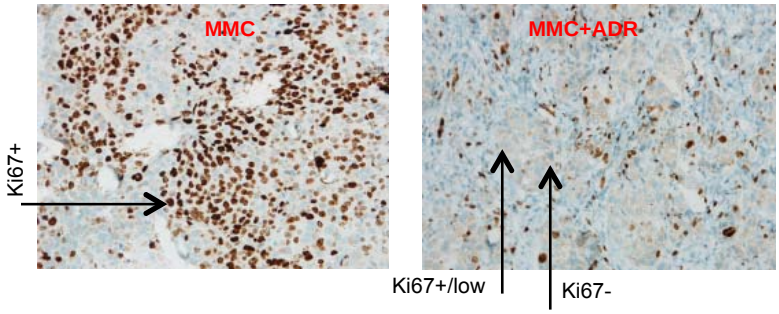


Fig. 6. ADR treatment induces indolent and quiescent types of tumor dormancy, in vivo. FVBN202 mice were challenged with MMC ($3x10^6$) in the mammary gland region; after tumors reached 30-50 mm^3 animals were treated with ADR (MMC+ADR; 20 mg/kg; i.v.), or remained untreated (MMC). Tumor growth was monitored for 4 weaks, and tumors were removed and subjected to IHC analysis of Ki67.



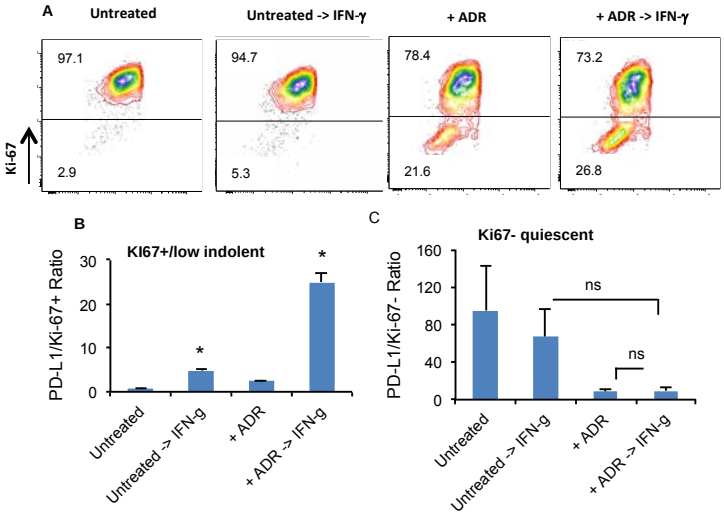


Fig. 7. IFN-g induces upregulation of PD-L1 on Ki67+ MMC and Ki67+/low indolent MMC but not on Ki-67- quiescent MMC. MMC tumor cells were treated for 3 consecutive days with ADR (1uM) or left untreated. Three weeks later ADR-treated (+ ADR) and untreated MMC (untreated) cells were stimulated with IFN-g (50ng/ml) for 12-16 hours (+ADR -> IFN-g, and Untreated -> IFN-g) to induce the expression of PD-L1, or remained untreated to serve as controls. Cells were subjected to a three-color staining FITC-FVS, PE-Ki-67 and Brilliant Violet 421-PD-L1 antibodies. A) Representative data from triplicate experiments gated on Ki67+ cells in MMC tumor cells. B) Ratio of PD-L1 MFI to the frequency of Ki-67+ cells in untreated and ADR-treated MMC cells, with and without IFN-g stimulation. C) Ratio of PD-L1 MFI to the frequency of Ki-67- cells in untreated and ADR-treated MMC cells, with and without IFN-g stimulation.. Data were quantified using flow cytometry and are representative of viable tumor cells. Error bars represent ± SEM.

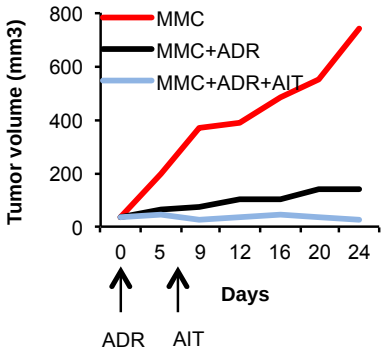


Fig. 8. Adoptive immunotherapy (AIT) of FVBN202 mice induced regression of ADR-induced indolent tumor. MMC tumor-bearing mice either served as control (MMC), or injected with ADR (20 mg/kg, i.v.) on day 0 to establish indolent tumors in vivo. All mice were then injected with cyclophosphamide (CYP, 100 mg/kg) on day 5 for the depletion of endogenous T cells. ADR-treated mice either remained untreated (MMC+ADR) or received AIT on day 6. Tumor volume was measured with digital calipers, and calculated by length x width^2 /2).

References

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2. Kmieciak M, Payne KK, Idowu MO, et al. Tumor escape and progression of HER-2/neu negative breast cancer under immune pressure. J Transl Med 2011;9:35,5876-9-35.

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4. Beatty GL, Paterson Y. IFN-gamma can promote tumor evasion of the immune system in vivo by down-regulating cellular levels of an endogenous tumor antigen. J Immunol 2000;165(10):5502-8.

5. Kmieciak M, Basu D, Payne KK, et al. Activated NKT cells and NK cells render T cells resistant to myeloid-derived suppressor cells and result in an effective adoptive cellular therapy against breast cancer in the FVBN202 transgenic mouse. J Immunol 2011;187(2):708-17.

6. Payne KK, Keim RC, Graham L, et al. Tumor-reactive immune cells protect against metastatic tumor and induce immunoediting of indolent but not quiescent tumor cells. J Leukoc Biol 2016.

What opportunities for training and professional development has the project provided?

- A PhD student, Kyle Payne, who was working on tumor dormancy was graduated in 2015.
- A first year MD student, Nicholas Schmedding, conducted his summer research training on tumor dormancy.
- A Bachelor student, Alex Liakos, conducted his undergraduate dissertation research on tumor dormancy.
- A new PhD student, a new MS student and a new undergraduate student were rotated in the lab of Dr. Manjili, and then joined the lab working on tumor dormancy.
- A visiting scientist, *Yibin Xie*, MD, from Peking Union Medical College, Beijing, China has been working on tumor relapse following conventional therapies.

How were the results disseminated to communities of interest?

1) Concepts that are proposed in this project were used to formulate two graduate level lectures- advanced immunology and molecular biology of cancer- related to cancer dormancy.
2) As an invited speaker, Dr. Manjili gave a talk on "*Immunotherapy for cancer dormancy*". The 2016 Controlling Cancer Summit, London, UK, May 17-19, 2016.
3) As an invited speaker, Dr. Manjili gave a lecture on tumor dormancy in the University of Connecticut, Title: "*The inherent premise of immunotherapy for cancer dormancy*", Hartford, CT, May 5, 2016.
4) As an invited speaker, Dr. Manjili gave a lecture on tumor dormancy in the University of South Carolina, Title: "*Current status and future prospects of immunotherapy: Targeting cancer dormancy*", Columbus, SC, September 4, 2015.
5) As an invited speaker, Dr. Manjili gave a lecture on tumor dormancy in the Cancer Cell Signaling group meeting, Massey Cancer Center, Title: *Immune-mediated tumor dormancy and inflammation,* September 2015

What do you plan to do during the next reporting period to accomplish the goals?

Since transient, but not permanent, inhibition of autophagy by CQ during ADR treatment prolonged tumor dormancy, we will perform in vivo studies in FVBN202 mice to determine if ADR+CQ could induce a stronger immune responses in animals, and result in prolonged tumor dormancy compared with ADR alone. We will also perform gene array analysis to determine whether function of CQ in prolonging ADR-induced tumor dormancy is beyond a transient inhibition of autophagy. We will perform T cell depletion studies in vivo in order to determine whether immunogenic tumor dormancy and the host immune response are responsible for prolonging tumor dormancy. Tumor-specific immune responses will also be monitored ex vivo. Contribution of the tumor IFN-g Ra in tumor dormancy or recurrence will also be determined.

4. IMPACT:
What was the impact on the development of the principal discipline(s) of the project?

Dr. Manjili provided expert commentary on "Accelerating Progress against Cancer" in a Twitter Chat hosted by ABC News (April 19, 2016)

What was the impact on other disciplines?

The results had an impact on environmental science by linking how chemicals can cause tumor dormancy or escape from dormancy and result in recurrence. As a moderator, Dr. Manjili disseminated the results of tumor dormancy during the roundtable group that assessed data needed to better inform the low-dose mixture theory, Low-dose mixtures and cancer highlighted at NIEHS symposium, Durham, North Carolina, August 2015. (http://www.niehs.nih.gov/news/newsletter/2015/9/spotlight-mixtures/index.htm)

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Papers

1. Payne KK, Keim RC, Graham L, Idowu MO, Wan W, Wang XY, Toor AA, Bear HD, **Manjili MH**. Tumor-reactive immune cells protect against metastatic tumor and induce immunoediting of indolent but not quiescent tumor cells. *J Leukoc Biol* 2016 Feb 29. pii: jlb.5A1215-580R. [Epub ahead of print] PMID: 26928306
2. **Manjili MH**, Payne KK. Prospects in cancer immunotherapy: treating advanced stage disease or preventing tumor recurrence? *Disc Med* 19: 427-431, 2015. PMID: 26175400

Presentations (invited speaker):

1) Manjili MH. "*Immunotherapy for cancer dormancy*". The 2016 Controlling Cancer Summit, London, UK, May 17-19, 2016.
2) Manjili MH. "*The inherent premise of immunotherapy for cancer dormancy"* Hartford, CT, May 5, 2016.
3) Manjili MH. "*Current status and future prospects of immunotherapy: Targeting cancer dormancy*", The University of South Carolina, Columbus, SC, September 4, 2015.

4) Manjili MH. *Immune-mediated tumor dormancy and inflammation,* Cancer Cell Signaling group meeting, Massey Cancer Center, September 2015

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Savannah Butler
Project Role:	Lab specialist/graduate student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Ms. Butler has performed in vitro studies of chemotherapy-induced tumor dormancy
Funding Support:	DoD
Name:	Kyle Payne
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	Mr. Payne has performed in vivo studies of chemotherapy-induced tumor dormancy, immunotherapy, and in vitro studies of tumor dormancy. Wrote two papers.
Funding Support:	DoD, AAI fellowship award
Name:	Hussein Aqbi
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	Mr. Aqbi has performed in vitro and in vivo studies associated with chemotherapy- and RT-induced tumor dormancy, and immune response studies.
Funding Support:	DoD, PhD scholarship

Name:	Yibin Xie
Project Role:	Visiting Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Dr. Xie has performed in vivo studies of breast cancer dormancy.
Funding Support:	DoD, fellowship award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS: The partnering PI, Dr. David Gewirtz, is submitting an independent progress report

9. APPENDICES: Documents attached

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Prospects in Cancer Immunotherapy: Treating Advanced Stage Disease or Preventing Tumor Recurrence?

Masoud H. Manjili and Kyle K. Payne

Abstract: Human vaccines against infectious agents are often effective in a prophylactic setting. However, they are usually not effective when used post-exposure. Rabies vaccine is one of the exceptions, which can be used post-exposure, but is effective only when used in combination with other treatments. Similar results have been obtained with cancer vaccines and immunotherapies. Cancer immunotherapies generally prolong patients' survival when they are used during advanced stage disease. The potential of immunotherapy to cure cancer could be revealed when it is applied in a prophylactic setting. This article provides a brief overview of cancer immunotherapeutics and suggests that immunotherapy can cure cancer if used at the right time against the right target; we suggest that targeting cancer during dormancy in order to prevent tumor recurrence as advanced stage disease is potentially curative. [*Discovery Medicine* 19(107):427-431, June 2015]

Cancer Immunotherapies: Premises and Challenges

Recently, there have been dramatic advances in the field of cancer immunotherapy. However, these advances generally have been limited to increasing patients' survival for a limited period of time when administered in a therapeutic setting against advanced stage disease. In April 2010, the U.S. Food and Drug Administration (FDA) approved the first therapeutic cancer vaccine. This vaccine, sipuleucel-T (Provenge, manufactured by Dendreon) is designed to stimulate an immune response to a prostate tumor antigen, prostatic acid phosphatase (PAP). In a clinical trial, sipuleucel-T extended survival of patients with metastatic prostate cancer by a median of 4.1 months (Kantoff *et al*., 2010). Blockade of immune checkpoint molecules has also prolonged survival of patients with advanced cancer. For instance, anti-CTLA-4 ipilimumab therapy resulted in a 3.5-month gain in overall survival in patients with stage III or IV metastatic cutaneous melanoma (Hodi *et al*., 2010). Cumulative response rates for anti-PD-1 antibody among patients with non-small-cell lung cancer, melanoma, and renal-cell cancer were 18%, 28%, and 27%, respectively. Responses were durable such that 20 of 31 responses lasted 1 year or more in patients with 1 year or more of follow-up (Topalian *et al*., 2012).

Ex vivo expansion of tumor reactive T cells administered therapeutically has shown promise in some patients with advanced tumors. In 2006, adoptive immunotherapy (AIT) utilizing normal circulating lymphocytes transduced with a retrovirus encoding a MART-1-specific T cell receptor (TCR) resulted in objective regression of melanoma lesions in 2 of 15 patients (Morgan *et al*., 2006). In one patient, AIT resulted in 89% reduction of the liver tumor mass, at which time it was removed by surgery, and the patient remained disease free 21 months later. In another patient, AIT resulted in the regression of the hilar mass, and the patient remained disease free 20 months later (Morgan *et al*., 2006). AIT utilizing lymphocytes genetically engineered to express a chimeric antigen receptor (CAR) against the B cell antigen CD19 also mediated regression of an advanced B cell lymphoma in one patient with progressive lymphoma. No information was provided as to whether the patient remained relapse-free (Kochenderfer *et al*., 2010). The use of AIT in patients with metastatic melanoma utilizing tumor-infiltrating lymphocytes (TILs) grown in IL-2 resulted in tumor regression in 49% of patients (Dudley *et al*., 2002). When AIT was combined with total body irradiation (TBI) objective responses increased to 72%. Among treated groups, 20% had complete tumor regression and over 10 years relapse-free survival (Rosenberg *et al*., 2011). Thus far, of the 34 complete responders in the National Cancer Institute (NCI) trials, one has recurred (Rosenberg and Restifo, 2015).

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Despite the remarkable recent advances in cancer immunotherapy, the ability of immunotherapy to treat common carcinomas, which account for a majority of all cancer deaths, is limited. The application of immunotherapy to highly proliferative tumors renders the tumors prone to immunoediting and subsequent immunological escape during cell division. An important point to consider is that human vaccines against infectious diseases also are not effective in a setting of established disease. The rabies vaccine is an exception; however, it is ineffective as a single agent or at the onset of clinical illness.

Successful human vaccines against infectious diseases suggest that vaccines and administration of immunotherapy can be effective in a prophylactic setting either prior to exposure to infectious agents including pathogen-associated cancers or during the incubation period or dormancy after the exposure. For instance, the rabies vaccine can be used as post-exposure prophylaxis because the incubation period or dormancy for rabies is 1-3 months which provides a window for vaccination. Yet, it should be combined with anti-rabies immunoglobulin injections into the wound in order to control the infection and allow for the vaccine to work. Prophylactic cancer vaccines have also been successful. The FDA has approved two vaccines, Gardasil and Cervarix, that protect against HPV infection which is the leading cause of cervical cancer worldwide (Doorbar, 2006). HPV infection is also responsible for some vaginal, vulvar, anal, penile, and oropharyngeal cancers (Lowy and Schiller, 2006). The FDA has also approved a prophylactic cancer vaccine against HBV infection, which is a cause of liver cancer. Today, most children in the United States are vaccinated against HBV shortly after birth (Mast *et al*., 2005).

Cancer Therapies and Tumor Recurrence

Tumor dormancy in the form of residual disease is evident in almost all cancers, particularly breast cancer (Manjili, 2014). Up to 30% of patients with early stage breast cancers who have no evidence of metastasis will end up with distant recurrence of disease less than a decade after the treatment of primary cancer (Almog, 2010). Therefore, adjuvant chemotherapy is an option after surgery in order to kill cycling residual tumor cells. Yet, chemotherapy has shown limited success as it reduces metastatic recurrence by only 30% at 10 years (Demicheli *et al*., 2005). This is because while many tumor clones undergo apoptosis in the presence of chemotherapy other tumor clones escape from apoptosis, become indolent, and lie dormant (Almog, 2010; Demicheli *et al*., 2005; Manjili, 2014). Similar escape mechanisms were reported as a result of immunotherapy. These include tumor antigen loss (Kmieciak *et al*., 2007; Kmieciak *et al*., 2013), HLA loss, tumor-induced immune suppressive mechanisms mediated by a suppressive type of myeloid regulatory cells (Mregs) namely myeloid-derived suppressor cells (MDSCs) and/or regulatory T cells (Tregs) as well as engagement of immune checkpoint pathways. Although reprogramming of tumor-sensitized immune cells can render them resistant to immune suppressor cells, their success in preclinical studies has been limited to a prophylactic setting (Kmieciak *et al*., 2011; Manjili and Payne, 2012; Payne *et al*., 2013). These escape mechanisms limit therapeutic application of immunotherapy as well as conventional therapies against cancer. Therefore, a major challenge in the treatment of cancer is to target and eliminate dormant tumor cells in order to prevent tumor recurrence as advanced stage disease.

Dormant breast cancer cells have been detected as disseminated tumor cells (DTC) that reside in distant organs, as well as circulating tumor cells (CTC) that can be detected in the bloodstream. In humans, DTC were isolated from bone marrow after removal of the primary lesion (Pantel *et al*., 1993; Suzuki *et al*., 2006). DTC that can resume proliferation and establish distant metastasis have been recently reported by Dr. Bissell's group (Ghajar *et al*., 2013). They showed that DTC reside on the endothelium of the microvasculature in the lung, bone marrow, and brain, which are common metastatic destinations of breast cancer. In addition, CTC have been detected in the bloodstream of breast cancer survivors several years after successful treatment of primary breast cancer (Sinha, 2012). Detection of 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 (Lucci *et al*., 2012; Meng *et al*., 2004; Sinha, 2012). Detection of CTC in breast cancer patients even 22 years after the completion of conventional cancer therapies suggest that: i) even recurrence-free cancer patients are at risk of tumor recurrence at any time, or ii) establishment of permanent tumor dormancy is feasible as a means to prevent tumor recurrence. Both forms of tumor dormancy, DTC and CTC, are different from metastasis. In fact, tumor dormancy is a step between treatment of primary cancer and recurrence as advanced stage disease. During dormancy, tumor cells maintain homeostasis and cellular integrity over prolonged periods of non-division, which is likely due to indolent growth defined by a balanced proliferation and death, and/or quiescent dormancy, defined by cell cycle arrest.

Immunotherapy of Dormant Tumor Cells for the Prevention of Tumor Recurrence

Primary cancers or advanced stage diseases harbor

highly proliferative tumor cells that can outnumber tumor-reactive T cells, and also secrete immune suppressive factors that dismantle immunotherapy of cancer. Therefore, cancer immunotherapy used against primary or advanced cancer likely will achieve prolonged patient survival, at best, rather than eliminating the tumor. Unlike highly proliferating tumor cells, dormant tumor cells are resistant to chemotherapy or radiation therapy due to their indolent nature, permitting their prolonged persistence *in situ*. Intriguingly, dormant tumor cells likely represent the best targets for immunotherapy as their secretion profile of immunosuppressive factors is dampened compared with proliferating tumors. Dormant tumor cells also represent a static target for immune cells, in contrast to proliferating tumors which may grow to skew the effector-to-target ratio in favor of the tumor. Therefore, the application of immunotherapy immediately after successful completion of chemotherapy, when tumor dormancy is likely established, can result in the prevention of tumor relapse by eliminating immune-vulnerable dormant tumor cells or facilitating permanent dormancy. Lessons learned from the application of the rabies vaccine during clinical latency suggest that cancer immunotherapy can be successful during tumor dormancy. Allogeneic stem cell transplantation against hematological malignancies is also effective during minimal residual disease or semi-dormancy, which is established as a result of prior therapies, rather than against active and advanced stage disease. This is because indolent tumor cells, which become chemo-refractory, remain sensitive to immunotherapy.

Mechanisms for cancer dormancy range from cell cycle arrest to immunoediting and angiogenic insufficiency (Teng *et al*., 2008; Uhr and Pantel, 2011; Yu *et al*., 1997). Fundamentally, these mechanisms vary between cellular quiescence and balanced proliferation and death (mitogenesis equally offset by apoptosis). Whereas cancer immunotherapy can control quiescent dormancy, it may induce immunoediting and result in the escape of dormant cells that are indolent due to their

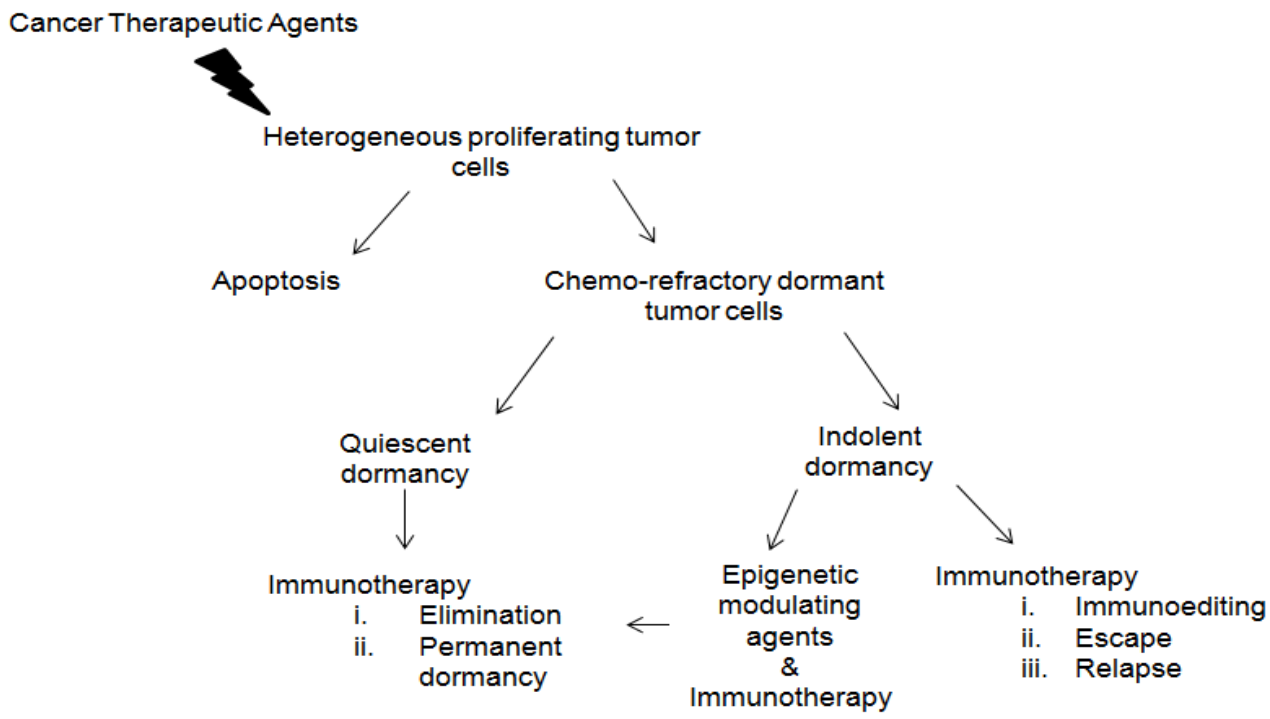

Figure 1. Immunotherapy of cancer dormancy. Tumors are comprised of heterogeneous cells, most of which undergo apoptosis upon treatment with cancer therapeutics. A residual population survives chemotherapy and becomes dormant. These dormant cells tend to be chemo-refractory cells, which may give rise to tumor recurrence. Such dormant tumor cells exist in a state of quiescent dormancy in which proliferation is arrested, or indolent dormancy defined by balanced proliferation and death. Quiescent dormancy represents the most vulnerable state to target tumor cells by immunotherapy, resulting in tumor cell elimination or permanent dormancy. Indolent dormant tumor cells retain immunoediting potential because of being able to change during cell division; effectively targeting such indolent cells immunologically likely will require combination therapy to maintain tumor-antigen expression to mediate successful elimination of the tumor cells by immunotherapy.

ability to undergo cellular division. Therefore, a major challenge in immunotherapy of tumor dormancy is to elucidate the mechanisms which induce quiescent dormancy and reduce indolent dormancy.

Conclusions

Heterogeneity of tumor cells results in a range of responses to therapeutic agents from apoptosis to inhibition of tumor cell proliferation. The latter establishes tumor dormancy (Figure 1). These dormant cells usually become resistant to conventional cancer therapies. However, they are the best targets for immunotherapy. Therefore, administration of immunotherapy after successful completion of conventional therapies, when tumors enter the dormant state, could prevent distant recurrence of the tumor in the form of advanced stage disease. There are two types of dormancy which include quiescent dormancy or cell cycle arrest, and indolent dormancy or balanced proliferation and death. Whereas the former is resistant to immunoediting, the latter can change during cell division under immune pressure and escape immunotherapy. Implementing strategies to promote the acquisition of quiescent dormancy in combination with immunotherapy, or the application of epigenetic modulating agents to overcome immunoediting of indolent dormant cells, could reduce the risk of relapse and associated mortality of cancer patients.

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Disclosure

Authors have no potential conflicts of interest to disclose.

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Article

Tumor-reactive immune cells protect against metastatic tumor and induce immunoediting of indolent but not quiescent tumor cells

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ABSTRACT

Two major barriers to cancer immunotherapy include tumor-induced immune suppression mediated by myeloid-derived suppressor cells and poor immunogenicity of the tumor-expressing self-antigens. To overcome these barriers, we reprogrammed tumor-immune cell cross-talk by combined use of decitabine and adoptive immunotherapy, containing tumor-sensitized T cells and CD25$^+$ NKT cells. Decitabine functioned to induce the expression of highly immunogenic cancer testis antigens in the tumor, while also reducing the frequency of myeloid-derived suppressor cells and the presence of CD25$^+$ NKT cells rendered T cells, resistant to remaining myeloid-derived suppressor cells. This combinatorial therapy significantly prolonged survival of animals bearing metastatic tumor cells. Adoptive immunotherapy also induced tumor immunoediting, resulting in tumor escape and associated disease-related mortality. To identify a tumor target that is incapable of escape from the immune response, we used dormant tumor cells. We used Adriamycin chemotherapy or radiation therapy, which simultaneously induce tumor cell death and tumor dormancy. Resultant dormant cells became refractory to additional doses of Adriamycin or radiation therapy, but they remained sensitive to tumor-reactive immune cells. Importantly, we discovered that dormant tumor cells contained indolent cells that expressed low levels of Ki67 and quiescent cells that were Ki67 negative. Whereas the former were prone to tumor immunoediting and escape, the latter did not demonstrate immunoediting. Our results suggest that immunotherapy could be highly effective against quiescent dormant tumor cells. The challenge is to develop combinatorial therapies that could establish a quiescent type of tumor dormancy, which would be the best target for immunotherapy. *J. Leukoc. Biol.* 100: 000–000; 2016.

Abbreviations: ADR = Adriamycin, AIT = adoptive immunotherapy, Aza = azacytidine, CTA = cancer testis antigen, CYP = cyclophosphamide, Dec = decitabine, FVS = fixable viability stain, FVS$^-$ = FVS-negative, i.d. = intradermally, IHC = immunohistochemistry, i.p. = intraperitoneally, i.v., intravenously, Ki67$^-$ = Ki67-negative, Ki67$^+$/low = low levels of Ki67, MDSC = myeloid-derived suppressor cell, MFI = mean fluorescence intensity, MMC = mouse mammary

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The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

MDSCs are key cellular suppressors of anti-tumor immune responses in breast cancer patients. Tumor-derived factors drive the accumulation of MDSCs in the bone marrow and secondary lymphoid organs and at the site of the tumor, thereby inhibiting the efficacy of cellular immunotherapy against established tumors. A number of strategies have been used to enhance immunotherapy of cancer by overcoming MDSCs. These strategies fall into 3 major categories that include MDSC deactivation, depletion of MDSCs, or conversion of MDSCs to APCs [1, 2]. The latter approach identified NKT cells as a key facilitator in promoting MDSC maturation into mature myeloid cells with anti-tumor immune stimulatory function. Therefore, it was suggested that the term M_{regs} better represents the plasticity of these cells, rather than MDSCs [3]. With the use of PBMCs of patients with early-stage breast cancer, we demonstrated previously that an optimal frequency of CD25$^+$ NKT cells within reprogrammed immune cells, cultured in the presence of MDSCs/M_{regs}, induced them to lose/down-regulate CD11b, which was associated with HLA-DR up-regulation. Such phenotypic modulation was shown to promote anti-human epidermal growth factor receptor 2/neu immune responses in vitro [4]. Therefore, inclusion of CD25$^+$ NKT cells in AIT should enhance the anti-tumor efficacy of adoptively transferred T cells by modulating MDSCs/M_{regs} to become immunostimulatory instead of immunosuppressive.

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Another barrier to successful cancer immunotherapy is that human cancers are usually poorly immunogenic, with the exception of melanoma. Therefore, the enhancement of the immunogenicity of tumor cells could make them better targets for immunotherapy. On the other hand, tumor immunoediting, such as loss of tumor antigens and engagement of the PD-1/PD-L1 pathway, is likely to occur in the face of robust anti-tumor immune responses. Therefore, the overcoming of tumor immunoediting and escape remains a major challenge for effective anti-cancer immunotherapies. To this end, it is critical to determine how tumors may or may not be prone to immunoediting and escape and how this tendency can be altered.

To address these challenges, we sought to modulate tumor-immune cross-talk by using reprogrammed T cells and NKT cells along with Dec. AIT, with reprogrammed, tumor-sensitized T cells and $CD25^+$ NKT cells, is expected to overcome MDSCs and establish memory responses [5], whereas Dec is expected to render tumor cells highly immunogenic by the induction of the expression of CTAs [6, 7]. Dec is an epigenetic therapy for acute myeloid leukemia, which may also inhibit the suppressive function of MDSCs [8]. We evaluated this combinatorial therapy against established primary tumors and against experimental metastasis. Furthermore, we identified 2 types of tumor dormancy, which included indolent dormancy characterized by $Ki67^+$/low and quiescent dormancy characterized by $Ki67^-$. We demonstrated that quiescent, but not indolent, dormant tumor cells were resistant to immunoediting.

MATERIALS AND METHODS

Mouse model

FVBN202 transgenic female mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used. These mice overexpress the nonmutated, nonactivated rat neu transgene under the regulation of the mouse mammary tumor virus promoter [9]. These mice develop premalignant mammary hyperplasia similar to ductal carcinoma in situ before the development of spontaneous carcinoma [10]. These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Tumor cell lines

The neu-overexpressing MMC cell line was established from spontaneous mammary tumors harvested from FVBN202 mice [11]. Tumor cells were maintained in RPMI 1640, supplemented with 10% FBS.

Ex vivo reprogramming and expansion of splenocytes

The reprogramming of tumor-sensitized immune cells was performed as described previously by our group [5]. In brief, FVBN202 transgenic mice were inoculated in the mammary fat pad with 3×10^6 MMC cells. Tumor growth was monitored by digital caliper, and tumor volumes were calculated by $v = (L \times W^2)/2$, where v is volume, L is length, and W is width. As described previously [11], splenocytes were harvested 21–25 d after tumor challenge, when the tumor had reached $\geq$1000 mm^3. Splenocytes were then cultured in complete medium [RPMI 1640, supplemented with 10% FBS, L-glutamine (2 mM), 100 U/ml penicillin, and 100 μg/ml streptomycin] and were stimulated with Bryostatin 1 (2 nM; Sigma-Aldrich, St. Louis, MO, USA), ionomycin (1 μM; Calbiochem, EMD Millipore, Billerica, MA, USA), and 80 U/ml/10^6 cells of IL-2 (PeproTech, Rocky Hill, NJ, USA) for 16–18 h. Lymphocytes were then washed thrice and cultured at 10^6 cells/ml in complete medium with IL-7 and IL-15 (20 ng/ml each cytokine; PeproTech). After 24 h, 20 U/ml IL-2 was added to the complete medium. The following day, the cells were washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml IL-2. After 48 h, cells were washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml IL-2. Twenty-four hours later, lymphocytes were again washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml IL-2. Lymphocytes were harvested 24 h later on the sixth day and were then used for in vitro studies or in vivo for AIT.

Adoptive cellular immunotherapy

Twenty-four hours before AIT, FVBN202 mice were injected i.p. with CYP (100 mg/kg) to induce lymphopenia. Individual groups of mice were challenged i.d. in the mammary gland region, with 3×10^6 MMC cells, or i.v. with 10^6 MMC. Individual groups of mice then received reprogrammed splenocytes i.v. at a dose of 70×10^6/mouse, 3 d after tumor challenge when the tumor became palpable (50–70 mm^3) or on the day of the i.v. tumor injection. Untreated tumor-bearing mice served as control.

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

MMC cells (3×10^6 cells/3 ml) were cultured in the presence of 3 μM Dec (Sigma-Aldrich) for 72 h. Medium was then removed, and cells were washed with sterile PBS and then treated with TRIzol (Life Technologies, Thermo Fisher Scientific, Grand Island, NY, USA), per the manufacturer's instructions. In vivo, FVBN202 mice, bearing primary tumor $\geq$1000 mm^3, were injected i.p. with a high-dose Dec (2.5 mg/kg), once daily for 5 d. Mice were euthanized, and tumors were harvested 3 d later, minced, and then treated with TRIzol, per the manufacturer's instructions. Contaminant DNA was then removed by DNase I digestion from the in vitro and in vivo specimens; RNA was then purified, followed by cDNA synthesis, as described previously by our group [12].

Real-time qRT-PCR for the detection of CTA expression

qRT-PCR was performed in triplicate wells using the SensiMix SYBR & Fluorescein Kit, according to the manufacturer's procedure (Bioline, Taunton, MA, USA), with the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). qRT-PCR was performed using primers specific for 6 murine CTAs and murine GAPDH. The reaction was initiated by a denaturing period of 10 min at 95°C, followed by 40 cycles of 95°C for 15 min, 60°C for 30 min, and 72°C for 15 min [6, 12]. Relative CTA expression was computed after normalization to GAPDH using the ΔΔ quantification cycle method.

IFN-γ ELISA

Reprogrammed immune cells were cultured in complete medium with irradiated (140 Gy) MMC cells or irradiated CTA-expressing MMC, induced by Dec treatment in vitro at a 10:1 ratio for 20 h. Supernatants were then collected and stored at −80°C until assayed. IFN-γ was detected using a mouse IFN-γ ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol [5].

Characterization of splenocytes and tumor-infiltrating leukocytes

Spleens and metastatic tumor lesions of FVBN202 mice were harvested when the animals became moribund and were then separately homogenized

(*continued from previous page*)

carcinoma, M_{reg} = myeloid regulatory cell, PD-1 = programmed death 1, PD-L1 = programmed death ligand 1, PI = propidium iodide, qRT-PCR = quantitative RT-PCR, RT = radiation therapy, TIL = tumor-infiltrating lymphocyte

into a single cell suspension as described previously [11] and below. Splenocytes were then characterized using flow cytometry. Reagents used for flow cytometry include the following: anti-CD16/32 antibody (clone 93), FITC-CD3 (17A2); FITC-CD11b (M1/70); FITC-anti-mouse IgG (Poly4053); PE-GR-1 (RB6-8C5); PE-PD-1 (RMP1-30); PE-CD25 (3C7); PE-Ki67 (16A8); allophycocyanin-CD49b (DX5); allophycocyanin-CD62 ligand (MEL-14); allophycocyanin-Annexin V; PerCP/CY5.5-CD4 (GK1.5); PE/CY7-CD8α (53-6.7); Brilliant Violet 421-PD-L1 (10F.9G2); Brilliant Violet 605-CD45 (30-F11); and PI, all of which were purchased from BioLegend (San Diego, CA, USA). BD Horizon V450-Annexin V and FITC-FVS were purchased from BD Biosciences. Anti-rat neu antibody (anti–c-Erb2/c-Neu; 7.16.4), was purchased from Calbiochem. All reagents were used at the manufacturer's recommended concentration. Cellular staining was performed as described previously by our group [11] or as recommended by the manufacturer (Ki67, FVS). Multicolor data acquisition was performed using a LSRFortessa X-20 (BD Biosciences). Data were analyzed using FCS Express v4.07 (De Novo Software, Glendale, CA, USA).

Isolation and characterization of lung metastases

Lungs were harvested from the "Control" and "AIT" groups after animals became moribund. Metastatic lesions were excised individually from the residual lung tissue and minced and digested in Trypsin-EDTA (0.25%; Life Technologies, Thermo Fisher Scientific) overnight at 4°C. The following day, the suspension was incubated at 37°C for 30 min, followed by gentle tissue homogenization to create a cellular suspension. The cell suspension was then washed twice with RPMI supplemented with 10% FBS. Residual RBCs were then lysed using ammonium-chloride-potassium lysing buffer, followed by an additional wash with RPMI 10% FBS. The cell suspension was then placed in cell culture and cultured with RPMI 10% FBS. Adherent metastatic tumor cells were then characterized for the expression of rat neu and PD-L1 using flow cytometry.

Characterization of metastatic tumor-infiltrating leukocytes

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

Establishment of ex vivo tumor cell dormancy

MMC cells were treated with 3 daily doses of ADR (doxorubicin hydrochloride, 1 μM/d for 2 h; Sigma-Aldrich). Residual, dormant MMC cells remained adherent to tissue-culture flasks, whereas the MMC cells susceptible to ADR therapy became nonadherent and were removed from the culture periodically. Assessment of viability, Ki67 expression, and IFN-γ-induced PD-L1 up-regulation by flow cytometry occurred 3 wk after the final treatment. Likewise, 3 daily doses of RT (2 Gy/d) were also used to establish dormant MMC cells. ADR and RT-induced dormant MMC cells were used in the cytotoxicity assay, 8 d after the final treatment.

IHC

Sections of formalin-fixed paraffin-embedded tissue from each tumor were stained with H&E to examine the histomorphology. Additional sections are then subsequently immunolabeled using the standard IHC technique, using the avidin-biotin peroxidase system with a purified anti-mouse Ki67 (BioLegend). Sections of lymph nodes were used as the positive control. Nikon Eclipse 80i light microscope was used to examine the H&E and IHC. The most intense labeling regions (hot spots) away from the edge of the tissue were evaluated using IHC-positive tumor cells as numerator and the overall tumor cells as the denominator. Representative images of the H&E and the corresponding hotspots were taken.

Statistical analysis

Statistical comparisons between groups were made using 1- and 2-tailed Student's t test. Time to death in the in vivo survival studies was calculated from baseline to the date of death. Mice were euthanized when they had a weight loss of ≥10%. Kaplan-Meier curves and log-rank tests are used to illustrate time to death and to test the difference between each group. $P \leq 0.05$ was considered statistically significant.

RESULTS

The reprogramming of tumor-immune cross-talk during immunotherapy fails to protect animals from an established primary mammary carcinoma

We have reported previously that AIT, using reprogrammed T cells and NKT cells in a prophylactic setting, protected animals against primary tumors and recall tumor challenge. This protection was associated with the presence of memory T cells, and $CD25^+$ NKT cells that rendered T cells resistant to MDSC-mediated suppression [5]. Here, we sought to determine whether AIT as a single therapy can protect animals against established primary mammary carcinoma by overcoming MDSCs. FVBN202 mice bearing primary tumors received AIT using reprogrammed T cells and $CD25^+$ NKT cells when the tumor had reached 50–70 mm^3 or remained untreated. As shown in **Fig. 1A** and **B**, AIT alone did not slow the rate of tumor growth (Fig. 1A) or improve overall survival (Fig. 1B) in recipient mice compared with untreated control mice. Then, we combined AIT with epigenetic modulation of tumor cells in vivo to enhance immunogenicity of tumor cells, as well as eliminate MDSCs. To do so, tumor-bearing animals received Dec before AIT. Use of Dec induced the expression of a panel of CTAs in tumor cells (Fig. 1C and D) and resulted in the elimination of MDSCs (Fig. 1E; $P = 0.034$). However, AIT still failed to protect animals from established primary tumors when compared with Dec alone (Fig. 1F and G). This failure was observed in spite of successful reprogramming of tumor-sensitized immune cells (Supplemental Fig. 1) and their enhanced reactivity against CTA-expressing MMC (Fig. 1H; $P = 0.0001$).

The reprogramming of tumor-immune cross-talk during immunotherapy prolongs survival of animals bearing metastatic tumor cells in their circulation

We have reported previously that administration of AIT along with another epigenetic modulator, Aza, was effective in prolonging survival of patients with multiple myeloma when treatment was delivered during minimal residual disease to prevent advanced stage disease [6]. Therefore, we sought to take a similar approach in our experimental model of breast cancer by administrating AIT and Dec when tumor cells were present in the circulation and before establishing lung metastasis. AIT alone had a marginal impact on the survival of animals,

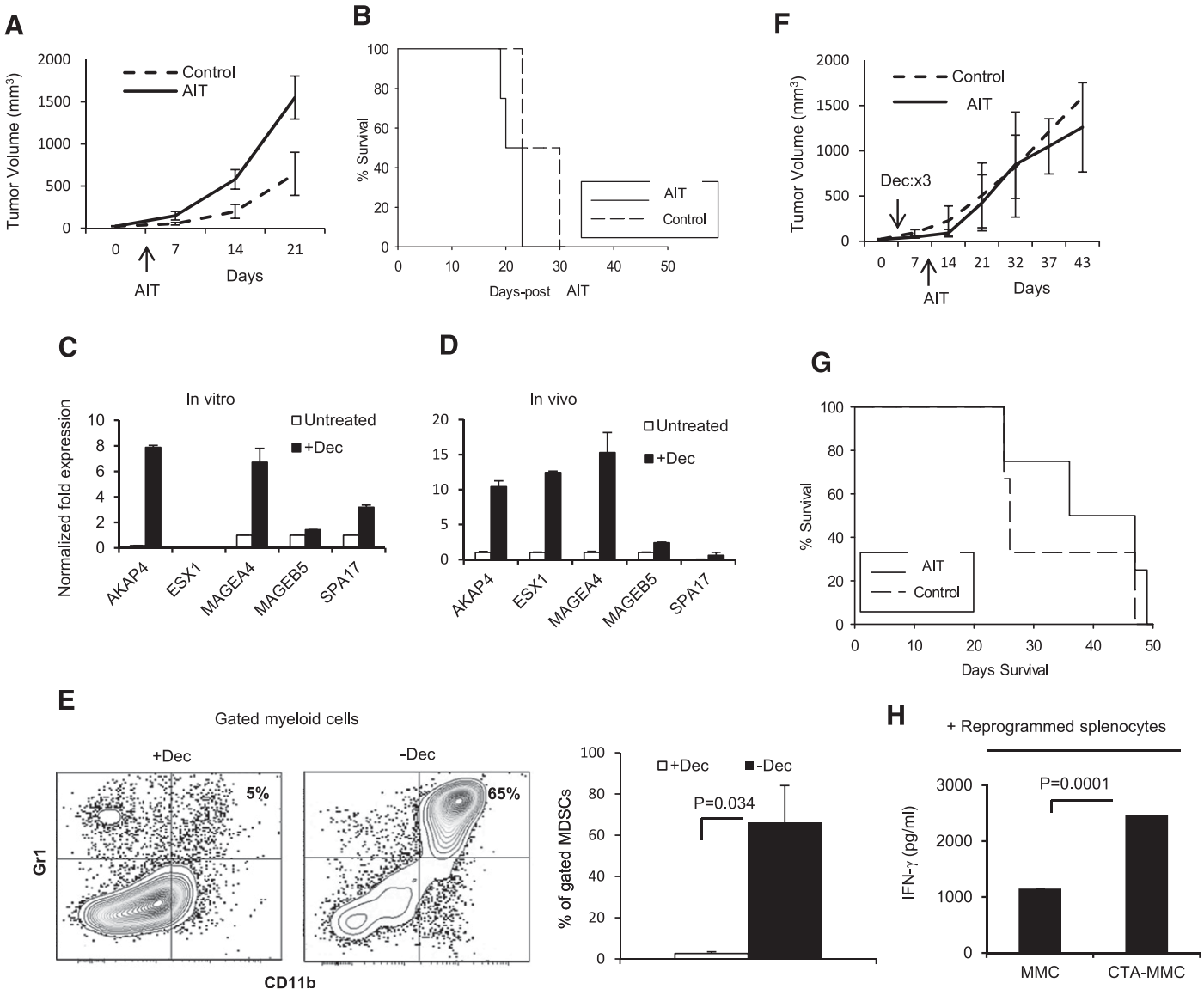

Figure 1. AIT, with or without Dec, fails to induce regression-established mammary carcinoma. (A and B) Animals were challenged with MMC (3×10^6) i.d. in the mammary gland region; upon the tumor reaching 50–70 mm^3, animals were conditioned with CYP (100 mg/kg). The following day, mice remained untreated (Control; $n = 4$) or received AIT ($n = 4$). (C) MMC tumor cells were cultured with (+Dec) or without Dec (Untreated; 3 mM) for 72 h; RNA was then extracted and converted to cDNA, followed by qRT-PCR, using primers specific for 6 murine CTAs. (D) Tumor-bearing (~1000 mm^3 i.d.) FVBN202 mice received 5 injections of Dec, 1/d (+Dec; 2.5 mg/kg; $n = 1$) or remained untreated ($n = 1$); the tumors were harvested 3 d later, and cDNA was generated to quantify CTA expression, which was normalized to GAPDH. AKAP4, A-kinase anchor protein 4; ESX1, ; MAGEA4, melanoma-associated antigen 4; MAGEB5, melanoma-associated antigen B5; SPA17, ESX1, Extraembryonic, spermatogenesis, homeobox 1; SPA17, Sperm Autoantigenic Protein 17. (E) Animals were challenged i.d. with MMC (3×10^6) in the mammary gland region; after tumors reached 50–70 mm^3, all animals were treated with Dec (every other day for 3 total injections; 2.5 mg/kg, i.p.; $n = 3$) or remained untreated ($n = 3$). Seven days later, mice were euthanized, and MDSCs were analyzed in the spleen. (F and G) Animals were challenged i.d. with MMC (3×10^6) in the mammary gland region; after tumors reached 50–70 mm^3, all animals were treated with Dec [every day for 3 total injections (×3); 2.5 mg/kg, i.p.]. Two days later, animals were conditioned with CYP (100 mg/kg, i.p.). The following day, mice remained untreated (Control; $n = 3$) or received AIT ($n = 4$), derived from a CTA^+ tumor-bearing donor. (H) MMC cells remained untreated (MMC) or were treated with Dec (3 mM; 72 h) to induce CTA expression (CTA-MMC). Tumor cells were then cocultured with reprogrammed splenocytes (1:10) for 20 h. IFN-γ was detected in the supernatant by ELISA. Data represent means ± SEM of duplicate wells.

whereas Dec alone resulted in prolonging the survival of animals (**Fig. 2**; P = 0.013). AIT + Dec was the most effective therapy that resulted in prolonging the survival of animals compared with the control group or Dec alone (Fig. 2; $P = 0.0001$ and $P = 0.037$, respectively). However, all animals eventually succumbed to metastatic tumors in the lungs.

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

To determine whether reprogrammed memory T cells were maintained in vivo, splenocytes of AIT recipients were collected when mice became moribund and cultured with MMC tumor

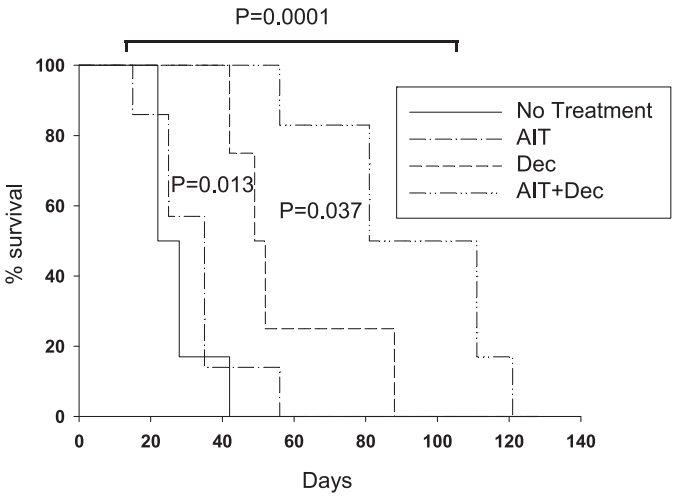

Figure 2. Combined use of Dec and AIT prolongs survival of animals and induces tumor immune editing. FVBN202 mice were challenged with 1×10^6 MMC cells i.v. Mice then remained untreated (Control; $n = 4$), received AIT ($n = 7$) on the same day as tumor challenge, received Dec (Dec; $n = 4$; 5 daily doses beginning on d 3 after tumor challenge), or received Dec and AIT (AIT + Dec; $n = 6$; AIT on the day of tumor challenge, followed by Dec beginning on d 3).

cells. As shown in **Fig. 3A**, tumor-reactive IFN-γ production by endogenous splenic T cells from the AIT group was greater than that produced by T cells from the control group ($P = 0.003$). To determine the impact of treatments on tumor immunoediting and escape, T cells and tumor cells in the tumor-microenvironment of the lung were analyzed. Metastatic tumor lesions were isolated from the lung at the end of the trial and analyzed for the expression of the tumor antigen, neu, and PD-L1. The tumor lesions isolated from the AIT group showed down-regulation of neu antigen on the tumor cells compared with control MMC tumor cells and the lesions isolated from the control group (Fig. 3B, upper; $P = 0.00003$ and $P = 0.0008$, respectively). The AIT + Dec group showed similar trends as the AIT group. Additionally, 25% of MMC cells isolated from metastatic tumor lesions of the AIT group demonstrated total loss of neu expression compared with control MMC tumor cells and the lesions isolated from control group (Fig. 3B, lower; $P = 0.002$ and $P = 0.01$, respectively). Again, the AIT + Dec group showed similar trends as the AIT group. This suggests that metastatic MMC cells may eventually escape detection from neu-specific cellular immunity. Metastatic tumors of the control group that received no treatment did not show down-regulation or loss of neu antigen (Fig. 3B). As AIT was the major factor in neu loss/down-regulation, and the AIT-Dec group showed a similar trend, we looked at the expression of tumor PD-L1 expression in the AIT group. Interestingly, metastatic tumor cells from the control group had higher expression of PD-L1 in the tumor compared with the metastatic tumor cells from the AIT group or control MMC cells (Fig. 3C; MFI: 1360 vs. 390 ± SEM; $P = 0.011$). A similar trend was observed when MMC cells were cultured with IFN-γ or splenocytes of tumor-bearing animals (Fig. 3D).

The immune-suppressive function of PD-L1 requires engagement with PD-1, which renders immune cells unresponsive [13, 14]. Importantly, 40–50% of reprogrammed T cells and NKT cells that were used for AIT expressed PD-1 (Fig. 3E), but only $CD8^+$ T cells were observed to up-regulate PD-1 as a result of reprogramming (Fig. 3E; $P = 0.01$). Therefore, we also analyzed tumor-infiltrating T cells for PD-1 expression to determine the potential for the PD-1/PD-L1 axis to mediate T cell suppression within the tumor site. Interestingly, as seen in Fig. 3F, tumor infiltration of $CD8^+$ T cells into the tumor bed was greater in mice receiving AIT compared with untreated mice (14% vs. 3%, respectively; $P = 0.02$). However, expression of PD-1 on tumor-infiltrating $CD8^+$ T cells did not significantly increase following AIT compared with the control group (Fig. 3G). We did not observe $CD4^+$ T cell infiltration into the tumor lesions (data not shown). Splenic T cells and NKT cells that were isolated from the AIT and control groups when animals became moribund also expressed PD-1, although there was no statistical difference between the groups (Fig. 3H; 10% of T cells and 50% of NKT cells). Taken together, these data suggest that although AIT promotes the infiltration of $CD8^+$ T cells, the highly proliferative nature of the metastatic tumors may evade such anti-tumor immune responses by emerging with reduced expression of the tumor antigen, neu. Metastatic tumor of the Dec group also showed down-regulation of neu antigen (Supplemental Fig. 2A, left; MFI: 442 vs. 202 ± SEM), as well as total loss of neu antigen in 36% of tumor cells compared with the control MMC cell line containing a residual 5% of neu-negative cells (Supplemental Fig. 2A, right). As the control group did not show neu loss or down-regulation, but the AIT group did, we sought to determine whether neu loss or down-regulation in animals who received Dec could result from the contribution of an endogenous T cell response induced by Dec, which induces the expression of CTAs and therefore, could function as an in situ vaccination by eliciting endogenous T cell responses. To determine the contribution of Dec in neu antigen loss or down-regulation, we performed in vitro studies by treatment of MMC with Dec alone, where the endogenous immune response did not have any contribution. Dec treatment resulted in the quantitative down-regulation of neu expression but did not induce total neu loss (Supplemental Fig. 2B; $P = 0.008$). IFN-γ induced down-regulation of neu (Supplemental Fig. 2C; $P = 0.002$), and Dec did not recover neu expression. Thus, we then began to question whether residual tumor cells that remain after conventional cytotoxic therapy, which are generally dormant, also use similar escape mechanisms or if they were perhaps more sensitive to immune-mediated elimination.

To determine whether dormant tumor cells were resistant to immunoediting and escape, MMC tumor cells were treated with ADR to establish tumor dormancy ex vivo. Dormant tumor cells were then treated with a product of anti-tumor T cell responses—IFN-γ—to determine sensitivity of different dormant cells, quiescent and indolent, to immunoediting. We looked at the expression of PD-L1, as this is the most immediate immunoediting change that occurs as a result of IFN-γ treatment. A clinically relevant proliferation marker, Ki67, along with a viability marker (FVS), was used to detect

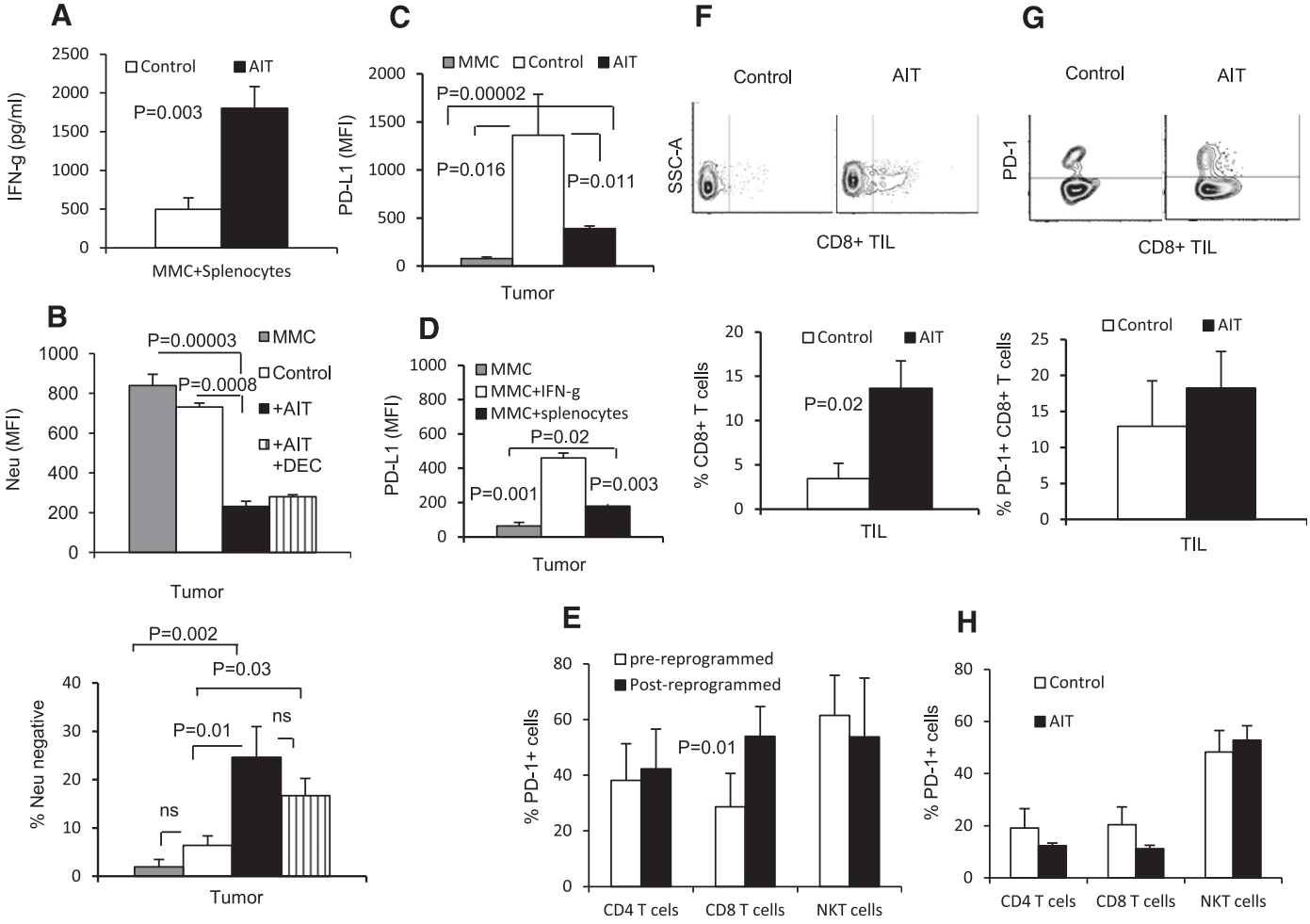

Figure 3. AIT promotes immunoediting of lung metastatic lesions. (A) Splenocytes were harvested from untreated mice (Control; $n = 5$) and AIT recipients ($n = 3$) and cultured in the presence of MMC cells (10:1) for 20 h. Supernatants were collected and subjected to IFN-γ ELISA. (B) Metastatic lesions in the lung of FVBN202 mice that remained untreated (Control; $n = 3$), AIT recipients ($n = 6$), and AIT + Dec recipients ($n = 4$) were harvested when mice became moribund. Tumor lesions were digested and analyzed. MFI of neu and frequency of neu loss (B) and MFI of PD-L1 (C) were then quantified using flow cytometry using MMC cell line (MMC) as an in vitro control. (D) MMC cells were cultured with IFN-γ or splenocytes of tumor-bearing mice, and PD-L1 was detected after 16–20 h ($n = 2$–3). (E) Spleens of FVBN202 mice bearing primary mammary carcinoma ($n = 4$) were harvested after tumors were ≥1000 mm^3. PD-1 expression was then quantified on the splenocytes, pre- and postreprogramming. (F and G) Metastatic lesions in the lung of FVBN202 mice that remained untreated (Control) and AIT recipients were harvested when mice became moribund. (F) The frequency of $CD8^+$ T cell infiltration metastatic lesion in the lung of control mice and the AIT group ($n = 3$) was determined on gated $CD3^+$ cells. SSC-A, Side-scatter-area. (G) Expression of PD-1 was determined on $CD3^+CD8^+$ cells (Control, $n = 1$; AIT, $n = 3$) by gating on $CD45^+$ viable leukocytes. (H) Spleens of FVBN202 mice that had received AIT ($n = 4$) or remained untreated ($n = 3$) and were i.v. challenged with MMC were analyzed by flow cytometry after tumor-bearing mice became moribund to quantify PD-1 expression. Data represent means ± SEM.

FVS^- viable, indolent tumor cells ($Ki67^+$/low) and quiescent tumor cells ($Ki67^-$). As shown in **Fig. 4A**, ADR induced apoptosis in the majority of MMC cells, such that by 3 wk after treatment, the number of FVS^- viable MMC cells was reduced from 77% to 31% ($P = 0.005$). The remaining residual viable tumor cells that escaped ADR-induced apoptosis entered a state of dormancy, as there was no significant increase in the number of tumor cells between 1 and 3 wk after completion of ADR treatment (Fig. 4B). To determine if dormant tumor cells could exploit immune escape mechanisms, we established ADR-induced tumor dormancy, followed by treatment of dormant MMC cells with IFN-γ, 3 wk after the completion of ADR treatment to provoke PD-L1 expression [15]. We evaluated the expression of PD-L1 on viable proliferating control MMC cells ($Ki67^+$), without (Untreated) and with IFN-γ (Untreated → IFN-γ) treatment, as well as on viable dormant tumor cells without (+ADR) and with IFN-γ (+ADR → IFN-γ) treatment. As shown in Fig. 4C, left, ADR-treated MMC showed a significant shift from $Ki67^+$ toward $Ki67^+$/low cells ($P = 0.026$), indicative of indolent tumor dormancy. ADR or ADR → IFN-γ treatment also resulted in a shift from $Ki67^+$ toward $Ki67^-$ quiescent cells, as shown by an increased frequency of $Ki67^-$ MMC (Fig. 4C; $P = 0.02$ and $P = 0.001$, respectively) and a decreased

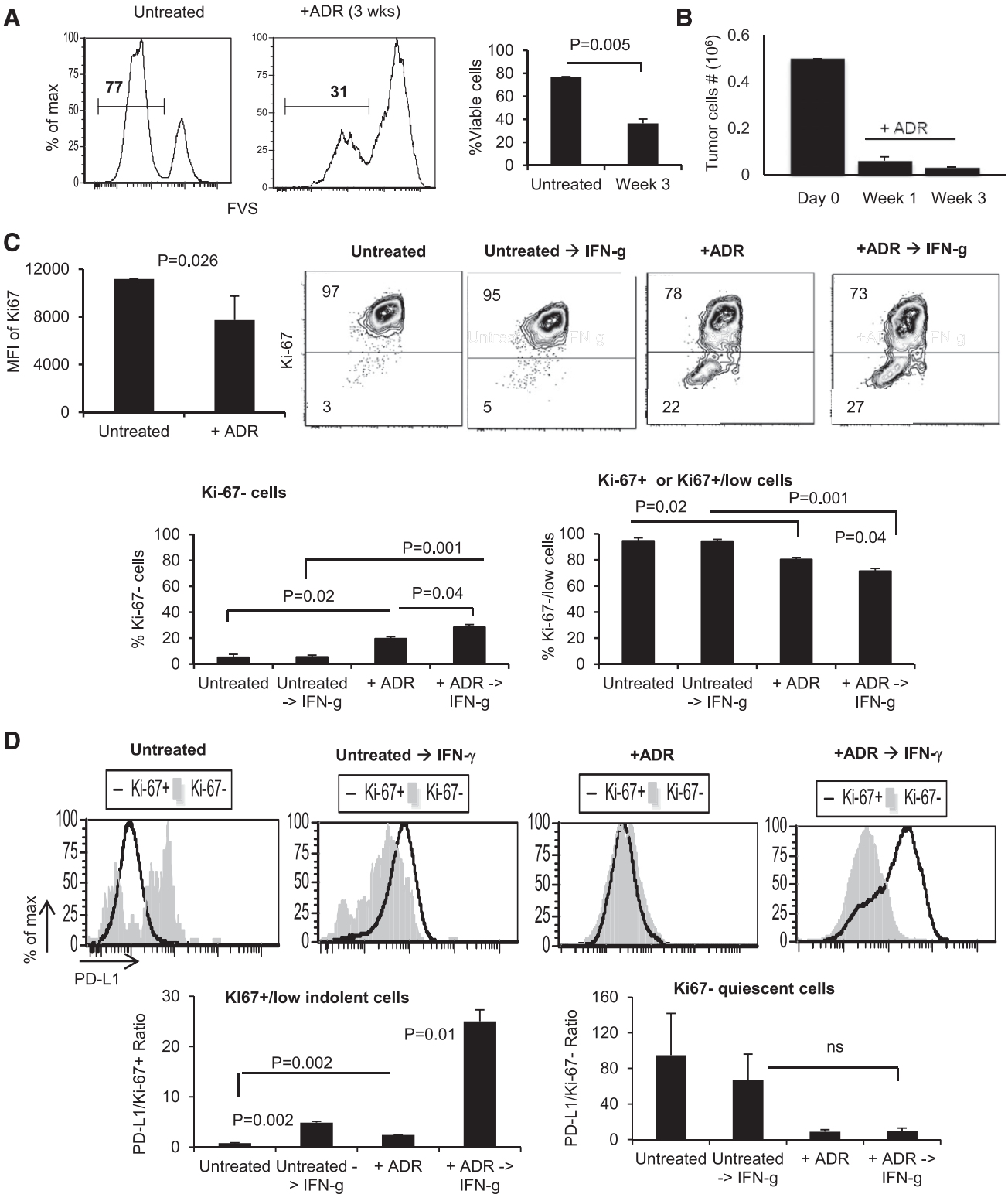

Figure 4. ADR treatment results in the emergence of indolent and quiescent tumor dormancy. (A) MMC tumor cells were treated with 3 daily doses of ADR (1 μM for 2 h) and then remained untreated for 3 wk. The frequency of viable MMC cells was determined by quantifying FVS^- cells using flow cytometry. (B) At wk 1 and 3 post-treatment, adherent and viable tumor cells were counted by trypan blue exclusion. (C and D) MMC cells were treated for 3 consecutive d with ADR (1 μM, 2 h) or left untreated. Three weeks later, ADR-treated and untreated MMC cells were stimulated with IFN-γ (50 ng/ml) for 12–16 h to induce the expression of PD-L1. (C) Emergence of Ki67 was determined in control MMC cells (Untreated), as well as ADR-treated cells (+ADR) ± IFN-γ stimulation. (D) The expression of PD-L1/cell was calculated by dividing PD-L1 MFI by the frequency of $Ki67^-$ cells in ADR-treated and untreated MMC cells ± IFN-γ stimulation. Data represent 3 independent experiments and means ± SEM.

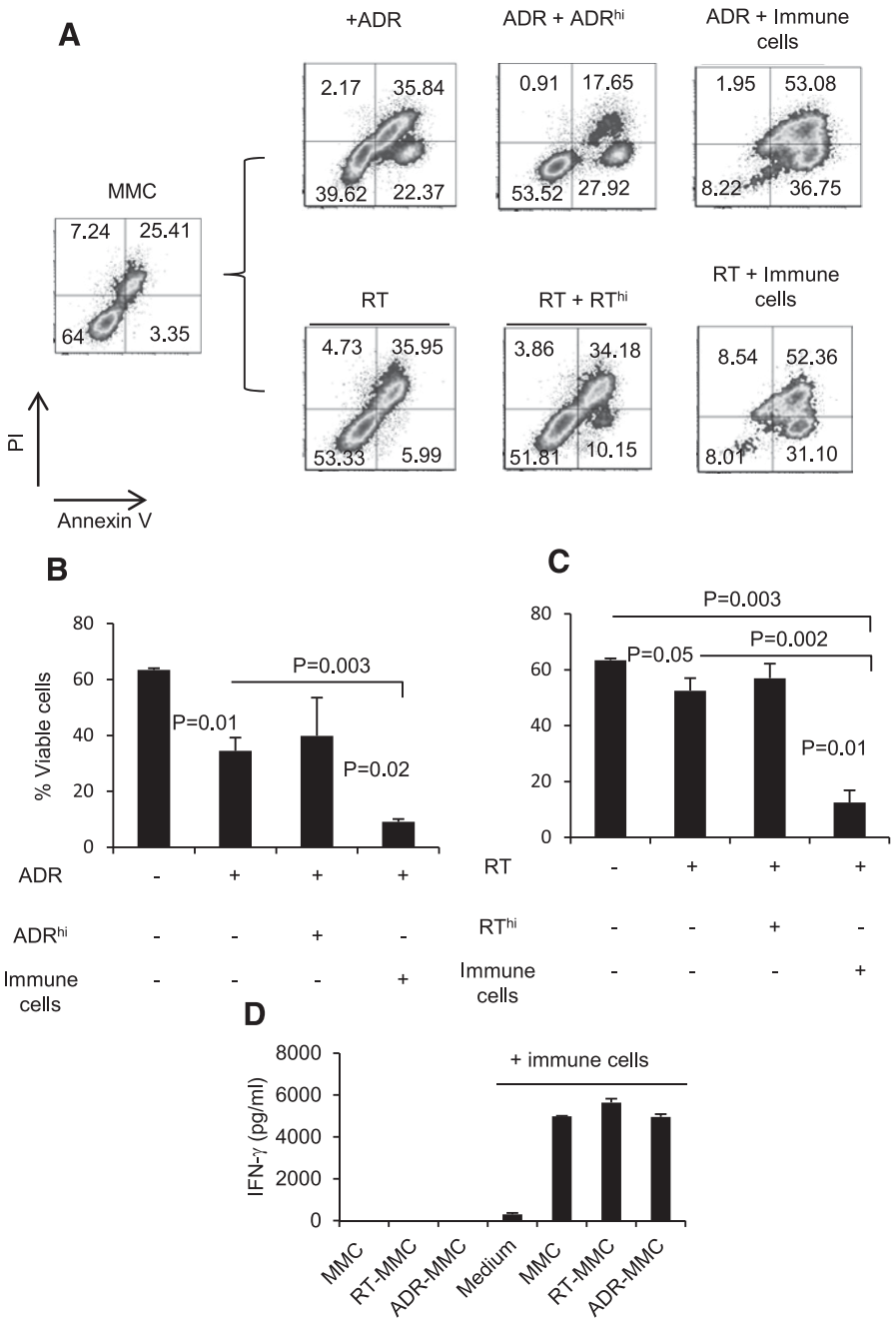

Figure 5. Immunotherapy displays cytotoxic function against treatment refractory dormant tumor cells in vitro. (A) MMC cells ($n = 3$) treated with ADR (1 μM, 2 h) or 2 Gy RT (RT-treated MMC) for 3 consecutive d and remained in culture for 8 d total to establish tumor cell dormancy in vitro. (B) On d 8, these dormant tumor cells were treated with a high-dose ADR (1 μM, 24 h; ADR-treated MMC + ADR^{hi}) or reprogrammed immune cells (ADR-treated MMC + immune cells; ADR-treated MMC + immune cells). Two days later, cells were stained with Annexin V/PI and analyzed by flow cytometry. Data represent 3 biologic repeats and means ± SEM. (C) On d 8, these dormant tumor cells were treated with 18 Gy RT (RT-treated MMC + RT^{hi}) or reprogrammed immune cells (RT-treated MMC + immune cells). Two days later, cells were stained with Annexin V/PI and analyzed by flow cytometry. (D) MMC tumor cells or dormant MMC cells (RT-MMC, ADR-MMC) were cultured in the absence or presence of the reprogrammed immune cells in a 10:1 ratio for 24 h. Control immune cells were cultured alone (Medium). IFN-γ release was detected in the supernatant using ELISA. Data represent 2 biologic repeats and means ± SEM.

frequency of $Ki67^+$ MMC cells (Fig. 4C; $P = 0.02$ and $P = 0.001$, respectively). IFN-γ treatment induced up-regulation of PD-L1 on $Ki67^+$/low indolent MMC (Fig. 4D, left; $P = 0.002$ and $P = 0.01$). Interestingly, $Ki67^-$ control MMC cells and $Ki67^-$ quiescent MMC cells did not up-regulate PD-L1 in the presence of IFN-γ (Fig. 4D, right).

Dormant MMC cells established by ADR or RT become resistant to higher doses of chemotherapy or RT but remain sensitive to immunotherapy

ADR chemotherapy usually induces tumor dormancy, which could lead to tumor recurrence. To determine the direct effect of ADR on tumor dormancy, we performed ex vivo experiments. ADR treatment increased the proportion of $Ki67^-$ tumor cells, which lasted for 3 wk. This trend was associated with reduced viability, 3 wk after treatment, which improved 6 wk after treatment (Supplemental Fig. 3; 77% > 31% > 50%). To determine whether dormant MMC cells established by ADR treatment remain sensitive to tumor-reactive immune cells, dormancy was established by treating MMC with 3 daily doses of ADR (1 μM/d for 2 h; Supplemental Fig. 4A); 8 days after the final treatment, MMC cells received a high dose of ADR (1 μM for 24 h) or were cultured with tumor-reactive immune cells for 48 h. ADR treatment induced apoptosis in MMC cells

(**Fig. 5A** and **B**; $P = 0.01$). Tumor cells that survived apoptosis became chemo refractory, such that additional ADR treatment at a higher dose (1 μM for 24 h) did not induce cell death (Fig. 5A and B; average 40% vs. 54%). However, they remained sensitive to tumor-reactive immune cells. In the presence of tumor-reactive immune cells, the frequency of viable ADR-treated dormant MMC dropped from 40% to 8% (Fig. 5A and B; $P = 0.003$). In fact, lymphocytes were more effective than a high dose of chemotherapy in inducing apoptosis in dormant MMC (Fig. 5A and B; $P = 0.02$). We also established dormant MMC by 3 daily doses of RT (2 Gy/d); again, surviving dormant cells became refractory to RT. An additional RT at a higher dose (18 Gy) did not markedly decrease the frequency of viable tumor cells (Fig. 5B and C; 53% vs. 52%). However, RT refractory MMC cells remained sensitive to tumor-reactive lymphocytes as the viability dropped from 53% to 8% (Fig. 5B and C; $P = 0.002$). In recapitulating our results with chemotherapy-induced tumor cell dormancy, tumor-reactive immune cells were more effective than high-dose RT at inducing apoptosis in dormant MMC (Fig. 5B and C; $P = 0.01$). To determine whether higher levels of apoptosis in dormant tumor cells were a result of their greater sensitivity to immune cells rather than a higher reactivity of the immune cells, IFN-γ ELISA was performed using reprogrammed immune cells cultured with MMC tumor cells or ADR- and RT-induced dormant MMC cells. As shown in Fig. 5D, tumor-reactive immune cells produced comparable levels of IFN-γ upon stimulation with MMC or dormant MMC (RT-MMC, ADR-MMC). To determine the establishment of $Ki67^-$ quiescent and $Ki67^+$/low indolent tumor cells, experimental animals bearing primary MMC were treated with ADR or remained untreated. Animals treated with ADR exhibited suppression of tumor growth (Supplemental Fig. 4B). Tumors of the ADR group showed a shift from $Ki67^+$ toward $Ki67^+$/low indolent and $Ki67^-$ quiescent cells (Supplemental Fig. 4C).

DISCUSSION

We developed an experimental metastatic mouse model by i.v. injection of highly proliferative MMC cells to FVBN202 mice. Animals in this model became moribund within 20-40 d and presented with lung metastases upon macroscopic inspection. This model represents the onset of advanced stage disease. We demonstrated that concurrent use of Dec with AIT using reprogrammed $CD25^+$ NKT and T cells prolonged survival of the experimental animals but failed to eliminate the tumor, as all mice eventually succumbed to metastatic disease in the lungs. Failure of tumor elimination was associated with down-regulation of the tumor antigen, neu, on metastatic tumor cells. Studies involving AIT without Dec treatment in vivo or Dec alone without immune response in vitro confirmed that total neu antigen loss and down-regulation were mediated by anti-tumor immune responses, whereas Dec alone only had the capacity to induce down-regulation of neu antigen. We have reported previously that treatment of neu-negative tumor cells (antigen-negative variant) with Dec resulted in the induction of neu expression at mRNA but not at protein levels [11]. Likewise, Dec treatment did not overcome IFN-γ-induced down-regulation of neu protein in MMC. Here, we also showed that a high dose of Dec induced down-regulation of the neu protein in vivo. These data suggest that high-dose Dec might have different effects on neu expression during mRNA transcription and protein translation. It was reported that a low dose of Dec could have only a hypomethylating effect when incorporated into DNA, whereas a high-dose Dec could also incorporate into RNA and show different effects [16]. Although Dec or Aza render tumor cells highly immunogenic by inducing the expression of highly immunogenic CTAs, this effect is usually transient in that tumor cells lose CTAs after the cessation of Aza therapy [6].

We hypothesized that targeting dormant but not highly proliferating tumor cells might overcome tumor immunoediting and escape. Therefore, we conducted studies to determine the sensitivity of dormant tumor cells to immunoediting and escape. We demonstrated that ADR treatment induced 2 types of tumor dormancy: 1) an indolent type of dormancy, characterized by the positive/low expression of Ki67; this type of dormancy is maintained through balanced proliferation and death, as these cells keep producing dead cells, whereas the total number of viable cells remains unchanged; and 2) a quiescent type of tumor dormancy that is characterized by lack of Ki67 expression ($Ki67^-$); this type of dormancy is maintained through total cessation of proliferation. We demonstrated that proliferating tumor cells, either untreated tumor cells ($Ki67^+$) or indolent tumor cells ($Ki67^+$/low), were susceptible to immunoediting and escape during cell division, but quiescent tumor cells ($Ki67^-$) failed to undergo immunoediting; in fact, they failed to up-regulate PD-L1 in the presence of IFN-γ stimulation. These results suggest that quiescent dormant cells could be the best target for immunotherapy.

It was reported that innate IFN-γ is essential for up-regulation of PD-L1 expression [17]. Intriguingly, an adaptive immune response following AIT resulted in a >3-fold inhibition in the induction of PD-L1 expression on tumor cells compared with the control group, although it was still significantly higher than MMC tumor cells in vitro before challenge. Similar results were obtained when tumor cells were cultured with IFN-γ or lymphocytes of tumor-bearing mice. These data suggest that a T cell-independent inflammatory response, which involves IFN-γ, has a greater impact than T cells on up-regulation of PD-L1. In addition, AIT was associated with a significant inhibition in the induction of tumor PD-L1 compared with no AIT control group, suggesting that the PD-1/PD-L1 axis is more active in tumor-bearing animals in the absence of AIT. To test this, we cocultured reprogrammed tumor-reactive T cells with MMC in the presence or absence of PD-1 blocking antibody; the anti-tumor function of reprogrammed T cells was not affected by PD-1 blockade in vitro (data not shown).

This is important, as reprogrammed T cells and NKT cells that were used for AIT expressed PD-1, and PD-1 expression was sustained after AIT. However, reprogrammed T cells also produce perforin and granzyme B [5], allowing them to induce apoptosis in tumor cells before they begin to up-regulate PD-L1 mediated by IFN-γ. These data suggest that AIT results in a

significant inhibition of tumor PD-L1 induction to the levels that might not be suppressive. Blockade of PD-1/PD-L1 by anti-PD-1 antibody did not have any effects on anti-tumor immune responses against MMC in vitro (data not shown). Therefore, prolonged survival in the AIT group could be associated with lower expression of PD-L1 in MMC when tumor cells were present in the circulation compared with the control group when tumors were established in the lungs. Given the high levels of PD-L1 expression on established tumors, administration of AIT as a single agent in a therapeutic setting is likely to fail in curing cancer, as it was evident in our therapeutic protocol (Fig. 1). Administration of AIT in a preventive setting, when tumor cells were in the circulation but before they establish lung metastasis, was highly effective, although animals succumbed to metastatic tumor, as their tumors begin to undergo neu antigen loss. Our data suggest that tumors use numerous mechanisms to change during cell division and escape from immunotherapy. These mechanisms were shown to overcome tumor immune surveillance and reduce the efficacy of immunotherapy [18, 19]. However, and intriguingly, dormant tumor cells that were established by chemotherapy or RT and that became chemo resistant or RT resistant remained sensitive to tumor-reactive immune cells. Our findings are consistent with the reports on the efficacy of AIT in patients with metastatic melanoma using TILs grown in IL-2. AIT, using IL-2-expanded TIL, resulted in tumor regression in 49% of patients [20]. When AIT was combined with total body irradiation, which was implemented to induce lymphopenia, objective responses increased to 72%. Among treated groups, 20% had complete tumor regression and >10 y relapse-free survival [21]. Thus far, of the 34 complete responders in the National Cancer Institute trials, 1 has recurred [22].

The results of this study suggest that administration of immunotherapy in a setting of advanced stage prophylaxis, i.e., after the completion of conventional cancer therapies, when tumor dormancy is established but before distant recurrence of the disease, could effectively target dormant tumor cells and prevent advanced stage disease. On the other hand, the application of immunotherapy to highly proliferative tumors renders the tumors prone to immunoediting and subsequent immunologic escape during cell division [23]. The challenge is to develop combinatorial therapies, i.e., AIT, following the administration of epigenetic modulators or small molecules that could induce cell-cycle arrest and establish a quiescent type of tumor dormancy so as to render dormant tumor cells resistant to immunoediting and escape from immunotherapy. The extension of knowledge gained from our preclinical studies to the clinical setting remains to be determined in patients with early-stage breast cancer or patients with minimal residual disease.

AUTHORSHIP

K.K.P. and M.H.M. contributed to the study's conception, design, experimental and analytical performance, and writing of the manuscript. R.C.K., L.G., M.O.I., and W.W. contributed to the study's experimental and analytical performance and writing of the manuscript. A.A.T., X.-Y.W., and H.D.B. contributed to the study's conception, analytical performance, and writing of the manuscript,

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DISCLOSURES

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Department of Defense. The authors declare no conflicts of interest.

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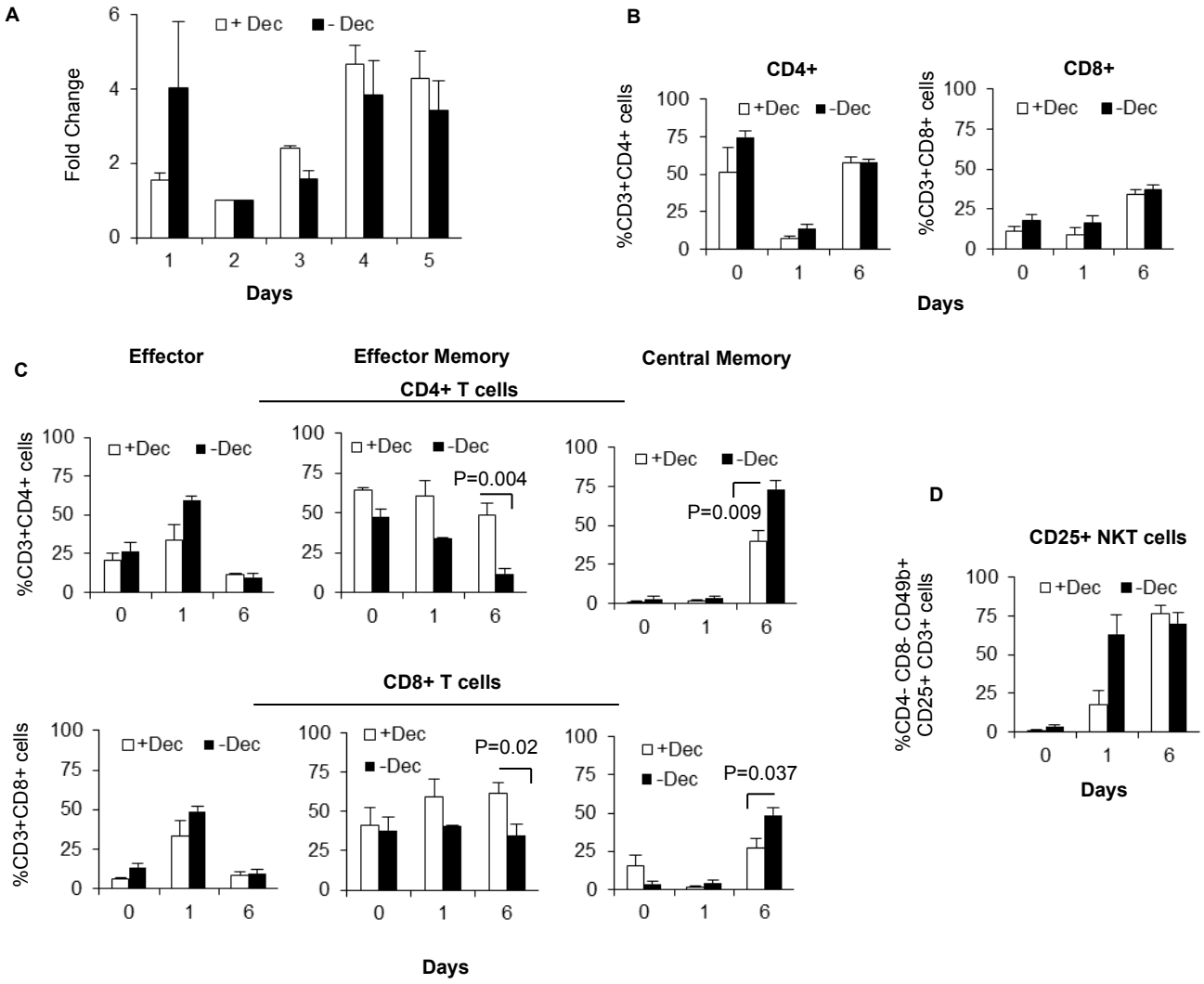
KEY WORDS:
adoptive immunotherapy · dormancy · escape · relapse
breast cancer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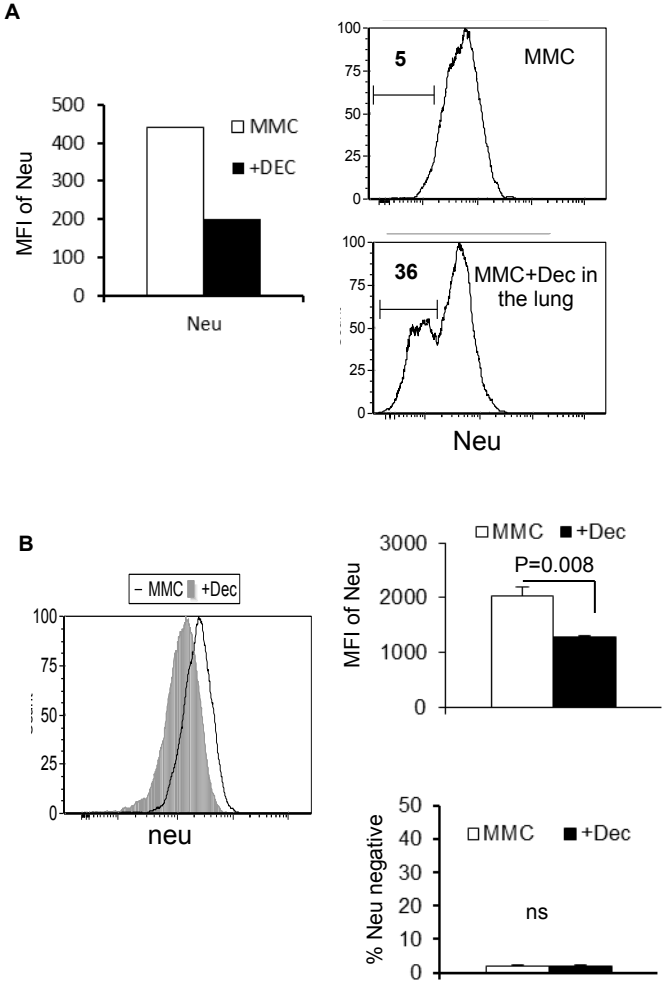
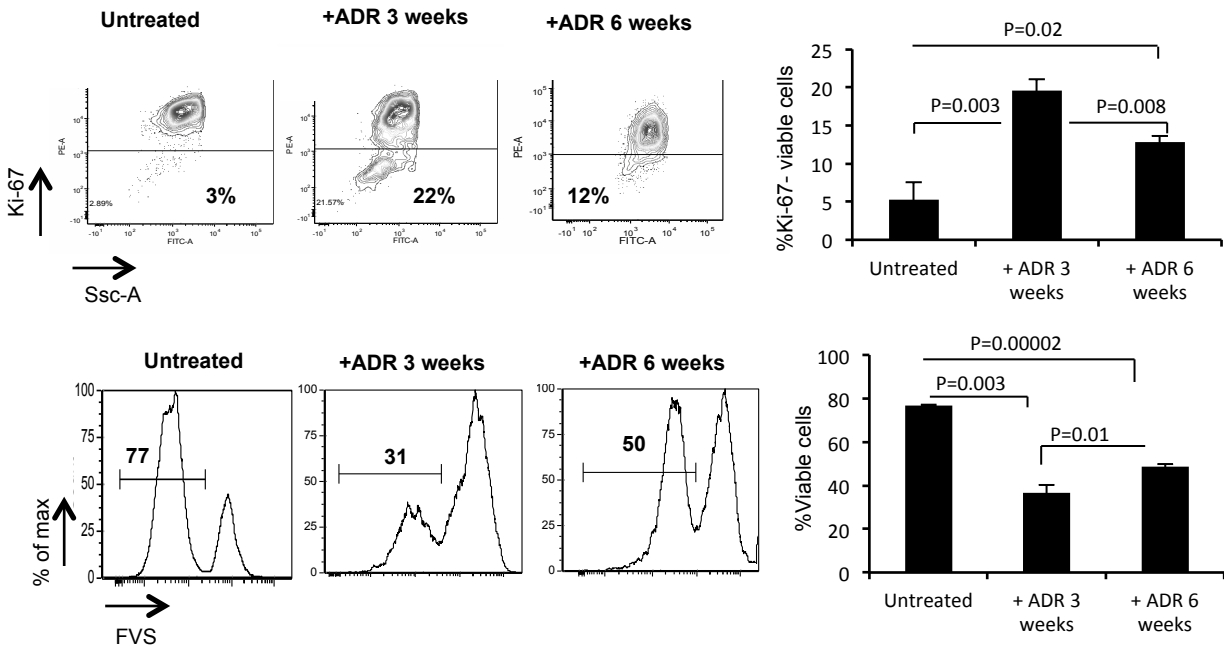
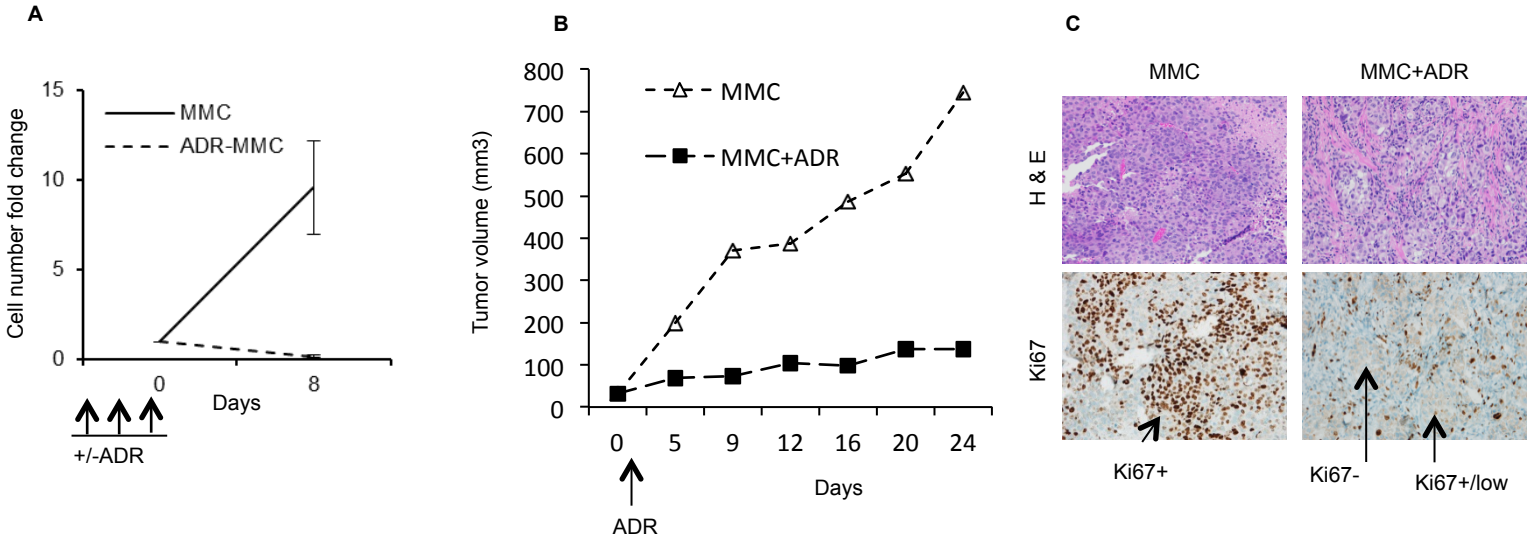
Supplemental Figure 1. Expansion and phenotypic reprogramming of tumor-reactive splenocytes is similar between animals bearing primary cancer with and without Decitabine preconditioning. FVBN202 mice were challenged with 3 x 10^6 MMC cells intradermally. A portion of the mice went on to receive five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



Supplemental Figure 2. AIT promotes immunoediting of lung metastatic lesions. A) FVBN202 mice were challenged i.v. with MMC cells (1 x 10^6); 3 days later they were injected with Dec (2.5mg/kg) once daily for 5 days or remained untreated (MMC). After the mice became moribund, metastases were excised from the lung and established *in vitro*. Neu median fluorescence intensity (MFI) and percentage of neu negative cells were quantified using flow cytometry 10-14 days after the animals had been euthanized. B) MMC cells were treated with Dec (Dec; 3uM) or remained untreated (MMC), *in vitro*. After 10 days of culture, neu expression was quantified using flow cytometry. C) MMC cells remained untreated or were treated with one dose of IFN-γ (50ng/ml) or Dec+IFN-γ, *in vitro*. Expression of neu was determined 7 days after the final treatment. Data represent mean MFI ± SEM of triplicates.



Supplemental Figure 3. Dormant tumor cells recover proliferative capacity as a function of time. MMC tumor cells were treated with 3 daily doses of ADR (1uM for 2 hs), then remained untreated for 3 weeks and 6 weeks, *in vitro*. At weeks 3 and 6 post-treatment, Ki-67 expression and viability were quantified within the population of adherent tumor cells. Data represent 3 independent experiments and mean ± SEM.



Supplemental Figure 4. ADR treatment induces tumor dormancy. A) MMC cells (n=3) treated with ADR (1uM, 2 hs) for 3 consecutive days and remained in culture for 8 days total, in order to establish tumor cell dormancy, *in vitro*. B) FVBN202 mice were challenged with MMC ($3x10^6$) in the mammary gland region; after tumors reached 30-50 mm^3 animals were treated with ADR (MMC+ADR; 20 mg/kg; i.v.), or remained untreated (MMC). Tumor growth was monitored for four weeks, C) Animals were sacrificed and tumor specimens were collected and subjected to H & E staining as well as IHC for Ki67. Arrows show Ki-67+ proliferating tumor cells (dark brown), Ki-67- quiescent tumor cells (blue color like background) and Ki-67+/low indolent tumor cells (weak brown). Figures show a 200X magnification.