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13. SUPPLEMENTARY NOTES

14. ABSTRACT Our finding can be broken down into three related components. I. <u>Autophagy Dependent Tumor Dormancy.</u> These studies indicate that use of pharmacologic agents such as chloroquine (now being tested in multiple clinical trials) could prolong chemotherapy induced cell arrest, which could contribute to a more durable disease remission. This type of transient blockade of autophagy does not affect breast tumor cell sensitivity to immunotherapy. In contrast, breast tumors that are intrinsically autophagy deficient may be more likely to contribute to recurrent disease; furthermore, a sustained knockdown of autophagy also renders the tumor cells resistant to immunotherapy. Consequently, transient inhibition of autophagy would be the preferred approach to enhance breast cancer sensitivity to chemotherapy.

<u>II. Senescence Dependent Breast Cancer Dormancy and Sensitivity to Senolytic Agents.</u> These studies demonstrate that cells in a state of senescence are capable of proliferative recovery, which may reflect a form of tumor dormancy. Furthermore, senolytic agents have the potential to attenuate or suppress proliferative recovery, suggesting their utilization to prevent breast cancer recurrence. Ongoing studies are designed to determine the impact of the senolytic agents combined with chemotherapeutics on the recognition and elimination of breast tumor cells by the immune system.

<u>III, Epigenetic regulation of tumor cell sensitivity to chemotherapy</u>. These studies indicate that pharmacologic inhibition of NURF (e.g. AU1) may be effective in sensitizing breast tumor cells to chemotherapy, through both direct (cell autonomous) and indirect (cell non-autonomous, immune mediated) pathways.

15. SUBJECT TERMS
Autophagy; tumor relapse; chemotherapy; immunotherapy; NURF; senescence; senolytics

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

2. **KEYWORDS:** tumor dormancy; disease relapse, chemotherapy, autophagy, senescence, immunotherapy.

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

The major goals of this project were to understand the role of autophagy in chemotherapy induced tumor dormancy and disease recurrence within the framework of the immune system.

What was accomplished under these goals?

This work developed two related models of breast cancer dormancy and disease recurrence in response to chemotherapy (primarily doxorubicin). The studies relating principally to autophagy have been published (Aqbi et al, 2018; Section I). Given that autophagy and senescence generally occur in tandem in response to chemotherapy and radiation, we also established the involvement of senescence in a model of tumor dormancy and disease recurrence (Section II), interrogating the capacity of the immune system to recognize and eliminate (or suppress the growth of) senescent tumor cells (manuscript in preparation). Finally, we identified a novel therapeutic strategy for enhancing the response to chemotherapy in breast cancer by suppressing the expression of the epigenetic regulator, NURF (Section III), which clearly promotes activation of the Natural Killer Cell component of the immune system, in large part by recognizing cells induced into autophagy (manuscript in preparation). Consequently, taken together, this grant has supported studies that have established the roles of autophagy and senescence in influencing the immune system response to breast cancer chemotherapy, which was the major overarching goal of this work.

I. Autophagy Dependent Tumor Dormancy

As shown in the attached manuscript (Aqbi et al, 2018), we investigated how autophagy is involved in the influencing the recurrence of mouse mammary carcinoma cells after cancer chemotherapy (doxorubicin) and in the response to immunotherapy. These experiments were performed utilizing both pharmacological and genetic approaches to inhibit autophagy. Unexpectedly, while a transient inhibition of autophagy prolonged chemotherapy-induced growth arrest (which we propose as a model of tumor dormancy), stable and prolonged genetic silencing of autophagy enhanced escape from growth arrest and appeared to accelerate tumor recurrence. Furthermore, while a transient blockade to autophagy did not affect sensitivity of the MMC cells to immunotherapy, prolonged autophagy inhibition rendered the MMC cells resistant to immunotherapy. These studies highlight the challenges involved in efforts to sensitize breast cancer (as well as other malignancies) to chemotherapy and radiation through autophagy inhibition. However, it does appear that use of pharmacologic agents such as chloroquine (now being tested in multiple clinical trials) could prolong chemotherapy induced cell arrest, which could contribute to a more durable disease remission. On the other hand, breast tumors that are intrinsically autophagy deficient may be more likely to contribute to recurrent disease.

II. Senescence Dependent Breast Cancer Dormancy and Sensitivity to Senolytic Agents

We developed an experimental protocol for the isolation of exclusively senescent tumor cells by flow cytometry, complemented by the monitoring of tumor cell outgrowth both *in vitro* and *in vivo* to generate evidence that (breast) tumor cells that recover from chemotherapy are derived from the senescent tumor population. Furthermore, we demonstrate that senolytic agent efficiency is exquisitely specific for this senescent cell population *in vitro* and propose that breast tumor cells entering a senescence state induced by chemotherapy or radiation *can be selectively eliminated by treatment with senolytic agents*. Additional studies are in progress (supported by alternative funds) to determine how the use of senolytics influences the capacity of the immune

system to recognize and eliminate any residual breast tumor cells. We propose that this approach could prevent (or significantly delay) proliferative recovery of breast cancer, and, by extension, disease recurrence.

Both preclinical studies and clinical trials are in progress combining ABT-263 and similar agents simultaneously with chemotherapy and for extended periods after chemotherapy (17-21 day cycles – (63)); however, our studies suggest that the *timing* of drug administration is critical; specifically, we propose that the maximum effect of senolytic treatment is on senescent cells, and therefore, *a sequential treatment regime to eliminate residual tumor cells after chemotherapy would maximize their benefit* ***(Fig. 1)***. Importantly, by using the senolytic agent after the primary chemotherapy (allowing the chemotherapy to partially clear from the patient), *toxicity to the patients would be highly reduced*, enabling higher doses or longer single senolytic treatment. Furthermore, by using a sequential treatment scheme, *the primary form(s) of chemotherapy could potentially be used at the lower, less toxic doses* that primarily induce senescence (64, 65). Finally, we show evidence of the importance of the immune system in restraining cell regrowth after promotion of chemotherapy-induced senescence, and hypothesize that *the effectiveness of the senolytic treatment post chemotherapy will be even more susceptible to the immune system*. Recognizing that it is unlikely that breast cancer can be completely eradicated, therapeutic modalities designed to prevent disease recurrence such as the one we describe, could transform this disease into a chronic, manageable one.

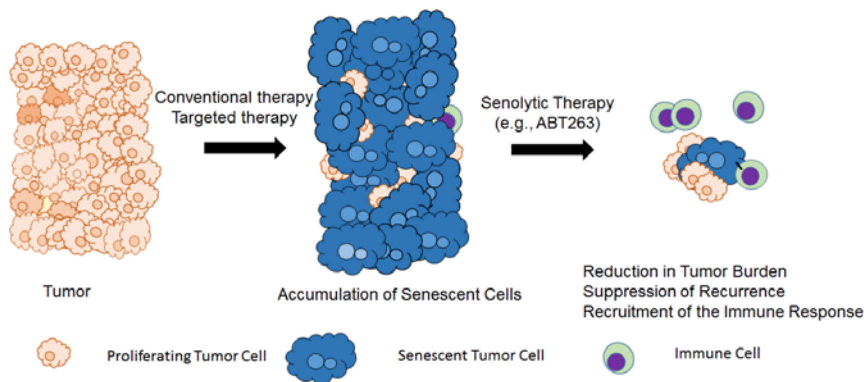

Figure 1. The use of Senolytic Therapy to Eliminate Senescent Tumor Cells. This model illustrates the proposed therapeutic approach. Tumors exposed to conventional/targeted therapies accumulate senescent tumor cells that persist after the end of treatment, which can contribute to cancer relapse. Sequential administration of ABT-263, or other senolytic agents, results in the selective elimination of residual senescent tumor cells and thereby tumor regression, delay in cancer recurrence and recruitment of an antitumor immune

Evidence for Proliferative Recovery from Chemotherapy-Induced Senescence - While data from multiple laboratories, using different experimental systems, collectively suggest that that tumor cells that have entered into an apparent state of senescence can give rise to proliferating progeny (13-20), we have attempted to more rigorously test this premise both *in vitro* and *in vivo.* To achieve this goal, we recently optimized an *in vitro* senescence recovery assay using human colon cancer cells with radiation treatments (14), and breast tumor cells treated with doxorubicin (as depicted in **Figure 3A**). Briefly, cancer cells are treated with chemotherapy (or radiation) for several days to promote senescence. Live cells are labeled with C_{12}-FDG (a substrate which becomes fluorescent when metabolized by SA-β-gal) (66), harvested and enriched by FACS based on the C_{12}-FDG intensity and enlarged cellular size. Purified cells are seeded *in vitro* to assess proliferative recovery and markers of senescence (e.g. expression of cytokines associated with the senescence secretory phenotype), to confirm the fidelity of our enrichment procedure (5, 14, 27).

We subjected both 4T1 and E0771 murine breast tumor cell lines to this protocol, and demonstrate that both models can recover from senescence **(Fig. 2A, D)**. The 4T1 triple negative breast cancer model (TNBC) (67) and the E0771 estrogen receptor positive (ER+) model (68) represent two major classes of breast cancer, and belong to different genetic backgrounds, BALB/c and C57BL/6, respectively. The *mass population* of 4T1 and E0771 breast tumor cells exposed to doxorubicin show the characteristic morphology and SA-β-gal staining indicative of senescence **(Fig. 2B, 2C, 2E, 2F)**. Proliferative recovery from senescence (reduced SA-β-gal staining) in the 4T1 and E0771 murine breast tumor lines is shown in **Fig. 2A, 2D and 2D inset, 2C and 2F)**. The number of SA-β-gal positive cells (cells with blue staining) at time points post doxorubicin exposure decreases over time **(Fig. 2C, 2F)**. It should be noted that the E0771 cells (69) are significantly more sensitive to doxorubicin than the 4T1 cells (69), entering senescence by exposure to 50 nM drug compared to the 1 µM needed to treat the 4T1 model (comparing **Fig. 2A** and **2D**). These studies show that the 4T1 and E0771 breast cancer models from two distinct strain backgrounds undergo senescence followed by proliferative recovery upon exposure to doxorubicin.

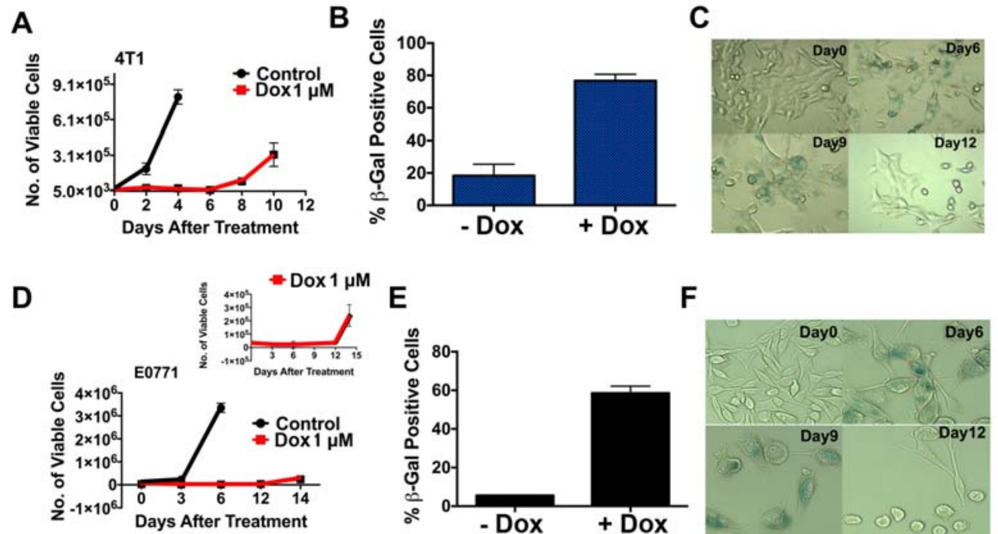

Figure 2. Recovery from Doxorubicin-induced senescence in mouse breast tumor cells. A. Recovery of 4T1 cells (mass culture) after induction of senescence by doxorubicin (Dox, 1µM) **B.** Quantitative analysis of senescence in 4T1 cells based on β-gal staining by Flow Cytometry 72 hrs after treatment removal **C.** SA-**β**-Gal staining of senescent 4T1 cells fixed at indicated time points **D.** Recovery of E0771 murine breast cancer cells in culture after induction of senescence by doxorubicin (50 nM for 24 hours) **E.** Quantification of senescence by Flow cytometry in E0771 48 hrs after drug removal **F.** Time course of SA-**β**-Gal staining of senescent E0771 cells fixed at indicated time points.

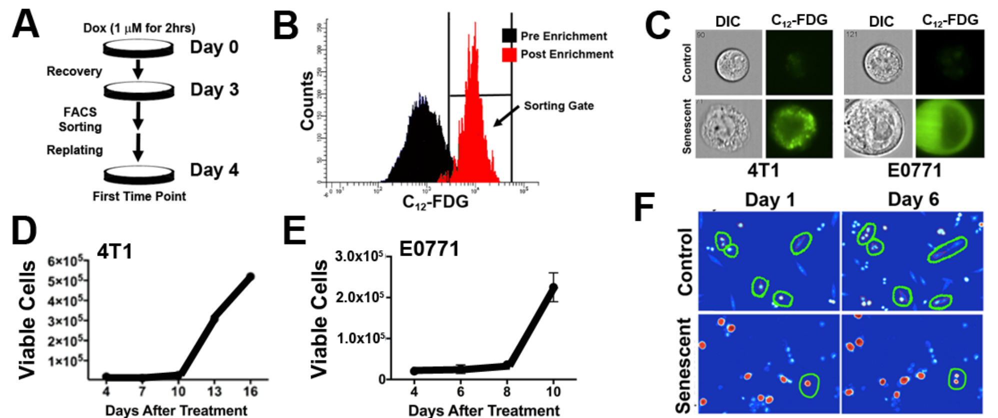

Figure 3. Proliferative recovery of sorted senescent 4T1 and E0771 cells. A. Diagram of the protocol used to sort senescent cells following exposure to chemotherapy. Cells are plated into 20 cm plates and allowed to adhere. Next day cells are treated with doxorubicin and allowed to recover for 72 hours after drug is removed. At this point cells are sorted; 25%-30% od senescent cells are isolated based on large size and highest intensity of C12 FDG staining **B.** A sample of sorted cells was analyzed by flow cytometry to verify purity of the SA-β-Gal positive population (97% of sample population is high SA-β-Gal positive) **C.** Sample of sorted cells was analyzed by ImageStream, both DIC and Fluorescent images of 4T1 and E0771 were taken to compare size and C12 FDG staining **D.** 4T1 cells sorted by the protocol described in figure 3A were plated in 6 well plates and cell number was determined at indicated time points **E.** Sorted E0771 were plated, monitored and cell number was determined at indicated time points **F.** Representative HSLCI tracks of control, untreated (upper panel) and high C_{12}-FDG-positive (lower panel) cells. Sorted E0771 day 5 after treatment and untreated controls were plated and observed by HSLCI. Senescent cells were considerably larger and more massive (1346 ± 92 pg) than untreated cells (814 ± 23pg). Controls were shown to undergo multiple mitotic events in the first day after plating. Mitotic events were also observed (but delayed) from senescent E0771 cells (lower panel).

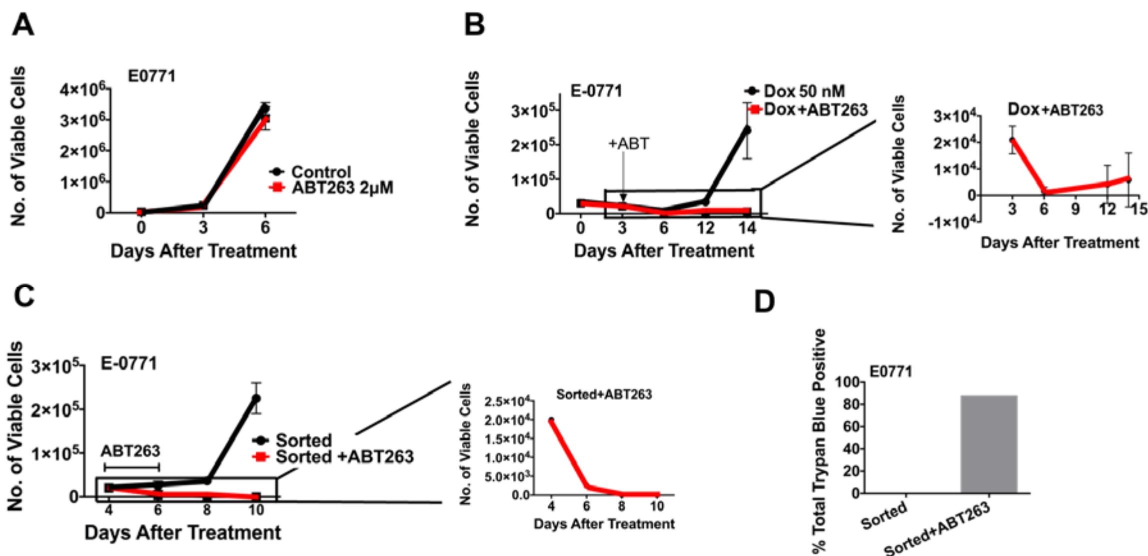

Figure 4. Selective elimination of senescent E0771 by ABT263 A. Control E0771 cells were plated in 6 well plates. Cells were either untreated and allowed to proliferate or treated with ABT 263 (2 uM) for 48 hours. Cell number was determined at indicated time points; no difference in proliferation rate was evident between treated and untreated cells **B.** E0771 cells were exposed 50nM doxorubicin (Dox) and were allowed to enter senescence for 2 days after drug removal; cells were then exposed to 2 uM of ABT 263 for 48 hours. Doxorubicin treated cells undergo growth arrest followed by recovery; cells treated with ABT-263 post senescence induction showed significant reduction in numbers (expanded scale in right panel) **C.** Sorted senescent cells were treated with 2 uM ABT 263 for 48 hours. Sorted senescent cells remained in growth arrest for several days and resumed proliferation. A pronounced decrease in cell number was observed in ABT 263 treated senescent cells, which did not resume proliferation (expanded scale in right panel) **D.** Cell death in ABT 263 treated senescent cells was determined by Trypan Blue staining (~87% next day after ABT removal).

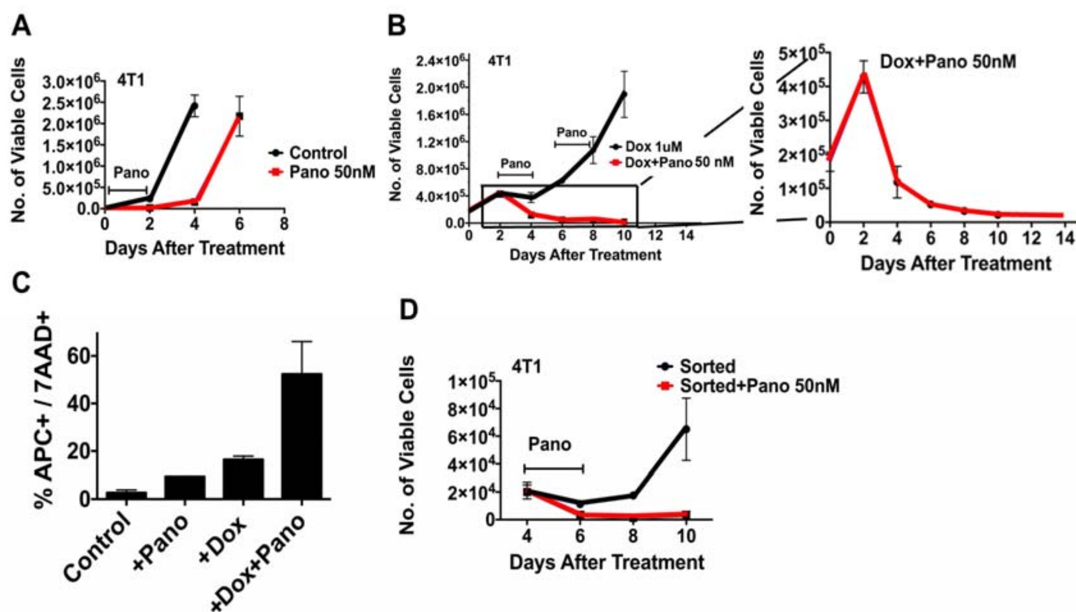

Figure 5. Selective elimination of senescent 4T1 by Panobinostat. A. Control and panobinostat (Pano) treated (50nM for 48 hours) 4T1 cells were observed and number of cells were recorded during time course progression. Panobinostat induced brief growth arrest but did not cause significant cell death and number of cells treated with Pano reached that of controls with only a slight delay **B.** 4T1 cells treated with doxorubicin (Dox -1 uM for 2 hours) were exposed to 50 nM pano for 48 hours 2 days after chemotherapy removal and then again 6 days after. Doxorubicin treated cells went through a phase of growth arrest and then robust proliferation (lower panel). Significant cell death was observed when senescent cells received Pano (upper panel). It is clear that panobinostat did not prolong doxorubicin induced growth arrest but caused great loss of cells. **C.** Quantitative analysis of apoptosis by flow cytometry using APC/7AAD dye **D.** Sorted senescent 4T1 were exposed to 50 nM Pano for 48 hr. The number of cells was determined at indicated time points, again showing elimination of senescent cells by Pano.

Next, we applied our sorting protocol to doxorubicin-exposed 4T1 and E0771 cells to enrich for senescent cells **(Fig. 3A)**. We validate our enrichment protocol by specifically showing histograms of cells pre- and post-enrichment based on their C_{12}-FDG staining, indicating that the enriched population is highly positive for SA-β-gal **(Fig. 3B)**. Senescence marker SA-β-gal staining by C_{12}-FDG was monitored using AmnisImageStreamX Mark II™ system **(Fig. 3C)**. Senescent cells sorting and replating demonstrates that recovery of the most highly SA-β-gal positive 4T1 and E0771 cells occurs after doxorubicin-induced senescence **(Fig. 3D, 3E)**. These studies confirm that ***senescence arrest can be succeeded by proliferative recovery*** in the 4T1 and E0771 models of breast cancer. Outgrowth from senescent cells was also demonstrable by real time biomass measurements using HSLCI (22); **Figure 3F** shows the replication of individual control cells and the delayed replication of senescence-sorted cells.

***Elimination of senescent breast tumor cells by treatment with ABT-263 (a cell autonomous strategy).* -** Our findings that a significant fraction of the tumor cell population enters into a state of senescence after chemotherapy suggested that senolytic agent such as the BCL-2/BCL-XL inhibitor ABT-263 could prove successful in the elimination of these senescent tumor cells (70). Preliminary experiments using ABT-263 (42, 71) show that ABT-263 promotes killing of the E0771 breast tumor cell line (and perhaps more importantly, prevents proliferative recovery) when the cells have entered into a state of senescence from doxorubicin exposure (**Fig. 4)**. As expected, ABT-263 does not interrupt the growth of *the untreated non-senescent tumor cells* **(Fig. 4A)** whereas ABT-263 dramatically impacts the recovery of both unsorted **(Fig. 4B and inset)** and sorted E0771 cells *induced into senescence* with doxorubicin **(Fig. 4C and inset)**. Note that close to 99% of the senescent E0771 cells are removed from the culture after a single ABT-263 treatment **(Fig. 4C)**. Trypan blue staining shows that the primary response of **doxorubicin + ABT-263** -treated E0771 cells is cell death **(Fig. 4D)**.

In practice, a single dose of a senolytic agent is unlikely to entirely eliminate a senescent population, which will be critical if senolytic agents are to be effective in mitigating against senescent-dependent tumor recovery, where theoretically a single senescent tumor cell has the potential to regrow and kill the patient. The effectiveness of multiple senolytic treatments on etoposide treated and sorted senescent cells is shown in **Fig. 5**. Using the 4T1 breast cancer model, a single exposure to panobinostat of non-senescent cells results in a modest growth delay from which the cells recover rapidly (**Fig. 5A**). Conversely, exposure of doxorubicin- treated 4T1 cells to panobinostat reduces the residual surviving population by ~ 96%. A second exposure of the residual population, as shown in **Panel 5B** - inset, results in the survival of a mere 1% of the cell population (i.e. a 99% reduction – as shown in expanded view). In **Panel 5C** we observe increased apoptosis of 4T1 cells treated with a combination of panobinostat and doxorubicin. In **Panel 5D** we observe similar results in the sorted purified doxorubicin-induced senescent 4T1 cells after treatment with panobinostat.

Immune Response to breast tumor cells induced into senescence by chemotherapy (cell non-autonomous effects). - Studies of proliferative recovery from therapy-induced senescence in tissue culture models are cell-autonomous. However, therapeutic responses are heavily regulated by the tumor microenvironment, in particular by the immune system (cell non-autonomous effects) (72, 73). To specifically study the immune response to breast tumor cells induced into senescence by doxorubicin, we have utilized comparative studies between immune competent and compromised mouse models as shown in **Fig. 6.** Towards this end, we implanted 1.0×10^6 4T1 or 5×10^5 EO771 untreated or FACS-purified senescent (**see Fig. 3A** - (14)) cells into the 4th mammary fat pad of immune competent syngeneic BALB/cJ (4T1) or C57/BL6 (EO771) mice or immune compromised NOD scid gamma (NSG) mice (74). Monitoring tumor growth over time, we observed a significant delay in recovery of tumor growth from senescent 4T1 cells (time to palpable growth between 25-31 days) compared to untreated controls (7 days) (**Fig. 6A**). In contrast to the findings in immune competent BALB/c mice, studies in immune compromised NSG mice (75) demonstrated a significant acceleration in tumor recovery for the senescent cells (between 15-19 days compared to the 25-31 days required for recovery in the immune competent mice) (**Fig. 6A**). With the lower inoculum of FACS-purified senescent EO771 cells, the difference was even more striking. While tumors became palpable in about 2 weeks after injection in the immune-deficient mice (**Fig. 6B**), only 2 out of 5 wild type mice had palpable tumors almost 3 months after injection (not visible in scale of **Fig. 6B**). In further support of the influence of the immune system in controlling senescent tumors, we observed a significant increase in the number of CD8+ cells detected by immunofluorescence in tumors derived from senescent 4T1 cells compared to parental untreated 4T1 cells (**Fig. 6C**). CD8+ T cells are major effector cells of the antitumor immune response, known to be stimulated by chemotherapy, with the ability to induce complete remission (76). In addition to a T cell response, we observe significant enhancements of NK cell killing to doxorubicin treated 4T1 cells in vitro compared to controls (**Fig. 6D**). Like T cells, NK cells are major suppressors of tumor growth, which specifically recognize and kill senescent tumor cells (57, 77).

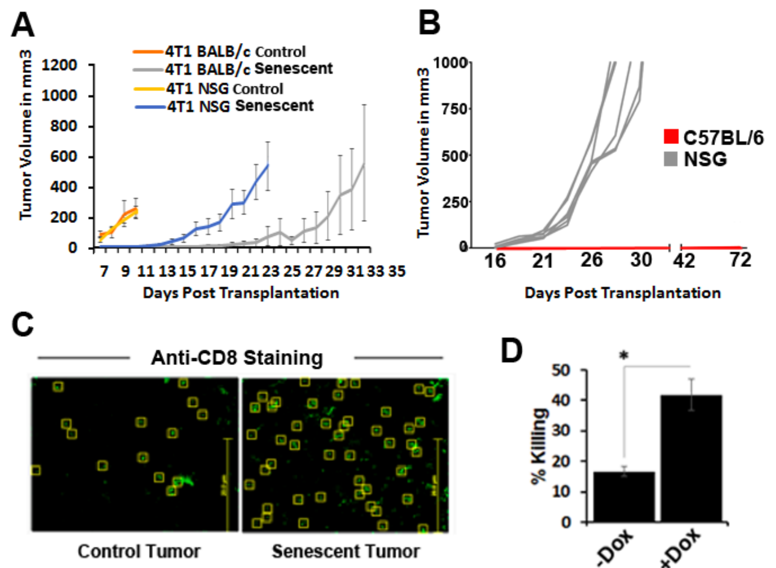

Figure 6. In-vivo recovery of senescent breast tumor cells and immune response. A. In-vivo study of BALB/c and NSG mice that received injections of sorted high β-Gal positive 4T1 shows senescent cells recovering and forming tumors with significant delay compared to non-treated controls. **B**. In-vivo study of C57BL/6 and NSG mice that received injections of sorted high β-Gal positive E0771 shows senescent cells recovering and forming tumors in NSG mice with markedly delayed recovery in immune competent mice. **C.** Immunofluorescence showing anti-CD8 staining in control and senescent tumors. CD8+ cells were identified using an Image J Macro. **D**. NK cells were obtained from the spleen of BALB/C mouse and cultured with addition of IL-2 cytokine for 24 hours in order to be activated. Non-treated and Dox treated cells were plated in 96 well plate

In summary, we present evidence from studies in cell culture as well as tumor bearing animals that breast tumor cells can recover from a chemotherapy induced senescence-like state *in vitro* and *in vivo* and, furthermore, that the immune system plays a central role in recognizing and eliminating senescent tumor cells and suppressing their recovery. We propose that this could represent a model of breast cancer dormancy and disease recurrence. We also present data indicating that senolytic agents can eliminate residual surviving breast tumor cells *in vitro*, which ultimately could provide a therapeutic strategy for suppressing breast cancer that recurrence after extended periods of tumor dormancy.

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III. Enhancement of tumor cell sensitivity to chemotherapy through both cell autonomous and cell non-autonomous (immune system) regulation by the suppression of an epigenetic regulator.

Due to the fact that breast tumor cells tend not to be highly immunogenic, studies were performed to determine whether silencing of an epigenetic inhibitor and chromatin remodeling complex, specifically, the nucleosome remodeling factor NURF, could enhance the response of breast tumor cells to therapy through both cell autonomous (tumor cell direct) and cell non-autonomous (immune system based) mechanisms. In mammals, NURF has 3 subunits: the essential and largest subunit bromodomain PHD-finger transcription factor (BPTF). Knockout of BPTF achieves specific depletion of NURF because BPTF is exclusive to NURF, and it is required for its function (1,2).

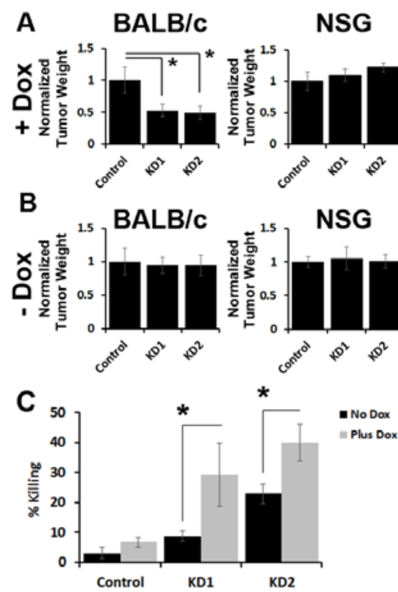

Fig. 1 ADR Enhances Antitumor Immunity to NURF Depleted Tumors. (A-B) WT (expressing a control shRNA) or BPTF KD (KD1 and KD2) 4T1 cells were inoculated into BALB/cJ or NSG mice and treated with **(A)** ADR 5mg/kg once a week for 3 weeks or a **(B)** PBS control. Tumors weighed 3 weeks (-Dox) or 4 weeks (+Dox) post inoculation. Normalized tumor weights are shown. Two-tailed t-test p values *=<0.05. SD error bars, N>6 tumors/group. **(C)** WT or BPTF KD 4T1 cells were treated with 50 nM ADR for 48 hours prior to coculture with mouse NK cells at an effector:target ratio of 10:1. Target cell killing was measured 24 hours after initial coculture by LDH release. Two-tailed t-test p values *=<0.05. SD error bars, N=3 replicates.

To test whether NURF depletion would improve the immunogenicity of breast tumors responding to ADR (doxorubicin or Adriamycin), we implanted BALB/cJ or NSG mice with wild type (WT; expressing a control shRNA) or BPTF KD (knockdown) 4T1 cells and treated the mice with ADR (5 mg/kg once a week for 3 weeks). This dose of ADR promotes tumor immunogenicity, but does not itself suppress tumor growth (3). Comparing the right and left panels of **Fig. 1A,** we observed an enhanced antitumor effect of ADR to BPTF KD cells in immune competent BALB/c mice, but not immune compromised NSG mice, supporting our hypothesis that NURF inhibition would enhance chemotherapy induced tumor immunogenicity. However, unlike the case in the 67NR and 66cl4 cells (not shown), BPTF KD alone does not result in suppression of 4T1 breast tumor growth (left panel of **Fig. 1B**). This is because to observe the enhanced antitumor immune response to NURF depleted 4T1 tumors, immune suppressive cells must be depleted (4). This, in fact, provides an advantage to experiments performed with 4T1 cells in tumor-bearing animals in that immune stimulation by ADR can be distinguished from the immune stimulatory effects of BPTF KD. In further support of the premise that NURF depletion enhances the therapy induced antitumor immune response, we do observe an enhanced NK cell cytolytic activity to ADR treated BPTF KD 4T1 cells compared to WT (control shRNA expressing) when co-cultured *in vitro* (**Fig. 1C**).

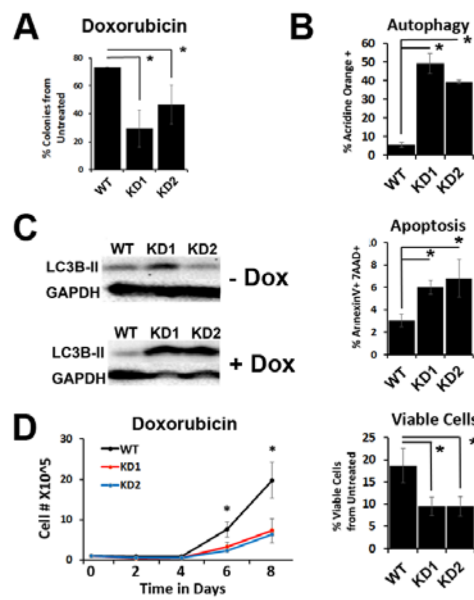

Fig. 2. BPTF Depleted 4T1 Cells Enter Autophagy After ADR Treatment. (A) Measurement of WT (control shRNA expressing) and BPTF KD (KD1 and KD2) 4T1 cell sensitization to 50 nM 24 hr ADR exposure by clonogenic survival. Percent of surviving cells are shown compared to untreated cells. **(B)** WT or BPTF KD 4T1 cells were treated with 50 nM ADR for 48 hours and flow cytometry was used to measure autophagy by acridine orange staining, apoptosis by annexin V staining and viable cells by trypan blue exclusion. **(C)** Western blotting for LC3BII from WT or BPTF KD 4T1 cells treated with 50 nM ADR for 48 hours. GAPDH was used as a loading control. **(D)** WT and BPTF KD 4T1 cells were treated with 50 nM ADR (Day 0) for 2 days, followed by recovery in growth media for 6 days. Viable cells were counted every 2 days. All panels are average, or representative, of 3 biological replicates.

We hypothesized that the observed enhancement of antitumor responses in BPTF KD cells exposed to ADR could be derived from two sources: (i) non-cell autonomous effects, specifically the generation of damage-associated molecular pattern (DAMP) molecules and immunogenic cell death (ICD) that enhance tumor cell immunogenicity and (ii) cell autonomous effects, specifically an increase in DNA damage via facilitation of the accessibility of DNA to the enzyme, Topoisomerase II. We further propose that the immunogenicity could be a consequence of chemotherapy-induced autophagy in the BPTF KD cells.

To test these hypotheses, we first asked whether BPTF KD could enhance ADR sensitivity in cell culture (i.e., a cell autonomous effect). These studies demonstrated reduced clonogenic survival of BPTF KD 4T1 cells compared to WT cells (expressing a control shRNA) when exposed to ADR (**Fig. 2A**). The reduced growth of ADR-treated BPTF KD 4T1 cells (between 40-60% reduction) coincides with a large increase (8-10 fold) in autophagy (initial studies based on flow analysis of acridine orange staining) and a smaller increase (~2 fold) in apoptosis (annexin V + 7AAD staining)(**Fig. 2B**). Consistent with these observations, BPTF KD results in increased LC3BII conversion indicative of the promotion of autophagy (**Figure 2C**), and delayed proliferative recovery after exposure to ADR (**Fig. 2D**).

ADR is known to induce DNA damage by poisoning Topoisomerase II (5). Using γH2AX levels as our endpoint, we observed increased DNA damage in ADR treated BPTF KD 4T1 cells relative to WT cells (expressing a control shRNA) by flow cytometry and Western blotting, suggesting enhanced Topo II poisoning (**Fig. 3A,B**). As a further control, we observed similar levels of autophagy and γH2AX in BPTF KD cells treated with γ-radiation, a DNA damaging agent independent of Topo II (**Fig. 3C,D**) (6) to which ***cells are not sensitized by NURF depletion*** (**see Fig. 5).** The enhancement of ADR sensitivity to BPTF KD cells is not simply a consequence of greater cellular drug accumulation, because we observe equivalent ADR accumulation into BPTF KD cells by flow cytometry (**Fig. 3E**).

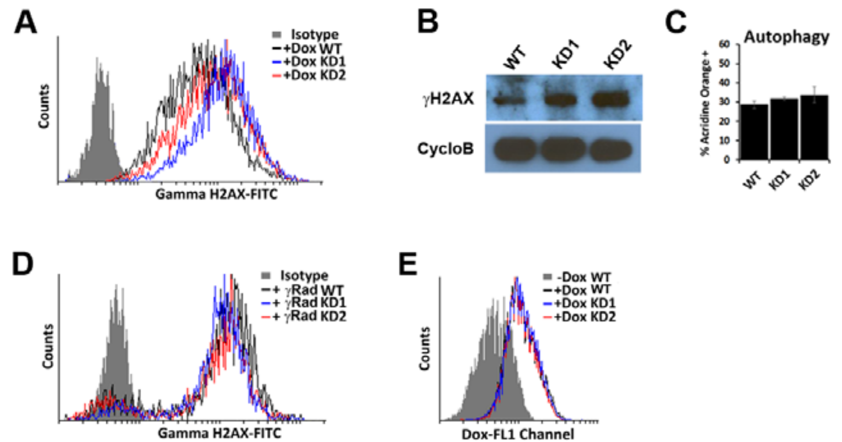

Fig. 3 BPTF Depleted 4T1 Cells Incur ADR Induced DNA Damage. (A) γH2AX was measured by flow cytometry after WT (control shRNA expressing) and BPTF KD (KD1 and KD2) 4T1 cells were treated with 50 nM ADR for 48 hours. **(B)** Western blotting of γH2AX from WT and BPTF KD cells treated with 50 nM ADR for 48 hours. Cyclophillin B was used as a loading control. **(C)** Autophagy was measured by acridine orange staining 48 hours after WT and BPTF KD 4T1 cells were treated with 5 Gy γ-radiation exposure. **(D)** γH2AX was measured by flow cytometry 30 minutes after WT and BPTF KD 4T1 cells were treated with 6Gy γ-radiation. **(E)** Flow cytometry measurement of 1 ug/ml ADR accumulation after 24 hours exposure to WT and BPTF KD 4T1 cells. Each panel representative of 3 biological replicates.

Figure 4 indicates that the reduced growth and enhanced autophagy observed in ADR treated BPTF KD 4T1 cells is also observed when WT 4T1 cells are pretreated with the NURF pharmacologic inhibitor AU1 (**Fig. 4A,B**) (7). This is a critical finding if we ultimately aspire to using pharmacological NURF inhibitors to enhance chemo-sensitivity in TNBC (triple negative breast cancer). Consistent with what we showed previously for BPTF KD 4T1 cells (4), AU1 treatment activates antigen processing and presentation genes *Lmp2*, *Lmp7*, *Tap1*, and *Tap2* that have key roles in regulating tumor cell antigenicity (**Fig. 4C**). These data suggest that NURF can be pharmacologically targeted for therapeutic benefit.

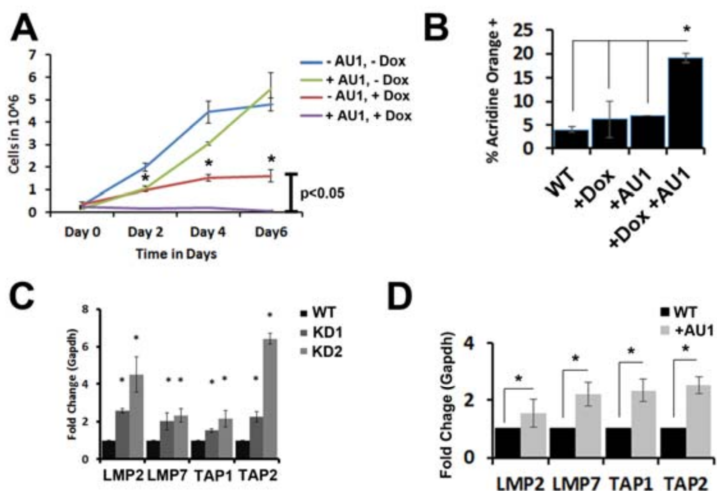

Fig. 4. BPTF Small Molecule Inhibitor AU1 Enhances Sensitivity to ADR and Improves Antigenicity. (A) WT 4T1 cells were pretreated with 10 μM AU1 for 2 days (Day -2) then treated with 50 nM ADR for 2 days, followed by recovery in growth media for 4 days. Viable cells were counted every 2 days. **(B)** WT 4T1 cells were pretreated with 10 μM AU1 for 2 days then treated with 50 nM ADR for 48 hours and flow cytometry was used to measure autophagy by acridine orange staining. **(C)** Lmp2, Lmp7, Tap1, and Tap2 expression measured from total RNA by qRT-PCR using Gapdh normalization from WT or BPTF KD 4T1 cells. **(D)** 4T1 cells were treated with 10 μM of the BPTF inhibitor AU1 for 12 days. Lmp2, Lmp7, Tap1, and Tap2 expression measured from total RNA by qRT-PCR using Gapdh normalization. Two-tailed t-test p values *=<0.05. SD error bars N=3 biological replicates.

We also asked whether enhanced sensitization to other chemotherapeutic agents (and ionizing radiation) might be observed with BPTF KD cells, potentially broadening the implications of our finding. To this end, we examined sensitivity to γ-radiation by clonogenic survival assay and to etoposide, and camptothecin using the standard MTT assay. We found that BPTF KD enhanced 4T1 breast tumor cell sensitivity to the Topo II poison etoposide, but not to the Topo I poison camptothecin or γ-radiation (**Fig. 5**). The selective enhancement of ADR and etoposide activity to NURF-depleted cells over camptothecin or radiation (**Fig. 5**) suggests functional connections between NURF and Topo II. ***However, it is critical to recognize that the lack of sensitization in cell culture does not exclude the possibility that an enhanced immune response might become evident in vivo through cell non-autonomous actions (e.g., DAMP secretion (8) mediated by autophagy).***

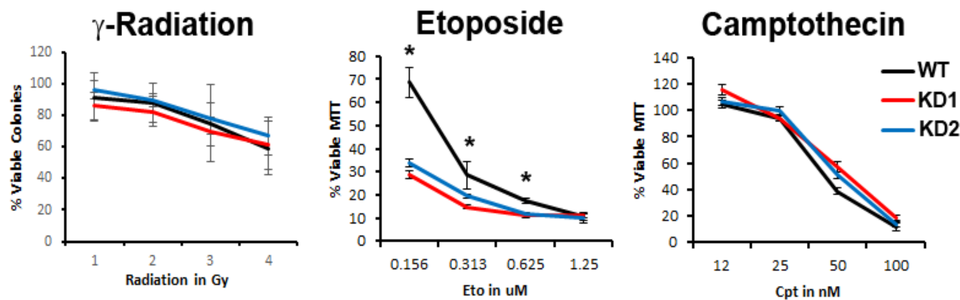

Fig. 5. BPTF Depletion Sensitizes Breast Cancer Cells to Other Chemotherapies. The effects of γ-radiation, etoposide, or camptothecin exposure on WT (control shRNA expressing) or BPTF KD (KD1 or KD2) 4T1 cell growth was determined by MTT assay (chemotherapies) or clonogenic survival (radiation). N=3 Replicates, *TTest p=0.05.

A seminal study by Michaud et al. has argued for the premise that the effectiveness of chemotherapy in cancer requires immune activation through the autophagy-mediated secretion of DAMPs (9). **Figure 2C** presents data indicating that the extent of autophagy is increased by 8- to 10-fold in ADR-treated BPTF KD cells compared to WT cells. These findings provide an opportunity to investigate the contribution of autophagy and autophagy-mediated secretion of DAMPS to chemo-sensitivity of breast tumor cells (8, 10). These studies are also relevant to the current clinical efforts to sensitize malignancies to chemotherapy and radiation via autophagy inhibition (11). If, in fact, autophagy inhibition i*nterferes* with tumor cell immunogenicity rather than the sensitization that is often observed in cell culture and in xenograft models (where, of course, the immune system involvement is thought to be minimal), then the strategy of autophagy inhibition could prove to be highly counterproductive. These findings will also allow us to define the cell non-autonomous impact of NURF depletion on autophagy and immune activation.

We have demonstrated that ADR selectively enhances autophagy in NURF-depleted cells and that NURF-depleted cells appear to have increased immunogenicity both in cell culture and in tumor-bearing animals. We further show enhanced sensitivity to ADR in cell culture as a cell autonomous effect that may be a consequence of increased DNA damage. A central question that remains is the nature of the chemotherapy-induced autophagy that improves breast tumor cell immunogenicity. The most common form of autophagy is cytoprotective in that its inhibition confers chemosensitivity or radiosensitivity (12). The cytotoxic form either directly promotes cell death (although this point is admittedly controversial) or is permissive for apoptosis (13). The cytostatic form mediates growth arrest (and cells recover proliferative function when it is inhibited). Finally, and most relevant to the current work, the nonprotective form is identified by the absence of any change in chemosensitivity or radiosensitivity when it is inhibited (12). In preliminary studies (**Fig. 6**) we demonstrate that autophagy inhibition by chloroquine fails to alter sensitivity to ADR in the 4T1 breast tumor cell line. This finding is consistent with previous work by our laboratory and others demonstrating that both ionizing radiation and cisplatin promote nonprotective autophagy in 4T1 breast tumor cells (14-16). Furthermore, the cell culture studies by Michaud et al (9) that reported the immune stimulatory effects of autophagy in fact identified nonprotective autophagy in that ATG5 knockdown in cell culture did not sensitize the tumor cells to mitoxantrone or oxaliplatin (two DAMP and ICD inducing chemotherapies).

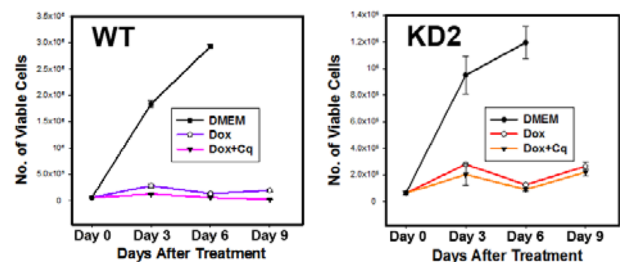

Fig. 6. ADR Induced Autophagy to BPTF Depleted Cells is Nonprotective. WT (control shRNA expressing) and BPTF KD (KD2) 4T1 cells were treated with 50 nM ADR (Dox) with or without 5mM Chloroquine (Cq) (Day 0) for 2 days, followed by recovery in growth media for 9 days. Viable cells were counted every 2 days. Panels are average, or representative, of 3 biological replicates.

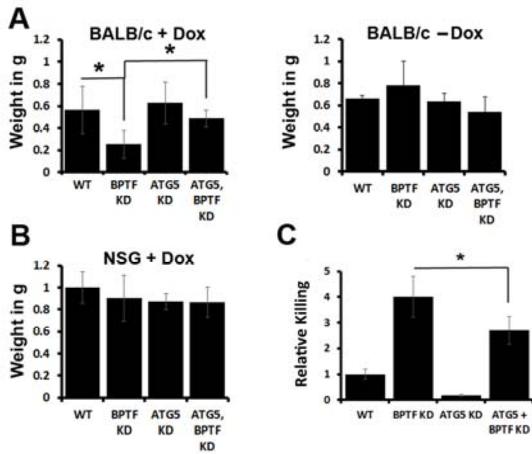

Fig 7) Autophagy is Required for the Antitumor Immune Response to NURF Depleted Tumors. (A-B) WT (expressing a control shRNA), BPTF KD, ATG5 KD or BPTF + ATG5 KD 4T1 cells were inoculated into BALB/cJ mice and treated with **(A)** doxorubicin 5mg/kg once a week for 2 weeks or a **(B)** PBS control. **(C)** Same experiment as above except NSG mice were used and treated with 5mg/kg once a week for 2 weeks. Tumors from A, B were weighed 4 weeks post inoculation. Two-tailed t-test p values *=<0.05. SD error bars, N>4 tumors/group. **(C)** WT (expressing a control shRNA), BPTF KD, ATG5 KD or BPTF + ATG5 KD 4T1 cells 4T1 cells were treated with 50 nM doxorubicin for 48 hours prior to coculture with mouse NK cells at an effector:target ratio of 10:1. Target cell killing was measured 24 hours after initial coculture by LDH release and expressed relative to WT. Two-tailed t-test p values *=<0.05. SD error bars, Representative of N=3 replicates.

Our findings from this award currently appear to be consistent with the hypothesis proposed by Michaud et al. (9) as the enhanced antitumor effects of doxorubicin to BPTF KD 4T1 tumors in immune competent BALB/c mice are eliminated when autophagy is inhibited by ATG5 shRNA silencing **(Fig. 7A)**. Furthermore, enhanced antitumor activity of doxorubicin to BPTF KD tumors *was not observed* in NSG mice demonstrating that the immune component is critical for the effect **(Fig. 7B)**. The importance of autophagy for the antitumor immune response to doxorubicin treated tumors correlates with its importance for NK cell mediated killing in vitro **(Fig 7C)**.

Summary and Overview Tumor cells reduce their immunogenicity in part through epigenetic mechanisms. Targeting these mechanisms (a cell non-autonomous approach) would represent a therapeutically viable strategy to reestablish antitumor immunity because, unlike genetic changes, which are permanent, epigenetic changes are reversible and are catalyzed by “druggable” enzymes. We have recently discovered that the epigenetic regulator and chromatin remodeling complex (CRC), the nucleosome remodeling factor (NURF), suppresses tumor cell antigenicity. NURF depletion from mouse models of TNBC enhances tumor cell immunogenicity, which we have shown can lead to complete regression in our metastatic breast cancer tumor models.

In efforts towards developing a novel and more effective therapy for TNBC, we combined NURF depletion with ADR, a standard of care cytotoxic chemotherapy that targets topoisomerase II (Topo II). NURF-depleted cells demonstrate enhanced sensitivity to ADR *in vitro* including increased DNA damage, reduced cell division, and suppressed proliferative recovery (cell autonomous effects). We further observe increased tumor cell immunogenicity, as demonstrated by increased sensitivity to the cytotoxic activities of natural killer (NK) cells *in vitro* and an enhanced antitumor response *in vivo*. Given the ongoing development of pharmacological agents that can target NURF, we believe that these findings can ultimately be translated to the clinic to improve the response of (triple negative) breast cancer to therapy.

Additional data and ongoing studies: In recent work, we observed that NURF depletion and the use of the pharmacologic NURF inhibitor, AU1,also sensitized the breast tumor cells to paclitaxel. We consider this to be a highly important observation demonstrating that sensitization is not limited to doxorubicin but also occurs for another drug commonly used in the treatment of breast cancer. Studies are also planned and in progress to determine whether the same outcomes will be evident using human breast tumor models, specifically the triple negative MDA-MB231 model of breast cancer.

Relevance and Implications Despite advances in cancer therapeutics over recent decades, developing effective strategies for the treatment of triple negative breast cancer (TNBC) continues to represent a significant challenge. Cytotoxic chemotherapies that often include ADR remain the standard of care in breast cancer; while generally successful, patients frequently relapse with therapy resistant disease. Immunotherapy has emerged as a treatment modality with great promise, but has seen limited success in breast cancer therapy due, in large part, to the low immunogenicity of breast cancer cells. In view of the fact that ADR has been shown to improve tumor immunogenicity by promoting the secretion of damage-associated molecular pattern (DAMP) molecules and immunogenic cell death (ICD), we have developed a unique strategy to enhance sensitivity to chemotherapy by both cell autonomous and cell non-autonomous mechanisms.

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

The project provided for the training and professional development of Dr. Theresa Thekkudan and Ms. Liliya Tyutyunyk-Massey, a PhD candidate in Dr. Gewirtz's laboratory.

How were the results disseminated to communities of interest?

The results have been disseminated in poster presentations at local (Massey Cancer Center, Virginia Commonwealth University) and national (American Association for Cancer Research) scientific meetings and in a recently published paper (Aqbi et al). Also, two manuscripts are currently in preparation that will describe the studies presented in Sections II and III.

What do you plan to do during the next reporting period to accomplish the goals? Although the current grant funding cycle has been completed, we are nevertheless working to complete the current studies for publication. As indicated above, the experiments with senolytics will be extended to human breast tumor cell lines. The studies relating to NURF inhibition are being extended to paclitaxel.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Autophagy Dependent Tumor Dormancy. These studies indicate that use of pharmacologic agents such as chloroquine (now being tested in multiple clinical trials) could prolong chemotherapy induced cell arrest, which could contribute to a more durable disease remission. This type of transient blockade of autophagy does not affect breast tumor cell sensitivity to immunotherapy. In contrast, breast tumors that are intrinsically autophagy deficient may be more likely to contribute to recurrent disease; furthermore, a sustained knockdown of autophagy also renders the tumor cells resistant to immunotherapy. Consequently, transient inhibition of autophagy would be the preferred approach to enhance breast cancer sensitivity to chemotherapy.

Senescence Dependent Breast Cancer Dormancy and Sensitivity to Senolytic Agents. These studies demonstrate that cells in a state of senescence are capable of proliferative recovery, which may reflect a form of tumor dormancy. Further, senolytic agents have the potential to attenuate or suppress proliferative recovery, suggesting their utilization to prevent breast cancer recurrence. Ongoing studies are designed to determine the impact of the senolytic agents combined with chemotherapeutics on the recognition and elimination of breast tumor cells by the immune system.

Epigenetic regulation of tumor cell sensitivity to chemotherapy. These studies indicate that pharmacologic inhibition of NURF (e.g. AU1) may be effective in sensitizing breast tumor cells to chemotherapy, through both direct (cell autonomous) and indirect (cell non-autonomous, immune mediated) pathways.

What was the impact on other disciplines?

The results presented in Section I have broad impacts to the field of **epigenetics** in that they characterize the nuclear functions for the NURF complex in chemosensitization, which has not been recognized previously. This work also has impact to the field of **immunotherapy,** strongly suggesting that inhibiting NURF is a novel means to enhance the immunogenicity of breast tumor cells, which has implications for the use of immunogenic modulators.

What was the impact on technology transfer?

None to report

What was the impact on society beyond science and technology?

None to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

We used both 4T1 and E0771 breast tumor cells in the studies relating to senescence as we determined that these cell lines are sensitive to senolytics. Our studies relating to epigenetic modulation were directed towards Natural Killer cells and T cells, which generate interferon gamma to more broadly understand the nature of the immune response to therapy. We determined that focusing our studies exclusively on IFNγ was too limiting, as many cancer cells can become IFNγ resistant as a means to adaption.

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

None to report.

Changes that had a significant impact on expenditures

None to report.

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

None to report.

6. PRODUCTS:

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

#3314 Synergistic effects of chemotherapy-induced autophagy and epigenetic remodeling. Liliya Tyutyunyk, Joseph Landry, Tareq Saleh, David Gewirtz. Virginia Commonwealth University, Richmond, VA.
Cytotoxic chemotherapy has been shown to enhance tumor cell immunogenicity by promoting the secretion of damage-associated molecular pattern (DAMP) molecules which, in turn, stimulates the immune response. These effects can be further stimulated by combining chemotherapy with an immunotherapy designed to enhance effector cell (CD8 T cell or NK cell) anti-tumor activity. Our studies demonstrate that knockdown of the epigenetic regulator NURF increases DNA damage (gamma H2AX staining) and autophagy (acridine orange staining) in breast tumor cells exposed to ADR and enhances growth inhibition as well as suppressing the capacity of the cells to recover and proliferate. Similar increases in autophagy were observed using a small molecule inhibitor of NURF,suggesting thatNURFcan be targeted therapeutically. Sensitization was not observed with ionizing radiation or cisplatin. Studies are in progress to assess the nature of autophagy in the NURF KD and NURF WT cells, distinguishing between the cytoprotective, cytotoxic, cytostatic and nonprotective forms. How NURF regulates ADR induced DNA damage is being investigated by mapping sites of damage genome wide. It is anticipated that enhanced cell autonomous tumor cell sensitivity in concert with improvements in tumor cell antigenicity (cell non-autonomous sensitization) achieved by NURFdepletion could improve anti-tumor immunogenicity, achieve tumor regression, reduce metastasis, and possibly achieve long term remission in breast cancer.
Proceedings of the American Association for Cancer Research Volume 58 April 2017

#5459 Autophagy and senescence as possible mechanisms leading to proliferative recovery and escape from treatment-induced tumor dormancy. Liliya Tyutyunyk, Theresa Thekkudan, Tareq Saleh, David A. Gewirtz. Virginia Commonwealth University, Richmond, VA.
Despite the ability of chemotherapy to eliminate the majority of tumor cells, some are able to escape cell death and proliferate. In this study we evaluated mechanisms of autophagy and senescence, that in theory allow cells to

escape apoptosis and might be responsible for cancer recurrence. Autophagy and senescence, either alone or in concert, may result in temporary growth arrest followed by proliferative recovery. With regard to autophagy, its function may be either cytoprotective, where autophagy inhibition results in sensitization to therapy or nonprotective, where autophagy inhibition does not alter sensitivity to chemotherapy and/or ionizing radiation. To determine the mode of autophagy in murine metastatic carcinoma 4T1 cell lines, autophagy was inhibited pharmacologically using chloroquine, and by genetic silencing of the Atg5 autophagy associated protein. Cells were treated with 1uM Adriamycin, stained with Acridine Orange to assess autophagy by fluorescent microscopy (autophagosomes appear orange-red) and autophagy quantified by Flow Cytometry. Induction and blockade of autophagy by chloroquine was confirmed by Western blotting for the appearance of LC3B and degradation of p62. Senescence was monitored by beta-galactosidase staining and quantified by Flow Cytometry based on C12FDG staining; senescence was confirmed based on the induction of p21 and p16. Adriamycin (ADR) exposure resulted in breast tumor cell death as well as prolonged growth arrest; some of the arrested cells eventually recovered and formed colonies. After exposure to ADR, 4T-1 cells were sorted based on intensity of beta-galactosidase staining and increased size (senescent cells experience changes in size and morphology). Beta--galactosidase positive cells were plated and monitored over time during which some of the cells recovered the capacity to proliferate. In separate studies with mouse mammary carcinoma cells, radiation also promoted cell death and prolonged growth arrest from which some cells were able to recover proliferative capacity. Our data suggests that although chemotherapy and radiation induce prolonged growth arrest and senescence, these features are not permanent and cells are able to escape and re-emerge from the senescent state to generate proliferating daughter cells. As pharmacologic inhibition of autophagy did not result in increased sensitivity to ADR in 4T1 cells (or to radiation in the mouse mammary tumor cells) or interfere with proliferative recovery, we postulate that inhibition of senescence associated pathways may block proliferative recovery and/or promote tumor cell killing in response to chemotherapy and/or radiation. Studies in progress are focused on modulation of c-myc and miR34 levels and the IL1/6/8 signaling axis as potential strategies for interference with senescence and suppression of residual (dormant) tumor growth and cancer recurrence.
Proceedings of the American Association for Cancer Research Volume 58 April 2017

1330 / 8 - Role of epigenetic remodeling in sensitizing triple-negative breast cancer cells to treatment through enhanced chemotherapy-induced autophagy. Liliya Tyutyunyk, Joseph Landry, David A. Gewirtz, Nga Dao.

Dysregulation of the epigenome is implicated in initiation and progression of variety of cancers and their acquired resistance to chemotherapy. As such targeting epigenetic regulators has the potential to modulate cancer cell biology and their sensitivity to chemotherapy and/or radiation. Our studies demonstrates that silencing of the epigenetic regulator Nucleosome Remodeling Factor (NURF) sensitizes breast tumor cells to chemotherapy and enhances the anti-tumor immune response. A screen of a variety of chemotherapeutic agents shows that NURF KD cells are selectively sensitized to Topo II inhibitors, which includes doxorubicin. NURF KD increases DNA damage (gamma H2AX staining) and autophagy (acridine orange staining) in breast tumor cells exposed to doxorubicin (Dox) and enhances growth inhibition as well as suppressing the ability of the cells to recover proliferative capacity. Increased autophagy as well as breast tumor sensitization to doxorubicin were observed using a small molecule inhibitor of NURF, suggesting that NURF can also be targeted pharmacologically. Our studies suggest that enhanced autophagy may be a primary contributor to chemo sensitivity in NURF KD cells. Studies are currently underway to confirm the role of autophagy in chemosensitization in- vitro and enhanced immune response in-vivo using autophagy deficient cells (through ATG silencing). Doxorubicin produces profound DNA damage and promotes immunogenic cell death enhanced by NURF KD. Increased cell autonomous antitumor effects by doxorubicin in concert with increased cell non-autonomous antigenicity could help to achieve tumor regression, reduce metastasis, and possibly promote long term remission in breast cancer.
Proceedings of the American Association for Cancer Research Volume 59 April 2018.

460 / 20 - Reversibility of chemotherapy-induced senescence in breast tumor cells in culture and in vivo. Liliya Tyutyunyk, Joseph Landry, Tareq Saleh, David Gewirtz.

Despite the ability of chemotherapy (and/or radiation) to eliminate the majority of tumor cells, some residual surviving cells may be able to escape cell death by entering into a prolonged growth arrest from which they can ultimately re-emerge and proliferative . In this study, we evaluated two mechanisms, autophagy and senescence, that in theory could allow tumor cells to escape apoptotic cell death, and which therefore might be responsible for cancer recurrence. 4T1 murine breast cancer cells exposed to the chemotherapeutic agent, Adriamycin

(doxorubicin), underwent both autophagy and senescence. Senescence induced by anticancer drugs or radiation is a state of prolonged growth arrest that is thought to be irreversible and is considered an alternative (but nevertheless desirable) response to apoptosis. Our studies, however, suggest that Therapy Induced Senescence (TIS) is reversible and serves as a mechanism of evading cell death. When subjected to acute treatment with Adriamycin, some of the 4T1 cells underwent apoptosis; however, a large population entered into a state of prolonged growth arrest that lasted for several days, after which time the cells recovered proliferative capacity and formed colonies. At the time points when cells were arrested, prominent Senescence Associated β-Galactosidase staining and polyploidy were detected, markers indicative of senescence. Inhibition of autophagy did not prevent the tumor cells from undergoing senescence and later escaping growth arrest. To further establish the reversibility of senescence, senescent cells that were isolated by Flow Cytometry (based on their enlarged morphology and detection of a fluorescent β-Gal substrate ($C_{12}FDG$)), plated and monitored over time; these cells were also shown to recover, as was the case from with the non-sorted senescent cells. Sorted and isolated senescent cells implanted into either NSG (immunodeficient) or syngeneic (immunocompetent) mice also recovered and developed into tumors; recovery was accelerated in the NSG mice, suggesting that the immune system may (at least transiently) recognize and eliminate the tumor cells either at senescence or during recovery from senescence. Senescent cells are characterized by a specific (though often heterogeneous) secretory profile (Senescence Associated Secretory Phenotype or SASP), some components of which have been shown to promote tumorigenesis and may assist in neoplastic escape after therapy. Furthermore, senescence and escape from senescence may represent a form of tumor dormancy and disease recurrence, respectively. Therefore, it is important to treat Therapy Induced Senescence as a deleterious outcome of treatment and identify strategies that might prevent senescent cells from neoplastic escape and induce cell death to completely eliminate residual tumors**. Proceedings of the American Association for Cancer Research Volume 59 April 2018.**

Papers

Gewirtz, D, Alotaibi M, Yakovlev V, Povirk LF. Tumor cell recovery from senescence induced by radiation + PARP inhibition. Radiat Res. 2016 Oct;186(4):327-332. *These studies have suggested that the promotion of senescence by therapy does not result in a sustained growth arrest. These findings are highly relevant to the issue of tumor dormancy and cancer recurrence. The methods developed in these studies, particularly the sorting of senescent tumor cells are currently being extended to* ***breast tumor*** *cells (findings to appear in the final progress report). [Submitted with previous report]*

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Toma W, Kyte SL, Bagdas D, Alkhlaif Y, Alsharari SD, Lichtman AH, Chen ZJ, Del Fabbro E, Bigbee JW, Gewirtz DA, Damaj MI. Effects of paclitaxel on the development of neuropathy and affective behaviors in the mouse. Neuropharmacology. 2017 Feb 22;117:305-315. *This paper develops a novel strategy to mitigate the peripheral neuropathy induced by drugs utilized to treat* ***breast****, ovarian and lung cancer. [Submitted with previous report]*

Galuzzi et al. Molecular definitions of autophagy and related processes. EMBO J. EMBO J. 2017 Jul 3;36(13):1811-1836. Epub 2017 Jun 8. Review. *This review attempts to integrate the literature relating to autophagy. [Submitted with previous report]*

Gewirtz DA, Tyutyunyk-Massey L, Landry JW. The potentially conflicting cell autonomous and cell non-autonomous functions of autophagy in mediating tumor response to cancer therapy. Biochem Pharmacol. 2018 Jul;153:46-50. Epub 2018 Feb 3. Review. (attached). *This review addresses the problems associated with utilizing autophagy inhibition as a strategy for chemosensitization and radiosensitization.*

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. 2018 Apr 24;9(31):22113-22122 (attached)

Grant Submissions/DOD Breast Cancer Program (2018)

Cell autonomous and cell non-autonomous sensitization of senescent breast cancer to senolytic agents

Targeting epigenetics with small molecules to achieve chemosensitization and enhance breast cancer immunogenicity

Grant Submissions NIH (2018)

1R01CA239733-01 Targeting therapy induced senescence to prevent disease recurrence in breast cancer

1R01CA239671-01 Sensitization of breast cancer to immune stimulating chemotherapies through suppression of a novel epigenetic regulator

Presentations

- **Website(s) or other Internet site(s)**
- **Technologies or techniques**
- **Inventions, patent applications, and/or licenses**
- **Other Products**

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Dr. David Gewirtz (3 years) - supported by Virginia Commonwealth University and this contract.
Ms. Liliya Tyutyunyk-Massey (18 months)- supported by Virginia Commonwealth University and this contract.
Mr. Tareq Saleh (12 months) – supported by the Kingdom of Jordan
Dr. Joseph Landry (18 months)- supported by Virginia Commonwealth University

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

National Institutes of Health Grant 1R01CA206028-01. Grant Period 3/01/2016 – 02/28/21. Co PI with Dr. Imad Damaj. 10% Effort. $280,293/annum. Mitigation of chemotherapy induced peripheral neuropathy. This project involves the utilization of nicotine and silent agonists of nicotine to suppress and/or prevent peripheral neuropathy induced by agents such as the taxanes and platinum based compounds utilized in the treatment of ***breast cancer***, ovarian cancer and lung cancer.

National Institutes of Health Grant CA219637-01A1. Grant Period 7/25/2018 – 06/30/22 Co PI with Dr. Imad Damaj. Peroxisome proliferator-activated receptor alpha agonists as potential treatment for chemotherapy-induced peripheral neuropathy. Direct Costs ~ $1,250,000. This project involves the utilization of PPAR alpha agonists (e.g. fenofibrate) to suppress and/or prevent peripheral neuropathy induced by agents such as the taxanes and platinum based compounds utilized in the treatment of ***breast cancer***, ovarian cancer and lung cancer.

What other organizations were involved as partners?

None

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS:

9. APPENDICES:

Gewirtz et al paper attached

Aqbi et al paper attached.

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Review

The potentially conflicting cell autonomous and cell non-autonomous functions of autophagy in mediating tumor response to cancer therapy

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A R T I C L E I N F O

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A B S T R A C T

Autophagy, a virtually uniform response to external stress such as that induced by chemotherapy and radiation, is generally considered to be cytoprotective in function, providing a foundation for multiple clinical trials designed to enhance therapeutic response via autophagy inhibition. However, this cell autonomous response can also be cytotoxic or nonprotective, with the consequence that autophagy inhibition would be counterproductive or ineffective, respectively. The non-cell autonomous function of autophagy remains quite controversial, with evidence both for and against autophagy-mediated activation of the immune system. If autophagy inhibition antagonizes the immune response, this would likely interfere with the potential sensitization resulting from suppression of the cell autonomous protective function. An additional complication, which has rarely been considered, is the nature of the contribution of therapy-induced autophagy in the tumor microenvironment, particularly the tumor stroma. Taken together, it is likely that the outcome of the current clinical trials, whether positive or negative, will be difficult to interpret given the complexity of the role of autophagy relating to the tumor cell (cell autonomous), the immune system (cell non-autonomous) and the tumor microenvironment.

1. Introduction

It has long been recognized that macroautophagy, herein referred to as autophagy, is a primary response of cells to conditions of stress [1,2]. Autophagy or "self-eating" reflects efforts of the cell to survive via the degradation of cellular organelles to generate energy and metabolic precursors [1,2]. One of the most provocative findings of the last decade relating to the tumor response to therapy has been the observation that most chemotherapeutic drugs as well as ionizing radiation promote autophagy in tumor cells [3–5].

Autophagy can have both cell autonomous and cell non-autonomous effects that will influence chemo- and radio-sensitivity. The most prominent cell autonomous effect of autophagy is cytoprotection, where autophagy inhibition leads to enhanced sensitivity of the tumor cell to the autophagy-inducing stress, often via the promotion of apoptosis [4,5]. Hundreds of papers have reported on cytoprotective autophagy, which has given rise to the premise that autophagy is a mechanism of drug and radiation resistance.

The cell non-autonomous effects of autophagy relate largely to the impact of drug/radiation induced autophagy on the immune system [6]. This has raised the question as to whether the promotion of autophagy contributes to activation of the immune response and is necessary for immune mediated elimination of the tumor cell. If, in fact, the immune response plays a critical role in the response to chemotherapy, as is now fully recognized by clinical efforts to utilize immune checkpoint inhibition as a therapeutic strategy, then the cell autonomous (cytoprotective) and the cell non-autonomous (immune promoting) functions of autophagy could be functioning in opposition. Consequently, efforts to modulate (suppress) autophagy in the clinic for patient benefit could prove to be counterproductive, particularly if the cell non-autonomous functions of autophagy are more relevant to the outcome of therapy; this could, in fact, be the case, based on evidence that the therapeutic benefits of many cytotoxic chemotherapeutic drugs are largely mediated by enhancement of an antitumor immune response [7].

2. The cellular response to radiation and chemotherapies

2.1. The cell autonomous effects of autophagy induction by chemotherapy

As indicated in the introduction, the bulk of studies in the literature report on the *cytoprotective* functions of autophagy, which are identified

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by sensitization to therapy when autophagy is inhibited by pharmacological agents such as chloroquine, bafilomycin or 3-methyladenine and by genetic silencing of autophagy regulatory genes such as ATG5, ATG7 or beclin [4,5,8,9]. In part, the protective actions of autophagy may derive from the inhibition of apoptosis [10]. The implicit assumption that chemotherapy and radiation induced autophagy is always cytoprotective has been the foundation for multiple clinical trials evaluating various therapies in combination with chloroquine or hydroxychloroquine, FDA-approved drugs that interfere with autophagy [11–14].

While these trials have provided information on the safety of chloroquine and hydroxychloroquine as adjuncts to therapy, overall the outcomes of these trials have not been encouraging [15]. Our own preclinical studies had anticipated these negative outcomes [8] because while chemotherapy and radiation will uniformly induce autophagy in tumor cells in culture the autophagy may not be cytoprotective and may actually be "nonprotective" [4,5]. That is, pharmacological or genetic inhibition of the autophagy does not uniformly alter sensitivity to therapy. Furthermore, there is also quite extensive evidence for the existence of a cytotoxic function of autophagy [16]. Given the absence of clinical biomarkers that could predict whether a particular therapeutic approach actually promotes the cytoprotective form of autophagy, patients in clinical trials could not be stratified to directly interrogate the relationship between the distinct forms of autophagy in their tumors and the impact of autophagy inhibition. In this context, it should be noted that the different functional forms of autophagy cannot be distinguished *a priori*, even in relatively uncomplicated cell culture studies, except by determination of the impact of autophagy inhibition on drug or radiation sensitivity.

2.2. The non-cell autonomous effects of autophagy induction by chemotherapy

For any autophagy inhibition strategy to successfully influence chemosensitivity or radiosensitivity, changes in the cell non-autonomous effects of autophagy, which influence the antitumor immune response, must be considered [6]. In many, if not most studies, these effects have not been taken into account because preclinical models (even the use of patient derived xenografts, which has recently become the norm for preclinical studies) traditionally rely on tissue culture and xenograft animal models, neither of which involves a functional immune response.

Using a variety of immune competent tumor models, mounting evidence has shown that therapy induced autophagy promotes several antitumor immune mediated effects, most prominently the secretion of immune stimulating danger associated molecular pattern (DAMP) signals [17], accompanied by immunogenic cell death (ICD) (Fig. 1) [18]. These combined processes are sometimes referred to as autophagic cell death [19], and it is through this process that select chemotherapies and radiation result in immune stimulating activities. These effects are context dependent as only select chemotherapies promote autophagic cell death [20], and are dependent on drug concentration and dosing schedules [21]. As an example, oxaliplatin, used at a concentration of 20 μM, induced ICD in prostate cancer models, whereas cisplatin at the same concentration induced apoptosis but not autophagy, and as a result an ICD response was not observed [22].

Therapy induced autophagy stimulates an antitumor immune response via the expression of immune stimulating DAMP secretory molecules such as ATP [17], calreticulin (CRT) and high motility group protein B1 (HMGB1) (Fig. 1) [23–25]. Translocation of CRT, an endoplasmic reticulum (ER) resident protein, to the plasma membrane is a result of ER stress induced by anti-tumor therapy [26]. Appearance of CRT on the surface of stressed and dying tumor cells leads to the cells' phagocytosis by antigen presenting cells and subsequent priming of CD4+ and CD8+ T-lymphocytes [27]. HMGB1, released by tumor cells post-mortem, acts as a chemokine binding to the TLR4 receptor on the

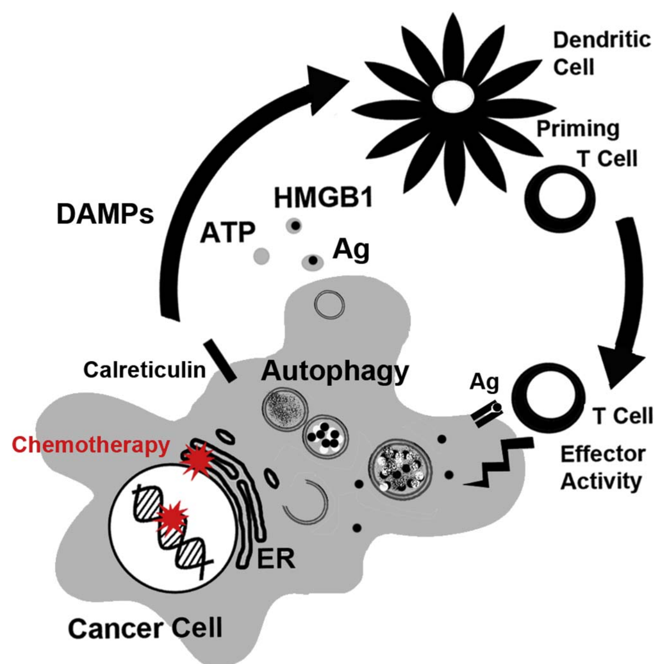

Fig. 1. Cell autonomous and cell non-autonomous effects of ICD inducing therapies. Select cytotoxic drugs and radiation promote damage to DNA and cellular macromolecules. In response, several signaling pathways lead to initiation of autophagy and assembly of autophagosomes. To clear these damaged proteins, and to recycle nutrients to the stressed cell, components of the tumor cell cytoplasm are sequestered into autophagosomes which eventually fuse with lysosomes for degradation and the completion of autophagy. Autophagy, accompanied by ER stress, leads to release of DAMPs that act as signaling molecules and attract and activate immune cells. In addition to DAMPs tumor specific antigens are released into the cytoplasm as a result of autophagy. Autophagosomes, with their antigenic cargo, are released by exocytosis or upon apoptosis and are engulfed by dendritic cells, delivering tumor specific antigens for subsequent cross-presentation to CD8+ and CD4+ T-cells. DAMP secretion and tumor antigen cross presentation enhance the antitumor immune response, potentially leading to immune clearance of residual tumor cells.

surface of the dendritic cells (DC), stimulating phagocytosis of dying cancer cells and eventual cross-presentation of tumor derived antigens [28]. ATP release depends on induction of autophagy [18] as well as on caspase-3 dependent cell death and functioning lysosomal protein LAMP1 [29]. Secretion of ATP serves as a signal attracting DC and macrophages to the tumor bed [30], and also activates DC and macrophages by binding to cell surface receptors (Fig. 1) [31].

In addition to its roles in promoting ICD and DAMPs, autophagy can also stimulate adaptive antitumor immunity by promoting antigen presentation by MHC class II molecules [32]. Specifically, autophagosome fusion with MHC class II loading compartments (late endosomes and lysosomes) facilitates the loading and presentation of intracellular epitopes, thereby enhancing recognition of tumor cells by CD4+ T-cells [32]. Fusion of nuclear/cytosolic antigens with Atg8/LC3, an autophagy associated protein, targets tumor antigens to the autophagosome for processing and presentation to both CD4+ and CD8+ lymphocytes [32].

Autophagy further appears to be instrumental in cross-presentation of tumor specific antigens to naïve T-cells by DC. Inhibiting the autophagic machinery by genetic or pharmacological intervention abolished gp100 peptide cross-presentation to cytotoxic T-cells *in vitro* and *in vivo* [33]. In this case, cells that succumb to ICD release autophagosomes that deliver cargo to MHC class I molecules for cross-presentation.

Lastly, autophagy has recently been shown to regulate the response to checkpoint blockade [34]. There is no debate that developing effective means of blocking immune checkpoint pathways has revolutionized the treatment of some forms of cancer [35]. Blocking these

pathways is thought to stimulate antitumor CD8 T-cell activity to tumors with a high mutation burden. The exact cellular mechanisms by which this occurs is not completely understood, and could involve regulating autophagy. In this context, it has recently been shown that suppressing the checkpoint ligand PD-L1 increases tumor cell autophagy [34]. Returning to a consideration of the cell autonomous impact of autophagy, if the autophagy induced by suppression of PD-L1 is cytoprotective, then checkpoint inhibition would protect the tumor cell from chemotherapy or radiation. It has also been proposed that induction of autophagy could be promoted in CD8 T-cells during checkpoint blockade [36].

The antitumor immune benefits of autophagic cell death are consistent with the long-held premise that the immune system is likely to play a central and fundamental role in the effectiveness of cancer therapy [7]. These studies are also particularly relevant to the clinical efforts to sensitize tumors to chemotherapy and radiotherapy through the pharmacological inhibition of autophagy [37]. However, if autophagy is, in fact, critical for an effective immune response, as proposed by Michaud et al. [17], then these efforts might be futile, since inhibition of autophagy in the tumors would collaterally suppress the immune response that is required for the effectiveness of these therapies, rather than enhancing responsiveness.

Given the growing recognition of importance of the immune system in promoting durable responses to cancer therapeutics, it is disconcerting that there have been few efforts to reproduce and validate the findings of the Kroemer laboratory that were published in 2011 [17]. A recent study by the Debnath laboratory argued that there was no difference in sensitivity to therapy in the context of immune competent and immune deficient tumor bearing animals, and that autophagy suppression had no direct therapeutic impact [38]. Unfortunately, the outcome of these findings remains somewhat inconclusive given that the chemotherapeutic agents utilized (5 mg/kg doxorubicin twice at 7 day intervals) were ineffective in interfering with the growth of the B16 melanoma cells. Studies by Ciampricotti et al. [39] also concluded that the antitumor effects of chemotherapy are independent of the adaptive immune response to poorly immunogenic spontaneous tumors rather than the highly immunogenic transplantable tumor models utilized by the Kroemer group. In response to these reports, Galluzzi and Kroemer make the argument that the experimental design of this work only serves to demonstrate that autophagy does not have a contributory role to chemotherapeutic sensitivity in studies involving non-immunogenic tumors [40,41].

It becomes apparent that the impact of autophagy promotion or inhibition to the immune system (i.e. the cell non-autonomous effects) cannot be stated with any degree of certainty. For instance, blocking the formation of autophagosomes by genetic or pharmacologic intervention was found to abrogate antigen cross-presentation, which is expected to dampen the immune response [33]. However, lysosomal acidification inhibitors that prevent degradation of autophagosomes were shown to be conducive to priming CD8+ T-cells [33]. Consequently, it appears that in some cases accumulation of autophagosomes is necessary for an enhanced immune response; however, the response may be attenuated or abrogated when the contents of autophagosomes are degraded during fusion with the lysosomes. In some studies, escape from the immune surveillance is attributed to induction of systemic autophagy by anti-tumor compounds. As an example, kidney and liver tumors treated with high doses of IL-2 showed regression upon inhibition of autophagy with chloroquine [42]. Although autophagy mediated release of HMGB1 can attract cytotoxic T-lymphocytes, under conditions of constitutively elevated levels promoted by systemic autophagy, HMGB1 also attracts T-regs [43] and MDSC [44], resulting in immunosuppression.

2.3. Cell autonomous and cell non-autonomous autophagy involvement in the abscopal effect in radiation therapy

One argument for the potential involvement of the immune system in the response to conventional therapeutics is the abscopal effect in radiation therapy [45,46]. Although actually quite rare in the clinic [47], evidence has accumulated from tumor bearing animal studies that irradiation of tumor cells, in context with disabling the immune suppressive effects of radiation therapy, can result in collateral suppression of tumor growth in non-irradiated distant tumor cells in the same animal [48]. The logical conclusion from these observations is that the immune stimulatory effects of radiation, possibly in the form of DAMPs and enhanced antigen cross presentation, has activated immune recognition of the non-irradiated tumor cells as well as the tumors directly exposed to radiation.

We and others have presented data indicating that radiation virtually always promotes an autophagic response in tumor cells [9]; consequently, it is quite feasible that radiation-induced DNA damage coupled with autophagy promotes the secretion of DAMPs. In this context, Ko et al. reported that radiation sensitization through autophagy inhibition is abrogated in immune competent animals [49]. These authors further argue that "autophagy inhibition reduces radio-responses *in vivo* due to deficient immunogenic signaling". This conclusion is based on the observation that autophagy inhibition through the silencing of ATG5 served to sensitize murine CT26 tumors implanted in immune deficient mice (essentially a cell autonomous effect that parallels the sensitization observed in cell culture); however, radio-sensitivity was *enhanced* in immune competent animals only when autophagy was functional (a cell non-autonomous immune effect that appeared to be autophagy dependent). These findings suggest that the cell non-autonomous functions of autophagy might have a significantly greater impact on radiation sensitivity than the cell autonomous effects [49]. However, these conclusions were not quite straightforward as radiation did, in fact, suppress tumor growth even when autophagy had ostensibly been blocked. These latter observations indicate that while the promotion of autophagy does appear to enhance susceptibility to radiation, autophagy *was not an absolute requirement* for growth inhibition by radiation in the tumor bearing animal. It is furthermore, difficult to draw broad conclusions from this work since, quite inexplicably, the capacity of ATG5 knockdown to suppress autophagic function in the CT26 cells was not validated. Furthermore, no data were provided that might have distinguished between growth inhibition and tumor cell killing by radiation, and the studies were presented in only a single experimental tumor model. Finally, our own studies have clearly identified a nonprotective function of radiation-induced autophagy [50], in which case radiation sensitivity is likely to be largely dependent on the cell non-autonomous functions of autophagy.

3. Conclusions

Currently the picture remains quite indistinct when we consider the potential for manipulation of chemotherapy or radiation induced autophagy for therapeutic benefit. If autophagy inhibition is to be a clinically effective strategy, then the autophagy in the tumor cell (the cell autonomous form) must *a priori* be of the cytoprotective form. If the autophagy is nonprotective, then autophagy inhibition will have little or no impact on drug or radiation sensitivity. If the autophagy is cytotoxic, then autophagy inhibition is likely to be counterproductive.

However, the ultimate outcome of this therapeutic strategy is likely to be dependent, in large part, on the cell non-autonomous functions of the tumor autophagy (limiting this discussion to the immune system and of necessity ignoring the role that autophagy might be playing in the overall tumor microenvironment). If the studies by Michaud et al. [17] are validated (and it must be noted that the findings were limited to oxaliplatin and mitoxantrone), then autophagy inhibition will actually interfere with drug action by obviating activation of the immune

system. If, however, autophagy and DAMP secretion fail to enhance the immune response, then autophagy inhibition will be effective or ineffective dependent solely on its cell autonomous functions.

Given the complexity of the dual impact of autophagy directly on the tumor cells and indirectly on the immune response, it is unlikely that the current clinical trials will be sufficiently informative to guide future clinical efforts; that is, the trials are unable to distinguish (and are not actually designed to address) the separate but complementary and interactive cell autonomous and cell non-autonomous functions of autophagy in determining sensitivity to chemotherapy and radiation.

Acknowledgements

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Research Paper

Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy

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ABSTRACT

Breast cancer 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.

INTRODUCTION

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 [1].

On the other hand, inhibition of autophagy by disruption of *Beclin 1* or deletion of *ATG5* increases the frequency of spontaneous malignancies [2] or liver tumor [3], 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 [4]. 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 [5]. Chloroquine (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.

RESULTS

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 1A, 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 1B, 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.

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 [6], 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 2A). 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 2B). In fact, MMC cells remained apoptotic by producing floater dead cells following ADR treatment (Supplementary Figure 1A) 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 (Supplementary Figure 1B). A transient blockade of autophagy by CQ did not affect susceptibility of tumor cells to ADR-induced apoptosis (Supplementary Figure 2). On the other hand, a transient blockade of autophagy during ADR chemotherapy, *in vivo*, did not prolong tumor dormancy in FVBN202 mice (Supplementary Figure 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 IFN-γ or MMC-reactive T cells three weeks after treatment with ADR or ADR+CQ. As shown in Figure 3, untreated MMC or dormant MMC treated with ADR or ADR+CQ all remained susceptible to IFN-γ treatment or T cells.

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 (Supplementary Figure 4A). 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 (Supplementary Figure 4B). Tumor cells remained intact for the expression of neu antigen, as well as cell proliferation *in vitro* and *in vivo* following knockdown of autophagy (Supplementary Figure 4C–4E). Flow cytometry analysis determined a lower level of viability in MMC compared with ATG5^{KD} MMC following ADR treatment (Figure 4).

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 4) 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 5A, 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 5B, $p < 0.001$). Interestingly, ATG5^{KD} MMC cells contained a higher number of polyploid-like cells following ADR treatment compared with autophagy-competent MMC (Figure 5B, $p < 0.03$).

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 6A, 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 6B). Finally, ATG5^{KD} MMC were found to be resistant to T cell-induced apoptosis compared with autophagy-competent MMC (Figure 7).

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 [7]. 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 was 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.

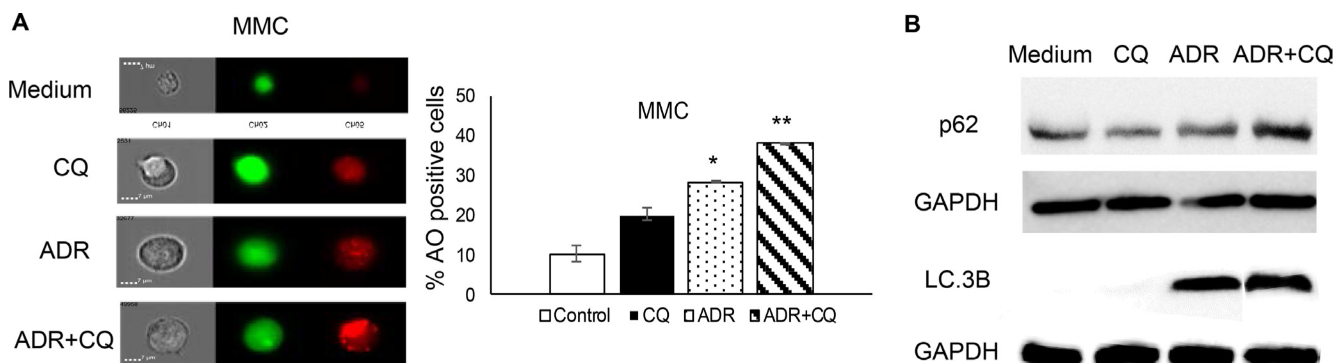

Figure 1: CQ blocks ADR-induced autophagy. MMC tumor cells received three daily doses of ADR alone (1 μM ADR for 2 hrs) (ADR) or in the presence of CQ (10 μM 3 hrs before ADR and 2 hrs during ADR treatment) (ADR+CQ), washed after each daily treatment and analyzed by acridine orange (AO) one day after 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).

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 [8]. 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 [9, 10]. It has been reported cancer stem cells play a role in tumor dormancy [11] and drug resistance [12], and that immunotherapeutic targeting of breast cancer stem cells inhibits growth of mammary carcinoma [13]. 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 [14]. Treatment-induced autophagy could lead to apoptosis [15] and tumor cell dormancy [16]. 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 [17]. Therefore, evaluation of apoptosis or tumor growth inhibition as a single factor without evaluating

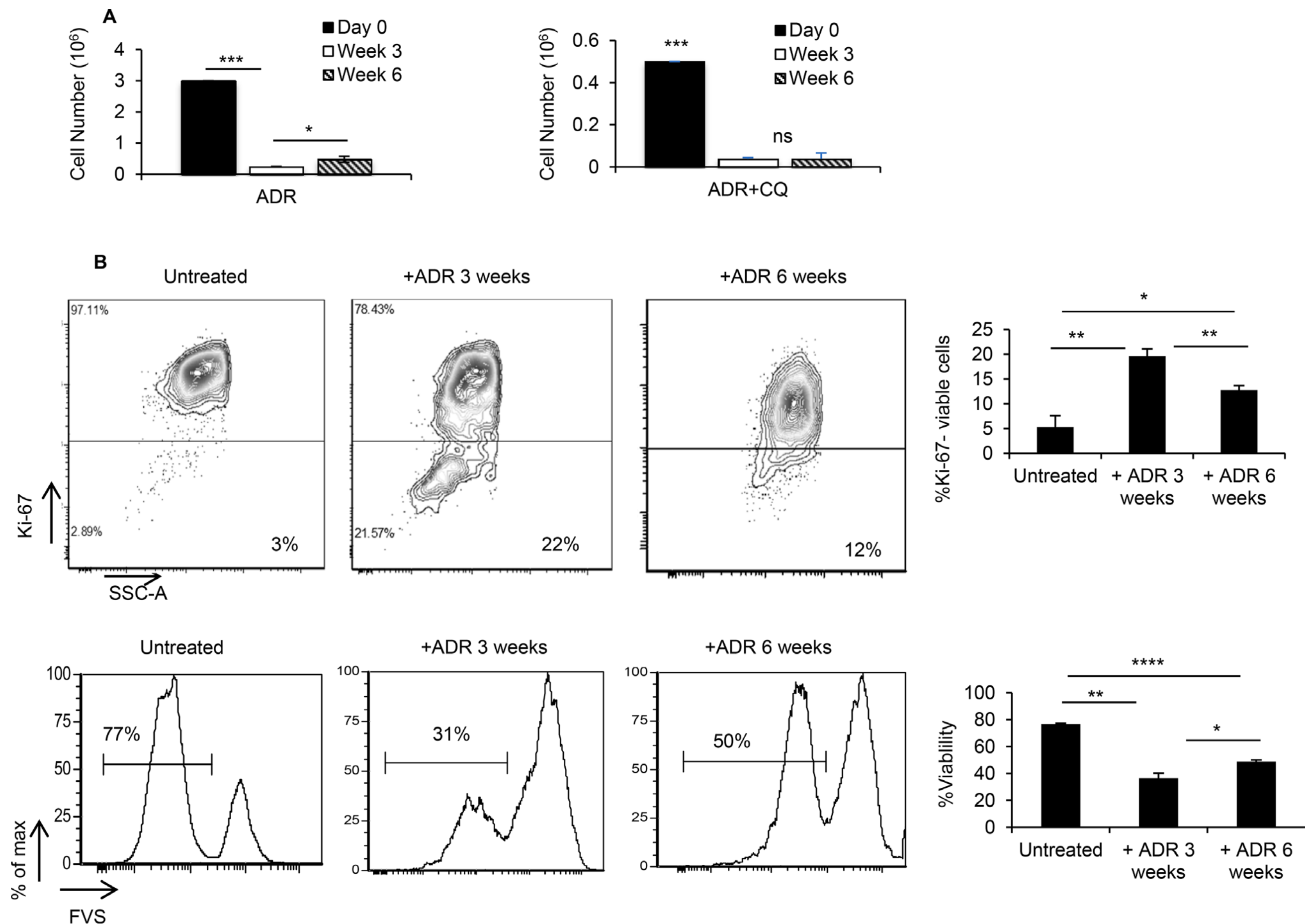

Figure 2: ADR-induced dormant tumor cells remain dormant in the presence of CQ. MMC tumor cells were treated with 3 daily doses of ADR (1 uM for 2 hrs), with one group receiving CQ (10 uM) 3 hrs prior to and during ADR treatment. Both groups remained untreated for 3 weeks and 6 weeks, *in vitro*. (**A**) Adherent viable cells were counted using trypan blue exclusion at various time points. Data represent 3 replicates ± SEM. (**B**) At weeks 3 and 6 post-treatment, Ki-67 expression (upper panel) and 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.

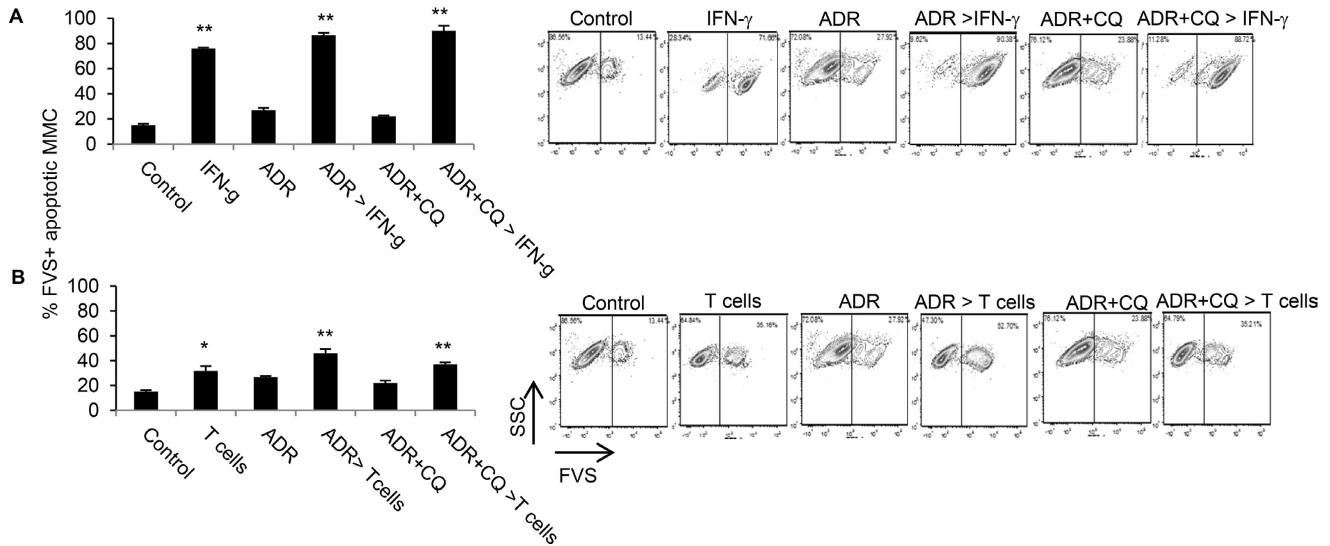

Figure 3: Dormant tumor cells established by ADR or ADR+CQ remain susceptible to immunotherapy. The *in vitro* tumor dormancy was established three weeks after three daily treatments of MMC with ADR or ADR+CQ. Untreated MMC cells were used as control. (**A**) Apoptosis was determined by FVS viability 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-g and analyzed two days later (50 ng/ml) (IFN-g), ADR-treated dormant MMC cultured with three daily doses of IFN-g (50 ng/ml) and analyzed two days later (ADR > IFN-g), or ADR+CQ-treated dormant MMC cultured with three daily doses of IFN-g (50 ng/ml) and analyzed two days later (ADR+CQ > IFN-g). (**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). Splenic T cells were collected from MMC tumor-bearing FVBN202 mice.

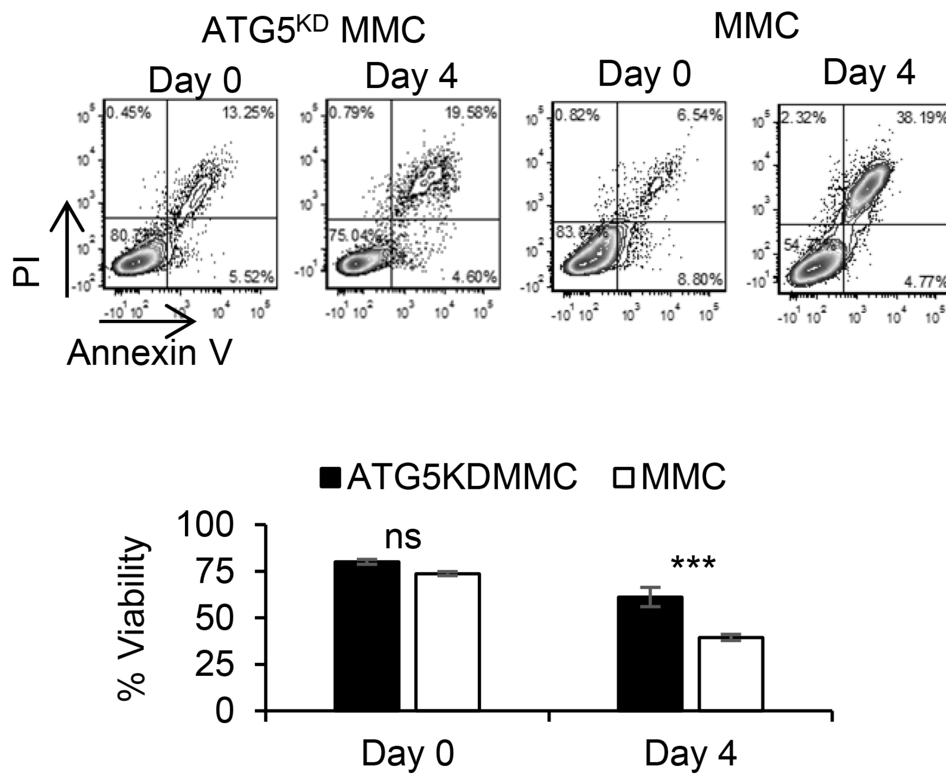

Figure 4: Autophagy knockdown tumor cells become less susceptible to ADR-induced apoptosis. Autophagy-deficient MMC (ATG5^{KD} MMC) or autophagy-competent MMC (MMC) were treated with a single dose of ADR alone (1 uM ADR for 2 hrs). Tumor cells were analyzed by Annexin V/PI staining prior to treatment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

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 [18]. In triple negative breast cancer, CQ sensitizes tumor cells to paclitaxel chemotherapy [19]. In several tumor models, CQ synergistically augmented sunitinib cytotoxicity on tumor cells [6]. 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 nuclei [20, 21]. To this end, cell-intrinsic autophagy protects the cell from genomic instability induced by the accumulation of toxins within the cell [22]. It has been reported that Beclin1 knockout mice fail to maintain genomic integrity by increasing DNA double stranded breaks and gene amplifications [20]. A higher expression of Beclin 1 in healthy breast tissue than in breast cancer suggests a deficiency in cell-intrinsic autophagy in tumors [23], 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 [24]. Expression of ATG5 in the tumor specimens is also associated with relapse-free survival in breast cancer patients [25]. 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 [26].

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.

MATERIALS AND METHODS

Tumor cell line

The neu overexpressing mouse mammary carcinoma (MMC) cell line was established from spontaneous mammary tumors harvested from FVBN202 mice [27]. Tumor cells were maintained in RPMI 1640 supplemented with 10% FBS.

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.

Antibodies

All antibodies were purchase from Biolegend (San Diego, CA, USA) unless otherwise stated. Antibodies were used as instructed by the supplier. Antibodies

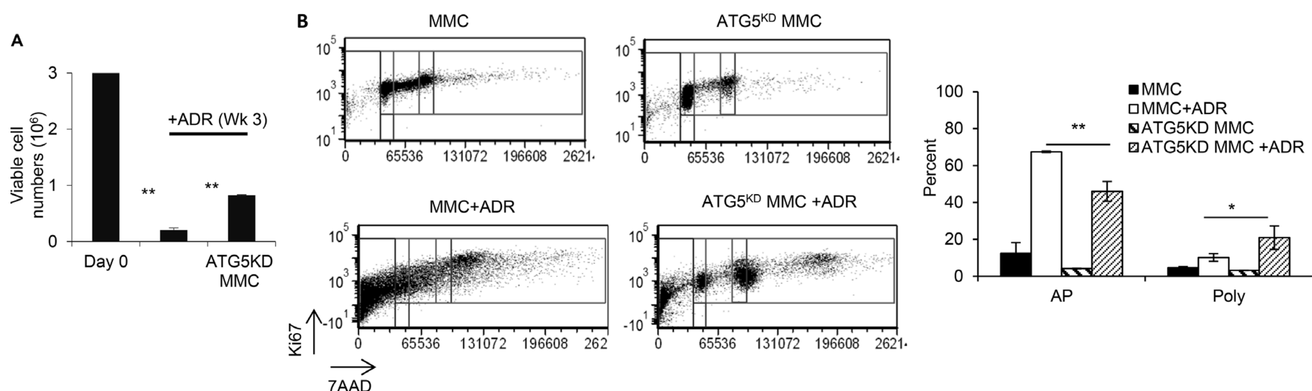

Figure 5: ADR-induced tumor dormancy in autophagy knockdown tumor cells with polyploid-like morphology compared with autophagy competent tumor cells, *in vitro*. MMC or ATG5^{KD} MMC tumor cells (3 million cells, Day 0) were treated with 3 daily doses of ADR (1uM for 2 hrs), and viable cells were counted 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.

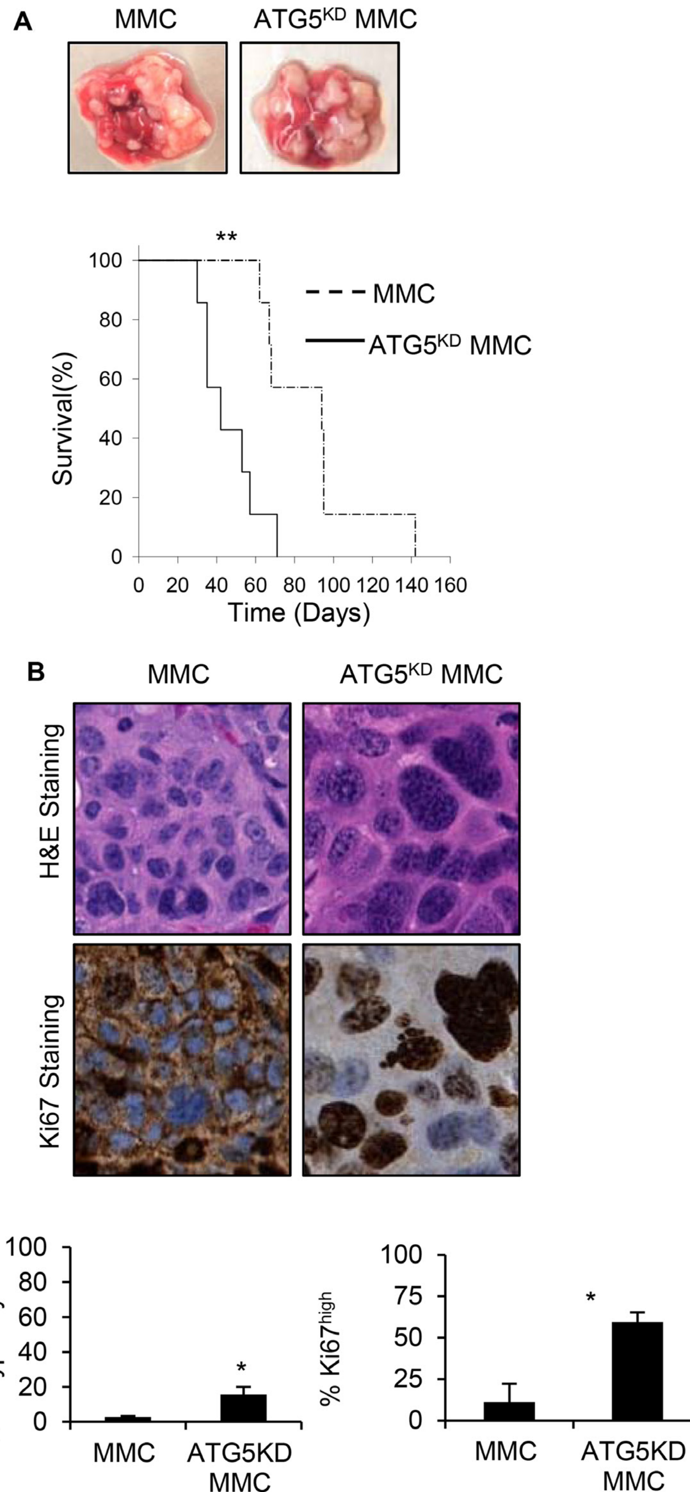

Figure 6: Earlier relapse of autophagy knockdown tumor cells with polyploid morphology compared with autophagy competent tumor cells, *in vivo*. (**A**) FVBN202 mice (n = 7) were challenged i.v. with 10^6 cells ADR-treated dormant control MMC (MMC), or ADR-treated dormant ATG5^{KD} MMC (ATG5^{KD} MMC). 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 0.02 mm^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 16 um 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 $p < 0.05$.

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, USA), FITC-FVS (BD Biosciences). All reagents were used at the manufacturer's recommended concentration.

Mice

FVBN202 transgenic female mice (The Jackson Laboratory; Bar Harbor, ME, USA) were used. These mice overexpress non-mutated, non-activated rat neu transgene under the regulation of the mouse mammary tumor virus promoter [28]. These mice develop premalignant mammary hyperplasia similar to ductal carcinoma *in situ* prior to the development of spontaneous carcinoma [29]. These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

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, 10 uM, 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.).

Cytotoxicity assay

Freshly isolated tumor-primed splenic T cells or *ex vivo* expanded splenic T cells were cultured with 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, 10 mM L-glutamine and 5 × 10^{-5} M 2-mercaptoethanol) with 20U/ml of IL-2 (Peprotech) in 6 well culture dishes. After 48 hs cells were harvested and stained for neu (anti-c-Erb2/c–Neu, Calbiochem), Annexin V and PI according to the manufacturer's protocol (BD Pharmingen). Flow cytometry was used to analyze the viability of neu positive cells [17, 30].

IFN-γ ELISA. Reprogrammed immune cells were cultured in complete medium with irradiated (140 Gy) tumor cells, ADR-treated dormant MMC or ADR+CQ-treated dormant MMC at a 10:1 ratio for 20 hrs. Supernatants were then collected and stored at −80°C until assayed. IFN-γ was detected using a Mouse IFN-γ ELISA kit (BD Biosciences), according to the manufacturer's protocol [30].

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, ****: < 0.00005).

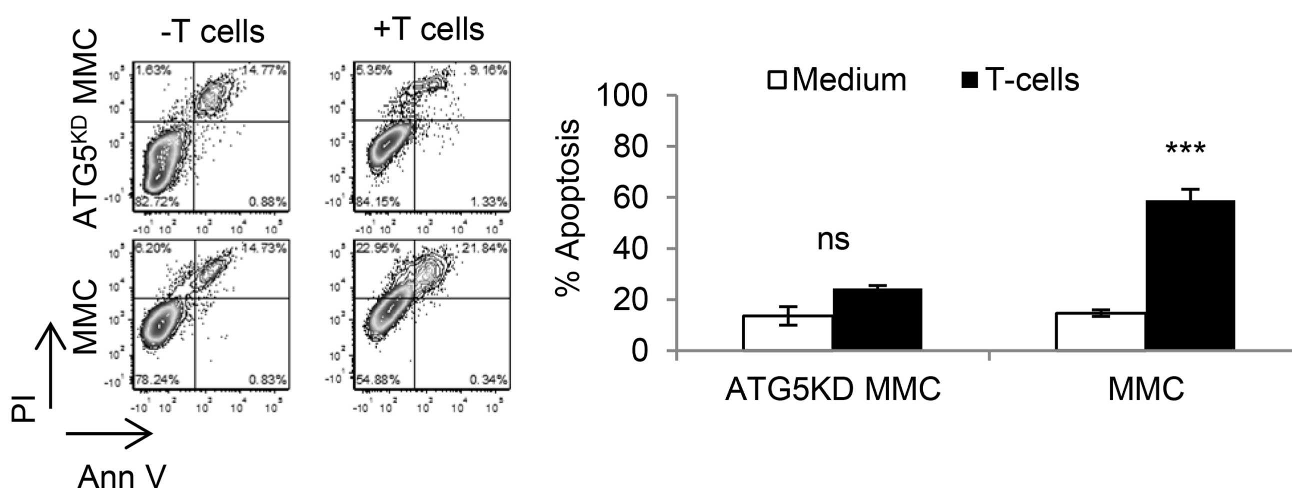

Figure 7: Autophagy knockdown tumor cells become resistant to T cell-induced apoptosis. Neu overexpressing autophagy-deficient MMC (ATG5^{KD} MMC) or autophagy-competent MMC (MMC) were co-cultured with MMC-sensitized T cells and then gated CD45-Neu+ tumor cells were analyzed by Annexin V/PI staining. Data represents triplicate experiments.

Abbreviations

ADR, Adriamycin, ATG5, Autophagy-related gene 5, BRCA1, Breast cancer gene 1, CQ, chloroquine, HSP70, Heat shock protein 70, IHC, Immunohistochemoistry LAMP2A, Lysosomal membrane associated protein 2 A, MMC, neu-overexpressing mouse mammary carcinoma, TNBC, Triple negative breast cancer.

Author contributions

M.H.M., D.A.G., K.K.P. contributed to the study's conception, design, experimental and analytical performance, and writing of the manuscript. H.F.A., L.T-M., T.T., R.C.K., S.J., S.E.B., T.M.S. contributed to the study's experimental and analytical performance and writing of the manuscript. D.B. contributed to statistical analysis and writing of the manuscript. H.D.B. and M.O.I. contributed to analytical performance and writing of the manuscript.

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CONFLICTS OF INTEREST

Authors have no potential conflicts of interest to disclose.

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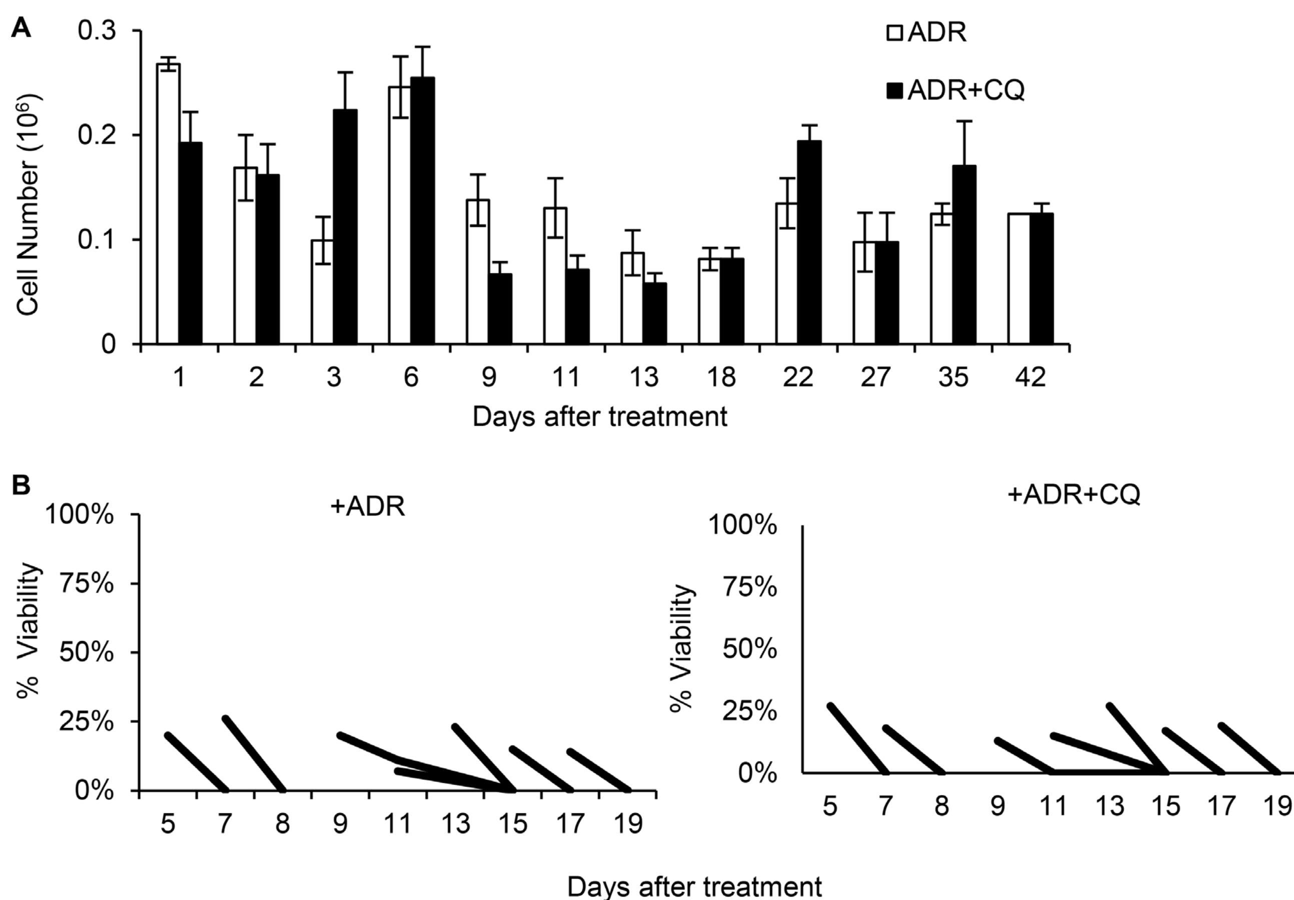
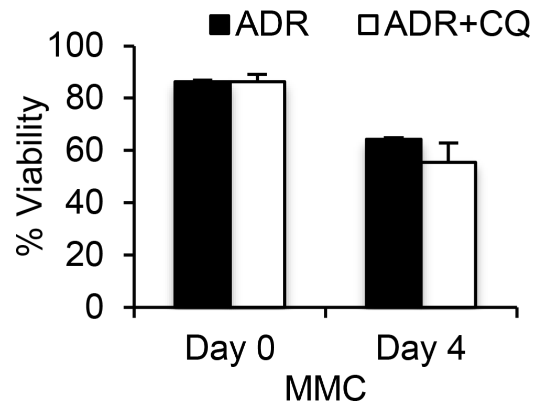
Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy

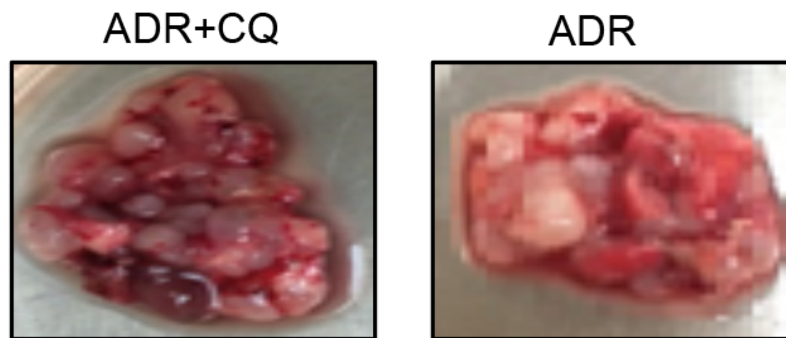
SUPPLEMENTARY MATERIALS

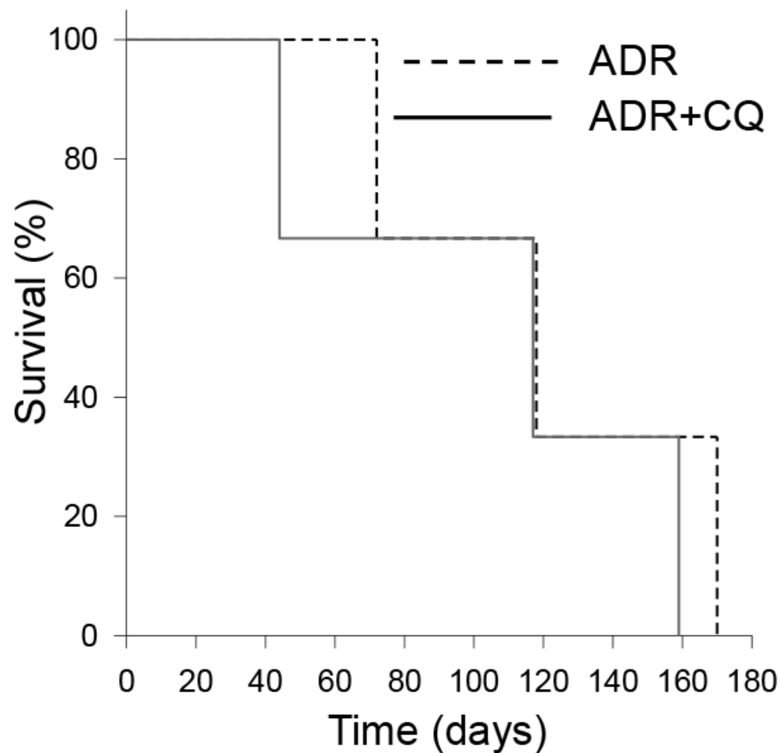


Supplementary Figure 1: ADR-induced dormant tumor cells produce floater apoptotic cells, *in vitro*. MMC tumor cells (3×10^6 cells/flask) were treated with 3 daily doses of ADR (1 uM for 2 hrs), with one group receiving CQ (10 uM) 3 hrs prior to and during ADR treatment. Both groups remained untreated 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.

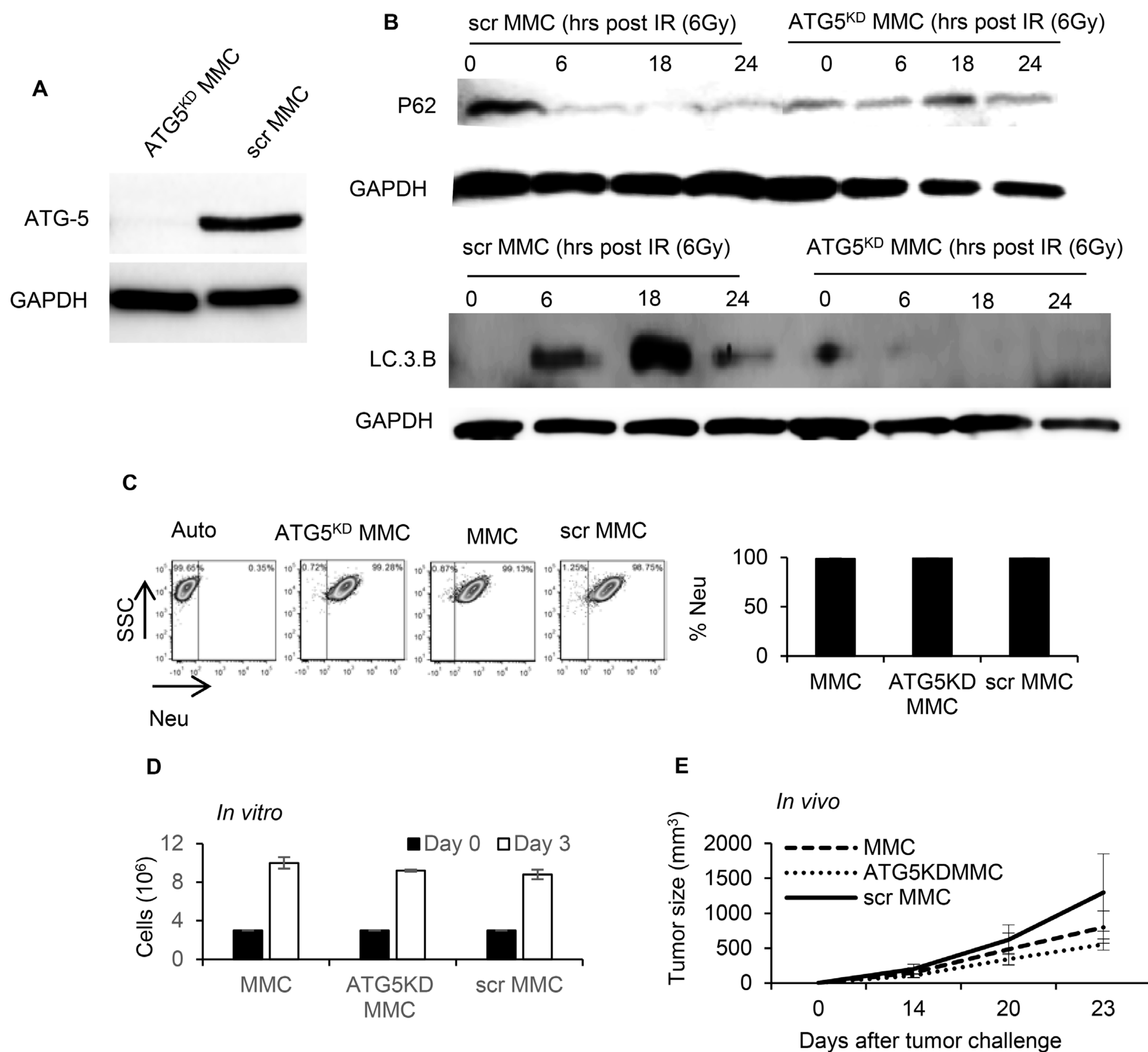


Supplementary Figure 2: A transient blockade of autophagy by CQ did not change the susceptibility of MMC to ADR treatment. MMC tumor cells were treated with ADR alone (1 uM ADR for 2 hrs) (ADR) or in the presence of CQ (10 uM 3 hrs before ADR and 2 hrs during ADR treatment) (ADR+CQ). Tumor cells were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





Supplementary Figure 3: 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 lung.



Supplementary Figure 4: 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.