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Treatment-Induced Breast Cancer Dormancy and Relapse

Rebecca Keim
Virginia Commonwealth University

TREATMENT-INDUCED BREAST CANCER DORMANCY AND RELAPSE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

REBECCA CAROLINE KEIM
Bachelor of Science, Virginia Commonwealth University, 2012

Director: MASOUD H. MANJILI, DVM, PHD
PROFESSOR OF MICROBIOLOGY AND IMMUNOLOGY

Virginia Commonwealth University
Richmond, Virginia
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ADR: Adriamycin

AMPKα1: Adenosine monophosphate-activated protein kinase α1

AO: Acridine orange

CEA: Carcinoembryonic antigen

CQ: Chloroquine

CRT: Calreticulin

CTC: Circulating tumor cells

DCs: Dendritic cells

DTC: Disseminated tumor cells

EDTA: Ethylenediaminetetraacetic acid

FACS buffer: 2mL PBS with 2% FBS, 0.1% sodium azide

FBS: Fetal bovine serum

GMF: Geometric mean fluorescence

HER-2: Human epidermal growth factor receptor-2

HMGB1: High mobility group box-1

Hyp-PDT: Hypericin-mediated photodynamic therapy

ICD: Immunogenic cell death

IFN-γ Rα: IFN-γ receptor-α

MMC: Mouse mammary carcinoma cell line

NOD-SCID: Non-obese diabetic severe combined immunodeficient

NSCLC: Non-small cell lung carcinoma

PBS: Phosphate-buffered saline

PD-L1: Programmed death-ligand 1

PI: Propidium iodine

POSTN: Periostin

ROS: Reactive oxygen species

RPMI+FBS: RPMI 1640 with 2mM L-glutamine and 10% fetal bovine serum

RT: Radiation therapy

SBRT: Stereotactic body radiation therapy

SEER: Surveillance, Epidemiology, and End Results Program

TGF-β: Transforming growth factor-β

TIM-4: T cell immunoglobulin- and mucin domain-containing molecule-4

TNBC: Triple negative breast cancer

TSP-1: Thrombospondin-1

Abstract

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When breast tumor cells encounter stress due to cancer therapies, they may enter a dormant state, escaping from treatment-induced apoptosis. Dormant cells may eventually regain proliferative capabilities and cause recurrent metastatic disease, which is the leading cause of mortality in breast cancer patients. We sought to determine if a high dose of radiation therapy (RT) or combined chemo-immunotherapy, with and without the blockade of autophagy by chloroquine (CQ), could overcome treatment-induced tumor dormancy or relapse. We found that autophagy contributes in part to treatment-induced tumor dormancy. We also found that

three therapeutic strategies were successful in inhibiting or preventing tumor relapse. These include: 18Gy/day RT, chemotherapy combined with the blockade of autophagy, and combined chemo-immunotherapy. Follow-up studies are needed to determine the feasibility of preventing tumor relapse by prolonging tumor dormancy versus eliminating dormant tumor cells.

Introduction

Breast cancer mortality and tumor recurrence

Conventional therapies for breast cancer, such as surgery, radiation therapy (RT), hormone deprivation therapy, and chemotherapy, are very effective at clearing primary tumors. In fact, according to a study done by the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute, for stage I and II tumors, the 5-year survival rate is over 98%. Even when breast cancer cells have reached stage III and spread to regional lymph nodes, there is still an 84.6% survival rate (1). However, regardless of stage, the survival rate declines over time. The overall 10-year survival rate is 84.3%, which then drops to 76% at 15 years (1). This is because even after the primary tumor has successfully been treated, there is still a chance of tumor relapse and resistance to therapy occurring. Additionally, not all subtypes of breast cancer are highly responsive to conventional therapies. For example, 10-17% of breast cancer patients are diagnosed with triple-negative breast cancer (TNBC), which lacks the hormone receptors for estrogen and progesterone, as well as low expression of human epidermal growth factor receptor-2 (HER-2) (2). These patients have a much higher mortality rate as they have fewer treatment options available. On average, the 5-year disease-free survival rate of TNBC patients is as low as 76.8% compared to the 98% of non-TNBC patients (2). Similarly, patients with later stages of breast cancer or relapsed and metastasized cancer have a poorer prognosis. According to the SEER study, once breast cancer cells have metastasized to other tissues, the 5-year survival rate drops to 25% (1).

For most patients, the primary tumor is very responsive to conventional therapies. In fact, most patients with earlier stages of breast cancer are thought to be cancer free after undergoing conventional treatment. However, in some patients, months, or even years after the completion of their initial therapy, the cancer relapses at distant sites where it has metastasized. This metastatic disease is the result of residual tumor cells from the primary tumor, which had escaped apoptosis induced by the initial treatment and then lay dormant before resuming proliferation. Generally, once a tumor has spread, it is usually more resistant to conventional therapies. Studies have found that relapsing tumor cells have a different phenotype than the original primary tumor, such as a change in MHC expression or a loss of HER-2 expression, which allows them to become resistant to certain therapies (3). However, the basis for tumor dormancy and relapse is poorly understood, making it difficult to effectively treat or prevent this metastatic disease.

Cancer Dormancy, Circulating Tumor Cells, and Tumor Relapse

In order for a tumor to relapse years after the primary tumor is considered cleared, residual tumor cells must escape treatment-induced apoptosis and become dormant until they are able to invade distant tissues and resume proliferation to form metastasis. Not much about this process is understood, although there are thought to be 3 main types of dormancy. The first is cellular dormancy, in which the tumor cells become quiescent and arrest in the G0 phase of the cell cycle. This is reversible, and allows the tumor cells to survive despite exposure to anti-cancer therapies (4). The second type is angiogenic dormancy. When a tumor cell resumes proliferation in a distant tissue, it requires more nutrients than the normal vasculature in the area

provides. Thus the tumor cells must induce angiogenesis in order to survive and continue growing. This requires the release of pro-angiogenic factors; however, not all tumor cells are capable of releasing a sufficient amount to overcome any anti-antigenic factors that may also be present in the microenvironment. Without a sufficient nutrient supply, the tumor is unable to grow past a certain point, so the cells remain in a balance between proliferation and apoptosis. In this state the tumor is undetectable, and is considered to be in a dormant-like state, until it can produce sufficient levels of pro-angiogenic factors (4, 5). The third form of tumor dormancy is immunologic dormancy. This form is similar to angiogenic dormancy in that it results in a state of equilibrium between proliferation and apoptosis in the tumor cells provided that tumors are immunogenic and able to induce anti-tumor immune responses. Unlike angiogenic dormancy however, the apoptosis is a result of the anti-tumor immune response. The immune response is still strong enough to inhibit the overall growth of the tumor. However, eventually, the constant immune pressure is enough to induce epigenetic changes in the dormant tumor cells, such as loss of tumor antigens or defects in the antigen presentation machinery, allowing them to escape and resume proliferation (4, 5).

Understanding the mechanisms behind dormancy is key to overcoming or preventing relapse from occurring in breast cancer patients. Unfortunately, this has proven difficult to study, as there are few dormancy models available. One group however, was able to establish such a model using breast cancer cells and cells found in the bone marrow. Using a 3D-collagen biomatrix, they cocultured the breast cancer cells with the bone marrow cells in order to mimic a bone metastatic microenvironment. They found that in the presence of the bone marrow cells, the breast cancer cells were unable to proliferate, exhibiting dormancy characteristics. When the coculture was disassociated, and the breast cancer cells were cultured alone, they were able to

resume proliferation (6). After establishing that it was in fact a dormancy model, the researchers then used their model to determine what mechanisms are involved in dormancy by inhibiting various pathways. The inhibition of certain pathways, such as receptor tyrosine kinase (RTK) and p38 signaling, resulted in a restoration of proliferation in the breast cancer cells, even in the presence of the bone marrow cells. They successfully showed how their model could be used to better understand the mechanisms involved in tumor dormancy (6). If other groups adopt similar models, it may be possible to determine several mechanisms involved in dormancy, and find more effective methods to combat dormancy and relapse.

Another group came up with a computer based model to simulate dormancy in order to determine the likelihood that metastatic cells are also cycling cells. Most anti-tumor therapies which target metastatic cells include adjuvant therapy, such as cyclophosphamide, methotrexate, and 5-fluorouracil, which primarily kills cycling cells (7). However, that is not an effective strategy if the metastatic cells are actually undergoing cellular dormancy and are quiescent, so it is important to determine what state these tumor cells are actually in. Unlike other computer simulations looking at dormancy, this model focused on the probability of the metastatic microenvironment establishing dormancy in the tumor cells. They found that it is highly probable that metastatic cells are in a quiescent state for extended periods of time. They compared these probabilities to *in vivo* data and found that their analysis is a very likely model of dormancy in patients (8). This model is important because it stresses the importance of finding therapies that will target quiescent cells, not just mitotically cycling tumor cells.

In a preclinical study on tumor dormancy, the ability to reawaken the dormant cells was examined. Suzuki et al. fluorescently labeled breast cancer cells and then injected into SCID/B17 mice. After the mice were sacrificed, the tumor cells were detected in and recovered

from the spleen and bone marrow, which did not show any signs of metastasis, and the lungs and lymph nodes, which are preferred metastatic sites. Only a few scattered cells were collected from the non-metastatic tissues, compared to the larger clusters of cells collected from the metastatic tissues. All the collected tumor cells were then cultured and monitored for proliferation. Even the tumor cells from non-metastatic tissues eventually began to proliferate after about 6-8 weeks. To see whether the collected tumor cells were still tumorigenic, despite not originally developing metastases, they were inoculated into fresh mice. In all mice, new primary tumors were seen developing within 2 weeks, regardless of which organs the tumor cells were initially collected from (9). This suggests that the ability of tumor cells to reawaken from dormancy and form metastases depends on the microenvironment in which they are growing. That would imply that there are certain mechanisms present in some tissues but not in others, which control tumor dormancy. If these underlying mechanisms are discovered, then maybe they can be exploited to prevent dormant cells from waking up and relapsing.

A recent study by Ghajar et al. looked specifically at what these mechanisms could be. They focused on disseminated tumor cells (DTC) in Non-Obese Diabetic Severe Combined Immunodeficient (NOD-SCID) mice in order to see what causes dormant tumor cells in distant tissues to shift from quiescence to proliferation (10). Interestingly, they found that the microvascular niche environment near the DTC was responsible for causing the shift. If the DTC were situated near mature microvessels that were not producing many angiogenic factors, then the DTC remained quiescent. This was partially due to the presence of thrombospondin-1 (TSP-1) in the vascular basement membrane, which helped suppress tumor growth. However, if the DTC were near activated neovascular tips, then they began to proliferate. This was due to periostin (POSTN) and transforming growth factor-β (TGF-β) which not only helped with

angiogenesis, but also woke the DTC from quiescence (10). This suggests that the endothelial cells of the tumor microenvironment and its related vasculature are responsible for regulating the phenotype of DTC.

Tumor cells are not dormant only at the primary site or in distant tissues. In order to metastasize to distant sites, the cells must first detach and enter the circulation. Once there, these circulating tumor cells (CTC) undergo cellular dormancy until they begin to colonize and new tissue and either relapse or switch to angiogenic dormancy. Because of their ability to spread to distant tissues, CTC are a hallmark of metastatic disease.

Several clinical studies have been conducted to look at the relationship between CTC, relapse, and overall patient survival. In one such study, blood samples were collected from patients who had undergone a mastectomy and appeared to be cancer free. CTC were found in patients anywhere from 7 to 22 years after their mastectomy. When grown in culture, collected CTC were also found to have a half-life of about 2 hours, which would suggest that they were being replenished by a replicating tumor somewhere else in the body. This tumor appears to be undergoing either angiogenic or immunologic dormancy, which is why the patients appeared asymptomatic (11). Another similar study collected blood samples from stage I-III breast cancer patients at the time of their surgery to look for the presence of CTC. After determining that 24% of their patients had CTC, the group then looked to see whether these patients experienced different recurrence and survival rates than those without CTC. The presence of CTC was found to be related to both earlier recurrence and an overall lower survival rate, suggesting that CTC could be used as a valuable prognostic tool in patients of all stages (12).

A third group compared CTC levels between breast cancer patients of different stages to see how early CTC could be detected, and whether there was a correlation between the amount

of CTC found and staging. CTC were found even in patients with stage I cancer. The amount of CTC recovered from a patient was directly related to their stage, as well as lymph node status. And just as the previous groups had found, there was a direct correlation between amount of CTC recovered and overall survival rates. In addition to comparing CTC levels between patients, the group also cultured the collected CTC and found that not only did the cells proliferate, they also had very distinct populations, such as stem cells, progenitor cells, and those of an epithelial lineage (13).

Similar results have been shown with other types as cancers as well. Hofman et al. found that regardless of staging, patients with non-small cell lung carcinoma (NSCLC) who also had CTC had lower long term survival rates than those that did not. These patients were also at a higher risk of relapsing than those of a similar stage who did not have any CTC (14). In another study with NSCLC, it was found that in patients with later stages, there are very high levels of CTC, supporting the idea that CTC levels can serve as a strong prognostic factor, regardless of cancer type (15).

Inducers of Tumor Dormancy

Although the exact factors that lead to tumor dormancy are not well understood, conventional anti-tumor therapies, such as chemotherapy and RT have been shown to have a dual function in that they can induce both tumor cell death and tumor dormancy. Chemotherapy is a very effective therapeutic strategy that invokes very high levels of tumor cell death. However, it has been found that if tumor cells are exposed to chemotherapy for long periods of time, they eventually grow resistant to the toxic effects of the drug. Those residual resistant cells

then escape, become dormant and eventually form metastatic cancer (16, 17). It is also thought that due to the heterogeneous nature of tumor cells, it is possible that different clones respond differently to the treatment such that some clones undergo apoptosis whereas other clones become quiescent but remain viable. The latter are the ones that become dormant and eventually cause relapse (17). RT has been shown to induce a balance between tumor cell death and growth or growth arrest, resulting in a dormant state (18). Gao et al. found a TLR9-dependent pathway that stimulated tumor growth after RT. When this pathway was blocked, tumor cells took longer to relapse. When it was not blocked, it was triggered by soluble factors found in the microenvironment following RT, which activated STAT3, promoting inflammation and revascularization. It was suggested that if RT is combined with inhibition of TLR9/STAT3, then it may be possible to reduce the rate of tumor relapse (19).

Similar to conventional therapies, immunotherapy also has a dual function in that it can induce tumor cell death as well as tumor cell dormancy. Strongly immunogenic tumors are the result of the expression of highly antigenic proteins, which trigger a strong T cell-mediated immune response. A strong anti-tumor immune response could then induce epigenetic changes in the tumor resulting in antigen loss or lead to immunoselection of the antigen-negative tumor cells, which would then begin to proliferate (20). Our group has previously shown that one method of tumor escape via immunoediting is through the down regulation of the neu antigen, as a result of methylation of the neu promoter following treatment with IFN-γ, a product of the immune response (21). This occurs because neu-specific CD8+ T cells are capable of inhibiting tumor growth, but fail to completely eliminate them (21).

In a later study, our group also found that patients with HER-2/neu negative breast cancer generally had a stronger HER-2/neu specific IFN-γ producing T cell response than HER-2/neu

positive patients. This suggests that the stronger immune response led to the down regulation of the neu antigen, which would allow the tumor to escape neu-targeted therapies (22). A study on B cell lymphoma supported our findings by showing that CD8+ T cells were necessary for the maintenance of dormancy; when the IFN-γ was neutralized by monoclonal antibodies, the tumor cells were unable to remain dormant. This suggests that CD8+ T cells support tumor dormancy through the secretion of IFN- γ (23). Our group has also recently discovered that the levels of IFN-γ receptor-α (IFN-γ Rα) expression on tumor cells will determine the outcome of tumor immune surveillance such that low levels of IFN-γ Rα expression support tumor dormancy under immune pressure until tumor cells undergo epigenetic changes, lose target antigens and relapse (24).

Wu et al. found that some tumor cells were able to escape immune pressure without losing their tumor antigens or MHC class I expression. The research group initially induced poorly tumorigenic tumors in normal and nude MMTV- C3H/HeN mice using UV irradiation. After collecting the primary tumor from the initial mice, the group then reinjected a new group of mice and repeated the cycle 3 times. With each reinoculation, the tumor grew more aggressively. Both the primary and late, aggressive cells were grown in culture to compare their MHC class I expression as well as their susceptibility to IFN-γ. The primary tumor initially had low levels of MHC class I expression and was resistant to CTL-mediated killing, however, with increasing exposure to IFN-γ, upregulation of MHC class I expression was observed. In contrast, the late tumor cells initially had higher expression of MHC class I and were more sensitive to CTL-mediated killing. With prolonged exposure to IFN-γ, however, the late tumor cells became resistant to any further CTL-mediated cell death, despite expressing high levels of MHC class I (25). This suggests that prolonged exposure to IFN-γ may result in resistance to

further immune pressure and tumor dormancy. This was also demonstrated by our group (22, 24).

The adaptive T memory response has also been found to have a role in the induction and maintenance of dormant tumor cells. It was found that CD8+ memory T cells actively controlled tumor cell growth in the bone marrow by invoking a strong antitumor response whenever the memory cells were restimulated with tumor antigen. Each time the tumor cells began to resume proliferating, the memory cells would be restimulated, and induce another immune response, resulting in the death of some, but not all, the tumor cells. This established a cycle of immunologic dormancy in the tumor cells, resulting in a balance between tumor cell death and proliferation (26).

Finally, one study found that the cytokines produced by CD4+ T cells could control whether tumor cells became dormant or more tumorigenic. Rip1-Tag2 mice were injected with tumor cells and IFN-γ producing Tag-Th1 cells. Under normal conditions, the CD4+ T cells were able to arrest tumor growth and angiogenesis, resulting in tumor dormancy. However, when the mice were also treated with an anti-IFN-γ monoclonal antibody, tumor growth and angiogenesis were actually promoted. Similar results were seen when transgenic *TNFR1-/-* mice were injected with the same tumor and T cells, but not the anti-IFN-γ monoclonal antibody (27). This suggests that both IFN-γ and TNF are needed to prevent the growth of tumor cells and that a balance between dormancy and growth is controlled by the cytokines produced by the adaptive immune response.

Mechanisms of Tumor Dormancy

While not a lot is known about tumor dormancy, it is understood that some underlying mechanisms are needed to maintain tumor cells in a dormant state. Angiogenic dormancy is not true dormancy in that the individual tumor cells are not in a state of growth arrest. However in quiescent dormancy, the tumor cells are actually arrested and must maintain this arrest themselves. Different mechanisms have been proposed as the primary means for maintaining tumor cells in a dormant state.

One such mechanism is senescence, which is defined as the state in which cells are no longer able to divide. They do remain viable and metabolically active, however (28). Senescent cells tend to become flat and enlarged and have increased expression of certain markers such as p53 (29). Senescence is generally a delayed response to stress, and involves many mechanisms, such as epigenetic regulation and a DNA damage response (30). Senescence is also closely linked to aging (29). Traditionally, senescence has been considered irreversible; however, more recent studies have shown that cells are in fact able to recover the ability to proliferate under certain conditions, such as suppression of p53 (28, 29).

Senescence is thought to be closely linked to another possible mechanism of dormancy, autophagy. Like senescence, autophagy occurs naturally in cells. Its primary role is to maintain homeostasis in healthy cells by degrading potentially harmful components of the cell, such as old organelles and misfolded proteins. This helps prevent the buildup of reactive oxygen species, while also recycling necessary components to be reused in the production of new proteins. Autophagy does this through the creation of the autophagosome, which sequesters the products to be degraded. Once this double-membraned vesicle is formed, it then fuses with the lysosome,

forming the autophagolysosome. This highly acidic vesicle, as well as its contents, is then degraded. This mechanism is regulated by several different molecules, including mTOR, AMPK (31), and Beclin-1, which allows tumor cells to avoid CTL-mediated lysis (32).

Because of its role in maintaining homeostasis, autophagy in a natural, healthy state is thought to be cytoprotective. However, this is not always the case. Four different types of autophagy have been proposed (33). The first is the cytoprotective function, which maintains homeostasis and allows tumor cells to resist apoptosis in response to stress or cancer therapy. It is thought that blocking this function will sensitize these cells to the apoptosis inducing function of cancer therapies. The second is the cytotoxic function, which promotes tumor cell killing. This function is usually not seen with conventional anti-tumor therapies (33). The third function is a non-protective form of autophagy. With this type, the cells appear to be autophagic, however, when autophagy is blocked, the cells do not appear to be more or less sensitive to treatment. The fourth type of autophagy is the cytostatic function, which leads to an inhibition of growth in the tumor cells, although the cells do remain viable. However, if this form of autophagy is blocked, it will actually protect the tumor cells from anti-tumor therapies (33).

In healthy cells, basal levels of autophagy are maintained in order to recycle intercellular components and to regulate metabolism. This protective autophagic response is beneficial because it clears away any harmful proteins and toxic free radicals. However, early cancerous cells have been found to undergo less autophagic degradation than healthy cells (34), suggesting that as levels of autophagy decline, normal cells are more susceptible to becoming tumor cells. This is due to the loss of the protective aspects of autophagy. This suppression of autophagy is only seen early on during tumorigenesis however, because as tumor cells progress and are faced with stressful conditions such as hypoxia and immune cells infiltration, they begin to up-regulate

autophagy to serve as protection against the immune response. What controls this shift between down-regulation and up-regulation of autophagy or shift from one type to another is still unclear. Understanding this process is vital, as it could eventually lead to new therapeutic strategies against cancer (34-37).

Autophagy and senescence as mechanisms for dormancy are thought to be very closely linked. Both autophagy and senescence have been seen as responses to conventional anti-tumor therapies. Both mechanisms can also serve cytoprotective as well as cytotoxic functions. But how these two are related is not well understood. One hypothesis is that autophagy and senescence induction are tied to similar pathways, and therefore one can lead to a more rapid induction of the other. On the other hand, some studies have suggested that autophagy and senescence have an inverse relationship. If one mechanism is inhibited, then the other is promoted. This type of relationship would imply that both mechanisms are cytoprotective and serve as backup responses to one another. Further studies are needed however, before a consensus can be reached on the nature of this relationship (28). Because the possible relationship between these mechanisms, it may be necessary to target both when studying possible anti-tumor dormancy therapies.

Impact of Autophagy on the Immune Response

Recent preclinical studies have found that not only does autophagy serve to protect against stressful conditions, but it also affects the immune response. In fact, autophagy is involved in antigen processing and presentation via lysosome-MHC class II pathway in order to elicit the immune response (38). Since autophagy can play a role in the differentiation and

activation of immune cells, defects in autophagy-related genes can lead to immune diseases or the promotion of tumor escape from the immune response (38).

In a study by Baghdadi et al., it was found that the chemotherapy-induced immune response can be suppressed by T cell immunoglobulin- and mucin domain-containing molecule-4 (TIM-4), which is expressed by tumor-associated myeloid cells. TIM-4 interacts with adenosine monophosphate-activated protein kinase α1 (AMPKα1), a metabolic regulator, which then activates autophagy-mediated degradation of tumor cells. This degradation results in much lower levels of antigen presentation, and thus there is less activation of the CTL response. When this TIM-4-AMPKα1 autophagy is blocked however, it increases the efficacy of chemotherapy by increasing the CTL response (39).

Autophagy also has an effect on the induction of immunogenic cell death (ICD), which helps stimulate the immune response. Autophagy is unnecessary for chemotherapy-induced cytotoxicity; however it is needed to induce ICD. Without autophagy, the tumor cells do not secrete ATP, which is a key danger signal and a major hallmark of ICD (40). In fact, if several autophagy-related genes, such as *ATG3*, *ATG5*, and *ATG7*, are knocked down, then the ATP secretion usually seen during ICD is abolished. ATP secretion is important for ICD because it is one of the strongest trafficking signals for macrophages and dendritic cells (DCs). It does this through binding the P2Y2 receptors found on most myeloid cells (41). Autophagy incompetent cells still undergo similar apoptosis and necrosis levels as autophagy competent cells; however, they do not release ATP, and therefore, cannot recruit immune cells to the tumor microenvironment or effectively induce anti-tumor immune response. Thus, tumor cells which are not autophagy competent can possibly escape triggering the immune response through ICD (42).

It has been shown that reactive oxygen species (ROS)-based endoplasmic reticulum stress also triggers ICD after hypericin-mediated photodynamic therapy (Hyp-PDT), although it was initially unclear whether this was also due to autophagy induction. When autophagy was knocked down in tumor cells, no change to ATP secretion was seen following Hyp-PDT. However, there was greater expression and membrane translocation of Calreticulin (CRT), another major hallmark of ICD. This suggests that in general, autophagy actually helps protect tumor cells from ROS produced by Hyp-PDT, since there was a better ICD response when autophagy was not occurring. Therefore, unlike chemotherapy-induced autophagy, ROS-induced autophagy allows the tumor cells to evade stimulating the immune response through ICD (43).

Strategies to Overcome Tumor Dormancy

Of the proposed strategies to overcome tumor dormancy, one method is to use stereotactic body RT (SBRT), in which the patient receives only a few fractions of RT at a much higher dose in an attempt to induce higher rates of apoptosis and necrosis in the tumor cells. The standard dose for RT in a clinical setting is 25 fractions of 2Gy over 5 weeks, for a total dose of 50Gy. It is unknown however, if dose and fractionation have an effect on the anti-tumor immune response, which would be important to know when designing an effective combination therapy (44, 45). SBRT uses a total of 50-60Gy split into 3-5 fractions of 10-20Gy each (46). It is thought that such a high amount of radiation at once would be toxic to patients, however early studies have suggested otherwise. 19.5-22.5Gy split into 3 fractions of 6.5-7.5Gy each has shown some potential in thoracic tumors, so Bondiau et al. created a clinical study to see the

effects in breast cancer patients. With 3 fractions in combination with chemotherapy, there were no signs of skin toxicity, and there was a promising pathologic response. It is still unknown how effective this therapy is in the long term compared to the standard 2Gy therapy, so further follow up is needed. However, because of the lack of toxicity, it is possible that a higher dose, such as 3 fractions of 10-20Gy, could be used (45). In a more recent study, they also found that treating patients with 25.5Gy delivered in 3 fractions of 8.5Gy has no adverse effects (47). It is thought that by treating patients with much higher doses, the tumor cells will be eliminated quickly, before they have a chance to escape and become dormant. That could prevent tumor cells from ever entering the circulation, thus preventing the threat of relapse entirely.

Another proposed strategy to overcome dormancy is to treat patients with combination therapies. In some cases, conventional therapies are able to stimulate the anti-tumor immune response, resulting in ICD. ICD has been shown to target treatment-resistant tumor cells, making this a very desirable therapeutic strategy (48). There are 3 major hallmarks of ICD, including secretion of ATP, cell surface expression of CRT, and secretion of high mobility group box-1 (HMGB1) (48). ATP serves as a major recruitment signal for DCs by binding the P2Y2 receptor found on myeloid cells. ATP also stimulates the secretion of IL-1β by myeloid cells, which is a pro-inflammatory cytokine and which helps with the maturation of both CD4+ and CD8+ T cells (48). CRT serves as an engulfment signal to DCs by binding to CD91, which is found on the surface of DCs and macrophages. Once a DC binds CRT to CD91, it ingests the entire cell expressing CRT through receptor-mediated endocytosis. This in turn facilitates presentation of tumor antigens to T cells. HMGB1 is a pro-inflammatory signal, which can bind to TLR4 on DCs and macrophages and stimulate the release of pro-inflammatory cytokines, and in turn stimulates the maturation of antigen presenting cells (41). The secretion of ATP occurs

independently from the expression of CRT and HMGB1 in ICD. In addition, it has been found that ATP secretion only occurs if the tumor cells were undergoing autophagy beforehand and it is caspase-dependent. Understanding the mechanisms behind ATP secretion and ICD can help improve the development of more efficacious ICD-inducing therapies (49).

Four different chemotherapeutic agents so far have been shown to fully induce ICD: cyclophosphamide, doxorubicin (Adriamycin-ADR), oxaliplatin, and mitoxantrone (16). While other chemotherapeutic agents are not able to do the same, most can induce at least some aspects of ICD. For example, one study found that docetaxel, a widely used chemotherapeutic drug, induced very high levels of expression of CRT, but little to no expression of ATP or HMGB1. While it is unable to induce ICD, docetaxel still induces immunogenic killing of tumor cells through the direct upregulation of other molecules, such as carcinoembryonic antigen (CEA) on the tumor cells. After treating MCF-7 breast tumor cells with docetaxel, greater rates of CEA-specific CTL-mediated tumor cell killing were seen. Even when the tumor cells became resistant to the toxic effects of docetaxel, they still experienced high levels of CTL-mediated cell death due to the increase of CEA and CRT expression caused by the drug (16). Therefore, despite the inability of most chemotherapeutic agents to induce classic ICD, they can still stimulate the immune system through upregulation of tumor antigens such as CEA, even after tumor cells have acquired a resistance to the drugs.

Several preclinical studies have shown that while RT and immunotherapy alone are not able to fully eliminate tumor cells; they have the potential to be very effective if used in combination. It has been shown that RT is able to counteract immune evasion by the tumor cells in various ways. First, RT causes the tumor cells to not only produce a more diverse pool of antigens, but to also express higher levels of MHC class I (50, 51), which increases the chances

of stimulating the immune system. RT has also been shown to cause dying tumor cells to secrete HMGB1 (50, 51). Even without inducing the release of CRT and ATP, this is an effective means to help stimulate the immune system. HMGB1 is a strong danger signal because it triggers the inflammatory response, so high expression of it by dying tumor cells helps stimulate the activation and maturation of DCs, which ultimately leads to greater activation of the adaptive immune response. Another receptor upregulated on dying tumor cells by RT is the death receptor Fas, which promotes CTL-mediate tumor killing through Fas-Fas-L pathway (50). Therefore, RT is effective at strengthening the immune response on a local level, which in turn leads to greater tumor cell killing overall (50, 51). One challenge to combining RT with immunotherapy however is that RT is also a strong inducer of non-immunogenic apoptosis. This type of cell death could potentially lead to tolerance because of the lack of an inflammatory response. When this occurs, DC maturation is not stimulated, so the adaptive immune response is not activated, resulting in immunological tolerance (50, 51). Therefore, it is necessary to find a way to overcome this tolerance when combining these two therapies.

Another type of combination therapy involving RT was proposed by Liang et al. after they found that with both BALB/c and C57/BL6 mice injected with tumors, higher doses of radiation induced a state of equilibrium in the tumor cells. In order to determine what factors contribute to maintaining stable tumors, the group then depleted CD8+ and CD4+ T cells, alone and in combination. They found that without CD8+ T cells, the tumor rapidly grew. They also found that initially, higher doses of radiation cause a greater infiltration of CD8+ cells, however local T cell numbers decrease over time, allowing the tumor to remain stable. So the group determined that it is necessary to either increase T cell activation or reduce T cell inhibition in order to overcome this stable tumor state post-RT. They proposed blocking programmed death-

ligand 1 (PD-L1) expression on the tumor cells using antibodies, as PD-L1 has been shown to inhibit a T cell response by binding to the PD-L receptor on activated T cells and causing them to become anergic. In their model, they were able to clear the tumors in their mice using that method, suggesting that by blocking negative immune regulators, it is possible to overcome tumor resistance to RT and immunotherapy (18).

A final proposed strategy to overcome dormancy is to block the mechanisms responsible for dormancy. When autophagy was inhibited in chemo-resistant tumor cells, the cells became resensitized to therapy and an increase in tumor cell death was seen. This led to autophagy becoming a major therapeutic target, with many groups studying the effects of blocking autophagy in combination with chemotherapy (35). The antimalarial drug chloroquine (CQ) has been found to be very effective at blocking autophagy. CQ blocks the fusion of the lysosome with the autophagosome, thus preventing the degradation of these vesicles (52). This results in a buildup of acidic vesicles within the cell. CQ is then combined with conventional or combination therapies, to help improve tumor cell killing. It is suggested that chloroquine will negate the cytoprotective effects of autophagy, and thus sensitize the tumor cells to treatment-induced apoptosis or necrosis. However, this is only an effective strategy against cytoprotective autophagy. If tumor cells are undergoing cytotoxic autophagy, blocking with CQ would actually do more harm than good, as it will instead lead to the protection of tumor cells. Therefore it is important to confirm that tumor cells are undergoing cytoprotective autophagy before utilizing this strategy.

When autophagy has been shown to be cytoprotective in tumor cells, preclinical studies have shown that blocking with CQ is very effective at enhancing tumor cell killing. In one study, when CQ treatment was combined with chemotherapy, 67NR and 4T1 breast cancer cell

lines became sensitized to treatments that targeted LY294002 or rapamycin treatment. Both the short term and long term viability and survival of the tumor cells significantly decreased (53). In another study that combined CQ treatment with RT, it induced a much stronger immune response against tumor cells, and offered protection against further tumor challenges in C3H mice (54). After the mice were injected with MCaK murine mammary tumor cells, they underwent RT. Only a portion of the mice were cured of their tumors, however, that number increased when the mice were also treated with CQ following RT. The group found that this was due to CQ pushing the tumor cells towards apoptosis, which RT alone was unable to do. CQ was also shown to increase the immunogenicity of RT in their model, by causing dying tumor cells to express danger signals such as H2-K^k. Finally, this group was able to show that CQ can help protect against future tumor challenges, by creating an anti-tumor vaccine. When CQ treatment was included in the preparation of the vaccine, almost half the inoculated mice were protected from tumors, while the mice injected with the CQ-free vaccine showed little to no tumor protection (54). This suggests that blockade of autophagy may be an effective method to improve the efficacy of conventional tumor therapies.

These proposed strategies to overcome dormancy have a lot of promise. Current cancer therapies are not effective enough at eliminating tumor cells and preventing tumor relapse, but if they are used in combination with each other or with new therapies that prevent or prolong dormancy, it may be possible to prevent tumor relapse. Our group was interested in the possible efficacy of these new strategies to overcome tumor relapse, so we studied the effects of a high dose of RT and a combination of chemotherapy and immunotherapy, since chemotherapy is immunogenic and its efficacy depends on the immune response (16). We also looked at the effects of blocking autophagy by CQ in combination with these therapies. We sought to

determine whether autophagy is the primary mechanism of tumor dormancy, and whether its blockade in combination with conventional therapies would be more effective at eliminating tumor cells or prolonging dormancy to prevent tumor relapse.

Materials and Methods

Tumor cell line

Mouse mammary carcinoma cell line (MMC), derived from spontaneous mammary carcinoma of FVBN202-transgenic mice as previously described (55), was used for this study. MMC were cultured in RPMI 1640 with 2mM L-glutamine (Life Technologies, Carlsbad, CA) and 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA) (RPMI+FBS) at 37ºC with 5% CO2. For passaging, the MMC were detached using 0.25% Trypsin/0.02% Ethylenediaminetetraacetic acid (EDTA) solution (Quality Biologicals Inc., Gaithersburg, MD). For flow cytometry analysis, MMC were detached with 2.5mM EDTA in phosphate-buffered saline (PBS).

Statistical Analysis

All data was analyzed using a one-tailed Student's *t*-test. P-values ≤ 0.05 were considered significant. Proliferation data was normalized to one million cells and then presented as fold change compared to day 0.

Experimental Design

For each treatment type, the MMC were treated once daily for either 1 or 3 days. Treating once allowed us to determine the immediate effects of each therapy while treating three times allowed us to determine the effects on the MMC following multiple treatments. Three doses were selected because during preliminary studies, no difference was seen between MMC treated for three days compared to those treated for five days.

For blockade of autophagy, CQ (Sigma-Aldrich, St. Louis, MO) was selected over other autophagy inhibitors because it is the only inhibitor that is already FDA approved and unlike genetic silencing, it is also translatable to a clinical setting.

Determining the effect of RT on tumor killing and tumor dormancy with and without blockade of autophagy

MMC were treated with the clinically relevant fractionated dose of RT (2Gy) or SBRT dose (18Gy) in the presence or absence of CQ (10μM), or CQ alone. For each round of treatment, MMC were first treated with 10μM of CQ and then incubated for 3 hours in the dark as previously described (56, 57). Cells were treated with CQ first in order to block any basal autophagy and to prevent the completion of any autophagy that is induced by RT. After 3 hours, the MMC were irradiated in a Cesium-137 irradiator, while CQ was still present in the culture. Afterwards, adherent MMC were washed with sterile PBS to remove CQ, and fresh RPMI+FBS was added to the culture. Any non-adherent floater cells in the media were spun at 1200 rpm for 8 minutes at 20 ºC and then added back to the culture flasks. Cells received 1 or 3 daily doses of 2Gy or 18Gy with and without CQ. One hour, 24 hours, or 72 hours after the final treatment,

any morphologic changes to the MMC were noted by visualizing the cells under a microscope at 25x. The MMC were detached with 2.5mM EDTA and counted using trypan blue exclusion (described below). At this time, the media was saved so that the floater cells could be included in the final count. Some cells were also then used for flow cytometry analysis in order to determine the frequency of apoptosis, autophagy, senescence, CRT expression, and cell cycle arrest, as described below.

Determining the effects of ADR chemotherapy, IFN-γ immunotherapy, and chemo-immunotherapy (ADR+IFN-γ) on tumor killing and tumor dormancy with and without blockade of autophagy

MMC were treated with either ADR (Sigma-Aldrich, St. Louis, MO) (1μM) (58), IFN-γ (Biolegend, San Diego, CA) (50ng/mL) (59), CQ (10μM) (56, 57), or a combination thereof.

Individual Treatments: For chemotherapy, ADR was added to the cell culture and the cells were incubated at 37ºC with 5% CO2 for 2 hours in the dark. The media was then removed, the adherent MMC were washed once with sterile PBS, and fresh RPMI+FBS was added to the culture.

For IFN-γ immunotherapy, IFN-γ was added to the culture and maintained approximately 19 hours at 37ºC with 5% CO2.

For blockade of autophagy, CQ was added to the cell culture and the cells were incubated at 37ºC with 5% CO2 for 5 hours in the dark. The media was then removed, the adherent MMC were washed once with sterile PBS, and fresh RPMI+FBS was added to the culture.

Combination Treatments: For a combination of CQ, ADR, and/or IFN-γ treatment, the MMC were always treated with CQ for 3 hours first. Then, while maintaining CQ in the culture, ADR was added for an additional 2 hours. After those 5 hours, the media was then removed, the adherent MMC were washed once with sterile PBS, and fresh RPMI+FBS was added to the culture. IFN-γ was then added to the cells and maintained in culture for the entirety of the experiment.

For any of the individual or combination treatments, if floater cells were required to be kept in culture, during the wash step, the media was collected instead of discarded and spun at 1200 rpm for 8 minutes at 20 ºC in order to collect the floater cells and add them back to the culture.

If the MMC received more than 1 round of treatment, then 24 hours after the initial start of treatment, another dose of CQ was added to the culture and the cycle repeated. Cells received 1 or 3 daily rounds of treatment. After either 24 hours or 48 hours following the treatment, any morphologic changes were noted by visualizing the cells under a microscope at 25x, the MMC were detached with 2.5mM EDTA, and then they were counted using trypan blue exclusion. At this time, the media was saved so that the floater cells could be included in the final count. Some cells were also used for flow cytometry analysis in order to determine the frequency of apoptosis, autophagy, senescence, CRT expression, and cell cycle arrest as described below.

Monitoring tumor dormancy and relapse in vitro

After MMC were treated with RT, ADR or combined ADR+IFN-γ and dormancy was established in vitro, cells were detached and analyzed for cell proliferation and apoptosis every 1-3 weeks. Morphologic changes were also monitored. A group of MMC were set aside to

monitor for long-term recovery of proliferation in the absence of further treatment. They were incubated at 37ºC with 5% CO2 and monitored daily. If there were very few viable cells present, the MMC were monitored visually until they showed signs of proliferation.

Trypan Blue Exclusion

In order to count the MMC and determine viability, the MMC were first detached using 2.5mM EDTA for 5 minutes at 37°C and 5% CO2, neutralized with an equal volume of RPMI+FBS, and then spun at 1200 rpm for 8-10 minutes at 20 ºC. After centrifugation, the supernatant was removed and the cell pellet was resuspended in RPMI+FBS. The MMC were either diluted 1:10 for a small pellet or 1:100 for a larger pellet with 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO). The mixture was then added to a hemocytometer (Hycor, Garden Grove, CA) and the MMC were counted. Any trypan blue negative (white) cells were counted as viable, while positive (blue) cells were counted as dead. Once all the MMC within the grid were counted, the total number of cells was determined by dividing the total number of cells by 9 and then multiplying by the dilution factor (10 or 100) times 10^4. To determine the percent viability, the number of viable cells was divided by the total number of cells.

Flow Cytometry

Data acquisition was performed using a Becton-Dickinson FACSCanto II flow cytometer. For each sample 10,000-50,000 events were collected. Samples were analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

Fluorochromes

To stain for apoptosis and necrosis, APC-conjugated annexin V (Biolegend, San Diego, CA) and propidium iodine (Biolegend, San Diego, CA) (PI) were used. To look at CRT surface expression, cells were first stained with purified mouse anti-mouse CRT (BD Biosciences, San Jose, CA), followed by FITC-conjugated goat anti-mouse IgG (Poly4053) (Biolegend, San Diego, CA). For autophagy staining, acridine orange (Invitrogen, Carlsbad, CA) (AO) was used. To stain for cell cycle arrest, PI was used. To look at senescence, bafilomycin A1 (VWR, Radnor, PA) and C12FDG (Life Technologies, Carlsbad, CA) were used. Antibodies were used at the concentrations recommended by the manufactures. Cellular staining was performed as described below.

Determination of Type of Apoptosis and Necrosis

To look at the viability, double staining of MMC was performed with annexin V and PI as previously described by our group (60). A third color was also used for some cells to look at surface expression of CRT. After detaching and counting the MMC, they were added to a round bottom polystyrene tube and washed twice in 2mL PBS with 2% FBS, 0.1% sodium azide (FACS buffer) at 1200 rpm for 8 minutes at 4ºC. The supernatant was removed and the pellet was resuspended in 200uL FACS buffer. The MMC were then stained with purified mouse anti-mouse CRT for 20 minutes on ice, in the dark and then washed twice in 2mL FACS buffer at 1200 rpm for 8 minutes at 4ºC. Next the supernatant was poured off and they were again resuspended in 200uL FACS buffer and stained with FITC-conjugated goat anti-mouse IgG for another 20 minutes on ice, in the dark. They were then washed once in 2mL FACS buffer at 1200 rpm for 8 minutes at 4ºC and then once again in 2mL of 1x annexin buffer (BD

Biosciences, San Jose, CA). The supernatant was removed and the pellet was resuspended in 200uL of 1x annexin buffer. The MMC were then stained with annexin V for 15 minutes on ice, in the dark. After the 15 minutes, PI was added, followed by 400uL of 1x annexin buffer, and the data were acquired using the cytometer immediately after.

Determination of Autophagy

In order to determine whether the MMC were autophagic or not, they were stained with AO. After the MMC were detached and counted, they were added to a round bottom polystyrene tube and washed twice in 2mL PBS at 1200 rpm for 8 minutes at 4ºC. The supernatant was poured off and the pellet was resuspended in 396uL of PBS. A working stock of AO was created by adding 1uL of AO to 99uL of PBS to create a 1:100 dilution. 4uL of this stock was then added to the MMC to create a final dilution of 1:10000 AO in PBS. The MMC were allowed to stain for 10 minutes on ice, in the dark, and then data were acquired using the cytometer immediately after (61).

To analyze the data, the geometric mean fluorescence (GMF) was determined for both the 488-610 red channel and the green channel using the FCS Express software (De Novo Software, Los Angeles, CA). In order to determine the GMF of autophagy, the mean for green was then subtracted from the mean for red.

Determination of Cell Cycle Arrest

To examine cell cycle arrest, two separate protocols were used. The two protocols were similar and produced similar results, but they differed in how the cells were fixed and permeabilized. In one, MMC were fixed and permeabilized in ethanol, while in the other, they

were permeabilized with sodium citrate (EMD, Billerica, MA) and Triton X 100 (Sigma-Aldrich, St. Louis, MO). The first protocol was useful when samples could not immediately be analyzed, because ethanol fixation allows the samples to be stored for several months. The second protocol did not allow long-term storage of samples, but produced clearer data because there was less loss of cells than with fixation by ethanol. These two protocols are described below. The data for both protocols were analyzed using the Multicycle feature of the FCS Express software (De Novo Software, Los Angeles, CA).

Ethanol fixation cell cycle protocol: Prior to staining, 70% ethanol was prepared and brought to 4ºC or colder. For each sample being stained, 4.5mL of ethanol was added to a conical polypropylene tube and kept on ice. To prepare the staining solution, to 10mL of 0.1% Triton X-100 in PBS, 2mg of DNase free RNase (Sigma-Aldrich, St. Louis, MO) and 200ug of 1mg/ml PI were added. This staining solution was prepared fresh each time this stain was performed. After the MMC were detached and counted, one million cells were suspended in 5mL of PBS in a conical polypropylene tube. They were centrifuged at 1200 rpm for 8 minutes at 4ºC and then the supernatant was immediately poured off. The pellet was then resuspended in 0.5mL PBS. This suspension was added to one of the ethanol tubes previously prepared and allowed to fix on ice for at least 2 hours. If needed, the cells in ethanol were stored at -20°C until the samples could be run on the cytometer. After fixation, the ethanol cell suspension was centrifuged at 1200 rpm for 8 minutes at 4°C and then the ethanol was completely decanted off. The MMC were then resuspended in 5mL PBS and allowed to sit for 1-2 minutes before centrifuging them again at 1200 rpm for 8 minutes at 4°C. After pouring off the PBS, the MMC were resuspended in 0.5mL of the premade staining solution and kept covered at 37°C for an hour. Immediately

before data were acquired on the cytometer, the MMC were transferred to round bottom polystyrene tubes and placed covered on ice.

Non-fixation cell cycle protocol: Prior to staining, the staining solution was prepared. The final concentrations used in the stock were 3.8x10-3M sodium citrate in water, 0.1% Triton X-100, 0.05 mg/mL PI, and 7K units/mL DNase free RNase. This staining solution was prepared fresh each time the stain was performed.

After the MMC were detached and counted, one million cells were suspended in 5mL PBS in a conical polypropylene tube. They were centrifuged at 1200 rpm for 8 minutes at 4ºC and then the supernatant was immediately poured off. The pellet was resuspended in 1mL of the premade staining solution and then allowed to sit, covered, at 4 ºC overnight. Before data was acquired using the cytometer, the cell suspension was transferred to a round bottom polystyrene tube.

Determination of Senescence

After the MMC were detached, they were resuspended in 1mL RPMI in a conical polypropylene tube. 100nM of bafilomycin A1 was added to the tube in order to induce lysosomal alkalinization. The MMC were allowed to incubate for 1 hour at 37ºC with 5% CO2. Next, 10µM of C12FDG was added the MMC were incubated for another 1-2 hours. The MMC were then washed with PBS twice at 1200 rpm for 8 minutes at 4ºC and resuspended in ice cold PBS at a concentration of $1x10^6$ cells/mL. The MMC were transferred to a round bottom polystyrene tube and then data were acquired using the cytometer immediately after (62).

Results

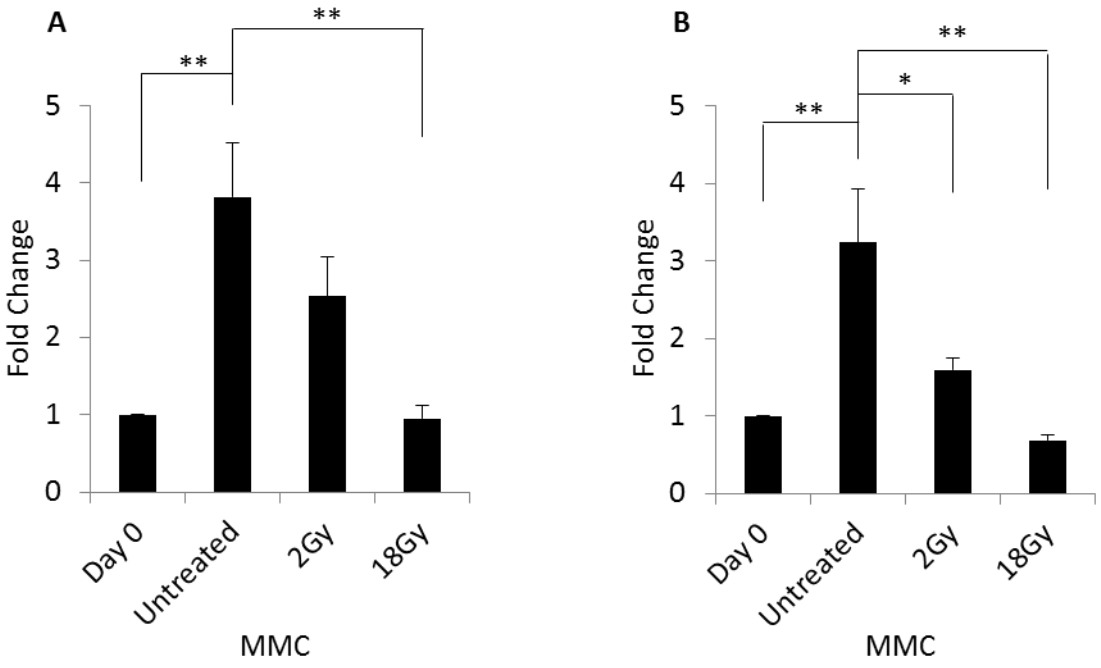
Dose-dependent inhibition of tumor cell proliferation by RT

In order to compare the effects of the clinically relevant fractionated dose of 2Gy/day to the SBRT dose of 18Gy/day, MMC were irradiated with one fraction of either dose per day for 1-3 days. 24-72 hours after the final fraction of RT, the MMC were detached and the total cells were counted using trypan blue exclusion to determine any changes in cell proliferation. After a single fraction of RT, only MMC treated with 18Gy showed a significant drop in proliferation compared to untreated MMC (Figure 1A, $p=0.009$). After three fractions of RT, both 2Gy and 18Gy treated MMC showed significant inhibition of proliferation (Figure 1B, 2Gy: $p=0.022$, 18Gy: $p=0.002$), although the inhibition was greater after 18Gy.

RT induces and expedites completion of autophagy in a dose-dependent manner

In order to determine whether RT-induced inhibition of tumor cell proliferation was associated with the induction of autophagy and/or senescence, the tumor cells were treated with either 2Gy or 18Gy as described in Figure 1. With just RT alone, it was not possible to visualize autophagy because of the RT-induced rapid completion of autophagy and resulting degradation of acidic vesicles. Thus, to detect autophagy, we also treated the cells with CQ three hours prior to and during RT in order to block the completion of autophagy, resulting in a buildup of acidic vesicles within the cell. This buildup in the presence of CQ shows the presence of autophagy. Cells were then stained with AO in order to quantify the accumulation of acidic vesicles through

Figure 1. RT results in a decrease in proliferation compared to untreated cells. MMC were treated with either 2Gy or 18Gy RT once per day for either one (A, n=3) or three (B, n=6) days. With three daily treatments, the floater cells were discarded on days 0, 1, and 2, and total cells were analyzed on day 3. The cells were detached 72 hours (A) or 24 hours (B) after the final fraction of RT, and the total cells were counted using trypan blue exclusion in order to determine any changes to proliferation. The data were normalized to one million cells and presented as fold change compared to Day 0.



flow cytometry. As can be seen in Figure 2A, presence of CQ revealed the occurrence of autophagy in all conditions after a single fraction of RT; however, RT+CQ showed a greater difference in levels of autophagy (2Gy+CQ: p=0.005, 18Gy+CQ: p=0.005) compared with baseline autophagy detected by CQ over untreated MMC (p=0.033). After three fractions of RT, 2Gy+CQ and 18Gy+CQ showed significantly higher levels of autophagy than their respective RT doses alone (Figure 2B, 2Gy+CQ: p=0.028, 18Gy+CQ: p=0.021). Three daily treatments of MMC with CQ did not show a significant level of autophagy compared with untreated (Figure 2B). In addition, MMC treated with 2Gy+CQ did not show significantly higher levels of autophagy than the baseline autophagy using CQ alone; however, 18Gy+CQ did (p=0.039), suggesting that multiple fractions of 18Gy RT induces greater levels of transient autophagy than that of 2Gy RT (Figure 2B). This data overall suggests that RT induces autophagy in a dose-dependent manner. The tumor cells were also stained for beta-galactosidase in order to detect senescence; however none was detected (data not shown).

After finding that RT can induce greater levels of autophagy in the tumor cells after multiple fractions, we wanted to know whether it would also result in morphological changes to the cells. Both 2Gy and 18Gy resulted in the enlargement of the tumor cells, which was more remarkable with 18Gy, regardless of the presence or absence of CQ. Cellular enlargement is known to be one of the features of autophagy (63, 64). This suggests that any changes seen are due to the induction of autophagy by RT rather than the completion of autophagy which was blocked by CQ (Figure 3).

Figure 2. RT induces autophagy in a dose-dependent manner. MMC were irradiated with 2Gy and 18Gy once per day for either one (A, n=5) or three (B, n=2) days. Because autophagy could not be detected in MMC treated with RT alone, the tumor cells were also treated with 10uM CQ three hours before and during each fraction of RT, in order block and visualize autophagy. With three daily treatments, the floater cells were discarded on days 0, 1, and 2, and total cells were analyzed on day 3. The cells were detached 1 hour (A) or 24 hours (B) after the final fraction of RT, stained with acridine orange, and analyzed through flow cytometry in order to measure accumulation of acidic vesicles. Levels of autophagy were detected by subtracting green fluorescence (non-acidic cellular components) from red fluorescence (acidic vesicles).

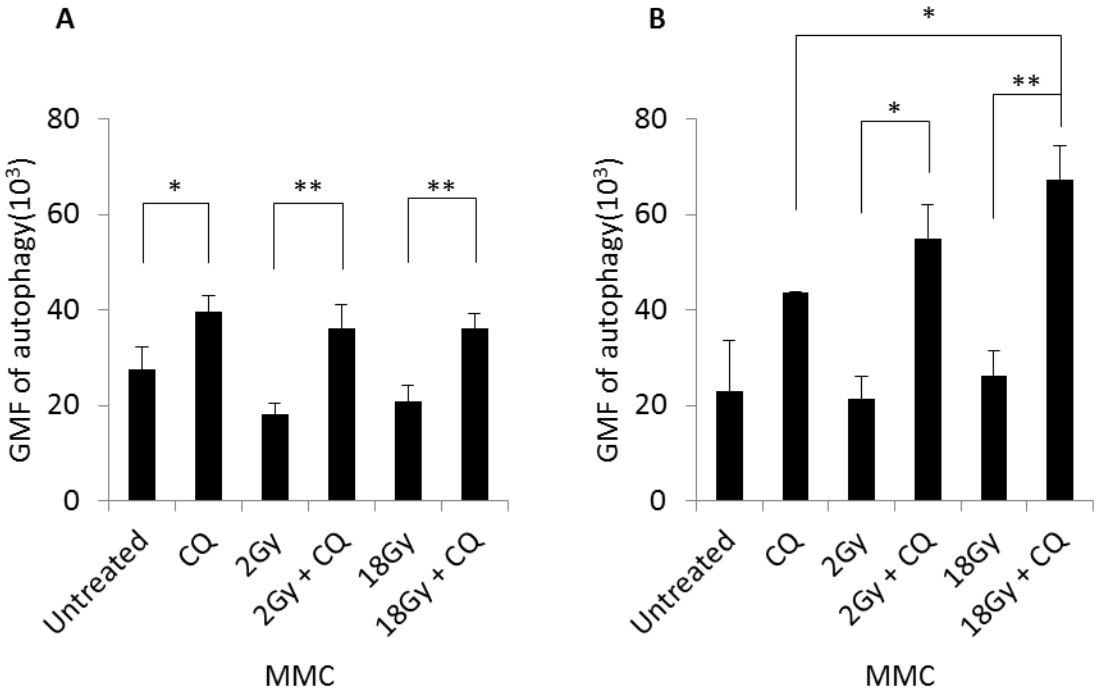
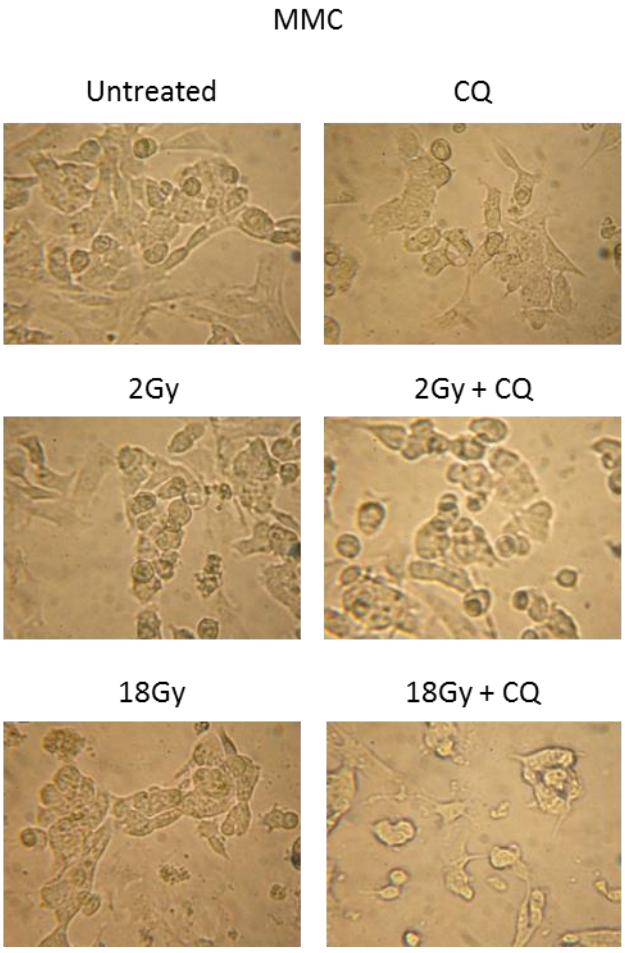


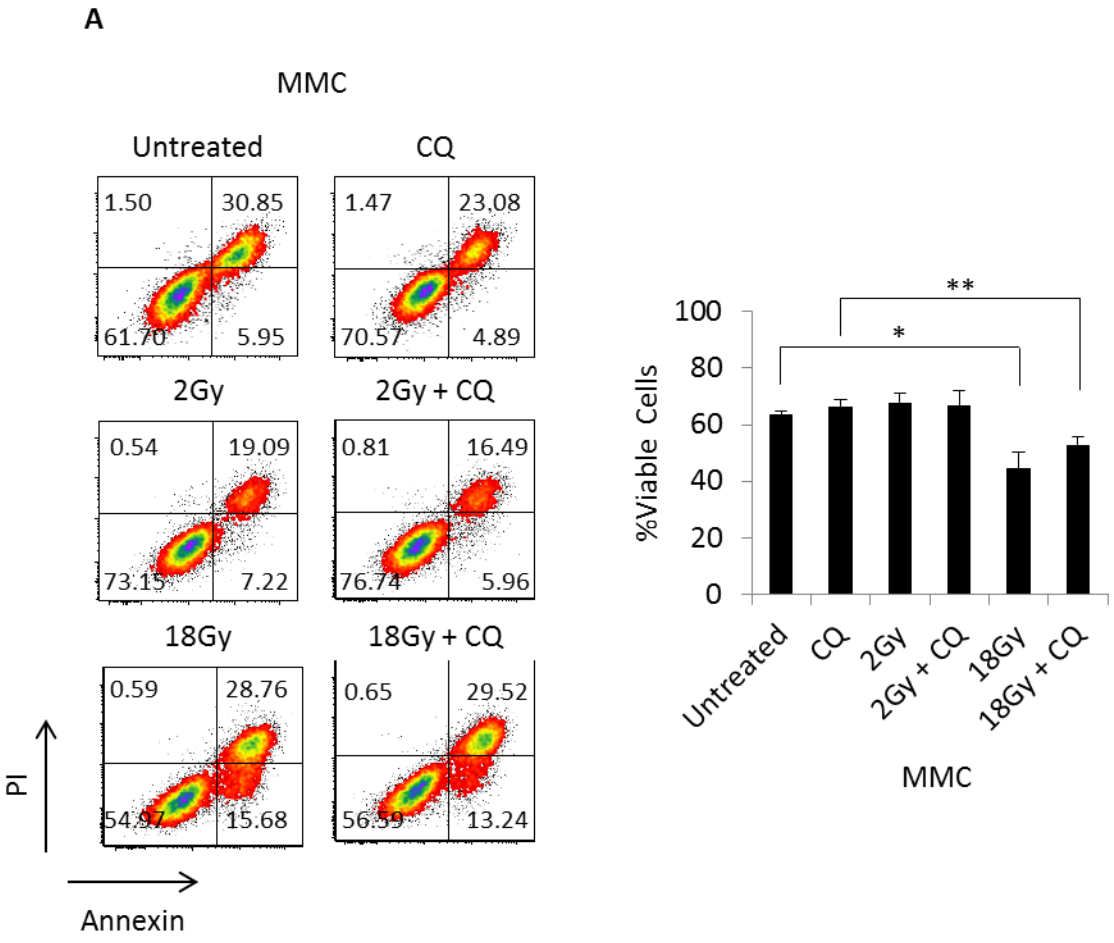
Figure 3. RT induces morphologic changes in MMC but CQ has no additional effect. MMC were irradiated with 2Gy and 18Gy, both with and without CQ. After three daily fractions of RT, the tumor cells were visualized under a microscope at 25x magnification.



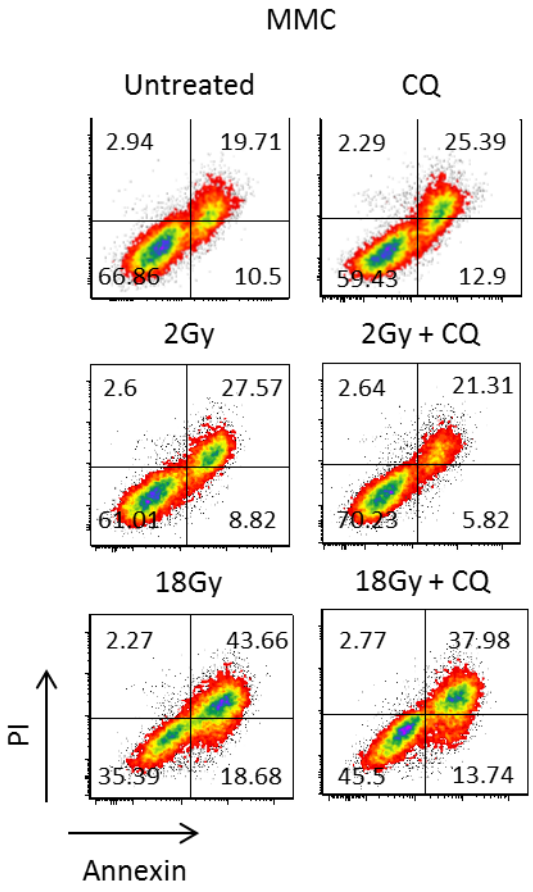
High dose RT induces apoptosis in tumor cells which is partially inhibited by the blockade of autophagy

In order to determine whether RT-induced autophagy is cytoprotective or cytotoxic and if blockade of autophagy could increase or decrease the apoptosis inducing function of RT, tumor cell apoptosis was determined in the presence or absence of autophagy blockade by CQ. After one fraction of RT, only 18Gy induced a significant drop in the viability of the tumor cells from an average of 63.6% to 44.6% (Figure 4A, 18Gy: p=0.016) which was slightly inhibited by CQ from an average of 63.6% to 53% (Figure 4A, 18Gy+CQ: p=0.008), when compared to the untreated MMC control, suggesting that RT induces a cytotoxic autophagy (Figure 4A). The drop in viability by 18Gy and 18Gy+CQ was due to an increase in Annexin V+/PI- early apoptosis, compared to the untreated MMC or CQ controls (Figure 4A, 15.68% vs. 5.95%, p=0.051 and 13.24% vs. 4.89%, p=0.057). Three daily fractions of 2Gy/day slightly induced Annexin V+/PI+ late apoptosis (Figure 4B, 27.57% vs. 19.71%) which was inhibited by blockade of autophagy in 2Gy+CQ/day (Figure 4B, 21.31% vs. 25.39%). 18Gy and 18Gy+CQ resulted in significantly lower viability in the tumor cells compared to the untreated MMC and CQ controls (Figure 4B, 18Gy: 38.97% vs. 66.08%, p=0.05, 18Gy+CQ: 44.62% vs. 67.01%, p=0.009). Blockade of autophagy by CQ slightly but not significantly improved the viability (Figure 4B, 2Gy+CQ: 68.32% vs.63.19%, 18Gy+CQ: 44.62% vs. 38.97%). 18Gy resulted in a significant amount of Annexin V+/PI+ late apoptosis (Figure 4B, 43.66% vs. 19.71%, p=0.012) and Annexin V+/PI- early apoptosis (Figure 4B, 18.68% vs. 10.5%, p=0.002), while blockade of autophagy in 18Gy+CQ resulted in a significant level of late apoptosis (Figure 4B, 37.98% vs. 25.39%, p=0.047). These data suggest that 18Gy is an effective inducer of apoptosis in MMC,

Figure 4. 18Gy/day RT induces apoptosis in tumor cells but addition of CQ has no added effect. MMC were irradiated with 2Gy and 18Gy once per day for either one (A, n=3) or three (B, n=2) days. Three hours before and during each fraction of RT, tumor cells were also treated with 10uM CQ in order block autophagy. With three daily treatments, the floater cells were kept in culture the entire time, and total cells were analyzed on day 3. The cells were detached 72 hours (A) or 24 hours (B) after the final fraction of RT, stained with APC-Annexin V and PI, and then analyzed for apoptosis using flow cytometry.



B


MMC
Untreated
CQ
2.94
19.71
66.86
10.5
2.29
25.39
59.43
12.9
2Gy
2Gy + CQ
2.6
27.57
61.01
8.82
2.64
21.31
70.23
5.82
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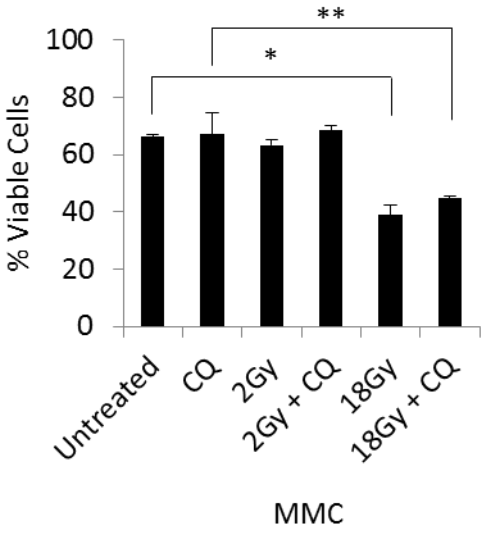
2.77
37.98
45.5
13.74
PI
Annexin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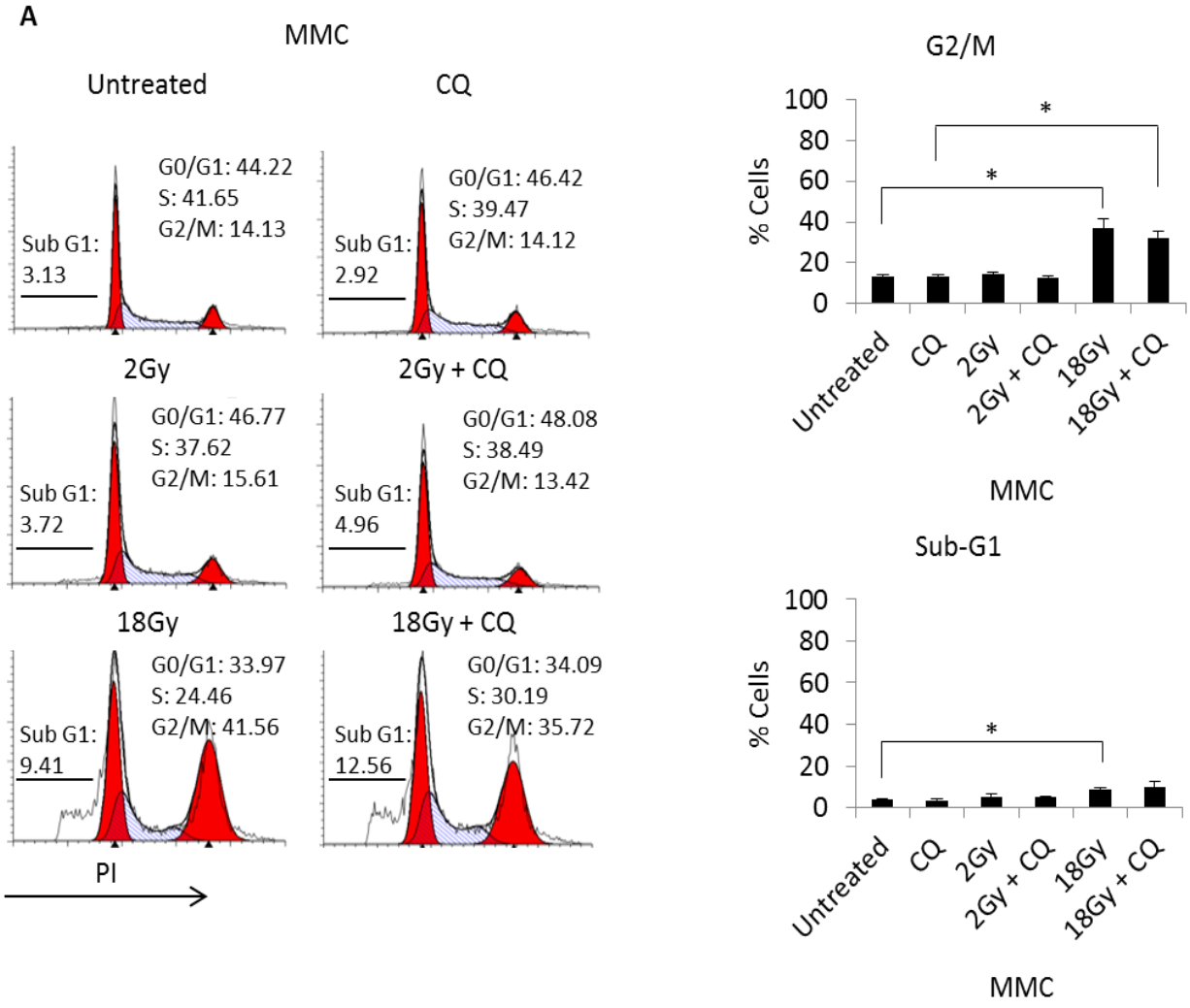
*
100
80
60
40
20
0
% Viable Cells
Untreated
CQ
2Gy
2Gy + CQ
18Gy
18Gy + CQ
MMC

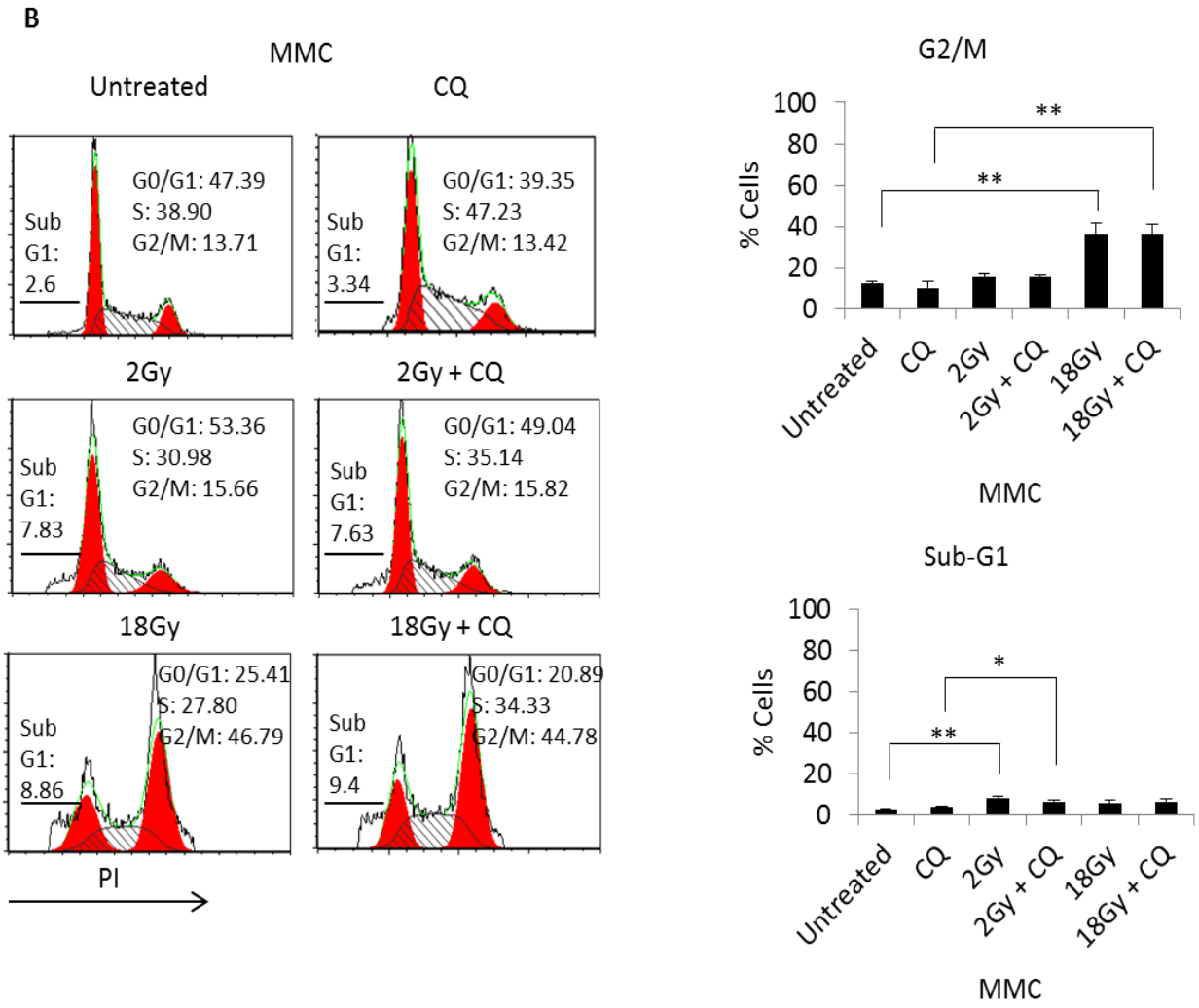
and that blockade of autophagy slightly protects the tumor cells from RT- induced apoptosis. In addition, the autophagy induced by 18Gy is not cytoprotective, since blockade of autophagy with CQ does not result in significantly less viability than RT alone.

RT induces G2/M and/or Sub-G1 arrest in a dose dependent manner

In order to determine whether inhibition of tumor cell proliferation and induction of apoptosis were associated with cellular arrest at a certain phase of the cell cycle, flow cytometry analysis was performed after PI staining of the cells. A single fraction of 2Gy, both with and without CQ, induced some arrest in the G0/G1 phase, although this arrest was not significantly greater than that seen in the untreated cells. G0/G1 are the growth phases of the cell cycle, so if more cells are arrested at this point, they would be viable, however not currently undergoing cell division, which would explain why 2Gy results in an inhibition of proliferation but not a significant drop in viability of the cells. 18Gy and 18Gy+CQ however, were seen to cause the MMC to arrest in G2/M, which was slightly reduced by CQ (Figure 5A, 18Gy: $p=0.018$, 18Gy+CQ: $p=0.016$). The G2 checkpoint is the final checkpoint after cells have undergone DNA replication in S phase but before they begin dividing in M phase. If cells arrest in G2, it suggests that there is DNA damage, likely caused by RT, and the cells are unable to divide as normal. 18Gy also resulted in a significantly greater level of Sub-G1 arrest in the cells which was reduced to an insignificant level by CQ (Figure 5A, $p=0.012$). After three fractions of RT, 2Gy and 2Gy+CQ are once again seen to induce some G0/G1 arrest. However, after 2Gy, both with and without blockade of autophagy, the tumor cells are also seen to arrest in Sub-G1 (Figure 5B, 2Gy: $p=0.001$, 2Gy+CQ: $p=0.007$). As with only a single fraction, 18Gy and

Figure 5. 2Gy/day RT induces some G0/G1 and Sub-G1 arrest, while 18Gy/day induces G2/M arrest. MMC were irradiated with 2Gy and 18Gy once per day for either one (A, n=2) or three (B, n=3) days. Three hours before and during each fraction of RT, tumor cells were also treated with 10uM CQ in order block autophagy. With three daily treatments, the floater cells were discarded on days 0, 1, and 2, and total cells were analyzed on day 3. The cells were detached 72 hours (A) or 24 hours (B) after the final fraction of RT, stained with PI and analyzed for cell cycle arrest through flow cytometry. The Sub-G1 population was analyzed separately from the other three populations.



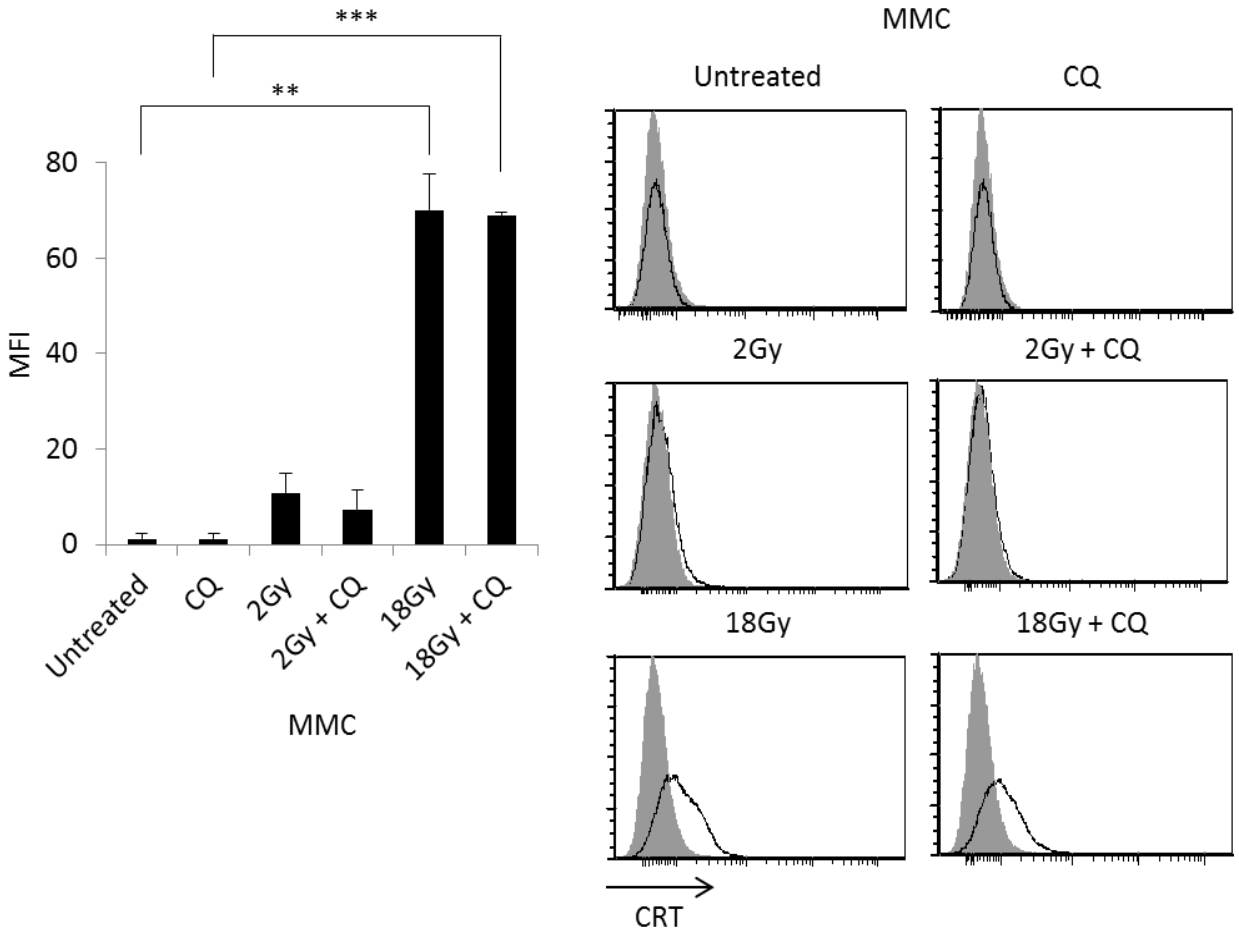

B
MMC
Untreated
CQ
Sub G1: 2.6
G0/G1: 47.39
S: 38.90
G2/M: 13.71
Sub G1: 3.34
G0/G1: 39.35
S: 47.23
G2/M: 13.42
2Gy
2Gy + CQ
Sub G1: 7.83
G0/G1: 53.36
S: 30.98
G2/M: 15.66
Sub G1: 7.63
G0/G1: 49.04
S: 35.14
G2/M: 15.82
18Gy
18Gy + CQ
Sub G1: 8.86
G0/G1: 25.41
S: 27.80
G2/M: 46.79
Sub G1: 9.4
G0/G1: 20.89
S: 34.33
G2/M: 44.78
PI
G2/M
% Cells
100
80
60
40
20
0
**
**
Untreated
CQ
2Gy
2Gy + CQ
18Gy
18Gy + CQ
MMC
Sub-G1
% Cells
100
80
60
40
20
0
*
**
Untreated
CQ
2Gy
2Gy + CQ
18Gy
18Gy + CQ
MMC

18Gy+CQ induce high levels of G2/M arrest in the tumor cells (Figure 5B, 18Gy: p=0.01, 18Gy+CQ: p=0.007).

RT induces immunogenic tumor cell killing in a dose-dependent manner

We next wanted to see whether the effects induced by RT on the tumor cells could also make them more susceptible to immune-mediated tumor cell killing. To do so, we looked at whether RT-induced apoptosis is also immunogenic. The three major hallmarks of ICD are the release of ATP, cell membrane translocation of the ER protein CRT, and release of HMGB1. We chose to focus on the cell membrane translocation of CRT, which could result in tumor cell uptake by antigen presenting cells. The subsequent presentation of tumor antigens would then result in the induction of the immune response. This is because antigen presenting cells express the CD91 receptor for CRT, and internalize apoptotic tumor cells that express the ligand CRT. CRT was selected as a marker specifically because of its role as an engulfment signal. Even if ICD is not fully induced in the tumor cells, membrane expression of CRT would facilitate phagocytosis of tumor cells by macrophages. We found that after three fractions of RT, only 18Gy/day resulted in a significantly greater overall level of membrane CRT compared to untreated MMC (Figure 6, MFI=70.11, p=0.006). 2Gy/day slightly increased membrane expression of CRT, although it was not significant (MFI=10.83). The blockade of autophagy by CQ slightly reduced levels of CRT expression in both 2Gy/day (MFI=7.41) and 18Gy/day (MFI=68.97). Baseline autophagy or its blockade by CQ did not induce CRT expression in MMC. While further studies are needed to look at the other markers of ICD in order to confidently determine whether ICD is in fact induced in the cells or not, this suggests that RT,

Figure 6. High dose RT induces significant cell surface expression of CRT. MMC were irradiated with 2Gy and 18Gy once per day for three days. Three hours before and during each fraction of RT, tumor cells were also treated with 10uM CQ in order block autophagy. The floater cells were kept in culture the entire time, and total cells were analyzed on day 3. The cells were detached 24 hours after the final fraction of RT, stained with APC-Annexin V, PI, and FITC-CRT, and analyzed for CRT expression using flow cytometry. Membrane translocation of CRT was detected by subtracting autofluorescence from the MFI. (n=2)

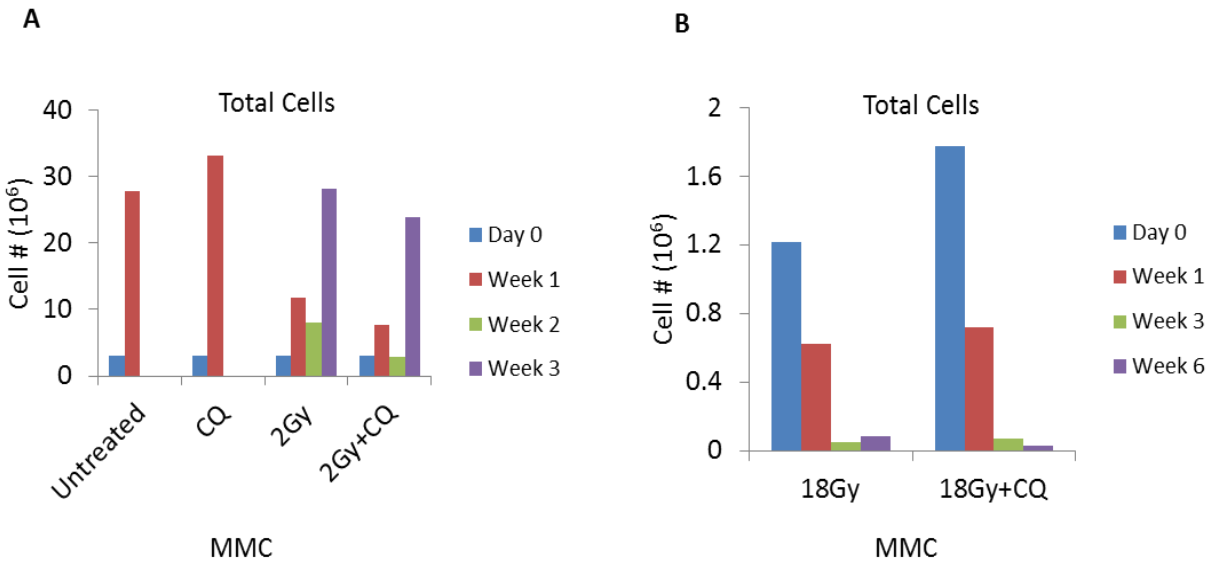


especially at a higher dose, has the potential to not only cause ICD in tumor cells, but also induce the expression of CRT on viable cells, thus making the cells more susceptible to the immune response.

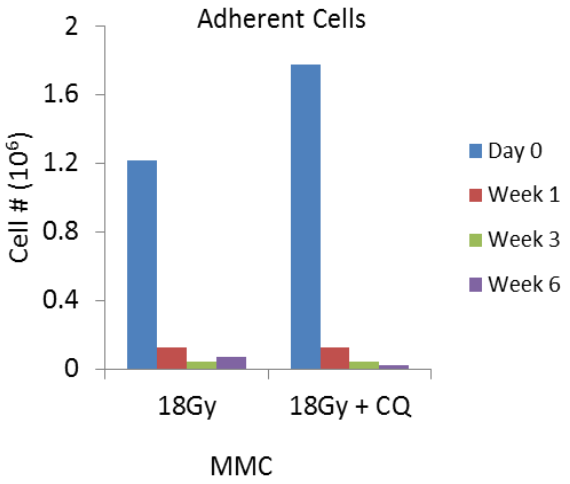
RT induces tumor cell dormancy in a dose dependent manner

We finally wanted to see the long term effects of RT on the tumor cells. After three daily treatments of MMC cells with 2Gy and 2Gy+CQ, we monitored the proliferation of the tumor cells over several weeks. While the untreated and CQ treated MMC showed about a 9- to 11-fold increase in proliferation after just 1 week, the 2Gy treated MMC cells showed about a 4-fold increase (Figure 7A). The 2Gy+CQ treated cells had an even greater inhibition of proliferation after 1 week, with only a 2.5-fold increase. The RT treated cells remained at a plateau phase of growth until week 3, when they experienced a similar 8- to 9-fold increase in proliferation as the control cells. These data suggest that while 2Gy/day can induce dormancy in the tumor cells and CQ supports RT-induced dormancy, it is very short term dormancy, and the tumor cells are able to recover normal growth rates within a month. Similarly, after three daily treatments of MMC with 18Gy and 18Gy+CQ, follow up studies were performed. Unlike 2Gy however, by three weeks, the 18Gy treated cells, both with and without CQ, did not resume normal proliferation. In fact, they had about a 0.04 fold change in cell number from day 0 (Figure 7B). Blockade of autophagy by CQ protected tumor cell dormancy such that 6 weeks after the treatment, 18Gy+CQ showed a lower number of cells compared with 18Gy RT alone (0.02 vs. 0.07 fold change from day 0). This trend was also seen when adherent (Figure 7C) and non-adherent floater cells (Figure 7D) were analyzed separately.

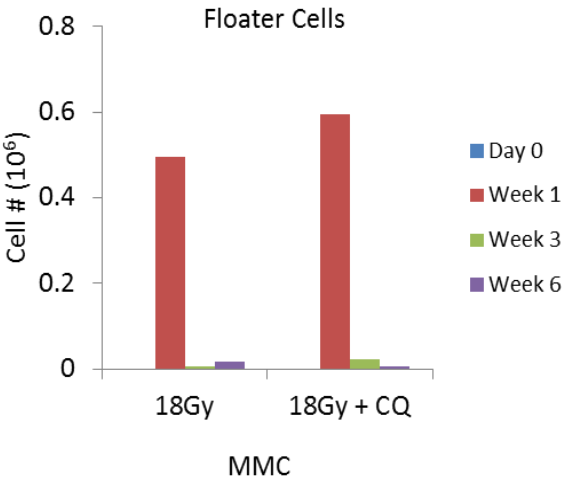
Figure 7. MMC are able to recover from dormancy induced by 2Gy/day but not by 18Gy/day. MMC were irradiated with 2Gy and 18Gy once per day for three days. Three hours before and during each fraction of RT, tumor cells were also treated with 10uM CQ for three hours in order block autophagy. The cells were detached 24 hours after the final fraction of RT, counted with trypan blue exclusion, and then replated in order to monitor their long term growth. Every 1-3 weeks, the tumor cells were detached and counted to detect any changes in proliferation. For 18Gy, floater cells and adherent cells were counted and analyzed separately. A) Long term growth of 2Gy and 2Gy+CQ treated cells. B) Long term growth of total 18Gy and 18Gy+CQ treated cells. C) Adherent cell counts of 18Gy treated cells during follow up. D) Floater cells counts of 18Gy treated cells during follow up. E) After being kept in culture for 1, 3, and 6 weeks, the 18Gy treated cells were detached, stained with APC-Annexin V and PI, and then analyzed for apoptosis using flow cytometry. Adherent and floater cells were stained separately from one another.



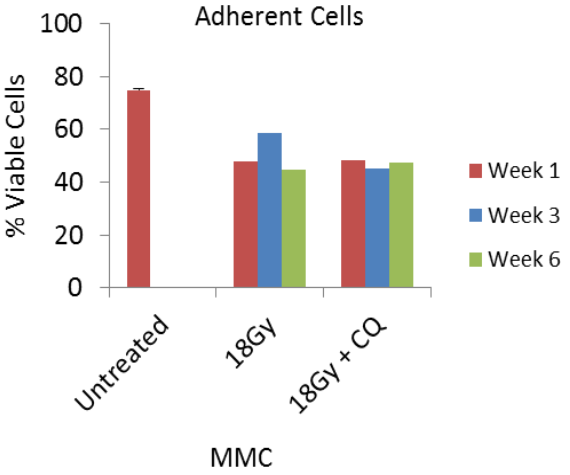
C

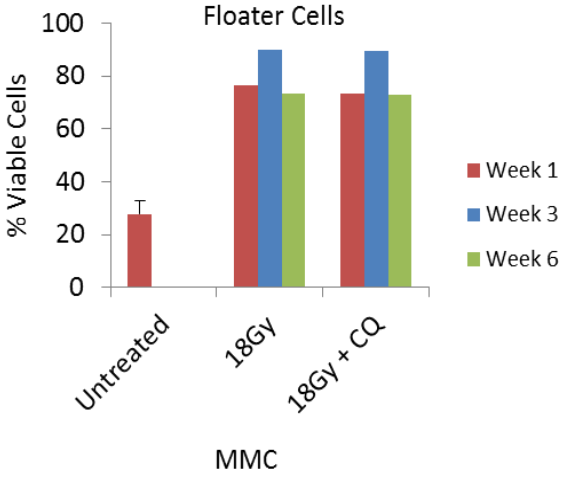

Adherent Cells
Cell # (10^6)
2
1.6
1.2
0.8
0.4
0
18Gy
18Gy + CQ
MMC
Day 0
Week 1
Week 3
Week 6

D


Floater Cells
Cell # (10^6)
0.8
0.6
0.4
0.2
0
18Gy
18Gy + CQ
MMC
Day 0
Week 1
Week 3
Week 6

E


Adherent Cells
% Viable Cells
100
80
60
40
20
0
Untreated
18Gy
18Gy + CQ
MMC
Week 1
Week 3
Week 6

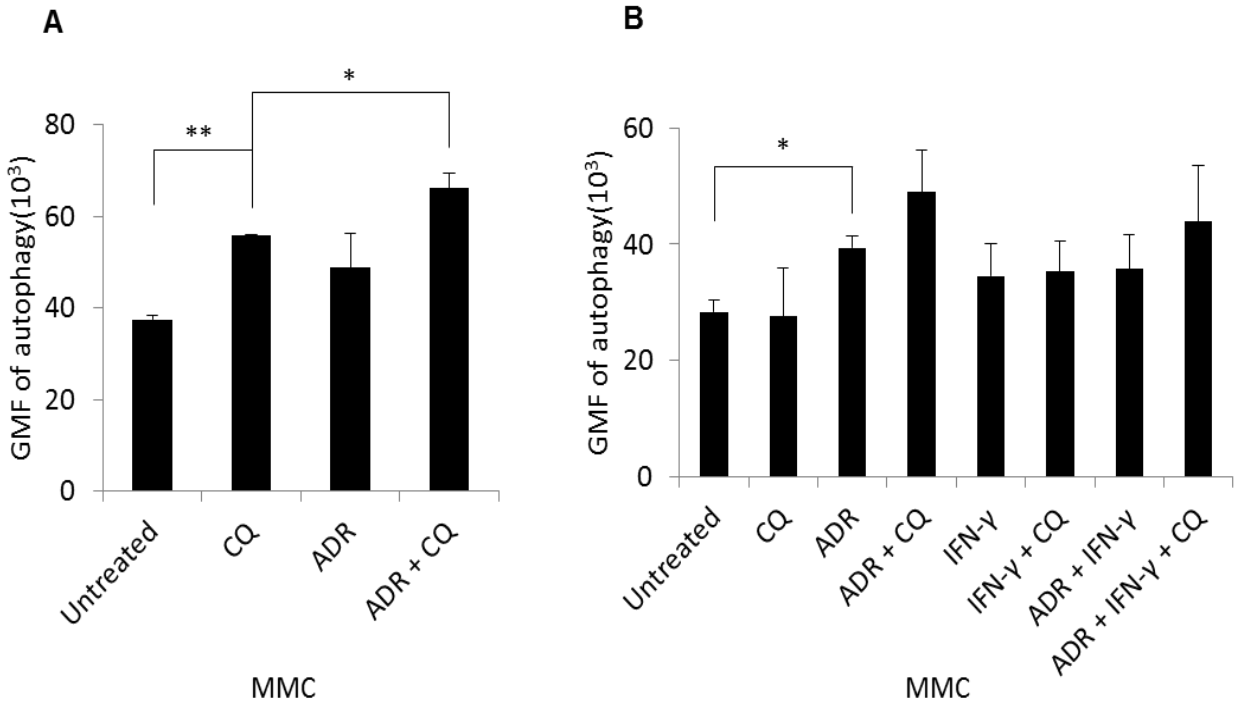

Floater Cells
% Viable Cells
100
80
60
40
20
0
Untreated
18Gy
18Gy + CQ
MMC
Week 1
Week 3
Week 6

We also wanted to determine whether tumor cells remain viable during dormancy. After being kept in culture for one, three, and six weeks, the 18Gy treated cells were stained for apoptosis. The floater cells were cultured and stained separate from the adherent cells to see if there were any major differences in viability. Any viable floater cells could be considered representative of CTC, because like CTC, they are quiescent and unable to attach or reside in distant tissues. Any viable adherent cells would be representative of DTC, because although they are also dormant, they are still able to attach to distant tissues. Both the adherent and floater tumor cells, with and without blockade of autophagy by CQ, remained viable during dormancy (Figure 7E). These data suggest that while tumor cells can eventually recover from 2Gy RT, they remain dormant and actually drop in cell number over time from 18Gy, and that CQ did not induce tumor relapse or overcome RT-induced tumor dormancy.

ADR, IFN-γ, or combined ADR+IFN-γ chemo-immunotherapy induces different levels of autophagy in tumor cells

After observing that RT induces autophagy in a dose-dependent manner in tumor cells, we wanted to see whether chemotherapy and/or immunotherapy would do the same. MMC were treated daily with ADR for 2 hours in the presence or absence of CQ (3 hours prior to ADR and 2 hours during ADR treatment). 1 hour or 24 hours after treatment, the cells were stained with AO. The presence of CQ revealed the occurrence of autophagy significantly at baseline levels (Figure 8A, $p=0.001$). ADR alone slightly increased the level of autophagy, and CQ resulted in detection of significant autophagy such that levels of autophagy in ADR+CQ was significantly greater than the baseline autophagy seen with CQ alone (Figure 8A, $p=0.045$). Because

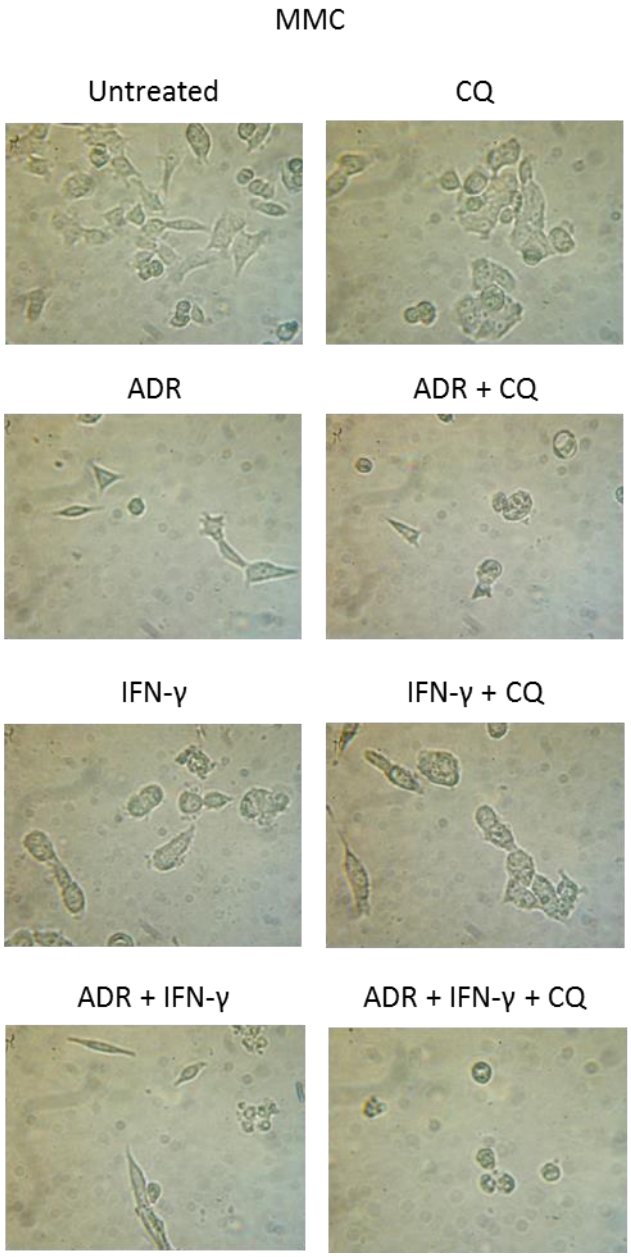
Figure 8. ADR induces autophagy. MMC were treated with ADR (A, B), IFN-γ (B), or combined ADR+IFN-γ (B) daily for either one (A, n=2) or three (B, n=2) days. Three hours before and two hours during ADR treatment, the cells were also treated with 10uM CQ in order block autophagy. With three daily treatments, the floater cells were discarded on days 0, 1, and 2, and total cells were analyzed on day 3. The cells were detached 1 hour (A) or 24 hours (B) after the final treatment, stained with acridine orange, and analyzed through flow cytometry in order to measure accumulation of acidic vesicles. Levels of autophagy were detected by subtracting green fluorescence (non-acidic cellular components) from red fluorescence (acidic vesicles).



autophagy was induced with a single treatment of ADR alone, we then wanted to see if autophagy would continue or increase after combined ADR+IFN-γ chemo-immunotherapy. After MMC were treated with CQ and/or ADR, IFN-γ was also added to the culture. 24 hours after three daily combination treatments, the cells were then stained with AO. As shown in Figure 8B, only ADR alone significantly induced autophagy above baseline levels ($p=0.033$). This level of autophagy was slightly increased following ADR+CQ whereas baseline autophagy in CQ alone did not show significant changes compared to untreated MMC. A slight increase in the level of autophagy was also detected in the presence of IFN-γ or ADR+ IFN-γ compared with baseline autophagy, but CQ did not enhance autophagy in the tumor cells treated with IFN-γ or ADR+ IFN-γ. These data suggest that presence of IFN-γ in combined ADR+ IFN-γ therapy inhibits ADR-induced autophagy. We also looked at senescence in these tumor cells by staining them with beta-galactosidase; however, senescence was not detected (data not shown).

We then wanted to see if autophagy induced similar morphologic changes following ADR and combination treatments as were seen with RT. As shown in Figure 9, ADR alone resulted in an elongation of the MMC. The blockade of ADR-induced autophagy by CQ (ADR+CQ) resulted in only some of the cells becoming elongated, while others became round. IFN-γ, in the presence or absence of CQ, appeared to reduce the cubical morphology of untreated MMC. The combination of ADR and IFN-γ resulted in similar morphologic changes to ADR alone, suggesting that IFN-γ did not have an additional effect on the morphological changes that were observed with ADR. When ADR+ IFN-γ treated cells were cultured with CQ, the cells became much smaller and rounder. The blockade of baseline autophagy by CQ did not affect the tumor cell morphology.

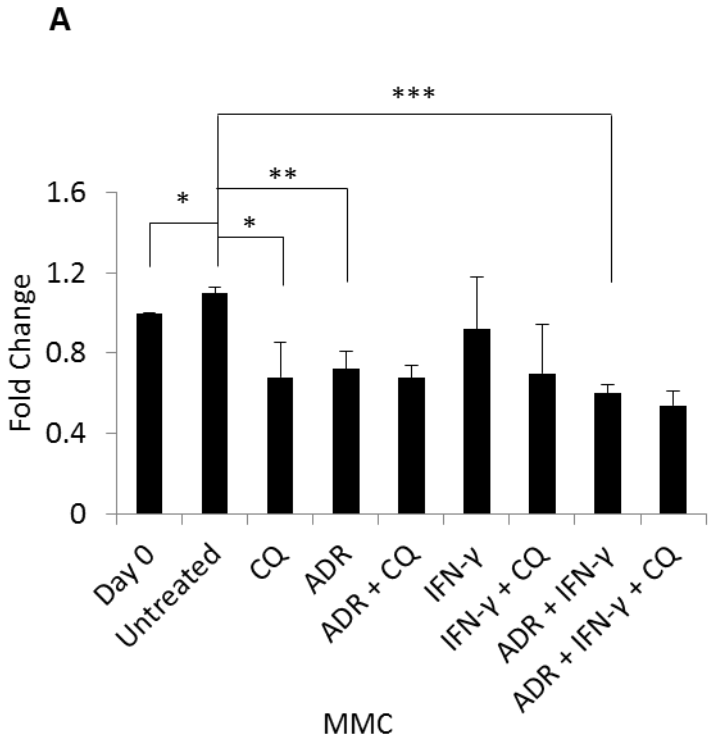
Figure 9. ADR or combined ADR+IFN-γ chemo-immunotherapy induce morphologic changes in MMC. MMC were treated with ADR, IFN-γ, or combined ADR+IFN-γ daily for three days. Three hours before and two hours during ADR treatment, the cells were also treated with 10uM CQ in order block autophagy. After the third treatment, the tumor cells were visualized under a microscope at 25x magnification.

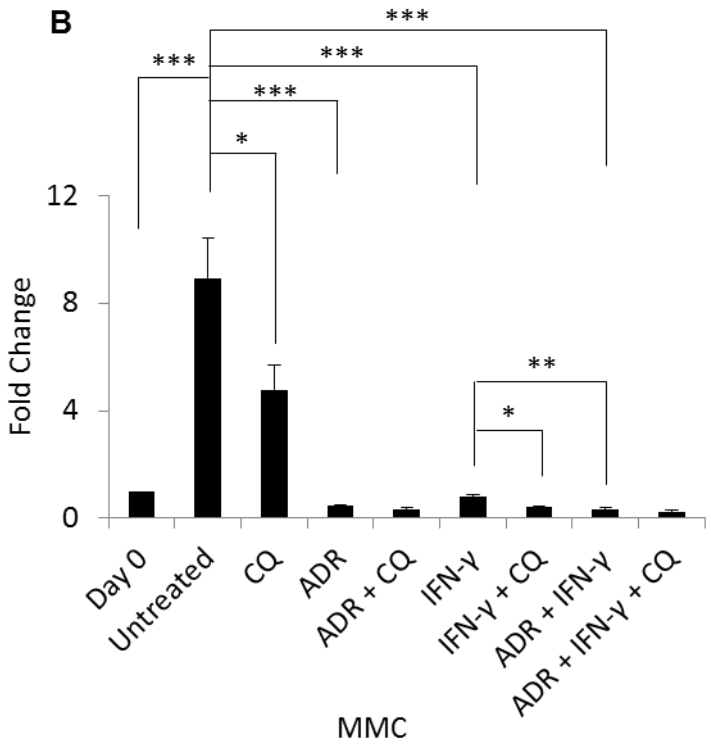


Dose-dependent inhibition of tumor cell proliferation by ADR, IFN-γ, or combined ADR+IFN-γ chemo-immunotherapy

In order to determine whether the induction of autophagy was associated with an inhibition of cell proliferation, the MMC were treated with ADR and/or IFN-γ both with and without the blockade of autophagy. After only a single treatment, both ADR and the combination of ADR with IFN-γ resulted in a significant drop in cell proliferation, compared to untreated MMC (Figure 10A). The inhibitory effect of ADR+IFN-γ was slightly higher than that of ADR alone (ADR: p=0.008, ADR+IFN-γ: p=0.0004). The blockade of baseline autophagy by CQ resulted in a drop in proliferation of untreated MMC (p=0.04), however CQ did not enhance but slightly reduced the inhibitory function of ADR or ADR+IFN-γ (Figure 10A). After three treatments of ADR, IFN-γ, or ADR+IFN-γ, a significant drop in tumor cell proliferation was seen in all conditions compared to untreated MMC (Figure 10B, ADR: p=0.0006, IFN-γ: p=0.0008, ADR+IFN-γ: p=0.0006). The combined ADR+IFN-γ resulted in a similar inhibition to ADR alone, however there was significantly more inhibition compared to IFN-γ alone (p=0.007). The addition of CQ resulted in a significant inhibition of proliferation of untreated MMC (p=0.027) or IFN-γ-treated MMC (p=0.011), however, it did not increase or decrease the inhibitory function of ADR or ADR+ IFN-γ.

Figure 10. ADR or combined ADR+IFN-γ chemo-immunotherapy inhibits tumor cell proliferation. MMC were treated with ADR, IFN-γ, or combined ADR+IFN-γ daily for either one (A, n=3) or three (B, n=4) days. Three hours before and two hours during ADR treatment, the cells were also treated with 10uM CQ in order block autophagy. With three daily treatments, the floater cells were discarded on days 0, 1, and 2, and total cells were analyzed on day 4. The cells were detached 24 hours (A) or 48 hours (B) after the final treatment, and the total cells were counted using trypan blue exclusion in order to determine any changes to proliferation. The data were normalized to one million cells and presented as fold change compared to Day 0.

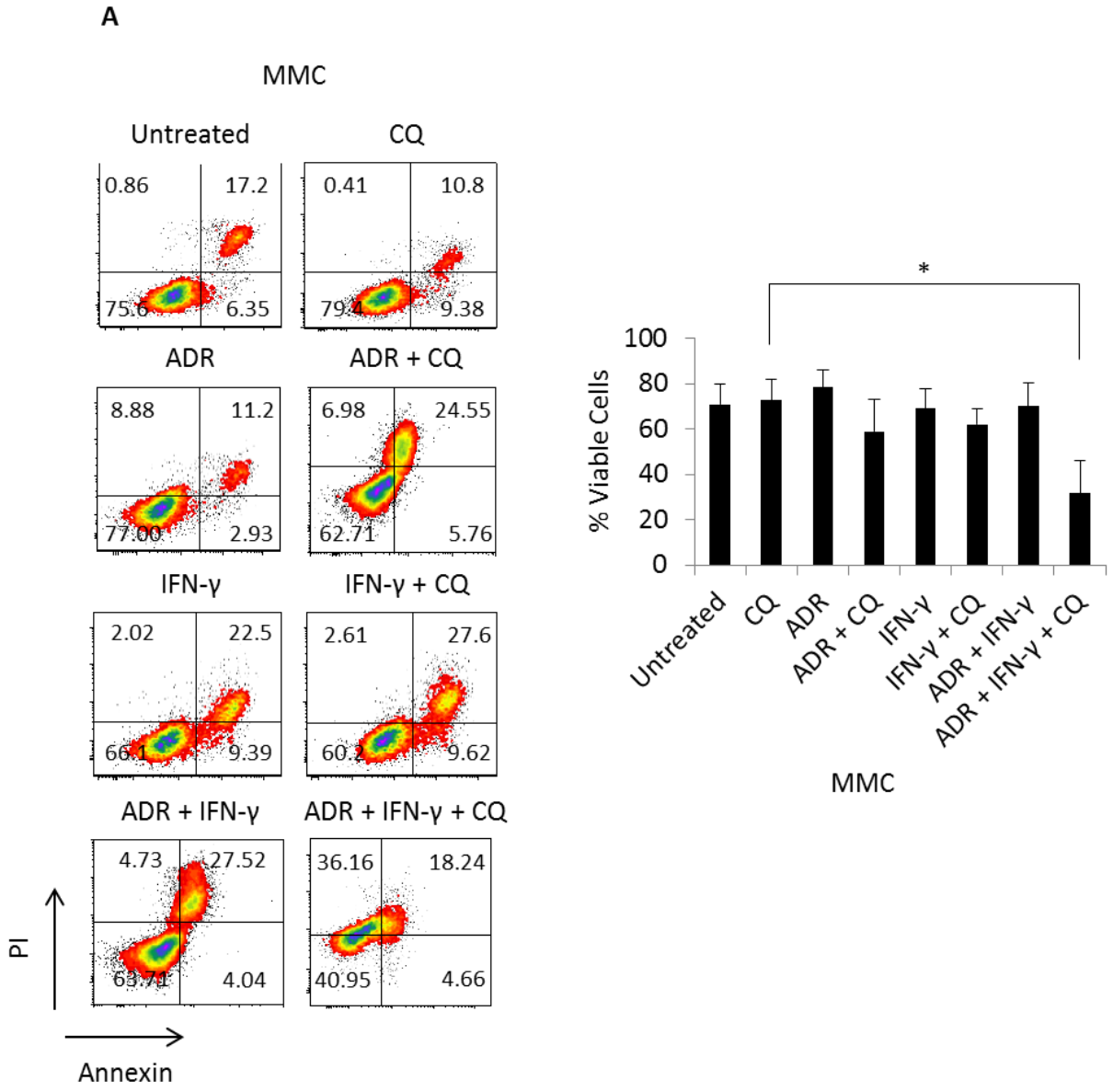


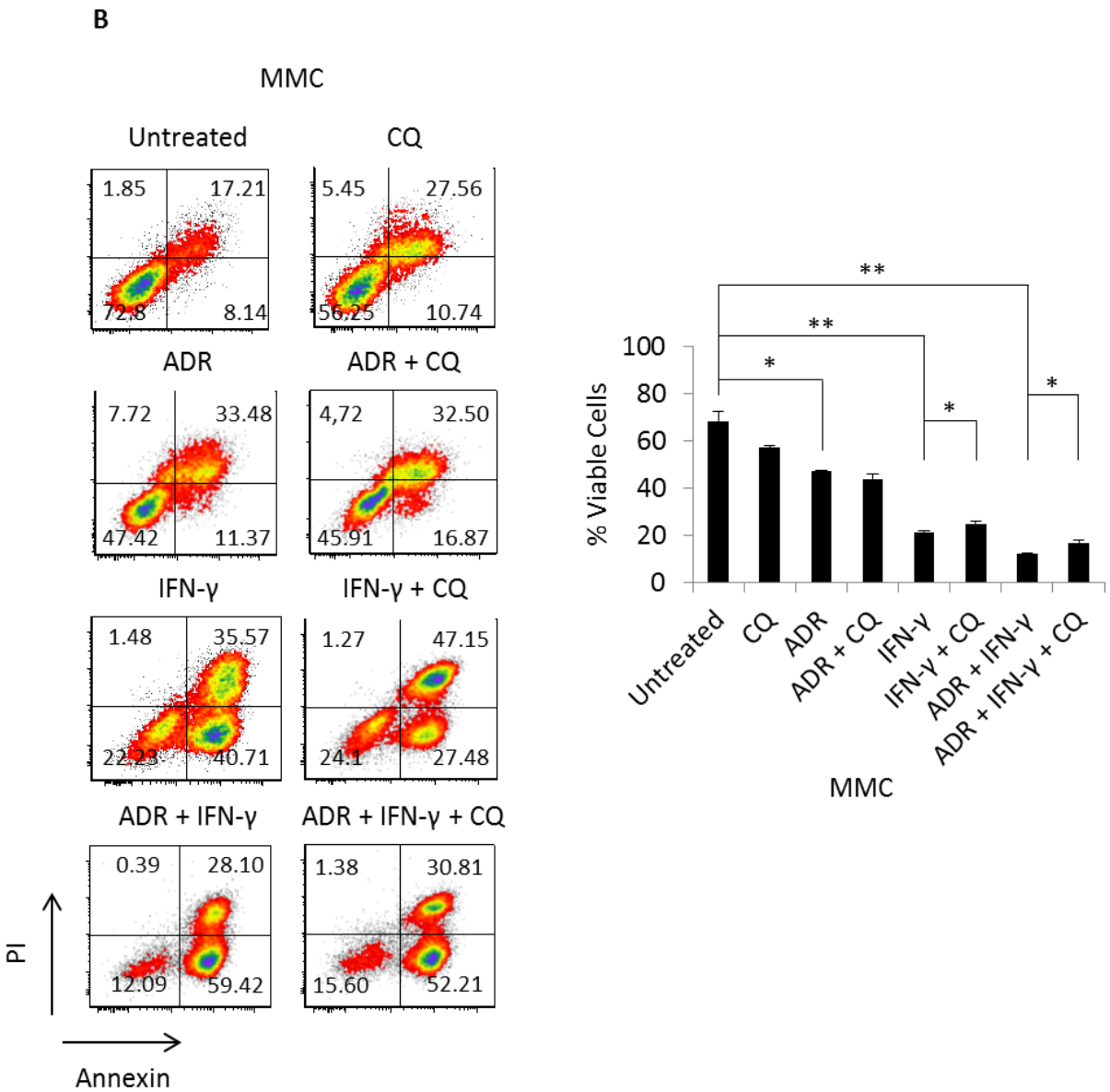

B
***
***
***
***
*
**
*
12
8
4
0
Fold Change
Day 0
Untreated
CQ
ADR
ADR + CQ
IFN-γ
IFN-γ + CQ
ADR + IFN-γ
ADR + IFN-γ + CQ
MMC

Blockade of autophagy exhibits variable effects on treatment-induced apoptosis depending on the treatment regimen

In order to determine if the blockade of autophagy could increase the apoptosis induced by ADR or combined ADR+IFN-γ chemo-immunotherapy, tumor cell apoptosis was determined in the presence or absence of CQ. After one treatment of ADR or combined ADR+IFN-γ chemo-immunotherapy, none of the treatments induced apoptosis. However, blockade of autophagy by CQ slightly reduced viability in ADR+CQ or IFN-γ+CQ treated MMC, and only the combination of ADR+IFN-γ with CQ induced a significant drop in viability of the tumor cells compared to the blockade of baseline autophagy by CQ, from an average of 72.7% to 31.7% (Figure 11A, p=0.037). Three daily treatments of ADR, IFN-γ, or combined ADR+IFN-γ chemo-immunotherapy resulted in a significant decrease in viability of the tumor cells compared to untreated MMC (Figure 11B, ADR: 68.39% to 47.11%, p=0.02, IFN-γ: 68.39% to 21.55%, p=0.004, ADR+IFN-γ: 68.39% to 12.34%, p=0.003). Among these treatments, IFN-γ had greater effect on tumor cell killing compared with ADR (p=0.0004), and combined ADR+IFN-γ chemo-immunotherapy had greater effect on tumor cell killing compared with IFN-γ (p=0.003). The addition of CQ did not increase or decrease the apoptotic effects of ADR alone; however, it did decrease the apoptotic effects of IFN-γ alone and combined ADR+IFN-γ (Figure 11B, IFN-γ: 21.55% to 25.04%, p=0.048, ADR+IFN-γ: 12.34% to 16.96%, p=0.039). Blockade of baseline autophagy by a single CQ treatment did not change tumor cell viability, but three daily treatments with CQ slightly reduced tumor cell viability.

Figure 11. Blockade of autophagy alters the apoptotic effects of combined ADR+IFN-γ chemo-immunotherapy in a dose dependent manner. MMC were treated with ADR, IFN-γ, or combined ADR+IFN-γ daily for either one (A, n=3) or three (B, n=2) days. Three hours before and two hours during ADR treatment, the cells were also treated with 10uM CQ in order block autophagy. With three daily treatments, the floater cells were kept in culture the entire time, and total cells were analyzed on day 4. The cells were detached 24 hours (A) or 48 hours (B) after the final treatment, stained with APC-Annexin V and PI, and then analyzed for apoptosis using flow cytometry.

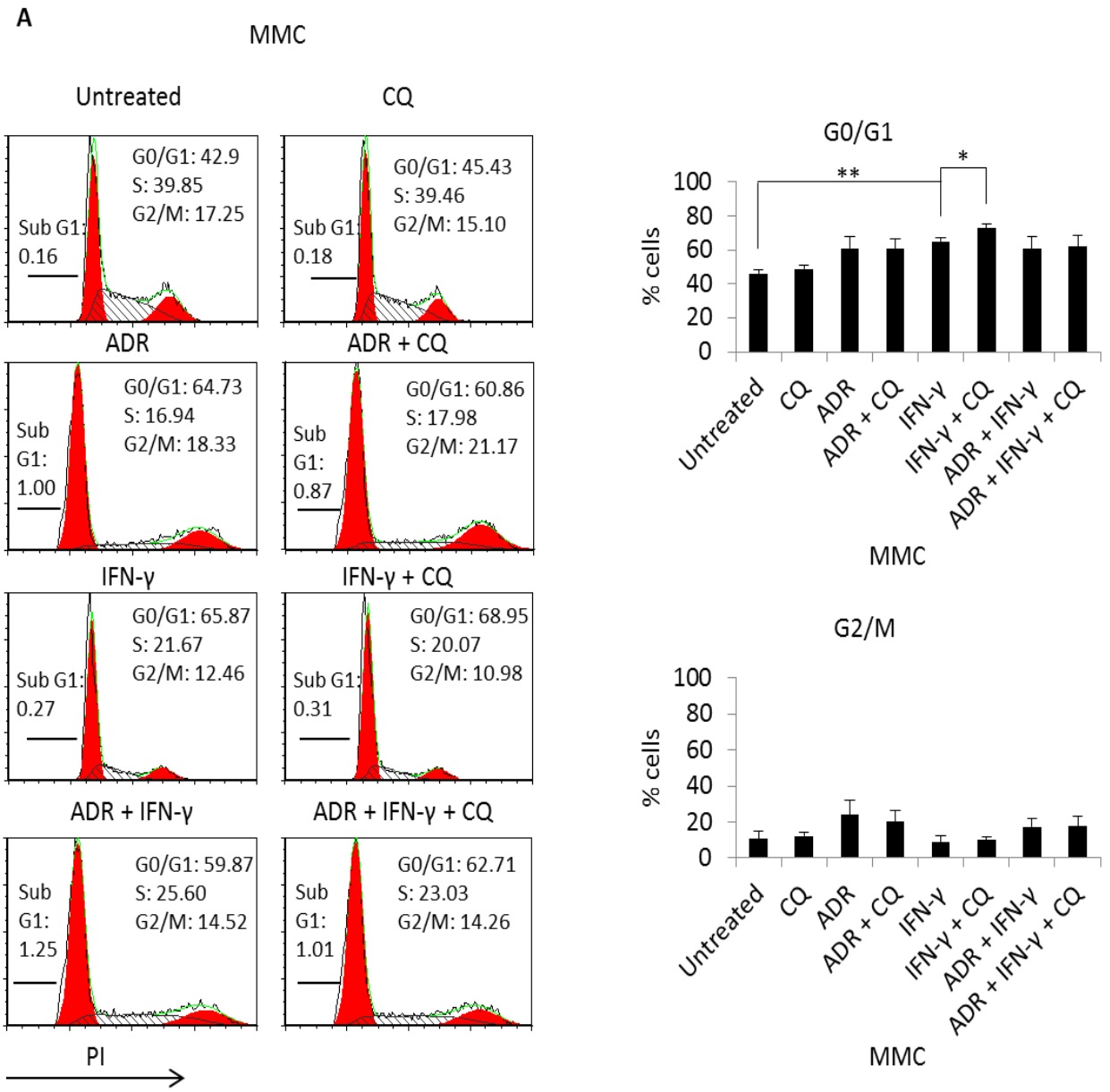


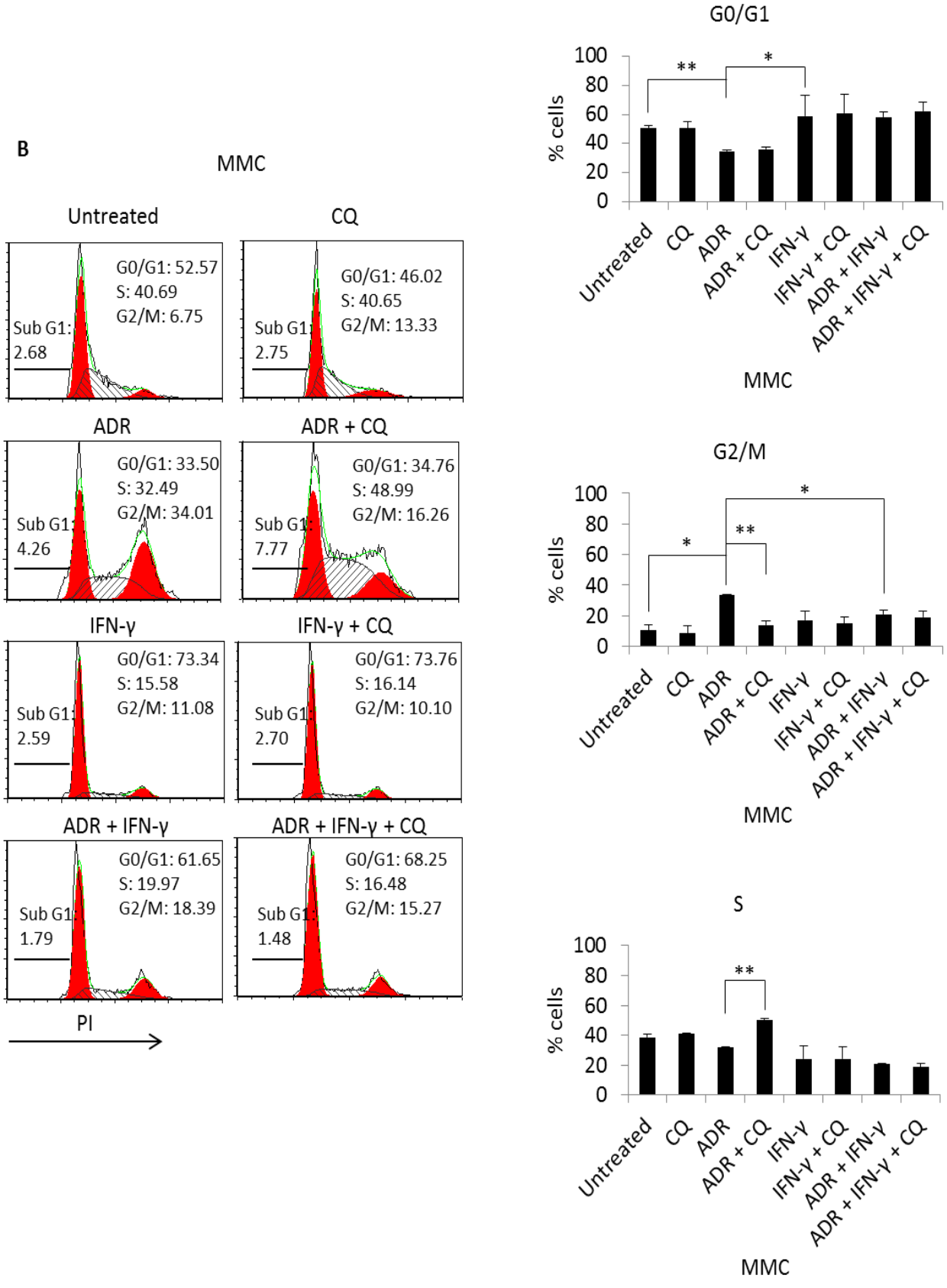

B
MMC
Untreated
CQ
1.85
17.21
72.8
8.14
5.45
27.56
56.25
10.74
ADR
ADR + CQ
7.72
33.48
47.42
11.37
4,72
32.50
45.91
16.87
IFN-γ
IFN-γ + CQ
1.48
35.57
22.23
40.71
1.27
47.15
24.1
27.48
ADR + IFN-γ
ADR + IFN-γ + CQ
0.39
28.10
12.09
59.42
1.38
30.81
15.60
52.21
PI
Annexin
% Viable Cells
100
80
60
40
20
0
**
**
*
*
*
Untreated
CQ
ADR
ADR + CQ
IFN-γ
IFN-γ + CQ
ADR + IFN-γ
ADR + IFN-γ + CQ
MMC

ADR induces G2/M arrest but IFN-γ or combined ADR+IFN-γ chemo-immunotherapy induces G0/G1 arrest

In order to determine whether the inhibition of tumor cell proliferation and induction of autophagy and apoptosis were associated with cellular arrest at a certain phase of the cell cycle, flow cytometry analysis was performed after PI staining of the cells. A single treatment of IFN-γ alone induced a significant amount of arrest in G0/G1 (Figure 12A, p=0.002). Blockade of autophagy by CQ resulted in significantly more G0/G1 arrest (IFN-γ+CQ vs. IFN-γ, p=0.043). Multiple treatments of IFN-γ alone, with and without CQ, only slightly increased G0/G1 arrest compared to untreated MMC, with and without CQ (Figure 12B). Multiple treatments of ADR alone resulted in significant G2/M arrest (Figure 12B, p=0.012). This was accompanied with a significant decrease in G0/G1 arrest (Figure 12B, p=0.008). The addition of CQ to ADR significantly reduced the percentage of cells in G2/M arrest (Figure 12B, ADR vs. ADR+CQ, p=0.009) which was comparable to CQ alone. Instead, a higher number of cells were arrested in S phase (Figure 12B, p=0.003). When IFN-γ was combined with ADR, there was a significant reduction in G2/M arrest compared to ADR alone (Figure 12B, p=0.02). The combined ADR+IFN-γ chemo-immunotherapy also resulted in greater G0/G1 arrest compared to ADR alone (Figure 12B, p=0.01), however, this arrest was not significantly greater than that seen with untreated MMC. The addition of CQ to combined ADR+IFN-γ chemo-immunotherapy neither increased nor decreased the effects of ADR+IFN-γ on cell cycle arrest. Blockade of baseline autophagy by CQ did not cause cell cycle arrest.

Figure 12. ADR induces G2/M arrest and IFNγ induces G0/G1 arrest in a dose-dependent manner. MMC were treated with ADR, IFN-γ, or combined ADR+IFN-γ daily for either one (A, n=3) or three (B, n=2) days. Three hours before and two hours during ADR treatment, the cells were also treated with 10uM CQ in order block autophagy. With three daily treatments, the floater cells were discarded on days 0, 1, and 2, and total cells were analyzed on day 4. The cells were detached 24 hours (A) or 48 hours (B) after the final treatment, stained with PI and analyzed for cell cycle arrest through flow cytometry. The Sub-G1 population was analyzed separately from the other three populations.




B
MMC
Untreated
G0/G1: 52.57
S: 40.69
G2/M: 6.75
Sub G1:
2.68
CQ
G0/G1: 46.02
S: 40.65
G2/M: 13.33
Sub G1:
2.75
ADR
G0/G1: 33.50
S: 32.49
G2/M: 34.01
Sub G1:
4.26
ADR + CQ
G0/G1: 34.76
S: 48.99
G2/M: 16.26
Sub G1:
7.77
IFN-γ
G0/G1: 73.34
S: 15.58
G2/M: 11.08
Sub G1:
2.59
IFN-γ + CQ
G0/G1: 73.76
S: 16.14
G2/M: 10.10
Sub G1:
2.70
ADR + IFN-γ
G0/G1: 61.65
S: 19.97
G2/M: 18.39
Sub G1:
1.79
ADR + IFN-γ + CQ
G0/G1: 68.25
S: 16.48
G2/M: 15.27
Sub G1:
1.48
PI
G0/G1
% cells
100
80
60
40
20
0
**
*
Untreated
CQ
ADR
ADR + CQ
IFN-γ
IFN-γ + CQ
ADR + IFN-γ
ADR + IFN-γ + CQ
MMC
G2/M
% cells
100
80
60
40
20
0
*
**
*
Untreated
CQ
ADR
ADR + CQ
IFN-γ
IFN-γ + CQ
ADR + IFN-γ
ADR + IFN-γ + CQ
MMC
S
% cells
100
80
60
40
20
0
**
Untreated
CQ
ADR
ADR + CQ
IFN-γ
IFN-γ + CQ
ADR + IFN-γ
ADR + IFN-γ + CQ
MMC

IFN-γ inhibits some of the ADR-induced immunogenic tumor cell killing

In order to determine whether ADR-, IFN-γ-, or ADR+IFN-γ-induced apoptosis is also immunogenic, the MMC were analyzed for membrane translocation of CRT through flow cytometry. ADR and IFN-γ alone resulted in significantly greater expression of membrane CRT (Figure 13, ADR: p=0.023, IFN-γ: p=0.015), although CRT expression was slightly lower in IFN-γ- treated cells compared to ADR-treated cells (MFI=26.32 compared to MFI=59.28). Combined ADR+IFN-γ chemo-immunotherapy slightly induced membrane expression of CRT (MFI=20.72). The addition of CQ neither increased nor decreased CRT expression following treatments.

Combined ADR+IFN-γ chemo-immunotherapy or the blockade of ADR-induced autophagy prolongs tumor dormancy and inhibits tumor relapse

Finally, we sought to determine whether MMC recover from tumor dormancy following the completion of treatments with ADR, IFN-γ, or combined ADR+IFN-γ chemo-immunotherapy. After three daily treatments of ADR and/or IFN-γ, we monitored the proliferation of the tumor cells over several weeks. By three weeks, ADR, IFN-γ, and ADR+IFN-γ treated cells all showed an inhibition of proliferation compared to day 0 (Figure 14A, ADR: p=0.0003, IFN-γ: p=0.001, ADR+IFN-γ: p=0.0004). The combined ADR+IFN-γ chemo-immunotherapy showed a greater inhibition compared to IFN-γ alone (p=0.007) but not to ADR alone. At three weeks, blockade of autophagy by CQ did not increase or decrease the inhibitory effects of any condition.

Figure 13. IFN-γ hinders ADR-induced CRT expression. MMC were treated with ADR, IFN-γ, or combined ADR+IFN-γ daily for three days. Three hours before and two hours during ADR treatment, the cells were also treated with 10uM CQ in order block autophagy. The floater cells were kept in culture the entire time, and total cells were analyzed on day 4. The cells were detached 48 hours after the final treatment, stained with APC-Annexin V, PI, and FITC-CRT, and analyzed for CRT expression using flow cytometry. Membrane translocation of CRT was detected by subtracting autofluorescence from the MFI. (n=2)

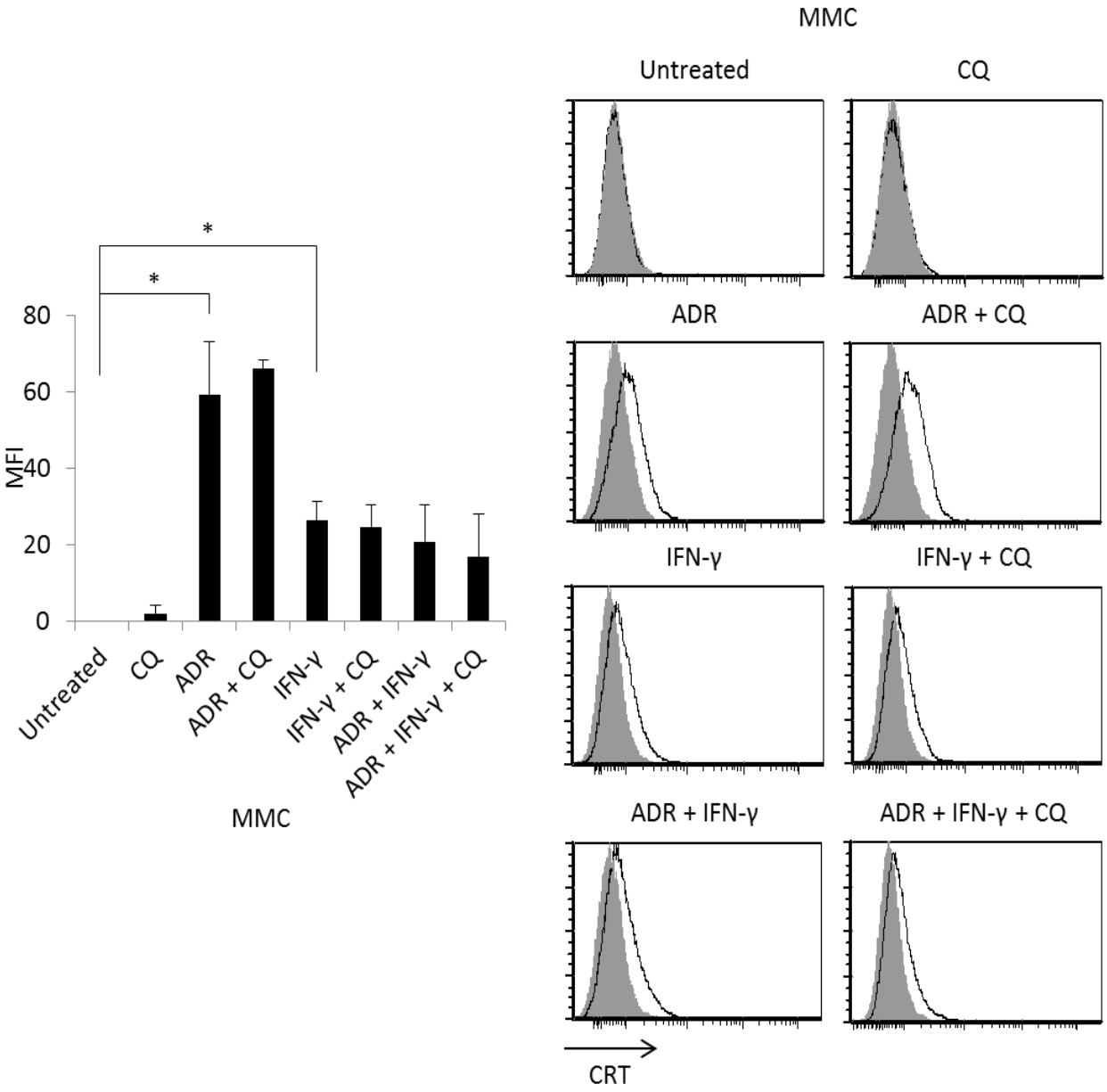
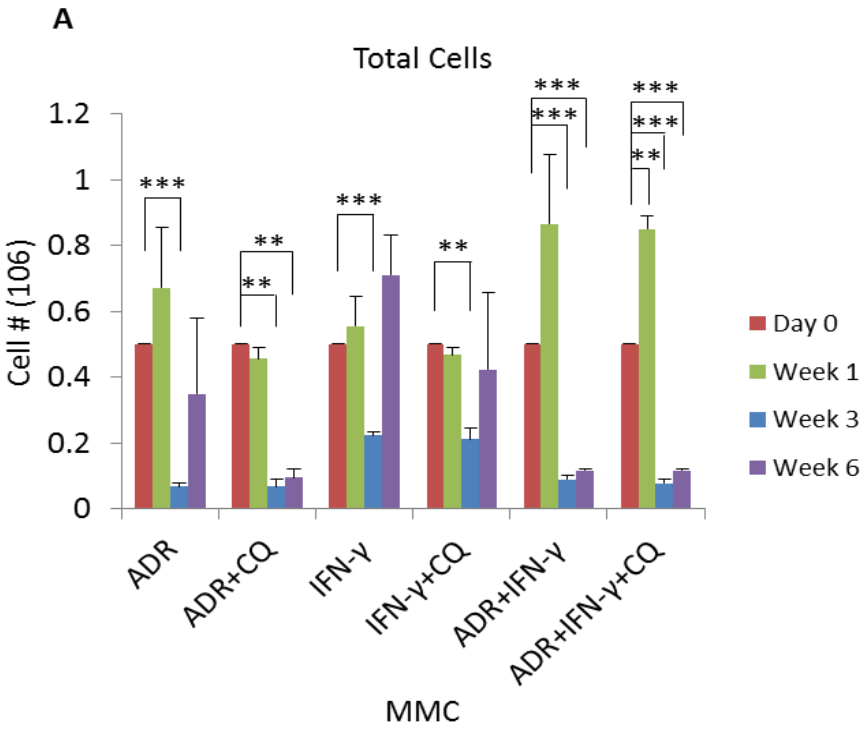
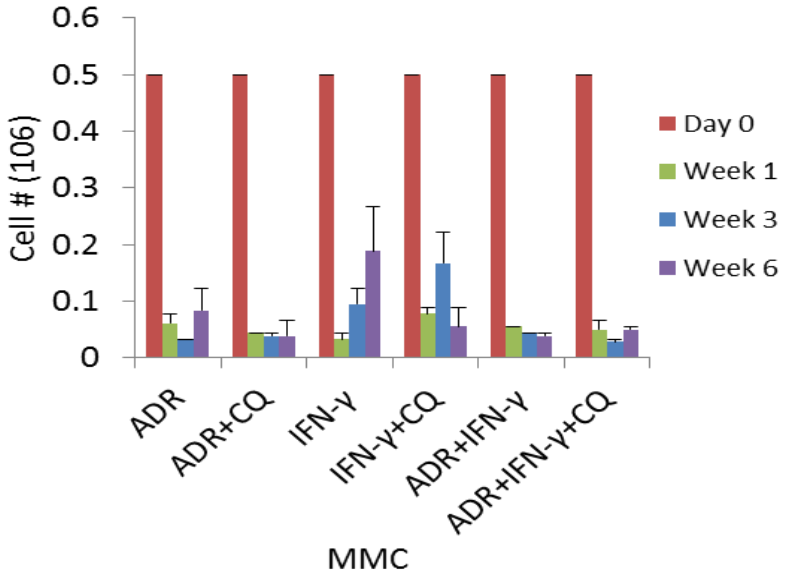


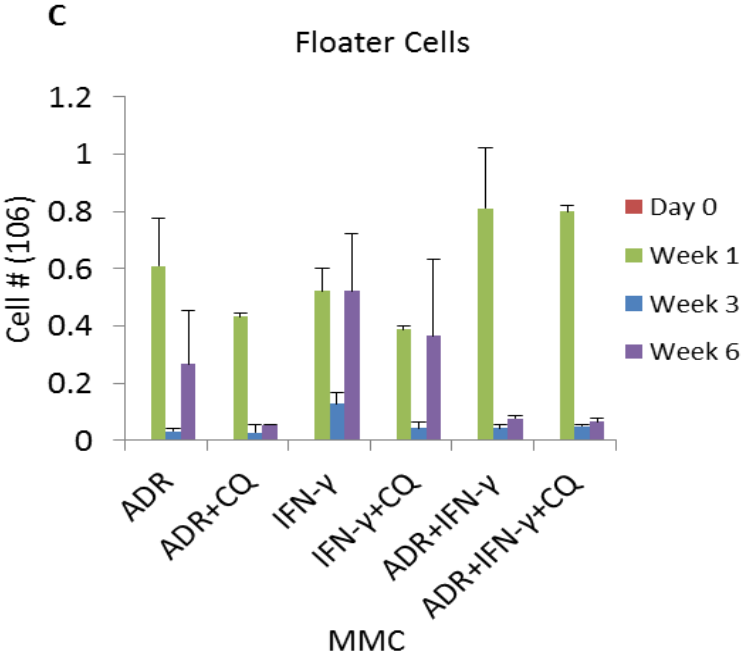
Figure 14. ADR and combined ADR+IFN-γ chemo-immunotherapy induce dormancy in tumor cells which may be recoverable. MMC were treated with ADR, IFN-γ, or combined ADR+IFN-γ daily for three days. For autophagy blockade, three hours before and two hours during ADR treatment the cells were treated with CQ. The cells were detached 48 hours after the final treatment, counted with trypan blue exclusion, and then replated in order to monitor their long term growth. Every 3 weeks, the tumor cells were detached and counted to detect any changes in proliferation. Floater cells and adherent cells were counted and analyzed separately. A) Total cell counts of tumor cells during follow up. B) Adherent cell counts of tumor cells during follow up. C) Floater cells counts of tumor cells during follow up. D) After being kept in culture for 1, 3, and 6 weeks, the cells were detached, stained with APC-Annexin V and PI, and then analyzed for apoptosis using flow cytometry. Adherent and floater cells were stained separately from one another. E) Morphology of the tumor cells 6 weeks after treatment.

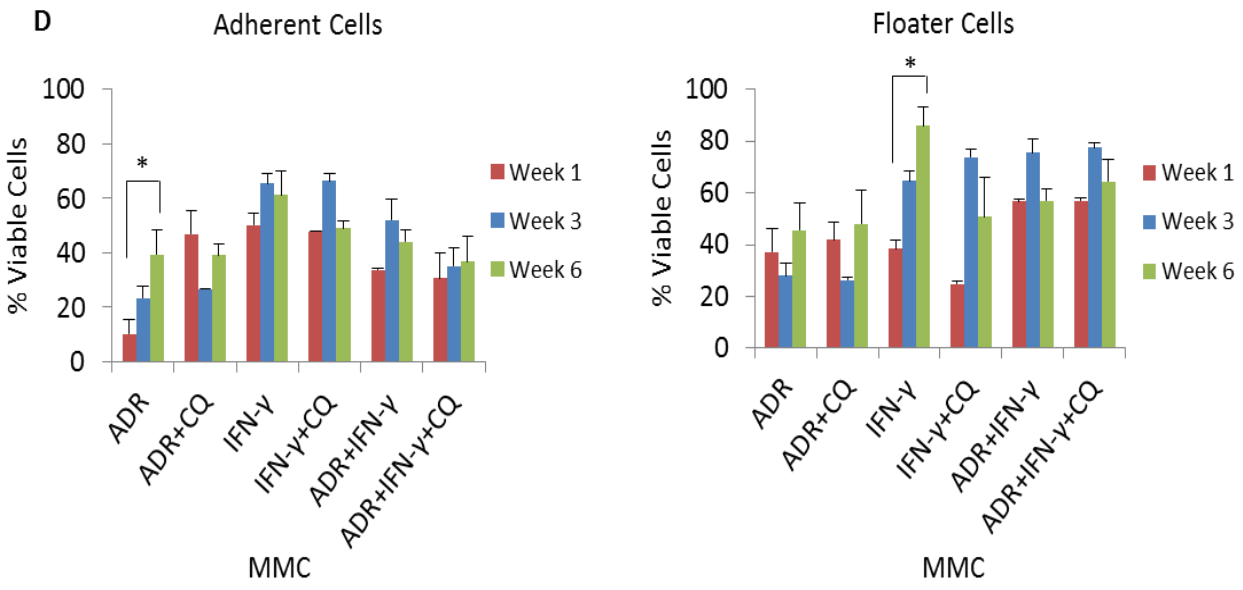


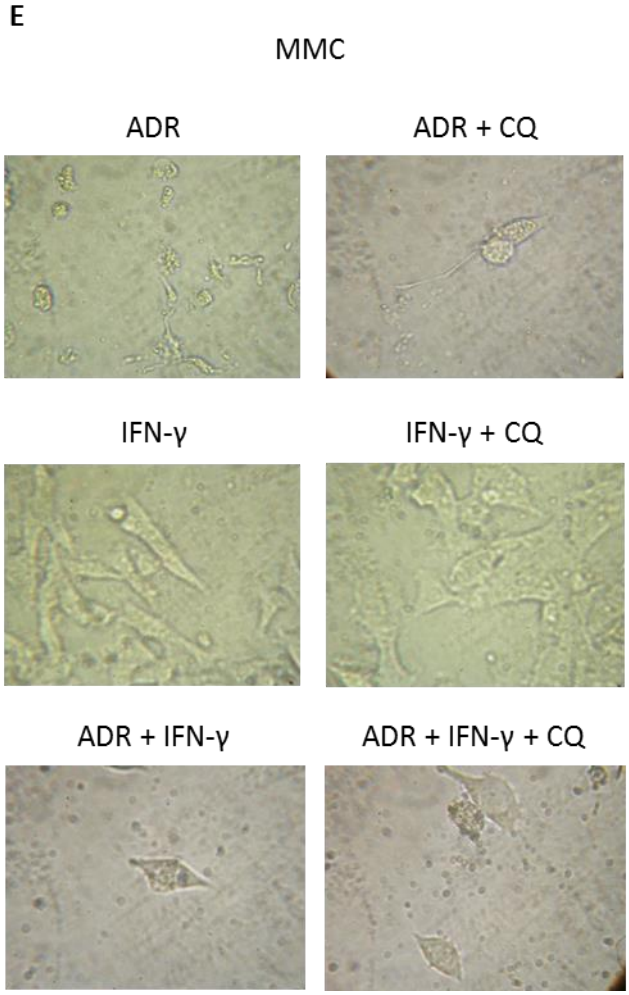
B


Adherent Cells
0.6
0.5
0.4
0.3
0.2
0.1
0
Cell # (106)
Day 0
Week 1
Week 3
Week 6
ADR
ADR+CQ
IFN-γ
IFN-γ+CQ
ADR+IFN-γ
ADR+IFN-γ+CQ
MMC

C


Floater Cells
1.2
1
0.8
0.6
0.4
0.2
0
Cell # (106)
Day 0
Week 1
Week 3
Week 6
ADR
ADR+CQ
IFN-γ
IFN-γ+CQ
ADR+IFN-γ
ADR+IFN-γ+CQ
MMC


D
Adherent Cells
Floater Cells
% Viable Cells
100
80
60
40
20
0
*
Week 1
Week 3
Week 6
ADR
ADR+CQ
IFN-γ
IFN-γ+CQ
ADR+IFN-γ
ADR+IFN-γ+CQ
MMC


E
MMC
ADR
ADR + CQ
IFN-γ
IFN-γ + CQ
ADR + IFN-γ
ADR + IFN-γ + CQ

After six weeks, ADR- and IFN-γ-treated cells began to resume proliferation, with no significant difference compared to day 0. Interestingly, blockade of autophagy maintained ADR-induced tumor cell dormancy and a significant inhibition of tumor cell proliferation compared to day 0 ($p=0.002$). The combination of ADR and IFN-γ resulted in a sustained tumor cell dormancy and significant inhibition of proliferation compared to day 0 ($p=0.0001$) which was similar inhibition to week 3. The addition of CQ to the combined ADR+IFN-γ chemo-immunotherapy neither increased nor decreased the inhibition of tumor cell proliferation (Figure 14A). When adherent and non-adherent floater tumor cells were analyzed separately, it was found that blockade of autophagy by CQ prevents resumption of adherent tumor cell proliferation in all conditions (Figure 14B), whereas CQ did not support IFN-γ-induced dormancy in floater tumor cells (Figure 14C).

We also wanted to see whether the tumor cells remain viable during dormancy. After being kept in culture for six weeks, the treated cells were stained for apoptosis. The floater cells, which were representative of CTC, were cultured and stained separate from the adherent cells, which were representative of DTC, to see if there were any major differences in viability. For every condition without CQ, there were still viable cells present regardless of whether they were attached or floating even six weeks following treatment (Figure 14D). Six weeks after the completion of treatments, significant increases in tumor cell viability were observed in ADR-treated adherent cells ($p=0.039$) and IFN-γ–treated floater cells ($p=0.028$) compared to those after one week. Such increases in tumor cell viability were inhibited by CQ. Tumor cell viability did not increase or decrease within 6 weeks after combined ADR-IFN-γ therapy.

Finally, we examined morphological changes in tumor cells. As seen in Figure 14E, six weeks after treatment with ADR, the tumor cells were very small and round. IFN-γ treated cells

were more confluent and had a very thin, elongated cytoplasm. Combined ADR+IFN-γ chemo-immunotherapy appeared to support cubic morphology similar to what is normally seen with untreated MMC; however the cells were very sparse. The addition of CQ to any of these conditions appeared to have no effect on cellular morphology, suggesting that the effects of CQ seen early during treatments are no longer seen after several weeks following treatment.

Discussion

The purpose of this study was to determine whether a high dose of RT or combined chemo-immunotherapy could completely eliminate tumor cells or prevent tumor relapse by prolonging tumor dormancy. We were also interested in determining the contribution of autophagy to treatment-induced tumor dormancy, and whether its blockade during conventional therapies would effectively eliminate dormant tumor cells or prevent tumor relapse, in vitro. To do so, we treated tumor cells with 2Gy/day and 18Gy/day RT, as well as ADR chemotherapy, IFN-γ immunotherapy, or a combination of ADR+IFN-γ chemo-immunotherapy. We also used CQ to block the completion of autophagy in the tumor cells during each treatment. We showed that while each therapy is effective at tumor cell killing, none are enough to completely eliminate tumor cells. Even with the blockade of autophagy, none of the treatment types were able to kill all tumor cells or prevent them from becoming dormant. However, 18Gy/day RT, combined ADR+IFN-γ chemo-immunotherapy, or ADR chemotherapy combined with the blockade of autophagy were the most effective therapies in inhibiting tumor relapse by prolonging cancer

dormancy. Sustained levels of tumor cell viability as well as the detection of some tumor cells in S phase during dormancy suggest that dormancy has been maintained by a balance of cell proliferation and cell death, rather than by a cessation of tumor cell proliferation. In fact, restoring such balance, which is similar to homeostatic proliferation of normal epithelial cells, could be an effective therapy for breast cancer. In other words, prevention of tumor relapse associated with an imbalance between cell proliferation and cell death could be as effective as the elimination of dormant tumor cells in overcoming mortality in breast cancer patients. This is important because circulating tumor cells that represent cancer dormancy have been detected in breast cancer survivors even 22 years after an effective therapy (11). In fact, maintaining cancer dormancy, if it is not possible to prevent it, is still beneficial to cancer patients. Our study did reveal which types of treatments are more effective at tumor cell killing, and which have the potential to be further developed into effective strategies to overcome tumor cell dormancy or tumor relapse.

We found that cancer therapies, including RT, ADR, IFN-γ, and combined ADR+IFN-γ chemo-immunotherapy, each had cytotoxic effects by inducing tumor cell apoptosis, as well as cytostatic effects by inhibiting tumor cell proliferation. The cytostatic effects resulted in the establishment of tumor dormancy, and were only partially mediated by the induction of autophagy, which differed in rates and types compared to baseline homeostatic autophagy. Baseline autophagy was a slow process which was detectable by the autophagy blocker, CQ. Daily use of CQ did not show a detectable accumulation of autophagic vesicles, because baseline autophagy is slow enough that new vesicles are not constantly produced to replenish the old. On the other hand, treatment-induced autophagy was a continuous process which was detectable even after continuous therapy in the presence of autophagy blockade. Additionally, unlike the

other treatments, RT actually expedited the completion of autophagy such that when autophagy was not blocked, the level of acidic vesicles was lower than that of baseline autophagy.

18Gy/day RT induced inhibition of proliferation, tumor cell apoptosis, CRT translocation to the cell membrane, and G2/M and Sub-G1 arrest. Each of these effects was partially inhibited by CQ, suggesting that RT induces different types of autophagy compared to baseline autophagy. For example, the inhibition of cytotoxic autophagy resulted in the partial inhibition of RT-induced apoptosis, while the inhibition of non-protective or baseline homeostatic autophagy resulted in the partial inhibition of tumor cell proliferation. The inhibition of baseline autophagy also resulted in the inhibition of normal cell proliferation, suggesting that baseline autophagy is involved in homeostatic cell growth a proliferation, as has been previously described (31).

With the combined ADR+IFN-γ chemo-immunotherapy, a unique impact on tumor cells was seen, in that it was not simply due to the addition of the anti-tumor efficacy of ADR chemotherapy and IFN-γ immunotherapy. ADR alone strongly induced G2/M arrest; however, IFN-γ shifted that arrest towards G0/G1 arrest during combined ADR+IFN-γ chemo-immunotherapy. In addition, it was found that while all treatments induced CRT expression on apoptotic tumor cells, IFN-γ was a weaker inducer than ADR, and even inhibited ADR-induced CRT expression during combined ADR+IFN-γ chemo-immunotherapy. Interestingly, the use of IFN-γ in the combined ADR+IFN-γ chemo-immunotherapy actually modified the biological impact of ADR by inducing a different type of autophagy such that CQ enhanced treatment-induced apoptosis early in treatment, but later after multiple doses, CQ was ineffective or even slightly reduced apoptosis. While apoptosis induced by IFN-γ alone was also slightly inhibited by CQ later during treatment, ADR alone-induced tumor cell apoptosis was increased by CQ. ADR-, IFN-γ- or ADR+IFN-γ-induced inhibition of tumor cell proliferation, which is one

characteristic of tumor dormancy, was slightly improved by CQ. As with 18Gy/day RT however, this enhanced inhibition of proliferation could also be due to the blockade of baseline or homeostatic autophagy. Altogether, these data suggest that chemotherapy and/or immunotherapy induce cytoprotective autophagy early during the treatment such that its inhibition by CQ increases apoptosis; however, tumor cell inhibition or dormancy induced by IFN-γ immunotherapy or combined ADR+IFN-γ chemo-immunotherapy is partly mediated by cytotoxic autophagy since blockade of autophagy later during treatment slightly enhanced tumor cell viability. Additionally, later in treatment, chemotherapy may induce non-protective autophagy, since its blockade did not alter the proliferation or the viability of the ADR-treated tumor cells. This suggests that each therapy induces a different type of autophagy, which supports the proposal that there are four types of autophagy: cytoprotective, cytotoxic, non-protective, and cytostatic autophagy (33). It is not clear whether the different types of autophagy were induced concurrently or sequentially.

Other studies have also shown that tumor dormancy and resistance to cancer therapies may not be entirely due to cytoprotective autophagy as is commonly thought. A recent study of leukemia found that idarubicin, a commonly used antileukemic drug, induces autophagy in REH tumor cells. Interestingly, when autophagy was blocked during treatment, the tumor cells were not further sensitized to idarubicin-induced cytotoxicity. Instead, similar to what we have shown, the tumor cells showed greater viability following autophagy blockade, suggesting that their model induced cytotoxic autophagy as well (65). It has even been shown that other therapeutic strategies used in conjunction with conventional therapies can actually switch treatment-induced autophagy from cytoprotective to cytotoxic autophagy. In a study using ZR-75-1 breast tumor cells, it was shown that RT alone induces cytoprotective autophagy such that

blockade with CQ improved treatment-induced cytotoxicity. However, when the tumor cells were also treated with Calcitriol, the active form of vitamin D3, the reverse was seen. RT still induced autophagy in the presence of D3, however, when autophagy was also blocked, the tumor cells became less sensitive to RT and viability increased, similar to what we saw in our own study (66). In addition, Bristol et al. found what appears to be non-protective autophagy in both an in vitro model, using 4T1 breast tumor cells, and in vivo model, using BALB/c mice. They irradiated the cells and mice with and without the blockade of autophagy using CQ, and found that although autophagy was induced in the tumor cells, addition of CQ had no effect on the viability of the cells (67).

Just as different types of treatment-induced autophagy were seen for each type of therapy, different tumor cell morphology and morphologic changes induced by blockade of autophagy were seen for each therapy as well. RT treated cells became enlarged and were not affected by CQ, while ADR treated cells became elongated and shrank, and were slightly altered by CQ, which made some cells round. IFN-γ treated cells appeared to only slightly lose the cubic appearance of untreated MMC and were also not affected by CQ. Combined ADR+IFN-γ chemo-immunotherapy induced similar morphologic changes to those seen following ADR chemotherapy; however CQ caused those cells to all become small and round. This variety of morphologic changes suggests that each therapy induces a different form of autophagy. This is further supported by the different effects seen when autophagy is also blocked using CQ. Autophagy has been associated with the enlargement of tumor cells because of the increase in autophagic vesicles within the cells (63, 64). In long term studies, the ADR treated cells became even smaller, while the IFN-γ treated tumor cells became more elongated with a spindle-like morphology similar to mesenchymal cells. The combined ADR+IFN-γ chemo-immunotherapy

treated cells appeared to regain the cubic epithelial cell morphology similar to that seen with untreated MMC. What is interesting is that the mesenchymal appearance of the IFN-γ treated cells suggests that they may have a more stem-like phenotype. This correlates to another study done by our group, in which we showed that IFN-γ treated tumor cells become dormant and quiescent. Once they relapse, they show a CD44+CD24- phenotype with spindle-like mesenchymal morphology and a down-regulation of the tumor antigen neu (21, 24). It is possible that this mesenchymal phenotype is what allows the IFN-γ treated cells to relapse, which could be why at six weeks those were the most proliferative cells in this current study.

From this study, we have found that the higher SBRT dose of 18Gy/day RT is much more effective than the lower clinical dose of 2Gy/day. The standard dosage of RT used for patients is a total of 50Gy split into 25 fractions of 2Gy each (44). Higher SBRT doses such as 18Gy/day are not currently used in a clinical setting as it is thought that it may be too toxic for patients. However, early clinical trials have shown that a total dose of 25.5Gy split into 3 fractions of 8.5Gy has no adverse effects on patients even after two years following RT (45, 47). In fact, those studies have suggested that it is safe to increase the dose even further. Even if it is not possible to treat patients with 18Gy at once, we showed that the effects of RT are dose dependent, so not only is a higher SBRT dose of RT much more effective at eliminating tumor cells, even 8.5Gy would be more effective than the 2Gy currently being used.

A single fraction of 18Gy inhibits proliferation, induces autophagy, and induces apoptosis more effectively than a single fraction of 2Gy. Those effects are even more apparent when the number of fractions of 18Gy is increased. Multiple fractions of 18Gy results in a much stronger induction of CRT translocation to the cell membrane, suggesting that not only is 18Gy more effective at inducing apoptosis, it is also able to induce ICD and thus stimulate the immune

system to further kill tumor cells. If this is the case, then treating patients with higher fractions of RT could lead to a more effective anti-tumor response from their immune systems in addition to inducing greater cell death. Further studies are needed however to see if 18Gy RT also results in the release of ATP and HMGB1, before it can be claimed that it induces ICD, however due to the high induction of autophagy by 18Gy, it is possible, as autophagy is a requirement for ATP release (40).

While it did result in more tumor cell death, 18Gy was unable to completely eliminate the tumor cells, and therefore did not overcome RT-induced tumor dormancy. However, it did prolong tumor dormancy and delayed tumor relapse compared with 2Gy/day did. MMC treated with 2Gy resumed proliferation levels equal to that of untreated MMC in about three weeks, while 18Gy-treated cells survived in a dormant state up to six weeks after the completion of RT. In addition, the blockade of autophagy by CQ could potentially prolong tumor dormancy even further because a lower number of tumor cells survived six weeks after a high dose of RT with CQ compared to without. This was because CQ enhanced RT-induced inhibition of tumor cell proliferation, without increasing apoptosis later during treatment. Longer studies are needed to see whether tumor cells treated with 18Gy do eventually recover, however, even if they do, this could still be a promising therapy, as it would at least prolong survival and give patients more time before relapse.

In addition to showing the potential efficacy of using a higher dose of RT, our study also showed how combining chemotherapy with immunotherapy could be an effective strategy to overcome tumor relapse. We found that this combined ADR+IFN-γ chemo-immunotherapy was effective at inhibiting proliferation and inducing apoptosis. It was slightly better than ADR or IFN-γ alone, though statistical significance was not achieved with the number of repeats that we

had. However, all treatments were less efficient than ADR in inducing CRT expression on apoptotic tumor cells and combined ADR+IFN-γ chemo-immunotherapy shifted the ADR-induced G2/M cell cycle arrest toward G0/G1 arrest. This could suggest that the addition of IFN-γ to ADR, similar to the blockade of ADR-induced autophagy by CQ, actually pushes the tumor cells towards a prolonged dormancy. Combined ADR+IFN-γ chemo-immunotherapy showed a superior anti-tumor efficacy such that tumor cells did not resume proliferation or relapse six weeks after the completion of treatment, whereas all other single treatments showed shorter tumor inhibitory effects up to six weeks when tumors began to proliferate. The only exception was the inhibition of autophagy by CQ during ADR chemotherapy, which generated similar results to combined ADR+IFN-γ chemo-immunotherapy in maintaining tumor dormancy, even after six weeks. This is likely due to both CQ and IFN-γ shifting ADR-treated cells away from G2/M arrest. CQ pushed the ADR-treated tumor cells towards S-phase arrest, which is indicative of an inhibition of DNA replication, and therefore growth arrest (68), while IFN-γ shifted the cells toward G0/G1 arrest, which is characteristic of prolonged tumor cell dormancy (4, 69).

Although 18Gy/day RT and combined ADR+IFN-γ chemo-immunotherapy were effective inducers of tumor cell apoptosis, their ability to inhibit tumor relapse and inability to overcome tumor cell dormancy is to be investigated in order to determine whether therapy needs to be improved to eliminate dormant tumor cells or if prevention of tumor relapse by prolonging dormancy could be an ultimate goal to eliminate mortality associated with tumor relapse in cancer patients. Regardless of how long the tumor cells remain in a dormant state, there is still a chance they could eventually resume normal proliferation rates and relapse. Many patients do not experience relapse until as many as 20 years after they have completed their initial treatment.

And once the tumor cells have relapsed, they are usually resistant to further therapy and thus lead to much higher mortality rates in breast cancer patients (1, 3). On the other hand, there are also cancer patients whose viable tumor cells were detected in the circulation without causing relapse even 22 years after the treatment (11).

Interestingly, the impact of both the single therapies and the combined ADR+IFN-γ chemo-immunotherapy on tumor dormancy was similar on both floater cells, which represent CTC, and on adherent cells, which represent tissue resident DTC. The only exception to this was the blockade of autophagy during IFN-γ treatment, which only prolonged dormancy in adherent tumor cells. Another interesting finding of our long term studies is that blockade of autophagy by CQ appears to sustain dormancy following ADR treatment or combined ADR+IFN-γ chemo-immunotherapy, both in adherent and floater cells, as well as following IFN-γ treatment, but only in adherent cells. This suggests that although blockade of autophagy may initially inhibit treatment-induced apoptosis, it eventually also inhibits relapse following certain treatments. This also suggests that ADR-induced autophagy is not cytoprotective, as its blockade helps maintain dormancy instead of hinders it.

In this study, we were unable to completely eliminate tumor cells; however we did show that it is possible to prolong dormancy. This is important because tumor cells usually develop survival or resistance mechanisms following treatments, which prevent complete elimination of cancer. However, once tumor dormancy is established, if we can sustain it such that relapse does not occur, that would still have major clinical implications. It may not be possible to completely eliminate residual tumor cells, but inducing life-long remission could be just as efficacious. Further studies are needed to determine the feasibility of this approach. It is also necessary to determine whether continuous blockade of autophagy during dormancy could result in the

elimination of dormant tumor cells. In the present research, we only blocked autophagy during treatment and prior to the establishment of dormancy. It would be interesting to see how CQ affects the tumor cells during dormancy, and if that would help prevent relapse for even longer. Other studies should also be done with combined chemo-radio-immunotherapy with and without the blockade of autophagy to see if a different combination is more effective at eliminating dormant tumor cells. These combination therapies should also be tested in vivo, because ICD induced by 18Gy/day RT or ADR chemotherapy could induce anti-tumor immune responses which were not addressed in our in vitro studies. In addition, RT and ADR are generally administered to patients sequentially. However, if used concurrently as we used ADR with IFN-γ in this study, RT+ADR may induce a higher rate of tumor cell death, or prove to be another effective method at maintaining dormancy. Finally, more studies are needed to better understand mechanisms of tumor dormancy beyond autophagy. As this study showed, autophagy may not be the primary mechanism as previously thought. There may be another, yet unknown, mechanism involved, or dormancy may involve a more complicated interplay between different types of autophagy. Once we better understand the mechanisms behind dormancy, we can determine how best to prevent it or how best to maintain it in order to prevent tumor relapse.

Future Directions

Further studies are needed to better understand tumor dormancy and relapse in our model. First, proliferation studies, such as staining for Ki67 or CFSE, need to be conducted in order to

determine whether the dormancy established by cancer therapies is due to a balance of cell death and proliferation, or due to an induction of quiescence in the tumor cells. In addition, longer follow up studies are needed to determine whether MMC treated with a high dose of RT, combined chemo-immunotherapy, or the blockade of ADR-induced autophagy eventually resume normal proliferation rates, or if they remain dormant. In our study, we only monitored the tumor cells for six weeks. It is possible that over time, those treatments will no longer maintain dormancy and prevent relapse, so it is important to know which types of therapy do eventually lead to relapse. It would also be useful to know whether the relapsing cells are primarily CTC, DTC, or both.

In this study, MMC were treated with chemotherapy and immunotherapy concurrently. However, IFN-γ appeared to negate some of the effects of ADR, including the high expression of CRT. Because of this, it may be more efficacious to use these therapies sequentially instead. For example, the MMC should be treated with ADR first, in order to receive all the tumor killing effects of chemotherapy. Once the cells become chemo-resistant, they can then be treated with IFN-γ. Because of the high induction of CRT by ADR, it is likely that the tumor cells would still be susceptible to immunotherapy. The same could be done with RT and immunotherapy, since a high dose of RT also results in a high expression of CRT on the cell membrane.

Once the in vitro efficacy of these treatments has been fully established, the next step would be to test these therapies in an in vivo model. Finally, these therapies need to also be tested on a human cell line, such as the SKBR3 human breast adenocarcinoma cells. If these therapies prove to be just as, if not more, efficacious in vivo and in a human cell line, then it is much more likely that they will translate well to a clinical setting.

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Vita

Rebecca Caroline Keim was born on April 7, 1990 in Arlington, Virginia and is an American citizen. She received her Bachelor of Science in Biology from Virginia Commonwealth University, Richmond, Virginia in 2012.