M2-like Macrophages Exert Cardioprotective Effects in Doxorubicin-induced Cardiotoxicity

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Background

Autophagy is a highly regulated intracellular metabolic process that occurs in all eukaryotic cells in which cytoplasm containing cellular contents such as misfolded or aggregated proteins, damaged organelles, and pathogens is delivered to the lysosome, a degradative organelle in the cell, to be broken down and recycled (Yang & Klionsky, 2009). More specifically, these cytoplasmic materials are isolated by the unique double-membraned phagophore to form an autophagosome organelle that mediates the movement and delivery of the materials through fusing its outer membrane with the endosome and hydrolytic enzyme-containing lysosome organelles that degrade the internal material. Once the macromolecules are degraded in the lysosome, its monomeric units such as amino acids are exported to the cytosol to be recycled (Mizushima, 2007).

One of the most prominent proteins that regulate cellular metabolism, and thus autophagy, is the mechanistic target of rapamycin (mTOR). mTOR has two distinct complexes, mTORC1 and mTORC2, that differ in key accessory proteins that allow for differentiation in functions. mTORC1 is primarily responsible for driving the majority of the cell's anabolic processes, increasing extracellular nutrient uptake, and inhibiting catabolic processes such as autophagy. The continuation of mTORC1's actions is heavily dependent on the availability of nutrients in the cell and additional second messenger signals, such as insulin, that indicate the organism's nutritional state (Deleyto-Seldas & Efeyan, 2021).

Once nutrient depletion or cellular stress is detected, mTORC1 is inhibited through a complex cellular pathway that activates a series of protein complexes. Two of these protein complexes are Unc-51 like autophagy activating kinase 1 (ULK1) and vacuolar protein sorting-34 (VPS34), whose dual-activation triggers recruitment of additional autophagy-related

proteins to the phagophore membranes and initiate autophagosomal maturation (Dunlop & Tee, 2014).

While autophagy plays a major role in maintaining cell homeostasis, it has also recently emerged in the cardio-oncology field as a potential protective mechanism against cardiotoxicity (Chen et. al 2022). In a study by Chen et. al, pre-starved mice who received Doxorubicin and had confirmed increased autophagic flux experienced a decrease in cardiomyocyte apoptosis compared to Doxorubicin-receiving mice who were not starved prior to chemotherapy administration and had decreased autophagic flux (Chen et al., 2022). This implies that autophagy could be a potential therapeutic target in order to reduce the harmful effects of Doxorubicin-induced cardiotoxicity.

The potential role of immune cells to modulate autophagy in the heart

Recent evidence suggests that innate immune cells are important to maintain homeostasis in the heart at all stages of development, from the fetal stage to adulthood (Lavine et al., 2018 ; Nicolás-Ávila et al., 2020). Macrophages are a critical component of the innate immune system and are derived from pluripotent hematopoietic stem cells in the bone marrow. Macrophages are primarily involved in defense against various pathogens and removing old, senescent, and dead cells (Lendeckel et al., 2022). During times of infection, inflammation, or tissue injury, macrophages will migrate to the site and generate consecutive ranges of cytokines, chemokines, matrix metalloproteinases, and growth factors to induce combinations of both inflammatory and anti-inflammatory impacts. Resident and monocyte-derived macrophages express a variety of pattern recognition receptors that identify both pathogen-associated molecular patterns and

damage-associated molecular patterns. Using these receptors, macrophages are able to recognize and ingest pathogens and cell debris using phagocytosis (Lavine et al., 2018).

Depending on factors such as microenvironmental stimuli and pathogen or antigen structure, macrophages can also differentiate into M1 and M2 subtypes to fulfill different roles (Lendeckel et al., 2022). M1 macrophages demonstrate strong cytotoxic effects against infected cells, eliminate pathogens during the course of infections, and contribute to strengthening the body's defense against infections. This is accomplished through an increase in the generation and release of proinflammatory or activating cytokines. M2 macrophages, however, have their primary roles in tissue repair and fibrosis. Unlike M1 macrophages, they are generally anti-inflammatory, and have been associated with cancer tumor progression and immunosuppression as a result (Lendeckel et al., 2022). Previous literature further suggested that M2 macrophages have a cardioprotective effect in heart failure (Gao et al., 2021). A study conducted by Liu et al. showed that this cardioprotective effect is maintained in doxorubicin-induced cardiotoxicity and is partly attributed to the mitochondrial transfer that occurs from the macrophages to the damaged cardiomyocytes (Liu et al., 2022).

Aim

The purpose of our study for the 2023 Laidlaw Undergraduate Research and Leadership Program was to investigate the effect of M2 macrophages on autophagic flux in the setting of in-vitro Doxorubicin-induced H9C2 cardiomyocyte damage. We will further validate the translational potential of M2 macrophages by investigating the impact on the heart and on the tumor in the tumor-mouse models treated with doxorubicin. We hypothesize that M2 macrophages can exert cardioprotective effects in the heart through modulating autophagy.

Materials and Methods

In this investigation, we induced the growth of M2-like macrophages through application of macrophage colony-stimulating factor (M-CSF) and cytokine interleukin 4 (IL-4) to macrophages derived from the bone marrow. Following this, we evaluated the impact of these M2-like macrophages on key autophagic signaling markers of H9C2 cells that had been subjected to Doxorubicin exposure of 30 minutes and 24 hours.

The generation of M2 macrophages

Female BALB/c mice were euthanized by cervical dislocation and bone marrow from both femurs and tibias were collected and treated with Red Blood Cell Lysis Buffer to remove red blood cells. The mixture was then pelleted at centrifuge settings of 1400 rpm for five minutes. The cells were washed three times in in cold Phosphate buffered saline (PBS) and cultured in a 10-cm cell culture dish containing 7 mL of pre-warmed complete RPMI 1640 medium (10% FBS, 1% Penicillin-streptomycin, RPMI 1640) and incubated for five hours at 37°C and 5% CO2 to remove stromal cells. After five hours, the stromal cells adhere to the surface of the dish, while the bone marrow stem cells and precursor cells remain free-floating or suspended in the media. The media was carefully collected without disturbing the adhered stromal cells and washed twice in PBS, and cultured in a 10-cm tissue culture dish with 10 mL Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. This step was crucial to ensure the purity of the bone-marrow derived macrophages (BMDMs) to remove stromal cells, which are indistinguishable from differentiated bone-marrow derived macrophages if allowed to grow. The cells were also treated with 20 ng/ml macrophage colony-stimulating factor (M-CSF) to stimulate macrophage production. The

medium was changed every three days. On the seventh day, the mature macrophages were treated with 20 ng/ml cytokine interleukin 4 (IL-4) to induce differentiation into M2-like macrophages.

Figure 1: Percent Viability of M2-like Macrophage Cell Cultures

	Mean % Viable	**Mean % Viable ± SD**
Culture #1	60.6%	60.6% ± 3.5%
Culture #2	82.6%	82.6% ± 6.1%

Figure 1. Percent viability of M2-like macrophage cell cultures that utilized two different cell collection methods. Cells were counted using a hemocytometer.

After 7 days of culture, the macrophages were collected, pelleted, and readjusted to a concentration of 1 x 10^6 cells/mL. Cells were immediately used for assays or cryopreserved in complete growth media containing 10% of DMSO. One important difference to note involves the collection of M2-like macrophages. In the first culture, the macrophages were manually scraped off the culture dish and counted before they were pelleted, which resulted in a viability of 60.6% ± 3.5% (Figure 1). This batch was used for the downstream operations and data analysis as displayed below. The second culture of M2-like macrophages had an extra 5 days of macrophage-formation before M2-like differentiation was initiated with IL-4. Additionally, the M2-like macrophages in the second culture that were removed using Cellstripper Nonenzymatic Cell Dissociation Solution™ (Corning, USA) were pelleted first before cell counting was performed. While there were the same amount of cells as the first culture, less dead cells were observed in this culture, which had a higher cell viability of 82.6% ± 6.1% (Figure 1).

Co-culture of M2 macrophage with murine cardiomyocytes

The cells were then co-cultured for 24 hours with H9C2 cells, a specific line of embryonic rat cardiomyocytes, that had either no Doxorubicin exposure, 30-minute Doxorubicin exposure, or 24 hour Doxorubicin exposure.

Fluorescence Activated Cell Sorting (FACS) to measure apoptosis, autophagy, and Doxorubicin fluorescence.

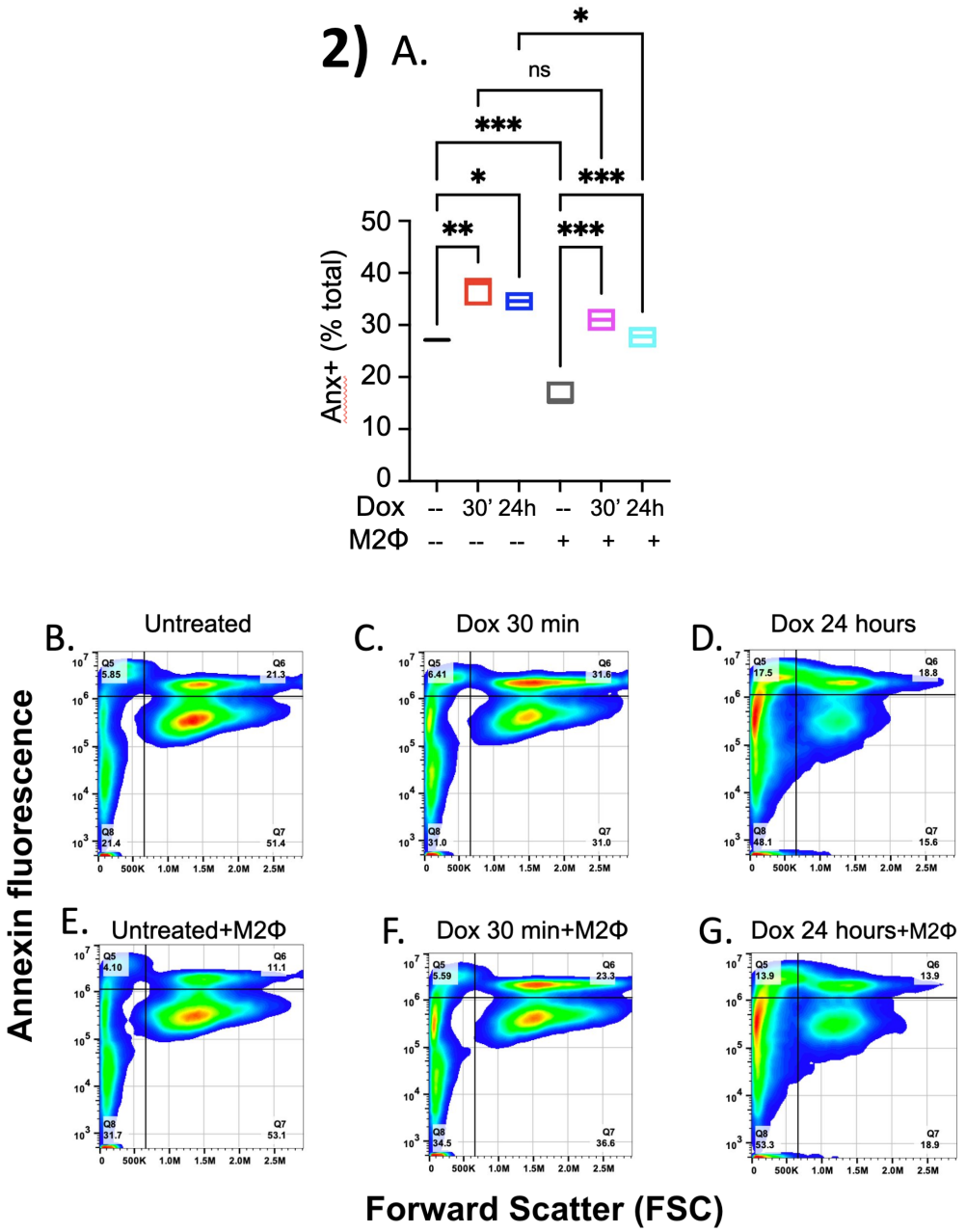
After 24 hours of co-culture, cells were treated with autophagy-detecting nanoparticles (ADN) manufactured in-lab and Cy5.5 to observe autophagic flux, Annexin V to evaluate apoptosis, and Sytox Blue for live-dead assay. The inherent doxorubicin fluorescence was detected to assess doxorubicin uptake or retention. FACS was performed on the Cytek® Aurora. FACS analysis was performed using FlowJo™ software.

Statistics methods

Statistical analysis and graphs generation were performed using Prism (GraphPad). All quantitative data were tested for normality. Analysis of variance (ANOVA) was used to compare the means and variance of three or more groups, followed by Tukey post-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$, ns=not significant. Floating bars graph depicts the minimum, maximum, and mean values.

Results

Apoptosis

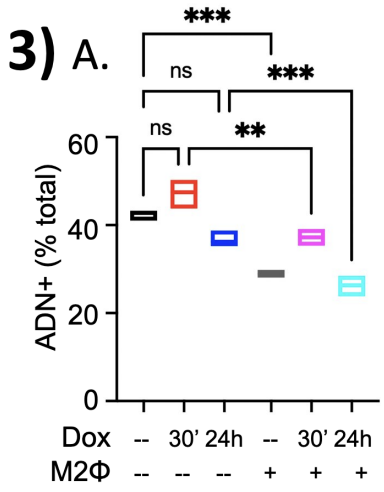


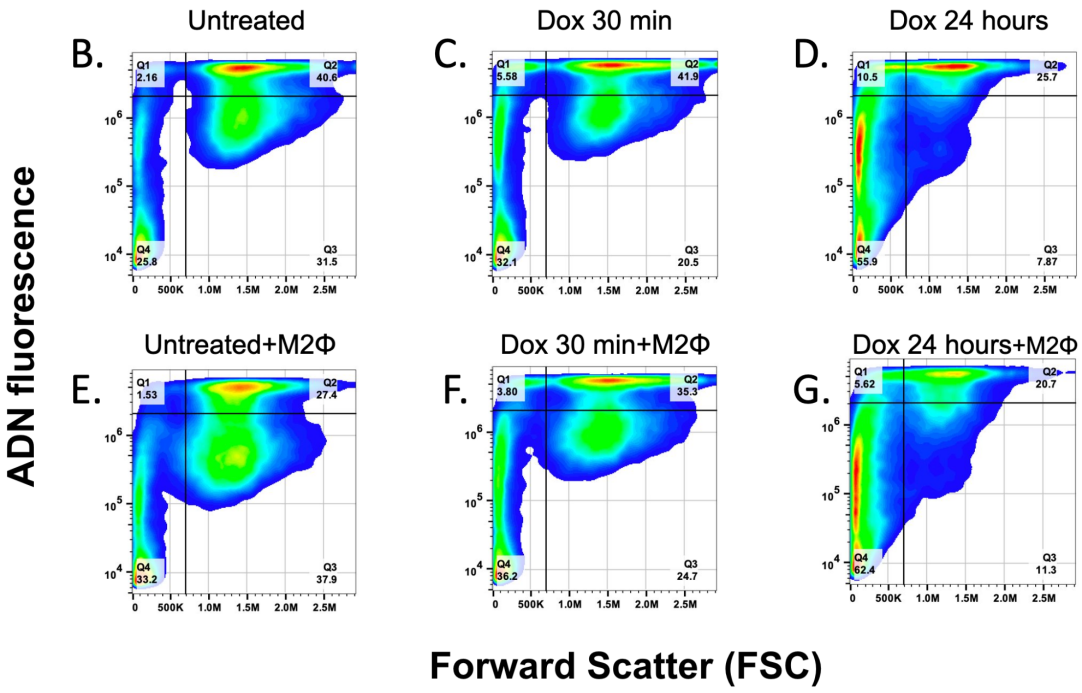
Figures 2A-G. H9C2 cells with AnnexinV fluorescence quantifying apoptotic signals from H9C2 cells.

Doxorubicin functions through inducing apoptosis in both tumor cells and normal cells, which can lead to cell death and cause myocardial damage. As demonstrated in Figure 2A, apoptosis reduction was observed through significantly decreased AnnexinV signal in all the co-cultures including M2-like macrophages. This can especially be seen at the 24 hour mark of

macrophage co-culture with Doxorubicin, where AnnexinV levels had returned to almost the baseline amount in the untreated group. Additionally, the 30-minute mark of macrophage co-culture with Doxorubicin had a higher AnnexinV signal than the 24 hour co-culture. These two observations could signify that there may be more early apoptotic cells present at the 30-minute mark whereas the 24 hour co-culture may have more necrotic cells than early apoptotic cells. Because necrotic cells are not stained by AnnexinV, this could lead to a decreased AnnexinV signal. However, dual staining to examine necrotic cell death is needed to confirm this. Figures 2B-G also show cell shrinkage as a result of Doxorubicin. Interestingly, in the presence of M2-like macrophages, while the Annexin-positive population decreased, the cells still shrank in all the co-culture samples. This was unexpected, since cell shrinkage is a hallmark characteristic of apoptosis.

Autophagy



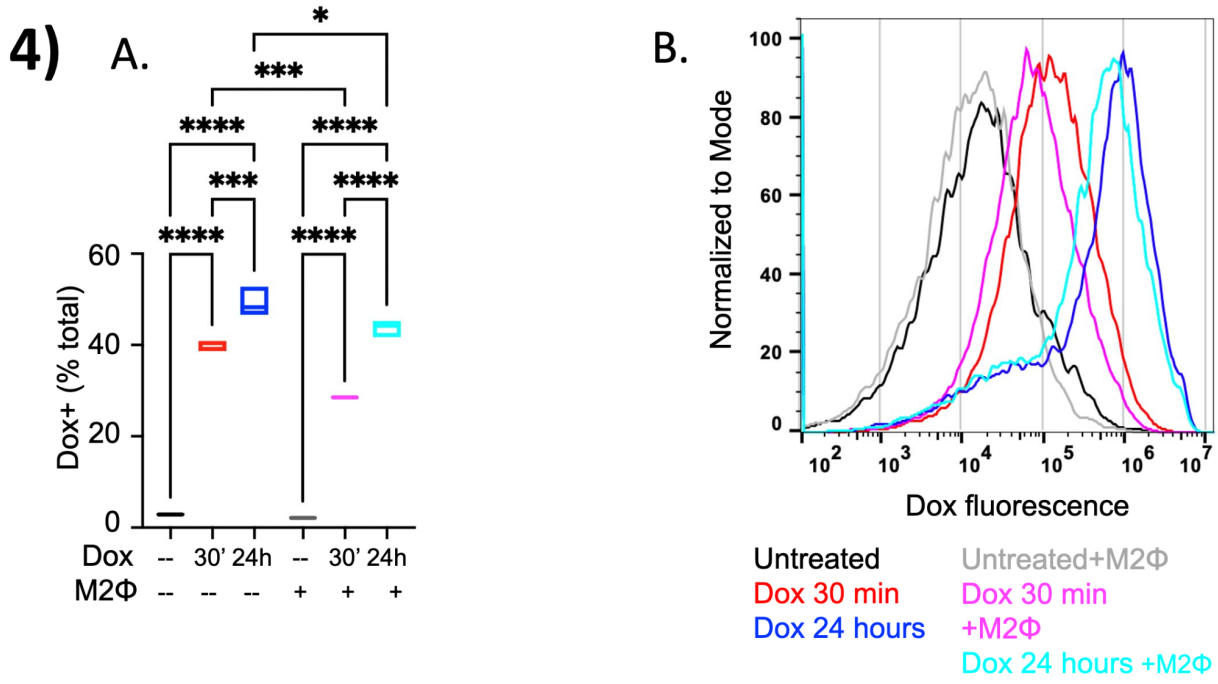


Figures 3A-G. H9C2 cells with Autophagy-detecting nanoparticle (ADN) fluorescence indicating autophagic signals from H9C2 cells.

Figures 3A-G present interesting results highlighting the potential effect of M2-like macrophages on autophagic flux. First, Figure 3A exhibits that co-cultures with M2-like macrophages resulted in a significantly decreased ADN-positive population compared to cultures without M2-like macrophages. This is especially true between the cultures with a Doxorubicin exposure of 30 minutes and 24 hours. Figure 3F had 39.1% of the total cell population as ADN-positive, whereas Figure 3G saw only 26.32% of the population being ADN-positive. This downregulated signal in the co-culture represents a significant decrease in autophagic flux, which could potentially indicate an interaction between M2-like macrophages and autophagy. Moreover, as seen in Figures 3B-G, as the ADN-negative population increased in the presence of M2-like macrophages, a leftward shift of the population, signifying decreasing cell size, was observed in all the samples containing Doxorubicin. For instance, Figure 3C had 37.68% of the total cell population being small, but Figure 3D saw this percentage rise to 66.4%. This trend

was maintained in all samples containing Doxorubicin regardless of the presence of M2-like macrophages. The cell size did not play a factor in the ADN-positive population, as the signal was downregulated regardless of cell size when in the presence of M2 macrophages. This pattern is supported when comparing Figures 3C and 3F with Figures 3D and 3G. This shows that M2-like macrophages are unable to rescue Doxorubicin-induced H9C2 cell shrinkage. Both of these described trends are seen in Figures 3B-G, especially Figure 3G: as the co-culture time continued, more cells became ADN- and less intact.

Doxorubicin Fluorescence



Figures 4A-B. Doxorubicin (Dox) fluorescence in co-cultured and non co-cultured samples at various time points to quantify Doxorubicin uptake by H9C2 cells.

Figures 4A-B provide a visual representation of the measurement of Doxorubicin fluorescence in all the samples, which provides insight into the Doxorubicin uptake and retention in the H9C2 cell samples. From the data presented in Figure 4A, it can be seen that the presence

of M2-like macrophages significantly reduces Dox fluorescence in all the samples. This is further supported in Figure 4B with the observable leftward shift of the Doxorubicin fluorescence peak of samples containing M2-like macrophages compared to samples without. The efflux mechanism of Doxorubicin after entering the cells is still a relatively unstudied field, but Menna et al. suggests that cardiomyocytes may utilize the physiological oxyferrous myoglobin ($Mb^{II}O_2$) and/or metmyoglobin (Mb^{III}) to degrade and diminish cellular levels of Doxorubicin (Menna et al., 2007). Thus, M2-like macrophages may interact with these cellular components to decrease the uptake and retention of Doxorubicin in H9C2 cardiomyocytes as a protective mechanism. It is also worth noting that the Doxorubicin uptake of cells was not as high as expected in all the samples. As a result, the possibility that the decreased Doxorubicin fluorescence in co-cultures containing M2-like macrophages was due to a general decreased Doxorubicin uptake cannot be ruled out.

Discussion

It can be concluded from the data presented above that M2-like macrophages can reduce autophagy and apoptosis without reducing the shrinkage of H9C2 cell size in the presence of cardiotoxic doxorubicin. This gives support to our hypothesis that M2-like macrophages can exert cardioprotective effects in the heart through modulating autophagy. This would be not completely unsurprising as recent literature shows the ability of M2-like macrophages to partake in cell-signaling activities (Messex et al., 2020). Moreover, the cell shrinkage phenomenon in the presence of Doxorubicin is not completely unexpected since there is literature showing that Doxorubicin may cause cell shrinkage through activating the downstream pathways that can cause cytoskeletal alterations that result in cell shrinkage (Green & Leeuwenburgh, 2002).

In conclusion, more evaluations are required to confidently confirm the translational potential of this as a viable cardioprotective strategy, especially because M2 macrophages have been shown to contribute to tumor growth due to its anti-inflammatory properties that lead to immunosuppression (Jayasingam et al., 2020)

Future directions

To confirm our results, replication of the experiment is required using the second batch of cultured M2-like macrophages. Due to a difference in collection method, the second batch had higher cell viability compared to the first batch used in the experiments, which will yield cleaner data and provide a more clear understanding into the role of M2-like macrophages on H9C2 cell Doxorubicin retention, autophagy, and apoptosis. It is also important to use FACS to confirm M2 differentiation into M2 macrophages using the two markers, anti-CCR2-APC and anti-CD206-PE, of M2-like macrophages.

Downstream experiments to further assess the impact and understand the potential protective mechanism of M2-like macrophages is also essential. The data presented here suggests that some potential mechanisms could include modulating autophagy and/or apoptosis, or altering the efflux mechanism of Doxorubicin from cells. Similarly, dual-staining for necrotic death and apoptosis would provide more insight into the decreased levels of AnnexinV in the 24-hour co-cultures.

It would also be interesting to examine if the anti-inflammatory cytokines that are released by M2 macrophages (IL-10, IL-13, TGF-β) themselves are sufficient to be cardioprotective, since both inflammatory and anti-inflammatory cytokines have been shown to be potentially cardioprotective (Hanna & Frangogiannis, 2020).

Because the M2-like macrophages were cultured in a separate compartment from the H9C2 cells in the co-culture, it would also be interesting to repeat these experiments with just the conditioned media to assess if it contains soluble factors that are able to replicate the protective effects of the macrophages.

Most importantly, additional experiments are required to assess if these results are translatable in-vivo. By using Doxorubicin-injected tumor mice, it would be valuable to assess whether M2 macrophage transfer can reduce apoptosis, modulate autophagy, and exhibit other effects on cardiomyocytes that are unable to be observed through in vitro experiments. These findings can have enormous implications for the potential of M2 macrophages in future therapies to counter the cardiotoxic effects of Doxorubicin in cancer patients worldwide.

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