EVALUATING CD47 EXPRESSION IN GLIOMA STEM CELLS AS AN IMMUNOTHERAPY APPROACH

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A Thesis

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ABSTRACT

Michael E. Geusz, Committee Chair

Although there are many therapies against cancer, they involve devastating side effects. Hence, we need to understand the nature of cancer cells and the unique markers that exist within these cells that allow them to evade the immune system. These cell properties could be exploited to our advantage. CD47 is a cell membrane receptor protein widely expressed in most cells and is a versatile and crucial target in the tumor microenvironment for creating novel therapeutic approaches for cancer treatment. However, few studies have examined CD47 in glioma. CD47 is found on the surface of multiple cell types, and it usually protects the cells from being removed by phagocytes. It has been found that most cancer cells have high CD47 expression that prevents them from being engulfed by macrophages or activated microglial cells, essentially acting as a "don't eat me signal." A second protein expressed on cancer cells, calreticulin (CALR), facilitates cell removal by phagocytes, serving as an "eat me signal."

In this project, we compared CD47 expression in glioma cancer stem cells (CSCs), which are negative for Hoechst 33342 nuclear staining (H-), and non-stem glioma cells (Hoechst-positive, H+) of the C6 cell line derived from a rat astrocytoma. We examined the colocalization of CALR with CD47 in both C6 cell types using immunocytochemistry and compared CALR and CD47 gene expression reported in the NCI-60 database of multiple human cancers.

We found a significant difference in CD47 expression, with more CD47 in the H+ cells than the H- cells, which could imply that GSCs are more susceptible to CD47 immunotherapy. The highest expression of CD47 (> 50% above signal range) appeared to be in exosomes related

to both cell types. We found a positive correlation between CD47 and CALR distribution in the H+ cells ($p = 0.0204$) and in both H+ and H- cells combined ($p = 0.0121$), suggesting that the cells might protect themselves from CALR-induced phagocytosis by increasing CD47. We also discuss the possible role of circadian clock control of these two proteins. Our data help provide valuable insight into how these cells might evade detection and death by immune cells.

I want to dedicate this thesis to the most important people who have supported and inspired me throughout this journey.

To my beloved Family: Adeeb, my darling husband, your unwavering love, encouragement, and belief in me. Your patience, understanding, and constant support have been my anchor during the highs and lows of this research. Your presence has given me the strength to pursue my dreams, and I dedicate this thesis to you with heartfelt gratitude.

To my supervisor, Michael E. Geusz:

I am immensely grateful for your guidance, mentorship, and expertise throughout this research journey. Your unwavering support, valuable insights, and constructive feedback have made me a better researcher. Thank you for believing in my potential and pushing me beyond my limits. This thesis is a tribute to your dedication and invaluable contribution to my growth.

Finally, to all the patients and their families who have been affected by glioblastoma:

This thesis is dedicated to you. Your courage, resilience, and strength in the face of adversity constantly remind you of the urgent need for innovative treatments. I hope that this research may contribute, in some small way, to the advancement of immunotherapy for the treatment of glioblastoma, ultimately improving the lives of those affected by this devastating disease.

With heartfelt appreciation and dedication, Sana Irshad

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I want to express my deepest gratitude to all the individuals who have contributed to completing this thesis. Your support, guidance, and encouragement have been invaluable throughout this research journey.

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I sincerely appreciate my thesis committee members, Julia Halo and Paul F. Morris, for their valuable input, feedback, and suggestions. Their expertise and constructive criticism have significantly enhanced the quality of this work.

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My dear friends thank you for your understanding, motivation, and encouragement. Your words of support and moments of levity have provided much-needed respite during these challenging times.

While I have attempted to acknowledge everyone who has played a role in this journey, I apologize for inadvertently missing anyone. Please know that your contribution is sincerely appreciated.

I am profoundly grateful for the guidance, support, and encouragement I have received throughout this research endeavor. This work was only possible with the collective effort and collaboration of all those mentioned above. Thank you for participating in this journey and for your significant contributions.

With heartfelt gratitude,

Sana Irshad

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Cancer stem cells

Cancer is a state of uncontrolled, abnormal division of cells. It can affect every and any cell type in the human body. When it affects the glial cells or supporting cells in the brain, it is called glioma. Gliomas can be classified according to the specific supportive cell type affected by it. Glioblastoma, a type of astrocytoma, is a brain cancer that affects the astrocyte cell type and causes abnormal, uncontrolled cell division of astrocytes.

The most frequent malignant primary brain tumors in adults are glioblastomas(Gimple et al., 2019). The prognosis for patients is still poor despite advancements in traditional glioma therapies such as surgical removal, chemotherapy, and radiation. Therefore, creating innovative therapies that increase impacted patients' survival chances is crucial. A growing corpus of research on the role of specific tumor-initiating cells, CSCs, in several malignancies, including pancreatic, colon, melanoma, brain, and colon cancer, is particularly intriguing. This cell subpopulation contains various traits exclusive to stem and progenitor cells. Importantly, these traits are also linked to chemo-radiotherapy resistance and higher relapse rates after treatment. (Li et al., 2018).

Glioblastoma stem cells

Within any cancer, a small population of cells accumulate mutations and have the properties of stem cells, which is the ability to self-renew and differentiate into different cell types within tumors(Venere et al., 2011). In glioma, these stem-like cells are known as glioma stem cells (GSCs). These stem cell populations are challenging to treat due to the following main traits:

1).Increased drug efflux pumps: such as ATP-binding cassette (ABC) transporters in their membrane, help them resist chemotherapy. These proteins can efflux known chemotherapeutic medications and other harmful substances from the cell. A large family of proteins known as ABC protein isotypes includes ABCG2 (also known as MDR1-multidrug resistance protein 1). These ABC transporters have received considerable attention in regard to drug resistance, particularly in glioma and breast cancer cells(Galli et al., 2004). We used this pump to our advantage in this study to differentiate between glioma stem-like cells and the other glioma cells of the C6 lineage by its ability to export the Hoechst 33342 nuclear stain.

2) Some malignant cells have a DNA damage repair pathway that is activated, which enables them to repair DNA damage brought on by chemotherapy medications(Gambelli et al., 2012). For instance, temozolomide (TMZ), an alkylating chemotherapeutic drug, can methylate the sixth carbon of guanine residues in DNA, causing DNA polymerase to add thymine rather than cytosine to the replicating strand. A DNA mismatch repair protein detects the error, which to fix it starts a futile repair cycle that ultimately results in cell death. However, some malignant cells overexpress the enzyme MGMT (methyl guanine methyl transferase), which enables the cell to remove the methyl group and resume replication, protecting the cell from the effects of TMZ and evading cell death(Hegi et al., 2008).

3) Pro-survival signaling pathway activation: The PI3K/Akt, Notch pathways, and increased expression of cluster of differentiation 47 (CD47) are a few of the pro-survival signaling pathways that are activated by GSCs. These help them survive by preventing apoptosis (programmed cell death) and fostering cell multiplication; these mechanisms support cell survival and aid in chemoresistance(Bleau et al., 2009). Our research focuses on one of these, the CD47 protein.

4) Modified cellular metabolism: GSCs exhibit metabolic modifications that enable them to endure stressful situations, such as chemotherapy exposure. They can change to different energy sources, such as glycolysis, to reduce their reliance on oxidative phosphorylation. Their resistance to chemotherapy may be influenced by these metabolic alterations(Y. Zhao et al., 2011).

5) Heterogeneity within GSC populations: Within the same tumor, diverse molecular and genetic features of GSCs can be found. Because of this heterogeneity, a small subset of GSCs may exist that are naturally resistant to chemotherapy and can repopulate the tumor(Denysenko et al., 2014).

CD 47

Although there are treatment options, glioblastoma is a particularly aggressive and invasive form of brain cancer with a dismal outlook (Schaff & Mellinghoff, 2023). Glioblastoma cells can elude immune identification and death through a variety of ways; the immune system plays a critical role in the regulation of tumor growth. One such method is the overexpression of CD47, a transmembrane protein that serves as a "don't eat me" signal to phagocytic cells like macrophages (H. Zhao et al., 2022). The purpose of this thesis is to investigate CD47's function in glioblastoma and its potential as a therapeutic target.

CD47 has a short, C-terminal domain (CTD), a 5-transmembrane (5-TM) spanning helical bundle domain, and a highly glycosylated, IgV-like extracellular domain (ECD) at the N-terminus (Fenalti et al., 2021a). CD47 consists of a 32 kDa polypeptide chain (Figs. 1 & 2). The five main domains are; An N-terminal signal peptide, an extracellular domain, a transmembrane domain, a cytoplasmic domain, and a C-terminal tail. The CD47 extracellular domain, which binds ligands, is made up of two tiny beta sheets sandwiched between a sizable, heavily

glycosylated loop. A number of disulfide bonds in the loop support its stability. It is well known that CD47's glycosylation significantly affects its functionality by modifying its interactions with other molecules (Fenalti et al., 2021a). CD47 has a single alpha-helix traversing the cell membrane as its transmembrane domain. It has been demonstrated that it controls CD47 signaling and functions and oversees attaching CD47 to the cell surface.(Brown et al., 1990; Fenalti et al., 2021b)

CD47's cytoplasmic domain is brief and enzymatically inactive. It interacts with a number of intracellular signaling proteins, including the cytoskeletal protein alpha-actinin and the Lyn protein of the Src family of kinases (Oldenborg et al., 2000). By using X-ray crystallography, the extracellular domain of CD47's crystal structure was identified, showing its intricate molecular architecture(Oldenborg et al., 2000). CD47 adopts a compact conformation, with the glycosylated loop serving as the molecule's core portion. The crystal structure demonstrates that the Ig-like domain of CD47 interacts with the V domain of the signal-regulatory protein alpha (SIRP) of macrophages through a complementary contact comprising important residues from both proteins. It also sheds light on the conformational alterations brought on by ligand interaction. It demonstrates that ligand interaction causes the stalk region of CD47 to rotate. This conformational change moves the cytoplasmic tail closer to the membrane, perhaps enhancing subsequent signaling events (Barclay & Van Den Berg, 2014).

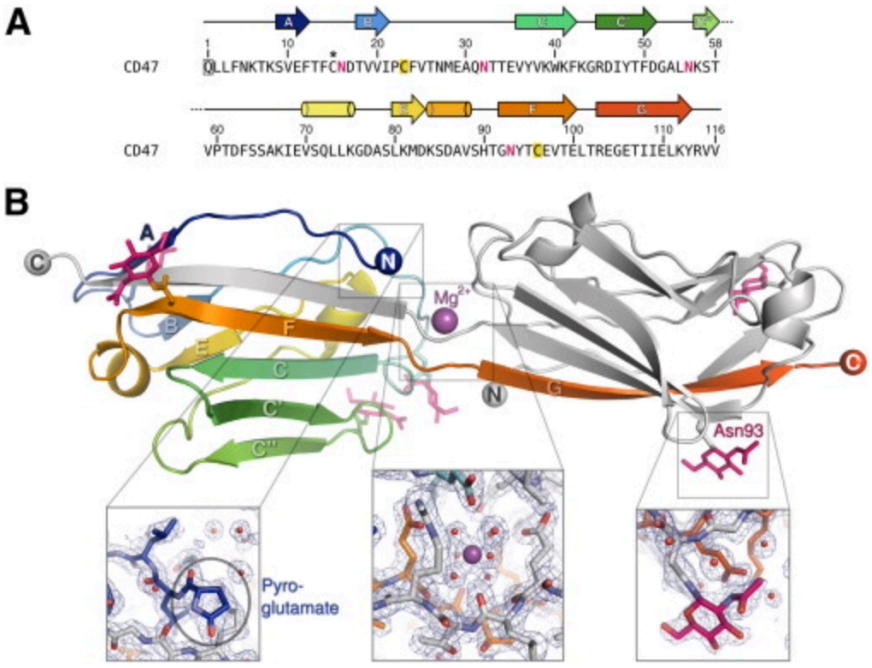

Figure 1. Molecular structure of CD47. *Note; arrows and cylinders represent β sheets and α helices, respectively* (Hatherley et al., 2008).

Note: this image is licensed under the creative commons attribution. Hatherley, D., Graham, S. C., Turner, J., Harlos, K., Stuart, D. I., & Barclay, A. N. (2008). Paired Receptor Specificity Explained by Structures of Signal Regulatory Proteins Alone and Complexed with CD47. *Molecular Cell*, *31*(2), 266–277. https://doi.org/10.1016/j.molcel.2008.05.026

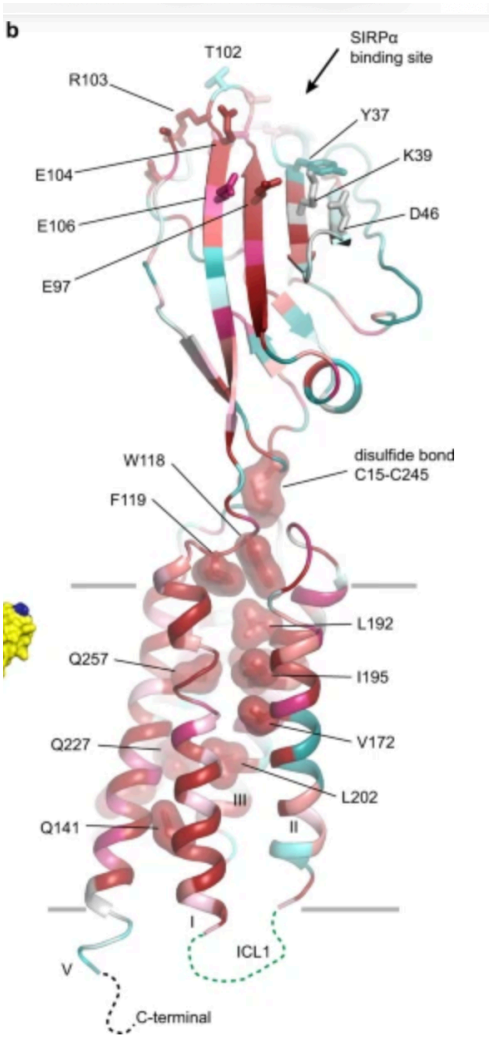

Figure 2. CD47 protein structure. *Notes; gray lines represent the approximate lipid membrane boundaries* Gray (Fenalti et al., 2021a).

Note: this image is licensed under the creative commons attribution. Fenalti, G., Villanueva, N., Griffith, M., Pagarigan, B., Lakkaraju, S. K., Huang, R. Y. C., Ladygina, N., Sharma, A., Mikolon, D., Abbasian, M., Johnson, J., Hadjivassiliou, H., Zhu, D., Chamberlain, P. P., Cho, H., & Hariharan, K. (2021b). Structure of the human marker of self 5-transmembrane receptor CD47. *Nature Communications 2021 12:1*, *12*(1), 1–14. https://doi.org/10.1038/s41467-021-25475-w

CD47 expression is increased in glioblastoma cells relative to healthy brain cells, and its overexpression has been associated with a worse prognosis in glioblastoma patients. When CD47 and the macrophage's SIRP interact, the macrophages' phagocytic activity is inhibited, which allows tumor cells to escape immune monitoring. Numerous studies have shown that inhibiting the CD47-SIRP connection can improve macrophage phagocytosis of glioblastoma cells, resulting in tumor cell death and enhanced survival in animal models (Fenalti et al., 2021a; Li et al., 2018).

CD47 in exosomes

Small, membrane-bound particles called extracellular vesicles (EVs) are produced from cells and are hypothesized to serve as cellular messengers that transport cargo from one cell to another (Whiteside, 2016). EVs come in a variety of subtypes with varying functions, loads, and sizes that can range from 30 to 1000 nm in diameter. The exosome, which ranges in size from 30 to 150 nm, is the smallest kind of EV (Fig. 3). The multivesicular body (MVB), a cellular structure where EVs are produced, is a result of endosome invagination. The vesicles are liberated when the MVB fuses with the plasma membrane and releases its cargo (Whiteside, 2016).

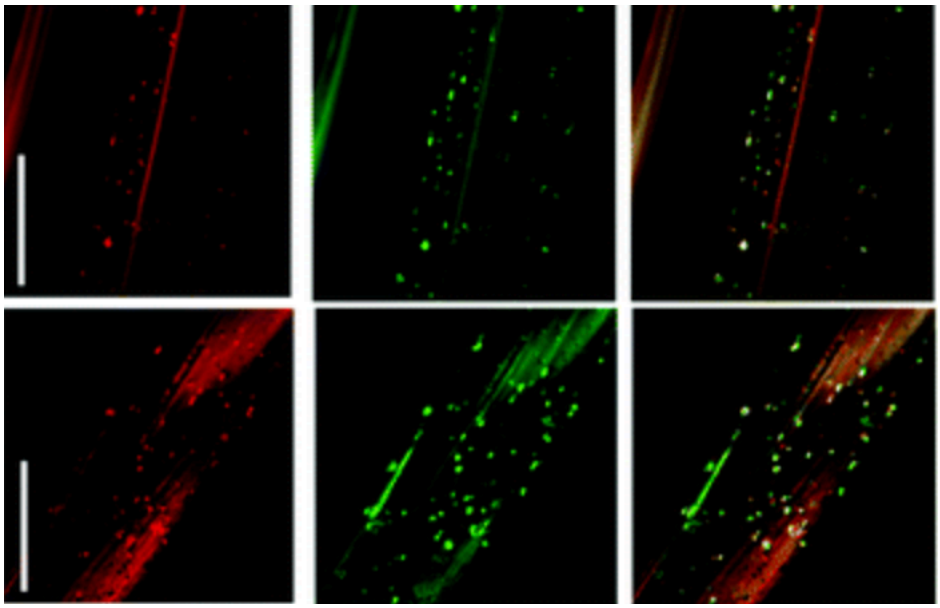

Figure 3. CD47 immunofluorescence labeling in fiber exosomes. *Note; Fiber immunofluorescence imaging showing immunolabeling procedure. The red color depicts the presence of the CD81 tetraspanin protein on exosome membranes bound to the polyester polymer fiber surface. Scale bar = 10 μm* (K. Jackson et al., 2022)

Note: this image is licensed under the creative commons attribution. K. Jackson, K., R. Powell, R., F. Bruce, T., & Kenneth Marcus, R. (2022). Facile, generic capture and on-fiber differentiation of exosomes via confocal immunofluorescence microscopy using a capillary-channeled polymer fiber solid-phase extraction tip. *Sensors & Diagnostics*, *1*(3), 525–533. https://doi.org/10.1039/D2SD00007E

CD47 is present in exosomes, and exosomes actually promoted a therapy targeting oncogenic Kras in pancreatic cancer (Kamerkar et al., 2017). The researchers detected CD47 in exosomes using mass spectrometry and discovered that CD47 in those exosomes actually shielded cells from being phagocytosed by monocytes and macrophages (Kamerkar et al., 2017).

CALR

Calreticulin (CALR) and CD47 are both involved in the body's immunological response (Feng et al., 2019). Although they perform various tasks, they are related by their part in immune system regulation. The protein CD47 is present on the surface of numerous cell types in the body, including cancer cells and red blood cells. Although the primary role of CD47 is to act as a "don't eat me" signal to the immune system, keeping immune cells from attacking and destroying the cells that express it, the intracellular protein CALR is involved in a number of cellular processes, such as calcium homeostasis and protein folding. CALR can, however, also be expressed on the cell surface in specific circumstances. As a result, CALR serves as an "eat me" signal to the immune system, encouraging the removal of the cells that express it (Feng et al., 2019).

Calreticulin is a calcium-binding protein primarily located in the endoplasmic reticulum (ER). It participates in several cellular functions, such as calcium homeostasis, signal transduction, and protein folding and quality control(Ron & Walter, 2007). For example, CALR improves quality control of protein folding: To provide appropriate folding and glycosylation of newly generated proteins, CALR serves as a chaperone protein by binding to newly made proteins after they enter the ER lumen. To maintain ER protein homeostasis, it also takes part in the identification and destruction of misfolded or unfolded proteins, in which case CALR is rapidly upregulated through induction of the CALR gene during the unfolded protein response (UPR) in response to ER stress (Hetz, 2012).

CALR also controls intracellular calcium ion levels, which is important for numerous cellular functions, such as muscle contraction, secretion, and apoptosis. It functions in

maintaining low cytosolic calcium because high cytosolic calcium can initiate apoptosis by activating a cascade of enzymes.(Hebert & Molinari, 2007)

CALR is also connected to a number of signaling pathways, including those involved in cell growth, apoptosis, and immunological responses. For instance, CALR can initiate signaling cascades by interacting with cell surface receptors such as CD91 and LDL receptor-related proteins. CALR has other roles in a variety of cellular activities, including antigen presentation, cell adhesion, and viral infection (Michalak et al., 1999).

Immunotherapy and the possibility of using CD47

Immunotherapy utilizes the immune system to find and eliminate cancer cells and is considered a fast-developing branch of cancer treatment. Numerous strategies, including monoclonal antibodies, small compounds, and bi-specific antibodies, have been investigated to use CD47 as a target for immunotherapy(Feng et al., 2019).

The use of CD47-blocking monoclonal antibodies, such as Hu5F9-G4 and CC-90002, is one of the most promising strategies (Reinhold et al., 1995). These drugs have demonstrated encouraging outcomes in preclinical research and early-phase clinical trials in a variety of malignancies, including lymphoma, leukemia, and solid tumors. These antibodies function by preventing CD47 from interacting with its receptor, SIRP, which enhances macrophage phagocytosis of cancer cells(Reinhold et al., 1995).

C6 glioma cell line

C6 is a rat glial cancer cell line that was previously isolated from an induced glioma formed from astrocytes. It is a well-studied cell line that has been utilized in comparative studies of many brain tumors, including glioblastomas, an astrocyte tumor (Giakoumettis et al., 2018). GSCs have been characterized in C6 monolayer cell cultures and tumorsphere cultures (Sharma

et al., 2014). C6 is tumor-forming in rats and contains a circadian clock in cell cultures and tumorspheres that regulates gene expression and the timing of epithelial-mesenchymal transition (EMT) of C6 cells into more motile cells with stem cell properties(De et al., 2020a). EMT is considered an important step in metastasis and production of CSCs of many cancer types.

Circadian rhythms in glioblastoma cells

The role of the circadian clock in cancer cells is unknown, but expression of the core genes that are part of the circadian timing mechanism alters cancer cell survival and EMT, suggesting that cancer therapies that directly target the circadian clock could improve patient outcome and survival (Malik et al., 2022). Although the regulation of many protein-coding genes is under circadian clock control, it is not yet known whether CD47 or CALR are rhythmically expressed in any cancer cell type. We explore the possible role of circadian rhythms in CD47 and CALR expression, which could have a large impact on targeted therapy against these two proteins at specific times of the day.

Specific aims

Aim 1

Use immunostaining and the C6 glioma cell line to compare CD47 protein expression in cancer stem cells and non-stem cancer cells.

After treatment with the Hoechst 33342 dye to identify GSCs and then immunostaining, CD47 expression was examined throughout C6 cells to statistically compare GSCs with non-stem cells. The relative expression of CD47 in exosomes was also compared.

Hypothesis 1: CD47 protein expression is higher in GSCs than in non-stem cells.

CD47 has been implicated in immune evasion and tumor progression. Comparing CD47 expression levels in GSCs to non-stem glioma cells could provide valuable insight into how

these cells evade immune destruction. Increased CD47 expression on GSCs could contribute to their resistance to phagocytosis and immune-mediated clearance. Given that in a previous study by Li et al., 2018, it was found that CD47 was higher in the cancer stem cells, we proposed finding similar results.

Hypothesis 2: More of the bright exosomes, which are in the upper 50% of the intensity range, are associated with GSC cell edges rather than non-stem cells.

In the literature by Kamerkar et al., 2017, we already know of the existence of CD47 in exosomes and that it possibly protects exosomes from being phagocytosed. Hence, finding an association of the CD47-rich exosomes to either GSCs or non-GSCs might suggest increased production of CD47-rich exosomes by the cell types and their possible role in immune evasion.

Aim 2

Examine CD47 and calreticulin (CALR) expression simultaneously in CSCs and non-CSCs to evaluate their co-localization.

The protocol for Aim 2 was identical to that for Aim 1, except that immunostaining was performed with a second primary antibody directed against CALR after immunostaining for CD47. We determined whether either protein is significantly elevated in either CSCs or non-stem cells and whether their co-expression is positively or negatively correlated. This relationship between the proteins also indicated whether the expression of the proteins is likely to be under circadian clock control in either cell type. The relative gene expression of CD47 and CALR was compared across nine human cancer types using a RNAseq database.

Hypothesis 3: CALR and CD47 expression intensity will be negatively correlated in GSCs and non-stem C6 cells.

Based on data from the CircaDB database, http://circadb.hogeneschlab.org, CD47 and CALR gene expressions were found to be negatively correlated to each other in normal liver tissue. For this reason, we predicted that the two proteins would be negatively correlated in both C6 cancer stem cells and non-stem C6 cells.

MATERIALS AND METHODS

Cell cultures

C6 cell cultures were expanded in 90-mm plastic petri dishes and then plated on glass-bottom 35-mm plastic petri dishes in 2 ml of medium consisting of DMEM, 10% fetal bovine serum, penicillin, streptomycin, 10 mM Hepes buffer (pH 7.2), and amphotericin B in a humidified 37ºC incubator. Bicarbonate levels of the medium were reduced to be compatible with CO_2 buffering in room air so that cells could be handled outside the incubator without effects on pH. The medium was exchanged every 2-3 days.

Identifying glioma stem cells in cell cultures

Hoechst 33342 is a nuclear stain that binds to the chromatin of both living and fixed cells, with the exception of CSCs, because of their increased ABC transporter activity that pumps out the stain(Zhou et al., 2001). This property distinguishes CSCs from non-stem cancer cells, which easily stain with Hoechst. The dye is imaged with near-UV excitation and DAPI filters for blue emission. When cells were about 75% confluent or allowed to grow longer and develop early tumorspheres, they were rinsed with medium, and treated with Hoechst at 10 μg/ml in medium for 15 minutes. Immunostaining procedures immediately followed this nuclear staining of live cells.

Immunocytochemistry

Cells were rinsed with phosphate-buffered saline (PBS) three times to remove unbound Hoechst dye and then fixed with cold methanol for 5 minutes. The cells were then rinsed three times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 minutes, rinsed with PBS, and then blocked with normal goat serum for 30 minutes, which was then replaced with a mouse

monoclonal primary antibody directed against rat CD47 (1:100 dilution, Santa Cruz) for one hour. The cells were rinsed with PBS three times and then given a secondary antibody (donkey anti-mouse conjugated with Alexa 568, 1:100, Life Technologies) for one hour. The cells were rinsed three times with PBS before imaging. For Aim 2, cells were treated with antibody against CALR conjugated to Alexa 448 (1:100, Santa Cruz) after treatment with the secondary antibody for colocalization with CD47.

Cell imaging

A confocal microscopy system was used to capture fluorescence and brightfield images of the cells. This system consisted of a DM13000B inverted microscope (Leica Microsystems, Buffalo Grove, IL, USA) equipped with a Spectra X LED light engine (Lumencore, Beaverton, OR, USA), X-Light spinning-disk confocal unit (CrestOptics, Rome, Italy), and a Rolera Thunder cooled-CCD camera with back-thinned, back-illuminated, electron-multiplying sensor (Photometrics). For data capture and analysis, we used Metamorph software that controls image acquisition (Molecular Devices, Sunnyvale, CA, USA) along with ImageJ software (NIH). Confocal images were collected in a Z-axis series with 20X, 40X and 63X objective lenses and standard DAPI, FITC, and rhodamine filter wavelengths. The 63X objective lens used oil immersion. Background intensity was subtracted based on autofluorescence intensity measurements from controls in which the primary antibody was omitted. Intensity measurements are in analog-digital units (ADUs) of the camera. The bias current of the camera sensor was compensated for by subtracting 1000 ADUs from the 16-bit raw images.

The images obtained were processed with ImageJ, and GSCs were identified using previously established methods (De et al., 2020b). Essentially, the GSCs were identified according to their round shape, small size, and their negative staining with the Hoechst 33342

nuclear stain. ImageJ software was used to quantify the intensity of fluorescence, indicating relative expression levels and cell locations of the two proteins, their degree of overlap, and the number of cells of each type expressing the proteins.

CD47 and CALR transcriptomics

RNAseq data was collected from the Cell Miner database version 2.6, Human genome version HG-19 (https://discover.nci.nih.gov/cellminer/queryLoad.do). Cell Miner provides analyses of the NCI-60 cancer cell lines used by the National Cancer Institute to screen candidate anticancer agents (https://dtp.cancer.gov/discovery_development/nci-60/cell_list.htm). Fragments per kilobase per million reads were compared as log2(FPKM + 1) by one-way ANOVA.

RESULTS

CD47 in C6 glioma stem cells and non-stem C6 cells

Aim 1

Use immunostaining and the C6 glioma cell line to compare CD47 protein expression in cancer stem cells and non-stem cancer cells.

CD47 Immunostaining in C6 cells

Hoechst staining of live C6 cells followed by fixation and immunostaining for CD47 was performed (Figs. 4 & 5). Cells were then located in brightfield images and identified as either H+ or H- (Fig. 6). These initial experiments verified that CALR was primarily at the edges of cells as expected and could be further quantified after subtracting the signal intensity caused by autofluorescence that was present in control cell cultures processed in the same way but with the primary antibody omitted.

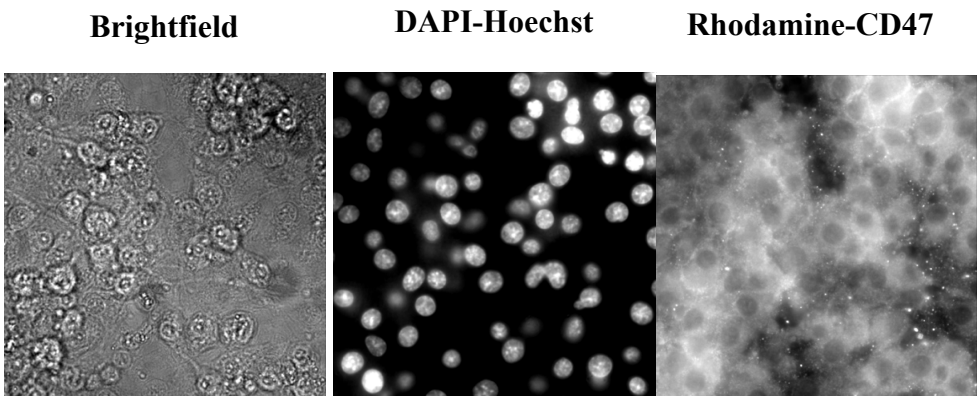

Figure 4. Raw confocal microscope images of C6 cells at 63X magnification.

Note; Left: Brightfield. Middle: Cell nuclei stained with Hoechst (DAPI channel). Right: Anti-CD47 monoclonal antibody and secondary antibody (rhodamine channel)

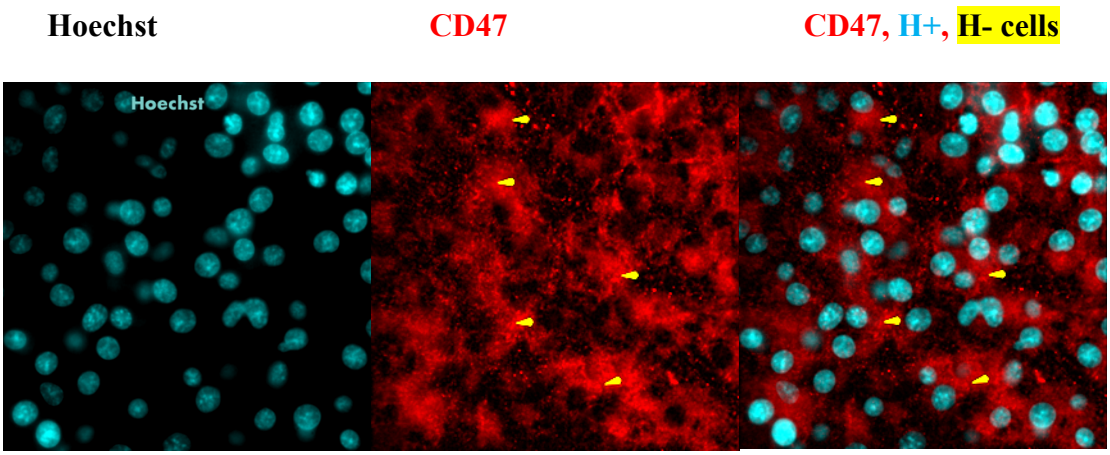

Figure 5. Elevated CD47 expression in Hoechst negative cells. ***Note;*** *Elevated CD47 immunofluorescence in Hoechst-negative cells after image analysis tagged with yellow arrows. Data from Figure 4 after subtracting threshold and identifying Hoechst-negative cells. Arrows indicate cells that do not stain with Hoechst but do have CD47 expression.*

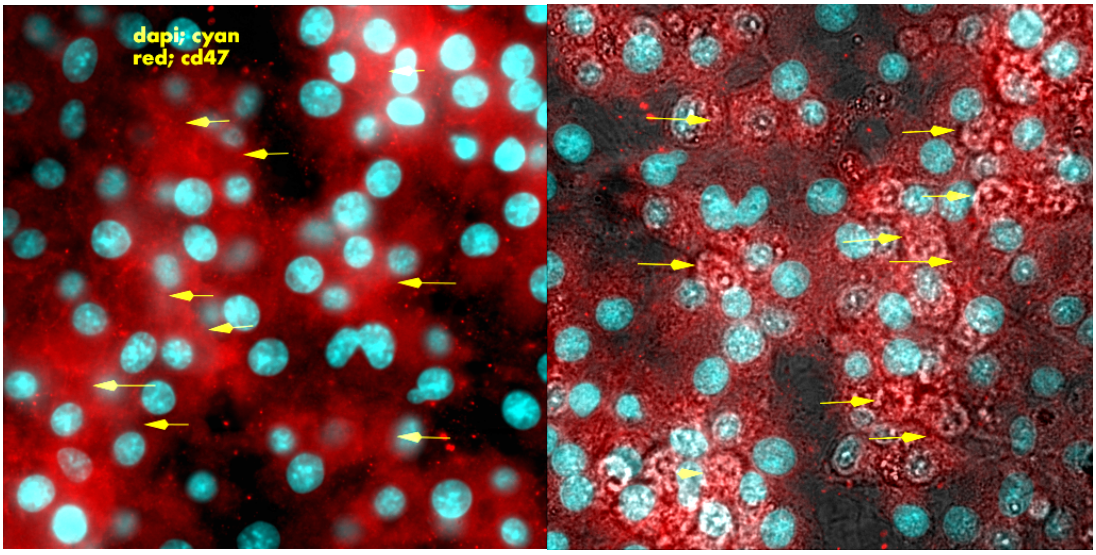

Figure 6. CD47 immunolocalization. *Note;* CD47 immunolocalization was observed mainly around the plasma membrane and in the extracellular areas. Left: Hoechst and CD47. Right: Hoechst, CD47, and Brightfield overlay. CD47 expression was examined in Hoechst-positive and negative cells (arrows) by visually identifying corresponding cells in the brightfield image.

CD47 Immunocytochemistry within C6 exosomes

CD47 immunostaining of the C6 cells revealed bright spots, mostly at the cell edges, which resembled CD47 protein expression in exosomes (Fig. 7). The brightest exosomes were considered those within the top 50% of the dynamic range of the fluorescence signal.

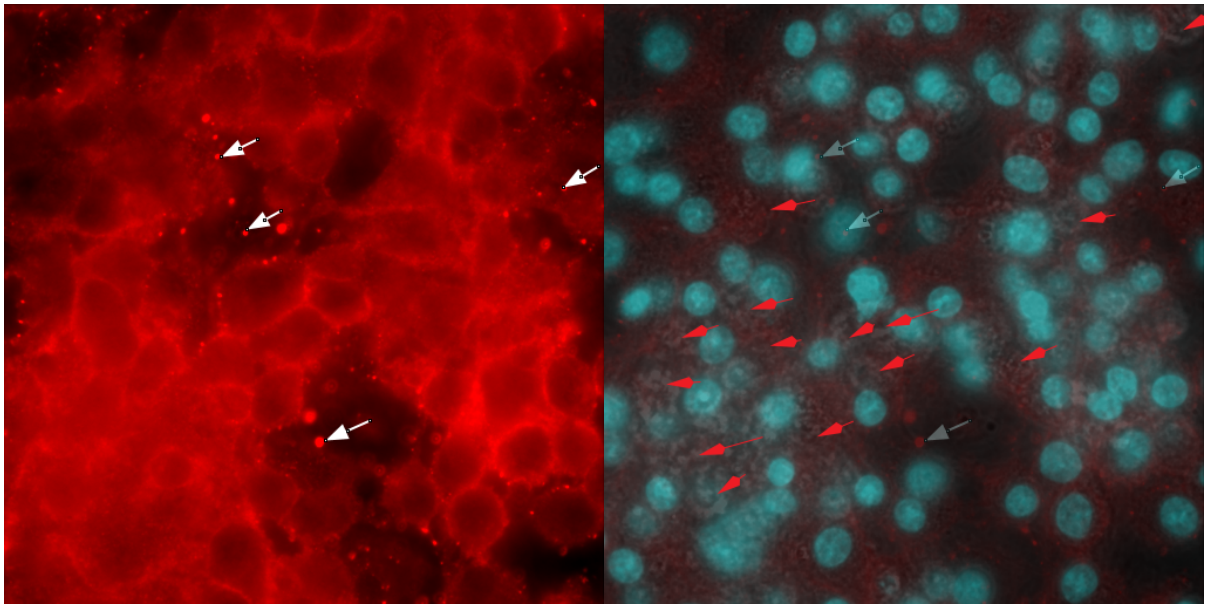

Figure 7.CD47 in exosomes *note; Left; A rhodamine channel image of CD47 proteins at 63x magnification after subtracting the signal from immunofluorescence according to the control data. White arrows point to the highest CD47 intensity after thresholding the CD47 signal to 50% of the dynamic range. The image on the right shows Hoechst-negative cells in the same image in an overlay of the Hoechst and Brightfield channels (red arrows).*

Next, we determined whether these bright spots were most closely localized to H+ or H- cells. After examining all available images, we concluded that they did not correspond with H+ or H- cells and appeared randomly distributed. Some did not appear to be associated with any cell at all. Given our data, it is not possible for us to prove that they are associated with H- cells as we hypothesized.

Total CD47 Distribution in Hoechst Positive and Hoechst Negative cells

Although we did not detect a difference in CD47 spots (presumed exosomes) between H+ and H- cells, we then compared overall CD47 expression in the two cell types.

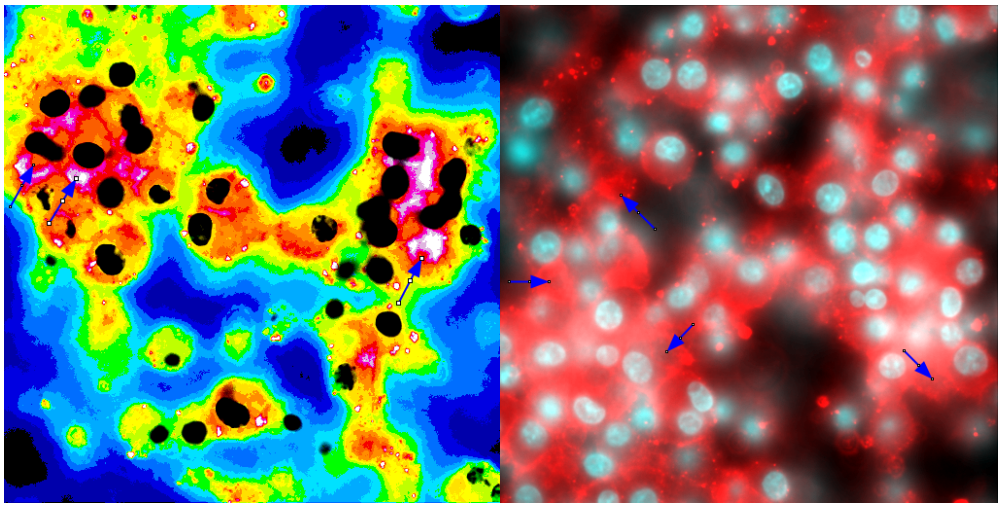

Figure 8. Pseudocolor distribution of CD47. *notes*; *arrows indicate H- cells from a previously overlayed image in which Hoechst-stained cell nuclei were marked as black. The white pixels in the image have the highest immunofluorescence intensity. Right: The CD47 expression is shown in red with Hoechst-stained nuclei in cyan and the H- cells (arrows). These images, along with cells identified in brightfield images, enabled a quantitative test of whether the CD47 expression is elevated in H- cells.*

Using all available 63X images, H- cells were identified in overlays of Hoechst staining, CD47 immunostaining, and brightfield images. A region-of-interest (ROI) was drawn around the cells in ImageJ. The maximum pixel intensity in each ROI was measured. A corresponding number of H+ cells was randomly selected, and the maximum pixel intensity was measured as with H- cells. The mean of the maximum intensity of the CD47 signal in H- and H+ cells was 12674 ±2582 (SD) and 15526 ±6662, indicating significantly higher CD47 expression in the H+ cells (T-test, $p=0.0328$, $n=30$ cells).

CD47 and CALR co-expression in C6 glioma stem cells and non-stem cells

Aim 2

Examine CD47 and calreticulin (CALR) expression simultaneously in CSCs and non-CSCs to evaluate their co-localization.

CD47 and CALR Immunostaining in C6 cells

We immunolocalized CALR in live C6 cells with monoclonal antibodies directed against CALR and conjugated with Alexa Fluor 488. Hoechst-negative cells with high CALR expression (H-/CALR+) were identified and were in the 5-25 µm diameter range of GSCs. These cells were previously characterized as GSCs by antibodies directed against stem cell marker proteins (Sharma et al., 2014). There was a wide range of CALR expression detected by immunostaining as shown in Figure 9. We used the top 50% of the immunofluorescence signal as a boundary to distinguish between high and low CALR expression.

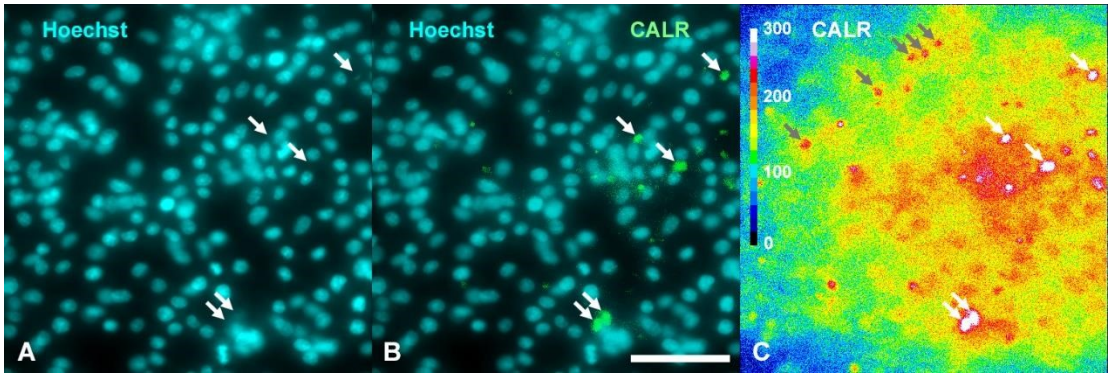

Figure 9. CALR expression in live C6 cells *note*; *High CALR in H- cells (white arrows) and low CALR expression in the non-stem cancer cells (H+) and some H- cells (gray arrows). The background-subtracted image in Fig. 8C indicates the over three-fold range of CALR immunofluorescence present in C6 cells. The intensity range is in digital camera units. Scale bar equals 50 µm.*

After confirming there was abundant CALR expression in live C6 cells, we examined CALR and CD47 with immunostaining in methanol-fixed cells to determine their relative expression within the entire cell (Fig. 10). The proteins certainly had multiple areas of colocalization, although not throughout the exact same areas. For example, exosomes did not show elevated CALR expression. The intensity of the expression of the two proteins was positively correlated (Fig. 11, Pearson's coefficient, r = 0.97).

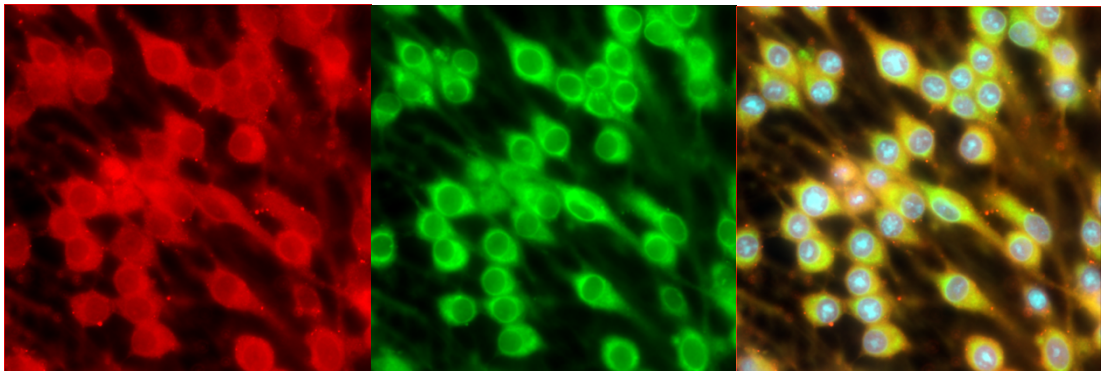

Figure 10. Colocalization of CD47 and CALR *Note; image from left to right showing CD47 distribution in the rhodamine channel, CALR in the FITC channel, and colocalization of the two proteins along with Hoechst-stained nuclei (blue). Yellow in the image on the right indicates areas of overlapping CD47 and CALR. Exosome-like structures are visible in the CD47 image at left, which do not show colocalization with CALR.*

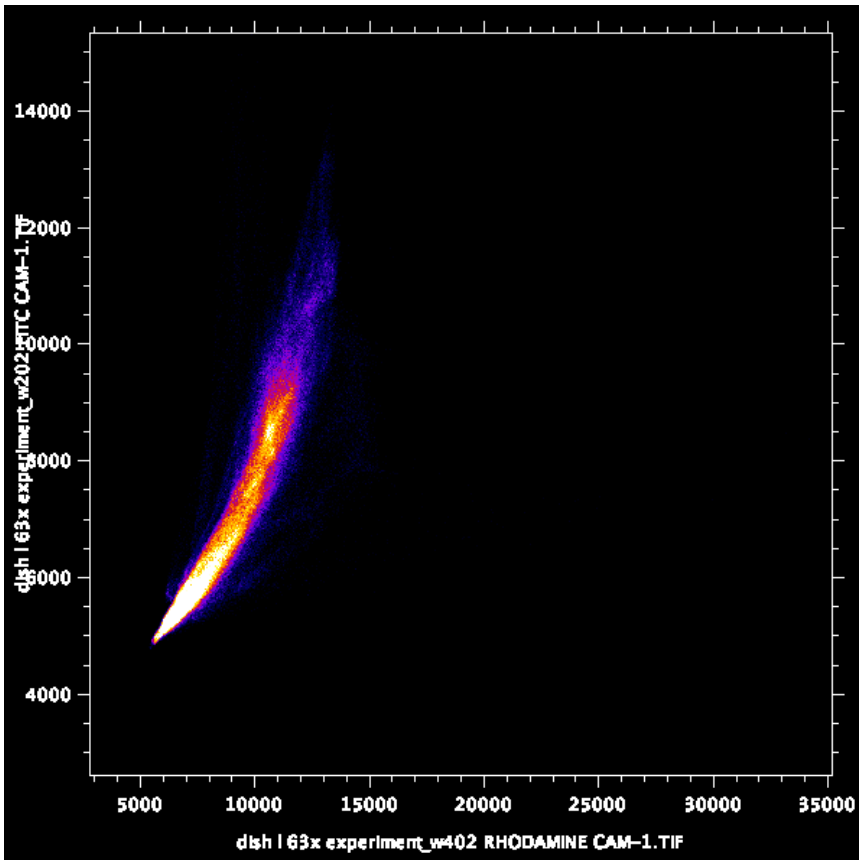

Figure 11. Scatter plot pixel distribution of CD47 against CALR *note; the red channel of CD47 is on the X axis, and the green channel of CALR on the Y axis are plotted against each other. Derived from ImageJ plugin Colocalization-finder.*

Comparing CD47 and CALR RNA Expression in other Cancer Cell lines

Although protein expression does not always reflect levels of corresponding gene activity, we examined whether CD47 and CALR expression is different in gliomas compared to other cell lines. One question this query addressed is whether there may be a selection for cancer cells that increase their CD47 expression as CALR expression increases, thereby protecting the cells from phagocytosis. CALR expression can increase in cells as they are exposed to stressful factors in their microenvironment, such as hypoxia or intracellular Ca^{2+} depletion.

CD47 expression was consistently lower than CALR expression in all nine cancer cell types represented in the set of NCI-60 cancer cell lines as provided in the Cell Miner database https://discover.nci.nih.gov/cellminer/queryLoad.do (Fig. 12). There was no significant difference between the ratios of CALR to CD47 expression across all the cancer cell types when compared by ANOVA (F = 1.616, p = 0.1434). Cancer cell lines derived from brain tumors were not different from the cell lines derived from other cancerous tissues. When comparing CD47 with CALR in all 60 cell lines, it was significantly lower than CALR by T-test (t = -29.97, p = 2.116×10^{-37}). CALR expression was not significantly different across cancer cell types (ANOVA, F = 2.008, p = 0.06408), but CD47 was (ANOVA, F = 2.532, p=0.0211).

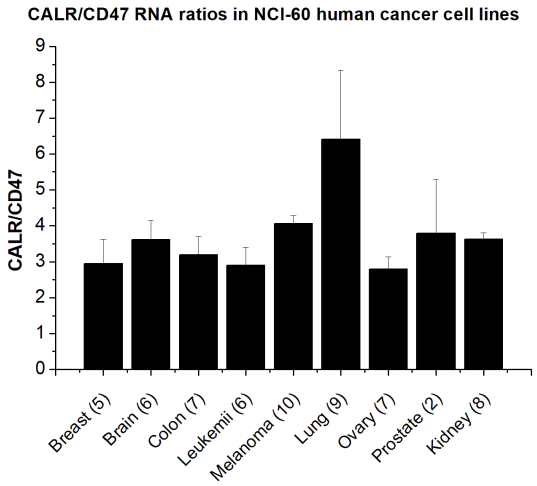

Figure 12. CALR/CD47 RNA rations in NCI- 60 cancer cell lines. *Note; The number of cell lines examined is shown after each cancer type. Error bars are standard errors of the mean.*

When examining all the cell lines together, CD47 showed a weak but not significant positive correlation with CALR expression by linear regression (R=0.2114 ±1.059 (SD), p=0.1049, n=60,) as shown in Figure 12. Clearly, transcripts for both proteins were highly expressed.

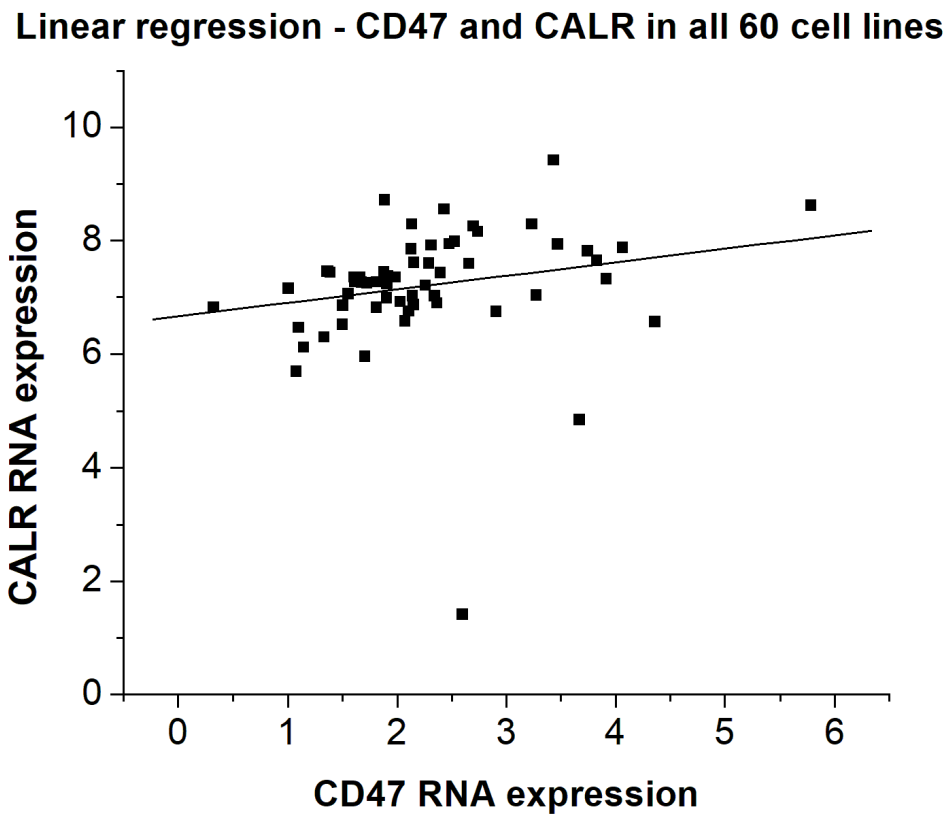

Figure 13. Linear regression graph of CD47 and CALR in all 60 cell lines. *Note; Shown are log2(FPKM + 1) values for the two gene transcripts and linear regression.*

DISCUSSION

CD47 distribution in Hoechst-positive and Hoechst-negative cells

The results showed that CD47 expression was higher in non-stem C6 cells (H+) than in GSCs (H-), which leads us to reject our Hypothesis 1 and suggests that the bulk of glioblastomas may, in this way, be protected from phagocytosis from macrophages or microglia.

In contrast, in a study on GSCs and CD47 by Li et al., 2018, it was found that CD47 was higher in the GSCs compared to the non-stem cells. And they suggested that the ability of GSCs to withstand phagocytosis and immune surveillance, which supports their survival and proliferation, is facilitated by high CD47 expression(Li et al., 2018). However, one major difference between our study and theirs is that they used different glioma cell lines, U138 and GL261, while we used the C6 cell line, which could be a major cause of such variation between study results.

Cellular heterogeneity of glioblastomas is well-known, and GSCs and non-stem cancer cells can display various characteristics even within the same tumor(Friedmann-Morvinski, 2014). This biological heterogeneity, where specific subsets of GSCs or non-stem cells may have different CD47 expression patterns, may be reflected in the observed variances in CD47 protein expression. A subset of GSCs and non-stem cells within the examined population may be represented by the observed differences, given the sample size of 30. Also, despite efforts to choose a representative sample, the population under study may nonetheless be naturally variable. Differences in the average maximum intensity of CD47 can be attributed to individual cells within a group exhibiting diversity in gene expression and protein levels. The observed

discrepancies could be the result of this variability, which the sample size of 30 may have captured in part.

If CD47 expression is subject to circadian regulation, it is conceivable that its levels might change over time(Xuan et al., 2021). The discrepancies in CD47 expression between GSCs (H-) and non-stem cells (H+) may be partially explained by the temporal fluctuations brought on by the circadian clock. This possible effect would suggest that at the study's evaluated time points, the non-stem cells (H+) expressed higher levels of CD47 expression.

The validity of these hypotheses would require additional experimental research. With regard to understanding how the circadian clock affects the dynamics of CD47 expression in GSCs and non-stem cells. Circadian rhythm profiling, gene expression investigations at various time points, or perturbation experiments modifying the circadian clock components would all be highly beneficial.

CD47 in exosomes

If the CD47-positive exosomes are the source of the bright areas of CD47 expression seen in this study, it may imply that glioma cells are actively secreting these vesicles into their surroundings. We tested Hypothesis 2, which stated that: exosomes with CD47 expression within the upper 50% of the intensity range are located near the edges of GSCs (H-) rather than non-stem cells (H+). However, no significant difference was detected. These exosomes could be attributable to their release from several cell sources, which could explain why they were not found to be higher near GSCs than non-stem cells.

The biology and immunomodulation of gliomas may be significantly impacted by the presence of CD47 on exosomes (K. Jackson et al., 2022). It is well established that CD47 and SIRP on macrophages interact, causing the "don't eat me" event that aids tumor cells in avoiding

phagocytosis and immune identification. Thus, by interacting with SIRP on immune cells, CD47-positive exosomes may have a similar immunosuppressive impact, encouraging immune evasion and tumor growth(Kamerkar et al., 2017).

CD47 and CALR expression in H+ and H- cells.

Based on these results, our Hypothesis 3 that CALR and CD47 expression levels in GSCs and non-stem C6 cells are negatively correlated is not supported but might be rather positively correlated. The comparison of CALR and CD47 H+ cells yielded a significant p-value ($p = 0.0204$). We also discovered a correlation between CALR and CD47 when the H+ and H- groups were combined ($r = 0.312$, $p = 0.0121$), but not in the H- group alone. This result is noteworthy and implies that there might be a difference in the expression and how the two proteins act in the two different cell types.

One possible interpretation of this result is that the GSCs may be more susceptible to CALR-induced phagocytosis when CD47 immunotherapy is used.

The association between CALR and CD47 expression goes beyond the expression of specific cell types and exposes a general difference in their expression levels across the examined cell populations, according to the observed substantial difference in the combined cell types. It is important to consider how circadian clocks and rhythms might relate to these proteins, given data showing a significant variation in the expression levels of CALR and CD47 in both H+ and H- cells together. Circadian clocks can control the expression of genes involved in a variety of biological functions, such as protein synthesis and turnover. It is probable that the circadian clock has an impact on the differential expression of CALR and CD47 seen in this work, causing fluctuations in their expression levels during a 24-hour cycle. The expression of CALR and CD47 may be influenced directly or indirectly by clock-controlled transcription

factors or clock-dependent signaling pathways due to the circadian regulation of gene expression.

However, understanding the temporal dynamics and regulatory mechanisms of CALR and CD47 expression in GSCs and non-stem C6 cells can be framed by considering the potential impact of circadian clocks and rhythms.

CALR expression in H+ and H- cells

The higher CALR expression in H- cells we observed confirms the higher CALR expression in H- cells observed in preliminary work during my collaboration with Eric Ntares in our lab, which also indicated a higher cell-surface CALR expression in H- cells. However, the previous measurements were of cell surface CALR protein using live cells, whereas these results were from fixed and permeabilized cells. Therefore, this novel finding indicates that overall CALR protein levels are higher in H- than in H+ cells. It agrees with one model explaining why surface CALR expression is elevated in cancer cells: Specifically, elevated ER stress in cancer cells is well known to induce the unfolded protein response and then CALR gene and protein induction, thereby providing a mechanism to cope with and recover from stressors such as ER Ca^{2+} depletion, cell damage from reactive oxygen species, or toxicity from chemotherapeutic agents(Hetz et al., 2011). The model considers the elevated CALR protein expression alone adequate for increasing cell surface CALR through the usual movement of proteins from the ER to the Golgi apparatus and then through intracellular vesicles to the cell membrane.

Incidentally, our results also indicate that the exosome-like structures we observed did express high CD47 expression but were devoid of detectable CALR, supporting the possibility that these extracellular structures lack the “eat-me” signal and serve in suppressing phagocytosis of both H+ and H- cancer cells. This result provides support for using immunotherapy

approaches that target not just cancer cells but also exosomes through the application of antibodies binding to CD47.

CONCLUSIONS

In conclusion, this thesis aimed to examine CD47 and CALR protein expression levels and any relationships between the proteins in GSCs (H-) and non-stem cells (H+). The findings have shed important new light on the biology and practical use of CD47 and CALR in relation to glioblastoma.

Contrary to the original hypothesis, the results showed that CD47 expression was higher in non-stem cells (H+) than in GSCs (H-). This study raises the possibility that non-stem cells use CD47-mediated immune evasion and tumor growth processes, which may affect the biology and immunomodulation of gliomas. Exosomes that are CD47-positive are a sign of active secretion by glioma cells, which may help to inhibit the immune system and advance the tumor.

In terms of CALR expression, GSCs (H-) showed higher levels than non-stem cells (H+). This result is consistent with the idea that increased ER stress causes CALR gene and protein expression in cancer cells, possibly assisting in cell recovery and stress management. The complicated interactions between these proteins and their functions in immune regulation and cellular responses are highlighted by the variable expression of CALR in different cell types and the absence of CALR in exosomes that are CD47-positive.

Given their well-known impact on the dynamics of gene expression, the potential role of circadian clocks in regulating CD47 and CALR expression was addressed. While speculative, the changes in CD47 and CALR expression levels could be attributed to the temporal fluctuations controlled by circadian clocks. To confirm and develop upon these ideas, additional research is required, such as circadian rhythm profiling and gene expression analyses at various time periods.

Overall, this thesis advances our knowledge of the dynamics of CD47 and CALR expression in GSCs and non-stem cells by highlighting the significance of functional ramifications, cellular heterogeneity, and putative circadian clock participation. In order to improve immune identification and phagocytosis of cancer cells, the findings have implications for the development of tailored therapeutics and immunotherapeutic techniques, including the employment of antibodies targeting CD47 and exosomes.

In conclusion, this study has shown the complex relationships between CD47 and CALR and their probable functions in the biology and immunomodulation of glioblastoma. More research is required to understand the underlying mechanisms and apply these discoveries in the clinic for better diagnostic, prognostic, and therapeutic approaches in glioblastoma patients. The knowledge acquired from this study has the potential to develop targeted strategies and customized medicine for the treatment of glioblastoma.

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