ATTEMPTING TO BLOCK CANCER’S IMMUNE EVASION “DON’T EAT ME” SIGNAL IN A COURSE-BASED UNDERGRADUATE RESEARCH EXPERIENCE

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ABSTRACT

In a semester-long Course-based Undergraduate Research Experience (CURE), 16 students attempted to kill breast cancer cells by blocking their immune evasion technique. The human breast cancer cell line MCF-7 upregulates cell surface CD47 to interact with signal-regulatory protein alpha (SIRPα) on THP-1 macrophages to evade phagocytosis, enabling cancer cell survival. Thrombospondin-1 (TSP1), a known high-affinity ligand of CD47, was used to outcompete SIRPα on THP-1 macrophages. By preventing the CD47-SIRPα interaction or “Don’t eat me” signal, an immune response was expected from THP-1 macrophages. Interestingly, there did not appear to be phagocytosis of the MCF-7 cells by THP-1 macrophages but there was a detectable production of pro-inflammatory cytokine TNFα, compared to controls. With mixed results, further optimization and experimentation are needed to confirm the effects of TSP1 on phagocytosis. Further understanding of ways to manipulate the CD47-SIRPα interaction between cancer and immune cells could lead to novel combinational immunotherapies.

INTRODUCTION

A growing problem in advancing research is a lack of laboratory space for students, individuals, and start-ups (Patel, 2021). At universities, many research faculty can only support a handful of students in their laboratories and there is low turnover when student researchers are working towards a thesis or dissertation. Course-based Undergraduate Research Experiences (CURE) have emerged as a possible supplement to direct research with a faculty member (Lopatto, 2007).
CUREs are designed to conduct novel research in a large classroom and/or laboratory training environment. As a result, students who complete CUREs should have a working knowledge of marketable laboratory techniques with the possibility of presenting their results at a conference or submitting them for publication. If research opportunities for undergraduate students continue to be restricted by one-on-one interactions with faculty, the lack of laboratory training beyond standard classroom material can have a detrimental effect on the number of prepared principal investigators (PIs) joining the workforce to conduct research on disease treatments and cures, including cancer.

<table>
<thead>
<tr>
<th>Original Hallmarks</th>
<th>New Hallmarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Avoiding immune destruction</td>
<td>1. Dedifferentiation and transdifferentiation</td>
</tr>
<tr>
<td>2. Reprogramming energy metabolism</td>
<td>2. Epigenetic dysregulation</td>
</tr>
<tr>
<td>3. Evasion of cell death</td>
<td>3. Altered microbiome</td>
</tr>
<tr>
<td>4. Genome instability and mutation</td>
<td>4. Altered neuronal signaling</td>
</tr>
<tr>
<td>5. Angiogenesis</td>
<td>6. Evasion of growth inhibitory signals</td>
</tr>
<tr>
<td>7. Growth signal autonomy</td>
<td>8. Unlimited replicative potential</td>
</tr>
<tr>
<td>9. Tumor-promoting inflammation</td>
<td>10. Invasion and metastasis</td>
</tr>
</tbody>
</table>

Table 1. Original and novel hallmarks of cancer

Cancer remains a worldwide leading cause of death. As the population grows, so will the number of cancer cases and deaths (Torre et al., 2016). About 10 million deaths and 19.3 million new cases caused by cancer were reported in 2020 (Sung et al., 2021). In 2020, the most diagnosed type of cancer worldwide was breast cancer, with 2.3 million new cases (11.7%; Sung et al., 2021). In 2040, 28.4 million cases of cancer are expected (Sung et al., 2021). The burden of cancer is expected to remain and grow worldwide unless novel screenings and therapeutics are available to detect and eliminate it.

There are fourteen hallmarks of cancer that enable immortality, growth, and dissemination as shown in Table 1 (Senga & Grose, 2021). Regarding the fourteen hallmarks, avoiding immune destruction is a challenge for the body’s cancer immune surveillance and therapeutics in mitigating breast cancer. The body’s immune system typically uses innate and adaptive immune responses to observe, detect, and extinguish budding cancer cells. However, cancer cells have developed strategies to escape
immune clearance by using immunosuppressive factors or decreasing their immunogenicity (Bates et al., 2018). Without mechanisms to eliminate the immune-resistant cells, they will continue to proliferate and be unchecked for regulation.

Macrophages are prominent players within the immune system that can be a target for novel therapeutic approaches. Macrophages have been shown to target tumor sites, directly phagocytose tumors, initiate a tumor’s microenvironment, and display noteworthy antigens (Sloas et al., 2021). A prominent checkpoint that acts to prevent phagocytosis by macrophages is cluster of differentiation 47 (CD47), which is a transmembrane protein expressed on all cells. When CD47 on a host cell interacts with a Signal Regulatory Protein Alpha (SIRPα) receptor on a macrophage, a self-cell signaling cascade informs the macrophage that the other cell is a healthy self-cell and to not mount an immune response (Chao et al., 2019). Unfortunately, cancer cells, including breast cancer cells, will overly express CD47 to mimic a healthy self-cell and continue to proliferate (Chao et al., 2019). Currently, anti-CD47 monoclonal antibodies have shown a significant response in diminishing tumor progression by enhancing macrophage activity (Takimoto et al., 2019). An example of a humanized anti-CD47 antibody showing such results in-vitro and in-vivo is CC-90002 (Narla et al., 2022). A concentration-dependent CC-90002 mediated phagocytosis was found in hematological cancer cell lines of acute myeloid leukemia (AML), acute lymphoblastic leukemia, and lenalidomide-resistant multiple myeloma (MM) (Narla et al., 2022). Moreover, immunodeficient mice with MM cell line-derived xenografts showed a similar dose-dependent antitumor activity of CC-90002 (Narla et al., 2022). The success of targeting and blocking the CD47-SIRPα interaction with monoclonal antibodies opens the door for exploring other known CD47 ligands as potential blocking agents. Testing for ligand interaction and the result on cancer cells involves techniques that are ever-evolving, while also not being too tedious for early researchers to master. This type of novel research is a natural fit to be conducted in a large classroom/laboratory CURE setting.

The Immunology Tech Lab CURE was created to teach students marketable laboratory techniques and contribute knowledge to the field through their results. In this CURE, alongside control experiments using anti-CD47 monoclonal antibodies, thrombospondin-1 (TSP1), which is a
known angiogenesis inhibitor and CD47 ligand (Kaur et al., 2021), was used to determine if it can outcompete the CD47-SIRPα interaction. As shown in Figures 1 and 2, TSP1 is a protein encoded by *THBS1*, and its expression decreases in the tumor microenvironment when malignant melanoma and breast carcinoma cell lines develop (Kaur et al., 2021). As expected, an increased expression of TSP1 has been shown to suppress carcinogenesis in squamous cell carcinoma, melanoma, cervical carcinoma, glioblastoma, and prostate carcinoma (Kaur et al., 2021). With this information about TSP1, the CURE students incubated TSP1-treated MCF-7 breast cancer cells with THP-1 macrophage to determine the presence of phagocytosis, via confocal microscopy, and immune response through detection of pro-inflammatory cytokines IL-1α and TNFα, via Enzyme-Linked Immunosorbent Assay (ELISA).

![Figure 1. Crystal structure of Thrombospondin-1.](image)

The figure displays color-coded domains of the 2D domain sequence in Figure 2. Teal is TSPN, magenta is VWFC, green is TSP1 repeat, red is EGF-like, black is TSP3 repeat, and yellow is C-terminal. Crystal structure of TSP1 (Jumper et al., 2021; Varadi et al., 2022).
Figure 2. Sequence, interactions, and effects of Thrombospondin-1’s domains. The layout displays the positioning of TSP1’s domains, and the below image lists their interactions and effects. There is one TSPN located between 47-270 AA. There is one VWFC located between 316-373 AA. There are three TSP1 repeats that are found at 379-429 AA, 435-490 AA, and 492-547 AA. There are two EGF-like domains that are found at 547-587 AA and 646-690 AA. There are eight TSP3 repeats that are found at 691-726 AA, 727-762 AA, 763-785 AA, 786-821 AA, 822-844 AA, 845-882 AA, 883-918 AA, and 919-954 AA. The C-terminal domain is found at 958-1170 AA.

Domains TSP1 ("UniProt: the Universal Protein Knowledgebase in 2023," 2023). The receptors/ligands and effects were found through the following sources: TSPN (Tisi et al., 2000), VWFC (Bonnefoy & Hoylaerts, 2008), TSP1 repeat (Guo et al., 1997; Gutierrez & Gutierrez, 2021), EGF-like domain (de Castro et al., 2006; Hulo et al., 2008; Sigrist et al., 2002; Sigrist et al., 2013; Sigrist et al., 2005), TSP3 repeat (Gutierrez & Gutierrez, 2021), and C-terminal (Gutierrez & Gutierrez, 2021).

We hypothesized that TSP1 will bind to the CD47 receptors on breast cancer cells, thus blocking the interaction of CD47 with SIRPα on macrophage, leading to an increase in phagocytosis of breast cancer cells and the creation of proinflammatory cytokines IL-1α and TNFα.

**Materials and Methods**

*Cell Lines:*

THP-1 macrophage(#TIB-202)and MCF-7 breast cancer (#HTB-22) cells lines were obtained from American Type Culture Collection (ATCC).
The THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium, the MCF-7 cells were maintained in Eagle's Minimum Essential Medium (EMEM), and both cell lines were supplemented with 10% fetal bovine serum (#16140071; ThermoFisher Scientific) at 37°C in a humidified atmosphere of 5% CO$_2$. The THP-1 cells were maintained at 1-6x10$^5$ cells/mL of media. The MCF-7 cells were treated with Trypsin/EDTA (#25-053-CI; Corning) and split when 60-90% confluency was observed under a light microscope.

**Labeling MCF-7 Cell Line:**

CellTracker Deep Red was obtained from ThermoFisher Scientific (#C34565). The CellTracker Deep Red dry powder was reconstituted in a stock dilution of 1 mM with dimethyl sulfoxide (DMSO; #317275-55ML; Millipore Sigma). The stock dilution was then turned into a working dilution with a concentration of 1 µM. MCF-7 cells were grown in a 24-well plate to a minimum of 50% confluency. The complete-EMEM media was aspirated from the MCF-7 cells. The cells were washed with serum-free media 2 times and 500 µL of pre-warmed CellTracker Deep Red working solution was added to each well at a concentration of 1 µM. The MCF-7 cells were incubated at 37°C for 30 minutes. Following incubation, the CellTracker Deep Red working solution was removed and 2 mL of EMEM complete media was added to each well.

**THP-1 Cell Differentiation:**

Approximately 1x10$^5$ THP-1 cells in 500 µL of RPMI 1640 complete media were suspended and added to a 24-well plate with round coverslips in each well. 500 µL of phorbol 12-myristate 13-acetate (PMA) solution (100ng/mL) in complete RPMI 1640 was added to the wells containing THP-1 cells while 500 µL of complete RPMI 1640 was added to control wells. The 24-well plate was placed in an incubator at 37°C in a humidified atmosphere of 5% CO$_2$ for 3 days to allow the THP-1 monocytes to differentiate to M0 resting phase macrophages and adhere to the cover slips. After 3 days, the cells were washed with serum-free media and maintained in complete RPMI 1640.

**Co-Culture of THP-1 and MCF-7 Cells:**

After culture, MCF-7 cells labeled with CellTracker Deep Red
were gently lifted using Trypsin/EDTA, counted and approximately 1 x10^4 cells were added to 2 mL centrifuge tubes. A thrombospondin-1 (TSP1) solution was added to each tube containing CellTracker Deep Red labeled MCF-7 cells to a final working concentration of 5 µg/mL of TSP1. The tubes were mixed well and then added to the 24-well plate with differentiated THP-1 macrophage growing on coverslips. The cell plate was tilted to mix the reagent and cells and then incubated for 24 hours at 37°C in 5% CO₂. After 24 hours, the cell supernatants were collected and preserved at -20°C for analysis by Enzyme-Linked Immunosorbent Assay (ELISA).

**Labeling of THP-1 Cells and Visualization of their Interaction with MCF-7 with Confocal Microscopy:**

After the removal of co-culture supernatants, 1 mL of phosphate-buffered saline (PBS; #BP3991; Fisher Scientific) was added and mixed in each well. The PBS was removed and 500 µL of 4% formaldehyde was added to each well and incubated for 20 minutes at room temperature. The 4% formaldehyde was removed, and each well was washed twice with 500 µL PBS. Then, 500 µL of solution containing monoclonal mouse anti-human CD14-PE conjugated antibody (R&D Systems) was added to the cells at a concentration of 10 µL/10^6 cells. The cells were incubated in the dark for 1 hour at room temperature. The anti-CD14-PE solution was removed, and each well was washed twice with 250 µL PBS. The coverslips, with macrophage attached, from each well were carefully removed and placed face down on a microscope slide. The cells on the slides were visualized using a Nikon Eclipse 90i confocal microscope using a 10x eyepiece and Plan Apo VC 60x oil immersion lens.

**Enzyme-Linked Immunosorbent Assay (ELISA) for Tumor Necrosis Factor alpha (TNFα) and Interleukin-1 alpha (IL-1α):**

Human TNFα (#KHC3011, Invitrogen) and Human IL-1α (#ab100560; Abcam) ELISA kits were used to analyze the supernatant from the co-culture of thrombospondin-1/MCF-7/THP-1 cells according to the manufacturer instructions. Standards, samples, and controls were added to a capture antibody-coated 96-well plate and incubated for 2.5 hours at room temperature. The solution was aspirated, and the wells were washed 4 times. A biotin conjugated detection antibody was added to
each well and incubated for 1 hour at room temperature. The solution was aspirated, and the wells were washed 4 times. A streptavidin horseradish peroxidase (HRP) solution was added and incubated for 45 minutes at room temperature. The solution was aspirated, and the wells were washed 4 times. A color-producing substrate specific for each ELISA kit was added and incubated for 30 minutes at room temperature in the dark. Stop solution was added, and the cell plate was scanned on a ThermoScientific Multiskan Skyhigh plate reader for absorbance (450 nm). A standard absorbance curve was created to evaluate the concentration of TNFα and IL-1α detected from samples.

**Results**

Confocal light microscopy was used to attempt to analyze the phagocytosis of the MCF-7 breast cancer cells by THP-1 macrophage. To visualize the cells on a confocal microscope, MCF-7 cells were labeled with a whole cell dye (CellTracker Deep Red) that shows as a red color, while the THP-1 macrophage were labeled with a cell surface receptor conjugated antibody (anti-CD14-PE) that shows as a green color. Analysis of phagocytic activity was determined by taking images of whole THP-1 macrophage and examining the cell borders to locate if MCF-7 breast cancer cells were found within or outside of the macrophages, as shown in prior published studies (Chao et al., 2011; Majeti et al., 2009). The modifications made to these techniques included adding Thrombospondin-1, investigating a breast cancer cell line, using CellTracker Deep Red and anti-CD14-PE to visualize the cells. When imaging the negative control samples containing

![Figure 3. Negative control.](image-url)

The labeled MCF-7 breast cancer cells (red), indicated by the arrow, are outside of the THP-1 macrophage (green), indicating that no phagocytosis occurred. The cells were visualized using a Nikon Eclipse 90i confocal microscope at 600x magnification.
a co-culture of THP-1 and MCF-7 cells, it appears that there are MCF-7 cells found mostly outside of the labeled cell surface of the THP-1 macrophage based on the color variation (Figure 3). This is consistent with prior knowledge that the interaction of CD47 on the MCF-7 cells with SIRPα on the THP-1 cell will result in no phagocytosis (Takimoto et al., 2019). In the images of the positive control samples containing a co-culture of THP-1, MCF-7 cells, and anti-CD47, there appear to be MCF-7 cells found mostly inside of the labeled cell surface of the THP-1 macrophage (Figure 4). Again, this result is consistent with prior knowledge that anti-CD47 antibodies will block the interaction of CD47 on the MCF-7 cells with SIRPα on the THP-1 cell and result in phagocytosis of the MCF-7 cells.

Figure 4. Positive control. Labeled THP-1 macrophage (green), MCF-7 breast cancer cells (red), and anti-CD47 were incubated together. The MCF-7 cells, indicated by the arrow, appear to be inside the THP-1 macrophage indicating phagocytosis. The cells were visualized using a Nikon Eclipse 90i confocal microscope at 600x magnification.

Figure 5. Experimental group. Labeled THP-1 macrophage (green), MCF-7 breast cancer cells (red), and TSP1 were incubated together. The MCF-7 cells indicated by the arrow appear to be outside of the THP-1 macrophage indicating that phagocytosis did not occur. The cells were visualized using a Nikon Eclipse 90i confocal microscope at 600x magnification.
Interestingly, there appear to be MCF-7 cells found mostly outside of the labeled cell surface of the THP-1 macrophage in the images of the experimental samples containing a co-culture of THP-1, MCF-7 cells, and TSP1 (Figure 5). This may signify that immune evasion by the MCF-7 cells potentially took place, and the CD47-SIRPα interaction was not blocked.

To further determine whether immune evasion did take place, ELISA was used to test the concentration of secreted factors in the THP-1 cells, THP-1 and MCF-7 co-culture, and THP-1 and MCF-7 co-culture with TSP1 (Figure 6). There was no detectable IL-1α in all three of the samples, which could signify that the concentration of IL-1α was not detectable in our samples at the working volumes used. This is further supported by the positive control, THP-1/MCF-7 and Anti-CD47, not containing detectable levels of IL-1α. Conversely, we did see detectable TNFα in the samples of THP-1/MCF-7 co-culture (1.3 pg/mL) and THP-1/MCF-7 co-culture with TSP1 (13.1 pg/mL). The difference in concentrations could signify that a baseline interaction is taking place in the THP-1/MCF-7 co-culture, while a larger immune interaction is possibly taking place in the THP-1/MCF-7 co-culture with TSP1 present.

**Conclusions and Future Directions**

As diseases become more prevalent there is an increased need for capable scientific researchers to find better treatment options, vaccines, and cures. In the university setting, the research training model of one-on-one interaction with a faculty mentor is straining to bring novel research experiences to as many students as possible. Unfortunately, there is a small maximum that any faculty mentor can train at one time because of the needs associated with one-on-one training. Interestingly, many research projects can easily be adapted to Course-based Undergraduate Research Experiences (CURE) taught as a formal class in larger classroom-type laboratories. The CURE format provides a higher volume of students with training on marketable and valuable research techniques each semester. In this CURE, a class of 16 students in one semester learned about immune evasion techniques of cancer cells and attempted, as a group, to reverse one of those techniques (CD47-SIRPα interaction), leading to the elimination of cancer cells.

As breast cancer becomes more prevalent, the need for new treatment options grows. While it is already known that anti-CD47
Figure 6. **ELISA.** The detection of IL-1α and TNFα in THP-1 cells, THP-1 and MCF-7 co-culture, and THP-1 and MCF-7 co-culture with TSP1.

antibodies can block the CD47-SIRPα interaction that allows cancer to evade the immune system, this study sought to find a new, potentially more effective way to block this interaction. In place of anti-CD47, a known high-affinity ligand for CD47, thrombospondin-1 (TSP1), was selected to test the ability of TSP1 to outcompete SIRPα on THP-1 macrophage for the CD47 on the MCF-7 breast cancer cells. Based on confocal analysis,
it appears that MCF-7 cells can be found outside of the THP-1 cells when TSP1 is present, which would signify that the interaction of CD47-SIRPα was still taking place and that TSP1 was not outcompeting SIRPα. Since the phagocytotic activity was dependent on qualitative observation, new methods to quantify the fluorescence intensity can be utilized in future studies to provide insight on the proximity of the MCF-7 cells to the THP-1 cells, such as the use of antibody-opsonized polystyrene beads (Babcock, 1999; Choy & Botelho, 2017). Moreover, the use of a surface receptor label for the THP-1 macrophage did not produce a uniform label detectable by confocal microscope. This made the location of MCF-7 cells in relation to the THP-1 cells hard to confirm. Therefore, the protocol can be improved for the next semester of CURE researchers by increasing the concentration of surface receptor label used, decreasing the working volume in the individual wells, or switching to a whole cell dye instead of a surface receptor targeting labeled antibody. Similarly, we expected to see varying levels of the pro-inflammatory cytokine IL-1α by ELISA, like we did in our TNFα ELISA. A possible solution to help measure a detectable level of IL-1α is to also decrease the working volume in the individual wells to help concentrate the cytokines present while also helping to bring the different cell types into a closer proximity to each other for immune interactions to take place. Once a protocol has been effectively established, future studies can employ statistical analysis by performing the assay in duplicates and triplicates and repeating the experiment at least two times for consistency. Ultimately, based on the results of confocal analysis and ELISA, it is not yet known if TSP1 is an effective inhibitor of the CD47-SIRPα interaction by outcompeting SIRPα. Additionally, activation of the THP-1 macrophages from an M0 to an M1 state and searching for the presence of more pro-inflammatory cytokines (IL-6, IL-8, and IL-12) in the supernatant could help improve future research examining the phagocytic ability of macrophages. Further experimentation must be conducted to optimize the methods and improvements detailed above.

Through this CURE, students were able to conduct novel research projects with hands-on training in a group setting, like students involved in one-on-one directed research with a single faculty mentor, while also learning to critically analyze results, determine experimental improvements to their research, and present their findings to various audiences in both written and oral forms.
References


