Assessing the Crosstalk between CD47 and SIRPα and its Role in Modulating Tumor Cell Growth

By
Juliane Baco
Massapequa High School, NY, USA

Abstract

CD47 has been found to be overexpressed in melanoma, leukemia and lymphoma tumors and to bind with SIRPα to avoid phagocytosis. However, in melanoma cells, blocking CD47 expression does not enhance the phagocytosis process. Surprisingly, we found and confirmed that melanoma cells express SIRPα, and so became curious as to if the CD47 and SIRPα interaction within tumor cells had a biological function to modulate tumor cell growth. To test, cell viability was assessed by MTT assay and flow cytometry. Gemcitabine was used as positive control, and the antibody against murine CD47 (clone MIAP301) was used. Results indicate that gemcitabine was effective in decreasing the viability of cells by around 50% in all tumor cells studied. In RAW264.7 cells and B16 cells, but not MC38 cells, cell viability was decreased upon administration of the CD47 blocking antibody. Flow cytometry data also confirmed the CD47 and SIRPα expression of the corresponding cells. The data suggests that there is a biological function in the crosstalk of CD47 and SIRPα and that it modulates cell viability, as well as a potential biological function of CD47 and SIRPα interaction in RAW 264.7 cells.

Background

CD 47 (Cluster of Differentiation 47) was formally identified as an IAP (integrin associated protein) and is known for its help in regulating integrin dependent mechanisms, and interacting with signal regulatory protein alpha (more commonly known as SIRPα) to promote the formation of filopodia on dendrites. However, it was found to be overexpressed in tumor cells. CD47 expression helps tumor cells avoid phagocytosis by binding to SIRPα which is overexpressed in phagocytes, such as macrophages. (Maurta) In tumor cells, the binding of CD47 from tumor cells and SIRPα on phagocytes will create an oversight of these tumor cells in the phagocytosis process, allowing tumors to grow and expand. In some tumor types, such as lymphoma and leukemia, that express CD47, blocking the binding of CD47 to SIRPα in tumor cells enhances phagocytosis of these tumor cells. However, in melanoma cells the binding of CD47 to SIRPα being blocked by blocking CD47 does not enhance the effects of phagocytosis in both in vitro and in vivo experiments.

MC38 is a colorectal cancer cell line known to respond to CD47 blockade. However, in B16, a melanoma cell line, treatment with CD47 blocking antibodies does not enhance phagocytosis. (Anderson) Furthermore, preliminary, along with other, data proves that SIRPα is expressed in melanoma cells, but not colorectal cancer cells

Since there is only a small amount of research conducted on the topic, this study first endeavors to reconfirm that SIRPα is expressed in B16 melanoma cells, and then determine whether or not the crosstalk between CD47 and SIRPα could be characterized as modulating cell growth and/or cell viability.

Procedure:

Experimental conditions and cell culture:

For this study, B16 (melanoma), MC38 (colorectal carcinoma) , and RAW 264.7 (macrophage II) cells (Sigma-Aldrich Mammalian Cell line) were grown, tested and analyzed. B16 and MC38 cells were grown in RPMI (Roswell Park Memorial Institute) media with 5% FCS (fetal calf serum) and cultured in 750 mL flasks over a period of 48 hours, while RAW 264.7 cells were grown in RPMI media with 10% FCS media over a period of 72 hours. This was done in order to ensure that there were enough viable cells to proceed with the analysis.

B16 and MC38 cells were then plated for 3 time conditions (0,24 and 48hours post administration) in separate 96 well plates, as seen in Figure 1. They were treated with no treatment, gemcitabine (a concentration of 4µl/ml as the positive control), and three concentrations. They were dosed with three doses of IGg2a, which is the isotype control antibody, and MIAP301, which is the clone CD47 blocking antibody. The doses were 1µg/ml, 10µg/ml, and 20µg/ml. This procedure was followed for both the B16 cells and MC38 cells, as well as the RAW 264.7 cells later in the experiment.

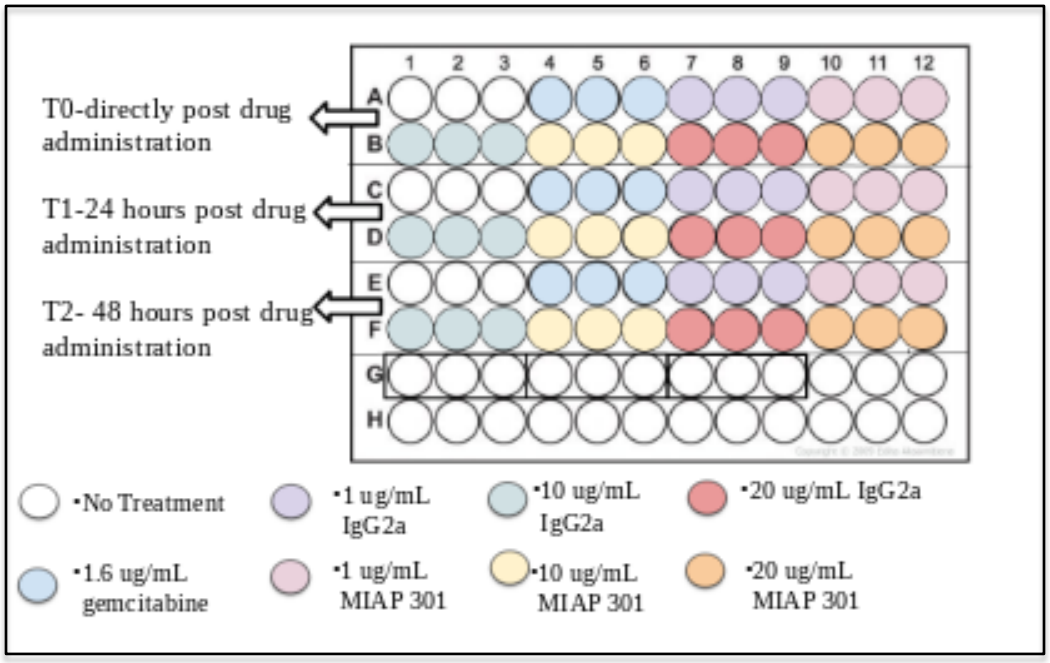

Figure 1: Experimental Design for 96 well plates and treatment administration plan

CCK 8 and Cell Viability Assay:

An MTT assay was done to assess the viability of the cells. CCK8, or cell counting kit 8 was done to assess the viability of the cells. When performing the assays, we followed the protocol for the MTT assay but replaced MTT with WST-8 which is clearer in measuring the external viability of the cell

rather than the interior. WST-8 is a tetrazolium salt, that produces formazan dye upon reduction in the presence of an electron carrier. This allows for the dehydrolization activity to be assessed. The shade of the dye is directly proportional to the cell viability. Direct assessment of the cell viability was measured by the dye shade 450.nm wavelength absorption using a Molecular Devices spectraMaxS i3 spectrophotometer.

Flow Cytometry to stain for K19, CD47, SIRPα and cell death:

Flow cytometry was also used to assess cell viability mainly for the expression of the remaining cells. Cells harvested from each time point after the drug treatment were stained for CD47, SIRPα, and Live/Dead amine dye, for 30 mins and then fixed using the Foxp3-fixation kit (eBioscience, Cat 00-5521-00) for 25 mins and then stained again with intracellular Ki-67 for 30 minutes, which is used to measure the growth factor of cell proliferation. After staining, cells with labels were run to allow manual analysis using LSR II machine and results were analyzed using FlowJo software.

Data:

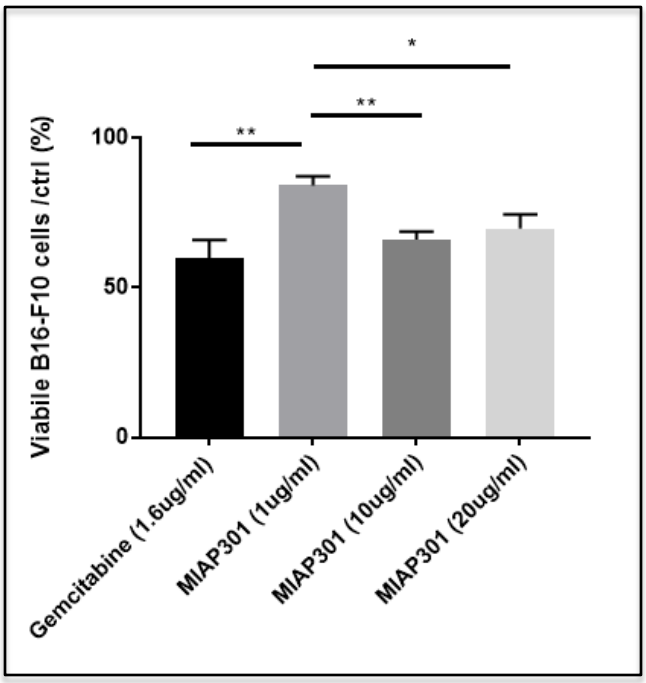

Figure 2: Percentage of Viable B16 cells/ctrl for gemcitabine and MIAP 301 concentrations

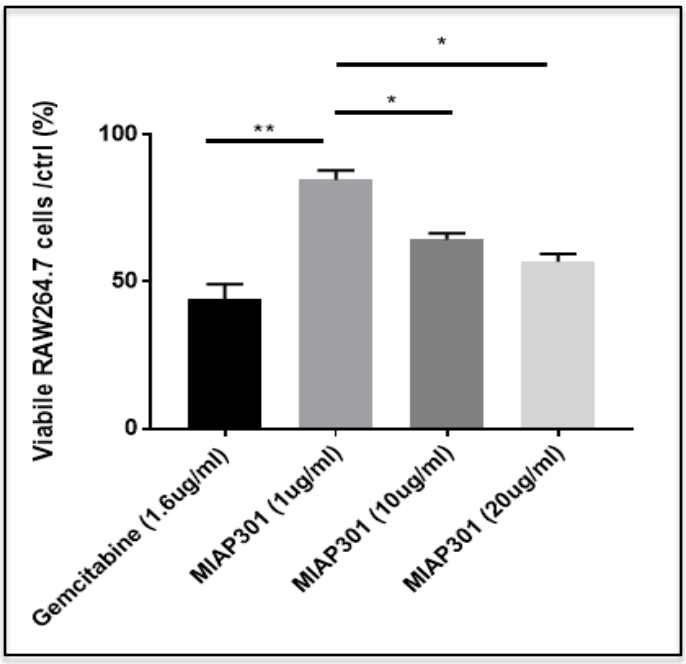

Figure 3: Percentage of Viable RAW 264.7 cells/ctrl for gemcitabine and MIAP 301 concentrations

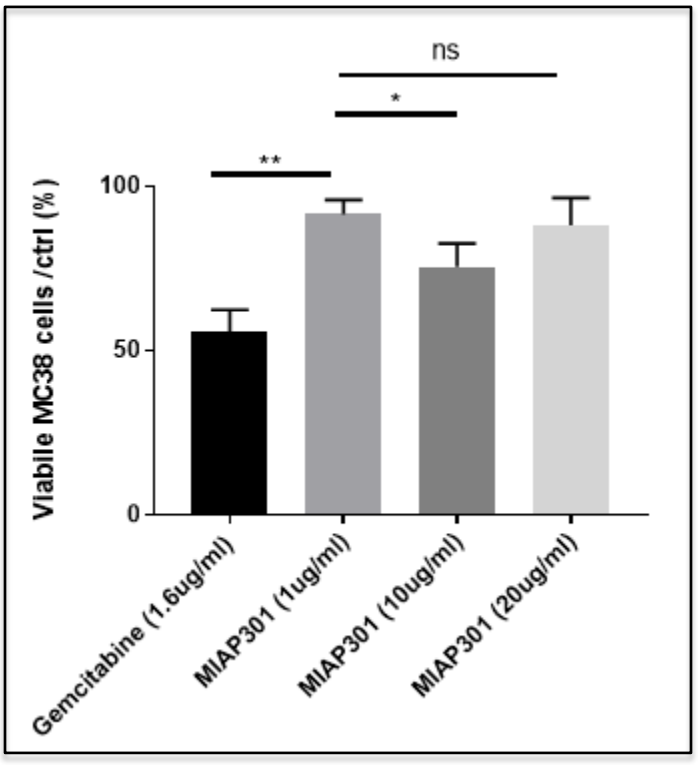

Figure 4: Percentage of Viable RAW 264.7 cells/ctrl for gemcitabine and MIAP 301 concentrations

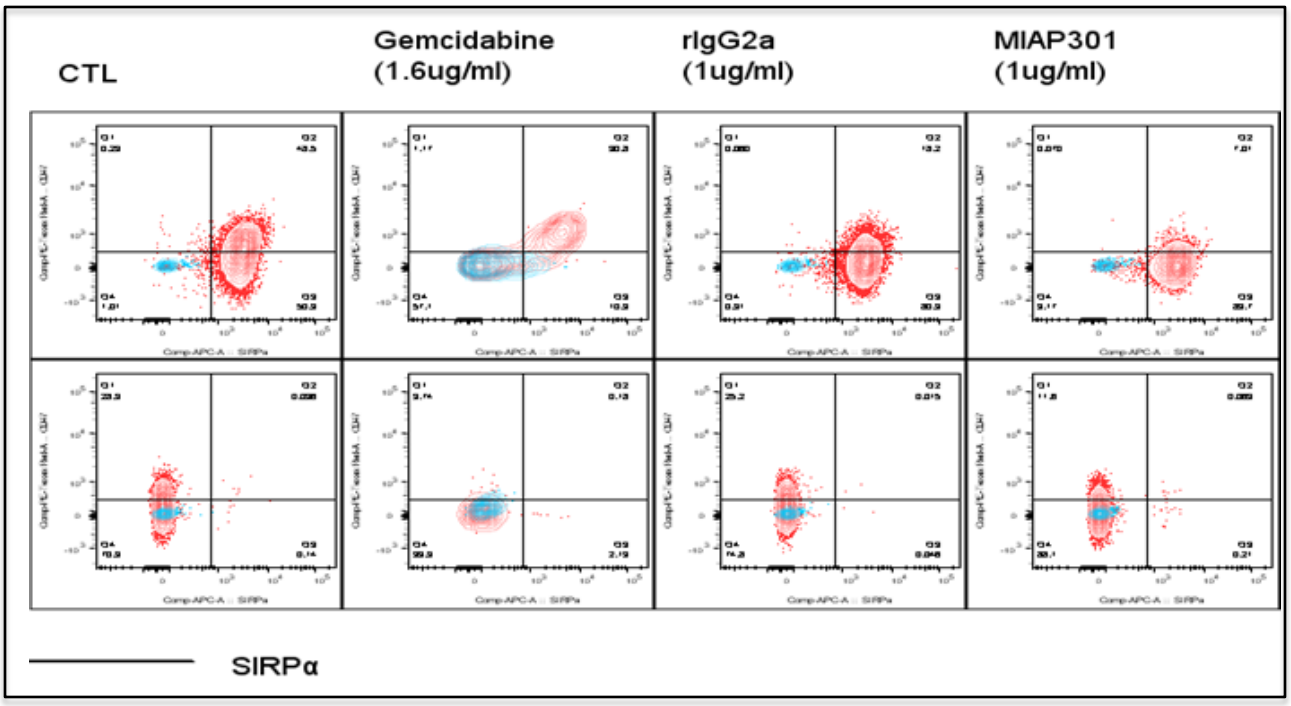

Figure 5: Flow Cytometry graphs with stain for SIRPα and CD47 for B16 (top line) and MC38 (bottom line) for the control group, Gemcitabine positive control, as well as 1 µg/mL doses for IgG2a isotype control and the MIAP301 clone CD47 blocking antibody

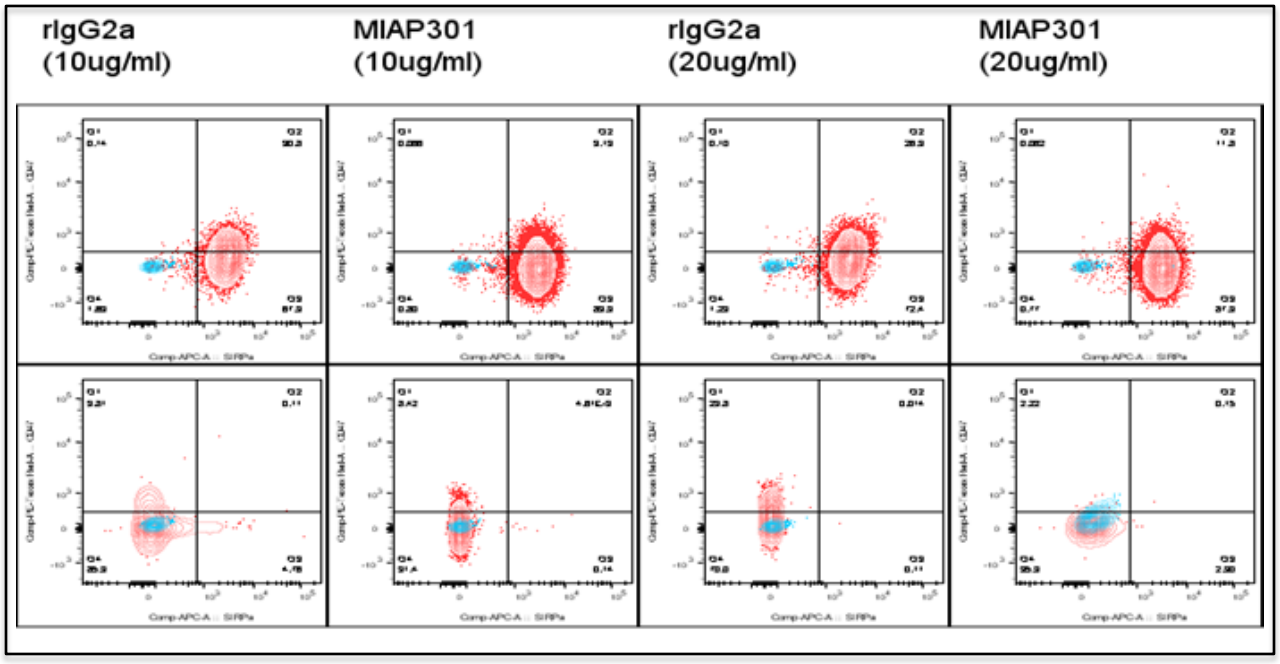

Figure 6: Flow Cytometry graphs with stain for SIRPα and CD47 for B16 (top line) and MC38 (bottom line) for 10 and 20 µg/mL and doses for IgG2a isotype control and the MIAP301 clone CD47 blocking antibody

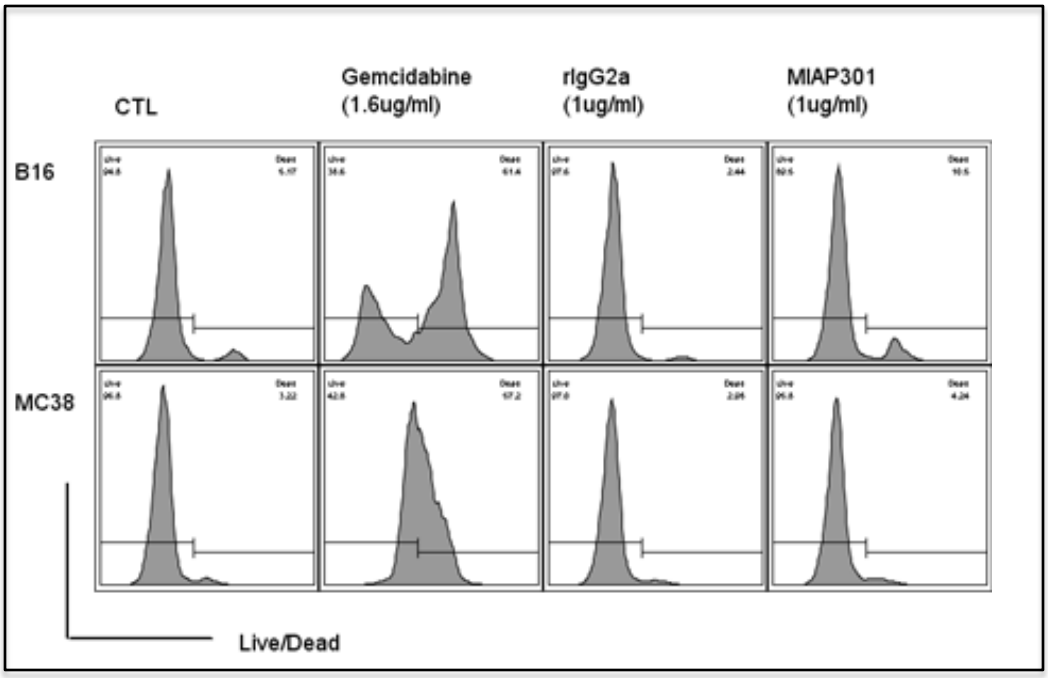

Figure 7: Flow Cytometry data stained for Live/Dead cells for B16 (top line) and MC38 (bottom line, for the control condition, gencidbine condition, as well as 1.0 µg/mL concentrations of the IGg2a isotype control and the MIAP301 clone CD47 blocking antibody.

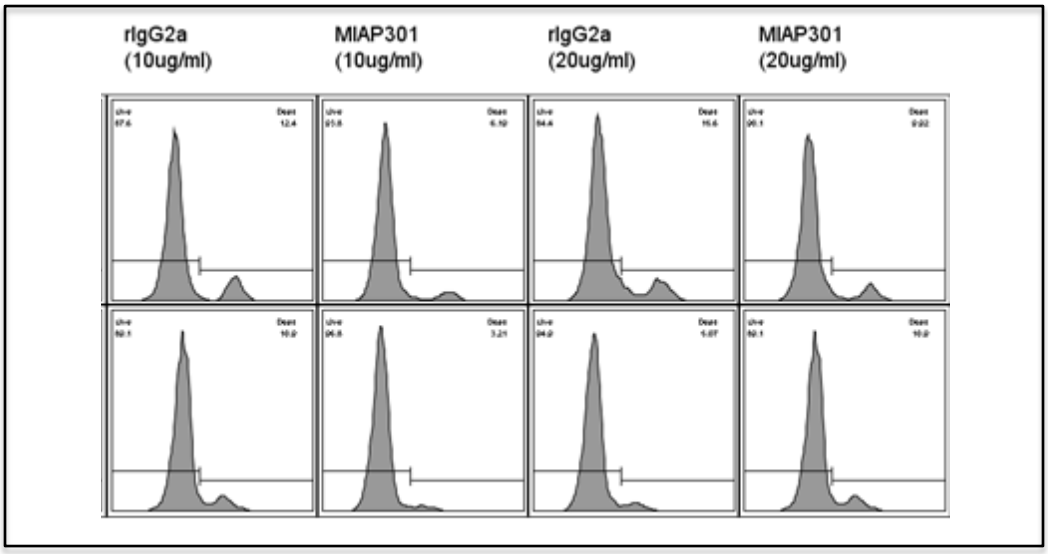

Figure 8: Flow Cytometry data stained for Live/Dead cells for B16 (top line) and MC38 (bottom line, for 10.0 µg/mL and 20.0 µg/mL concentrations of the IGg2a isotype control and the MIAP301 clone CD47 blocking antibody.

Data Analysis:

The cell viability assay provides a measure of the percentage of viable cells as a percentage compared to the control media. In the B16, MC38, and RAW 264.7 data, a 50% drop of cell viability was observed upon administration of gemcidabine. In the B16 assay, a 25% drop in cell viability was seen with the 1.0µg/mL dose of MIAP301, however when the doses were increased a greater decrease of cell viability was seen. A similar trend was observed in the RAW 264.7 cell viability assay, where an approximately 25% drop in cell viability was seen with the 1.0 µg/mL dose of MIAP301, however when the dosage was increased, there was a comparatively stark drop in the cell viability.

However, in the MC38 data, there was the expected drop in viability due to Gemcitabine, however any dose of the MIAP301 CD47 blocking antibody seems to have little to no effect on the viability of the cells. There was one abnormality in the data which indicated a small drop in cell viability at the dose of 10 µg/mL. However, this value is attributed to a plating issue when conducting the experiment, since it is only one specific trial that lowers the entire's sample's percentage of cell viability.

In the flow cytometry data, the control group stained for CD47 and SIRPα indicates high levels of CD47 expression. The B16 cells express high levels of SIRPα expression while the MC38 cells show little to no SIRPα expression. When gemcitabine is administered, we see a decrease in the percentage of cells that express CD47, as well as cells that express SIRPα. When the CD47 blocking antibody is administered, we see a continuous decrease (correlated with a continuous increase in concentration) in cells that express both CD47 and SIRPα, but no decrease in the percentage of cells that only express SIRPα.

The stain for live and dead cells shows that only the gemcitabine is actually successful in killing the tumor cells, clearly indicated in Figures 7 and 8. Any conditions that were treated with the MIAP301 CD47 blocking antibody showed little to no cell death upon treatment when stained.

Conclusion:

Over the course of this study, many points in the data sets lead to multiple conclusions. Firstly, the flow cytometry data reconfirmed that SIRPα is expressed in B16 melanoma cells, but not MC38 colorectal cancer cells. Secondly, it was observed that the treatment of the CD47 blocking antibody consistently successful in suppressing the growth of tumor cells. This suggests the existence of a biological function between CD47 and SIRPα in melanoma cells, furthermore suggesting that that the crosstalk between CD47 and SIRPα is required for optimal cell growth in tumor cells that express both proteins. And lastly, ideally the experiment would need to be repeated in order to ensure the discrepancy in the melanoma graph was in fact a plating error and nothing more.

Future investigations are needed to expand the understanding of the potential pathways mediated through the crosstalk of CD47 and SIRPα in tumor cell growth. Furthermore, in clinical applications consideration must be given to whether or not a tumor expresses both CD47 and SIRPα. If it does then it poses a biological function to modulating cell viability and must be taken into consideration when proceeding with treatment.

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