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Effects of SOCS1 and SOCS3 Peptide Mimetics on Macrophage Phagocytosis of Malignant Cells

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EFFECTS OF SOCS1 AND SOCS3 PEPTIDE MIMETICS ON MACROPHAGE
PHAGOCYTOSIS OF MALIGNANT CELLS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

COLT DYLAN CAPAN
B.A., Thiel College, 2015

2017

Wright State University

**WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL**

July 27, 2017

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Colt Dylan Capan ENTITLED Effects of SOCS1 and SOCS3 Peptide Mimetics on Macrophage Phagocytosis of Malignant Cells. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract

Capan, Colt Dylan. M.S. Microbiology and Immunology Graduate Program, Wright State University, 2017. Effects of SOCS1 and SOCS3 Peptide Mimetics on Macrophage Phagocytosis of Malignant Cells.

Macrophages play a key role in both the innate and adaptive immune system responses to foreign materials. Suppressors of cytokine signaling (SOCS) proteins are known as regulators of the immune response through various JAK/STAT pathways. This study examined the roles of SOCS1 and SOCS3 peptide mimetics on the phagocytosis of fluorescently labeled malignant target cell by RAW264.7 murine macrophages. The malignant cells used were Neuro-2a cells, a murine neuroblastoma cell line. A prominent “eat me” signal found in neuroblastoma cells is calreticulin (CRT), which permits macrophages to recognize and then phagocytize the malignant cells. When M1 (pro-inflammatory) polarized macrophages were treated with a SOCS1 peptide mimetic, an increase in phagocytosis was observed, but a SOCS3 peptide mimetic had no effect on phagocytosis. Neither SOCS1 nor 3 peptide mimetics showed a significant effect on the phagocytosis ability of the M2 (anti-inflammatory) polarized macrophages when target cells were stained with carboxyfluorescein succinimidyl ester (CFSE). When target cells were treated with anti-CRT, phagocytosis was decreased by both M1 and M2 polarized macrophages. When M2 macrophages were treated with SOCS3 peptide mimetic and target cells were blocked with anti-CRT, which initiates apoptosis, an increase in phagocytosis compared to unblocked target cells was observed. These results contribute

to our continuing understating of the primary functions of M2 macrophages in wound healing and clearance of apoptotic cells. Overall this study provides a mechanism by which SOCS1/3 peptide mimetics can enhance phenotypic specific phagocytosis of pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages.

List of Abbreviations

ATCC = American type culture collection

BSA = Bovine serum albumin

CD11b = Cluster of differentiation marker 11b

CFSE = Carboxyfluorescein succinimidyl ester

CRT = Calreticulin

IFN- γ = Interferon gamma

Il-10 = Interleukin-10

Il-13 = Interleukin-13

IL-4 = Interleukin-4

JAK = Janus kinase

KIR = Kinase inhibitory region

LPS = Lipopolysaccharide

M0 = Unpolarized macrophages

M1 = Macrophages polarized to the pro-inflammatory state

M2 = Macrophages polarized to the anti-inflammatory state

MAP-2 = Microtubule associated protein-2

merTK = Tumor associated macrophage receptor tyrosine kinase

N2a = Nuero-2a cell line

NB = Neuroblastoma

PBS = Phosphate buffered saline

PS = Phosphatidylserine

RAW264.7 = Macrophage cell line

RT = Room temperature

S1 = Suppressor of cytokine signaling 1

S3 = Suppressor of cytokine signaling 3

SOCS1 = Suppressor of cytokine signaling 1

SOCS3 = Suppressor of cytokine signaling 3

STAT = Signal transducers and activators

TGF- β = Transforming growth factor beta

TLR = Toll like receptors

TNF- α = Tumor necrosis factor alpha

μg = microgram

μL = microliter

μM = micromolar

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Introduction

Neuroblastoma (NB) is a form of childhood cancer that affects thousands of children every year. This form of malignancy is typically diagnosed in the early years of the child's life and has the highest mortality rate of any adolescent cancer (Cheung and Dyer, 2013). This form of childhood cancer progresses in the early stages of embryonic development. The malignant cells form from the neural crest and are most often found along the nervous system once they are detected (Heck *et al.*, 2009). Very rarely do NB tumors form in a hereditary manner; most cases that are studied are spontaneous mutations and these mutations have low incidence of occurring in the same gene. The most frequent gene that is mutated in both hereditary and spontaneous cases is the ALK gene (Pugh *et al.*, 2009). NB's have very efficient mechanisms, which they use to evade the immune system; these mechanisms include manipulation of both the innate and adaptive immune system.

Some methods used by NB's include the down regulation of cell surface markers that normally allow recognition by host T cells. They are also capable of recruiting macrophages to alter host immune cells to promote their own survival (Cheung and Dyer, 2013). Other cell types that play a vital role in the survival of tumors are natural killer cells, tumor-associated macrophages, dendritic cells, and myeloid suppressor cells. All of these cell types play different roles in the tumor cell environment including cytokine production, activation signaling and tumor phenotype editing that will promote an

environment for tumor survival (Whiteside, 2008). One cellular signal in particular has been at the forefront of research into the prognoses of tumor states and also looked at for new potential treatment options for various stages of tumor progressions. The signal of interest is calreticulin (CRT), found in five different types of cancers, acts as an “eat me” signal. “Eat me” signals are a signal that phagocytes, or macrophages, will recognize through specific cell surface receptors; this recognition allows the phagocytes to efficiently clear the cells from the cellular environment (Chao *et al.*, 2012). However, tumor cells have the ability to promote their own survival by up regulating the expression of cluster of differentiation (CD) 47, a “don’t eat me” signal that works to offset the amount of CRT expression so it can survive (Chao *et al.*, 2010).

Clearance of foreign materials, including tumors, from the cellular environment involves a multitude of signals to work in conjunction with one another. These signals include but are not limited to CRT, CD47, and phosphatidylserine (PS). Living cells tend to express both CRT and CD47, with CD47 having a greater expression to silence the “eat me” signal from CRT (Raghavan *et al.*, 2013). Conversely it has been shown that CRT has had higher levels of expression in patients that had neuroblastomas and survived (Hsu *et al.*, 2004). Cells that begin to undergo the early stages of apoptosis will express PS a signal that is universally recognized by phagocytes that are in the surrounding cellular microenvironment (Segawa and Nagata, 2015). Under normal circumstances PS is not overtly expressed on the cell surface and CRT will be expressed on malignancies. The expression of PS will be increased when the cell becomes stressed from an outside stimulant. If one of these signals is blocked or altered the opposing signal will then be expressed at a much higher ratio making it more easily recognizable by phagocytes

(Krysko and Vandenabeele, 2008). The manipulation of these signals present on tumor cells in a manner that promotes the clearance of tumors is at the forefront of cancer biology research.

Macrophages are considered to be one of the most important and dependable cell types in the immune system. Macrophages are relied on because they are able to tie in both the innate and adaptive immune systems. The prominent role they play in innate immunity consists of clearing cellular debris while also protecting the immune system from uncommon material such as, pathogens, bacterial infections, or tumors (Mosser and Edwards, 2008). The phagocytosis of these foreign materials will lead the macrophages to undergo various changes to their cellular morphology and physiology, which include the production of various cytokines, transduction of signals to help clear the material, and a change in cellular shape (Murray and Wynn, 2011). The production of innate immunity cytokines help recruit adaptive immune cells, these adaptive immune cells include T and B cells. Macrophages also have a role in the development of many inflammation diseases and autoimmune disorders.

The various roles macrophages play in the immune system include protecting the host from various contaminants, regulating immune responses, and assisting in the healing after a traumatic event. When conducting these functions of the immune system macrophages can generally be classified into two different categories: M1 or M2 macrophages (Qian and Pollard, 2010). Both types of macrophages are stimulated or polarized by different molecules that lead to their altered roles in the immune system. Typically these two distinctively different macrophages act in contradictory roles but will act together to protect the host. For example, M1 macrophages conduct their responses in

a pro-inflammatory pattern as where M2 macrophages will have an anti-inflammatory response to an immune challenge (Murray and Wynn 2011). Another hallmark difference between the two macrophage phenotypes is their difference in cellular shape when stimulated with phenotype specific cytokines. M1 macrophages will typically be round and appear flattened in shape with the appearance of vacuoles inside the cell. The M2 macrophages appear to be elongated in shaped without any vacuoles present (McWhorter *et al.*, 2013).

One of macrophages main contributions to an innate immune response is the engulfment of particles and for this reason they are referred to as a professional phagocyte (Flanngan *et al.*, 2012). Phagocytosis is a widely studied cellular process but because it involves so many different components and molecules a true understanding of the exact mechanisms involved has yet to be mutually agreed upon. There is a massive overlap of signals and receptors in phagocytosis, that are also involved in many other cellular functions this shows the importance of the process in relation to stabilization of the cellular microenvironment (Stuart and Ezekowitz, 2008). Toll-like receptors (TLRs) and pattern recognition receptors (PRRs) are two of the key components that have a multifaceted role in overall function. Both of there receptors, when bound by a pathogen or foreign material, trigger a downstream cascade that leads to the expression of cytokines which will then recruit even more cells to help clear pathogens (Akira *et al.*, 2006).

Eliciting a pro-inflammatory M1 phenotype response in macrophages requires the stimulation of macrophages with LPS and IFN- γ . This phenotype is most often associated with the phagocytosis, clearance of foreign materials, and is

tumorcidal (Mosser and Edwards, 2008). M1 macrophages typically have the responsibility of clearing pathogens and are involved in resistance to tumor growths (Martinez and Gordon, 2014). A high ratio of M1/M2 macrophages can result in the inhibition of cellular proliferation and an increase in tissue damage. Some disease states commonly associated with M1 macrophages are Alzheimer's disease, obesity, and various autoimmunity disorders (Mills, 2012).

The anti-inflammatory M2 phenotype macrophages have a more complex activation pathway compared to M1 macrophages; multiple subsets of M2 macrophages carry out their own functions in the microenvironment. M2 macrophages are characterized by their stimulation factors that also determine their functional role in the cellular microenvironment. M2a are considered alternatively activated by IL-4 and IL-13, M2b are characterized as type 2 stimulated by LPS, and M2c are classified as deactivated subtype stimulated by IL-10 and TGF- β (Rozer, 2015). Alternatively activated macrophages play a role in allergies and clearance of parasites, type 2 are known for their role as immune system regulator macrophages, and deactivated macrophages are important in tissue remodeling and matrix deposition (Martinez and Gordon, 2014). M2 macrophages that are actively producing IL-10 have phagocytic ability leading to the clearance of apoptotic cells that have a high ratio of PS signals (Xu *et al.*, 2006). Macrophages that are polarized to the M2c phenotype have an increased amount of merTK receptor that is associated with the clearance of apoptotic cells (Zizzo *et al.*, 2012).

Suppressors of cytokine signaling (SOCS) proteins are a family of proteins that assist in the regulation of cytokines and therefore affect the development and

differentiation of multiple cell types including macrophages. SOCS proteins are both stimulated by cytokines and regulate cytokine production; therefore, they act on target cells in a negative-feedback loop (Yoshimura *et al.*, 2007). SOCS proteins are capable of regulating the production of various cytokines by binding of the kinase inhibitory region (KIR) of the SOCS protein to Janus kinase/ signal transducers and activators of transcription, or more commonly known as JAK/STAT pathway. In this pathway the specific cytokines will bind their receptors, this binding of cytokine will then activate a cytokine specific JAK kinase and the substrates of the activated JAK kinases are the various STAT proteins. SOCS proteins disrupt this pathway by blocking the STAT protein from interacting with the activated JAK kinases (Tamiya *et al.*, 2011). Two of the most important SOCS proteins are SOCS1 and SOCS3; these two proteins are thought to play an extremely important role in inflammatory diseases and tumor development (Whyte *et al.*, 2011 and Carow and Rottenberg, 2014). Both of these proteins, SOCS1/3 have the ability to interact directly with JAK through their KIR. SOCS proteins play a very dynamic role in cellular function. The ratio of SOCS1 to SOCS3 proteins will determine the inflammatory state of macrophages in response to an immunological challenge (Yoshimura *et al.*, 2012). The current progress on SOCS protein interactions with the JAK/STAT pathway is leading to more studies involving different disease models. Progress in this field is contributing to a more well rounded understanding on the importance that the cellular microenvironment has on disease progression. Hopefully leading to a mechanism that explains a method to increase the clearance and removal of malignancies.

SOCS1 is most often associated with the inhibition of IFN- γ signaling, by acting as an alternative substrate to an activated JAK, blocking the ability of the STAT protein associated with IFN- γ signaling. SOCS1 is a key regulator in activation and maintenance of both M1 and M2 polarized macrophages. The ratio of SOCS1 to SOCS3 is at higher levels in M2 polarized macrophages and the ratio of SOCS3 to SOCS1 is higher in M1 macrophages (Whyte *et al.*, 2011). The role of SOCS1 has been studied most often through mice that have the SOCS1 gene deleted. These mice typically die within the first month from having constitutively active IFN signaling pathways. Leading to an increased activation of T cells and macrophages attacking various organs in an autoimmune disease manner (Kubo and Yoshimura, 2003). Other inflammatory cytokines and pathways that are affected in knockout mice are TNF, IL-12, nitric oxide, NF-kB and TLR signaling (Yoshimura *et al.*, 2007).

In terms of their mechanism of action SOCS3 acts in a very similar manner to SOCS1, the only difference is that different cytokine receptors are involved. SOCS3 typically acts as a negative inhibitor of cytokines, these cytokines include IL-6, IL-10, and IL-2. In many mouse models SOCS3 has been shown to play an important role in effecting inflammation and immune responses (Carow and Rottenberg, 2014). Current studies examining the effects of SOCS3 on the immune response involve a complete knockout of the SOCS3 gene. Upon knockout of the SOCS3 gene a greater amount of pro inflammatory cytokines was observed. Also, the deletion of SOCS3 enhances the amount of phagocytosis of M1 polarized macrophages (Qin *et al.*, 2012). Showing the importance of SOCS3 as an immune system regulator of phagocytosis in a murine model.

The current study focuses on the role that SOCS peptide mimetics have on the inflammatory properties of RAW264.7 murine macrophages. The main inflammatory property associated with murine macrophages is the process of phagocytosis, carried out by both M1 and M2c polarized macrophages. SOCS peptide mimetics can alter the percentage of target cells being cleared by inhibiting the production of certain cytokines and by increasing receptor expression. Based on current literature I hypothesize that the treatment of anti-inflammatory M2 macrophages with SOCS1 peptide mimetic will exhibit a pro-inflammatory property of increased phagocytosis of target cells. Similarly, SOCS3 peptide mimetic will decrease the inflammatory state of pro-inflammatory M1 macrophages to a more anti-inflammatory phenotype.

Hypothesis

Treatment of pro-inflammatory M1 macrophages with SOCS3 peptide mimetic will result in an anti-inflammatory phenotype leading to a decreased phagocytosis of malignant cells. Likewise, the treatment of anti-inflammatory M2 macrophages with SOCS1 peptide mimetic will then lead to a pro-inflammatory phenotype increasing phagocytosis as analyzed by flow cytometry. Malignant target cells, Neuro-2a, that have their “eat me” signal blocked will also show a decrease in the amount of phagocytosis.

Materials and Methods

Cell lines

The RAW 264.7 murine macrophage cell line (ATCC TIB-71) is an Abelson murine leukemia virus-induced tumor, adherent macrophage cell line produced from a male BALB/c mouse. RAW 264.7 cells were grown in either 25 cm² or 75cm² vented cap cell culture flasks and incubated at 5% CO₂ with a temperature of 37°C using a water-jacketed incubator. Cells were cultured in media containing Dulbecco's Modified Eagle's Medium (DMEM) and 10% fetal bovine serum (FBS). Cells were cultured in vented cap flasks in a ratio of 1:5, upon reaching confluency of 70%.

The Neuro-2a murine neuroblast cell line (ATCC-CCL-131) is an adherent neuroblastoma cell line produced in a strain A mouse. Neuro-2a cells were grown in an environment of 5% CO₂ and a temperature of 37°C using a water-jacketed incubator. Cells were cultured in either 25 cm² or 75cm² vented cap growing flasks with media that contained DMEM and 5% FBS. Further passages were subcultured in vented cap flasks in a ratio of 1:5, upon reaching 70% confluency. Vented cap flasks, growth medium, and fetal bovine serum were purchased from Fisher Scientific.

Polarization Treatment

RAW 264.7 macrophages were grown to approximately 70% confluency; cells were then removed from the growing flasks via a cell scraper and collected into 15 mL conical

tubes. Cells were centrifuged at 1700 revolutions per minute (4°C) for 5 minutes; supernatants were removed using a vacuum flask while the cell pellet was resuspended in 1 mL of complete growth medium. Cells were then counted and aliquoted into 25cm² growing flasks with roughly 1.0 X 10⁶ cells per flask. Cells were allowed to become adherent to the growing flasks when the appropriate polarization treatment was administered. RAW 264.7 macrophages achieved a M1 phenotype from treatment with 20 ng/mL IFN- γ (biolegend) and 100 ng/mL LPS (Chondrex) for 18 hours. M2 phenotype was obtained by a treatment with 10 ng/mL IL-10 (Biolegend) for 18 hours. After the 18-hour treatment time cells were prepared for a phagocytosis assay.

Cell Viability and Cell Counting

RAW 264.7 macrophages were aliquoted into 24-well plates at a seeding density of 0.1X10⁶ cells per well, cells were allowed to attach to the culture plates for two hours. After attachment to the cell culture plates the appropriate cytokines were added to achieve either M1 (LPS/IFN- γ) or M2 (IL-10) phenotype; SOCS 1/3 protein mimetics were added at a concentration of 35uM. Untreated cells were used as a control; all treatments were for a period of 18 hours. After the treatment period cells were scraped from the bottom of the 12-well plate using a cell scraper and collected into 1.5 mL microcentrifuge tubes. The supernatant was aspirated off after centrifugation at 1700 revolutions per minute (4°C) for a time limit of 5 minutes. The cell pellet was resuspended in 1 mL of complete growth medium and stained with trypan blue (Fisher Scientific) at a 2:1 ratio; cells were then placed on a hemocytometer to determine cell viability. Viable cells were translucent while the dead cells were stained blue when

examined using a phase contrast microscope. The following equation was used to determine cellular viability:

$$\% \text{ Cell Viability} = \frac{\text{Total viable Cells (unstained)}}{\text{Total Cells (viable and dead)}} \times 100$$

Immunofluorescent Staining

Cells were grown in vented capped growing flasks to approximately 70% confluency; cells were then counted and 0.05×10^6 cells were seeded onto slide chambers (Fisher Scientific). Cells were incubated at 37°C for 24 hours. Following incubation medium was removed and cells were washed twice with 1X PBS; after washing cells were fixed with 4% paraformaldehyde for 15 minutes at RT. Once cells were fixed to the slide chamber, cells were washed thrice with 1X PBS. For the staining of internalized proteins, cells were made permeable with 0.2% triton X100 for 10 minutes at RT. Next, cells were washed thrice with cold 1X PBS to remove permeabilization agent and nonspecific binding was blocked using a blocking solution containing 5% goat serum, 3% BSA, 0.5% tween20 in PBS for one hour at RT. Blocking solution was then removed and cells were washed thrice with 1% BSA solution, each subsequent wash step was done three times with 1% BSA. Next, cells were incubated with the respective primary antibodies for 1 hour at 4° C, followed by washing. In the dark, cells were then incubated with the fluorescent secondary antibody for 2 hours at RT followed by washing. After the last washing all liquid was completely aspirated off, silicone chamber was removed and the slide was left for 10 minutes at RT and then several drops of VectaShield (Vector Laboratories) was added to preserve the stained cells. A glass cover slip was then placed on top of the VecatShield and allowed to harden at RT for 20 minutes. Slides were then

analyzed using an Olympus Epi Fluorescence microscope with a real time spot color camera.

Table 1: Summary of Primary and Secondary Antibody Concentrations used to Confirm Cell Lines Used in the Current Study.

Antibody	Concentration	Company
Anti-CD11b (primary)	5 µg/mL	Abcam
Goat Anti-Rat IgG H&L conjugated to AlexaFluro 488 (secondary)	4 µg/mL	Abcam
Anti-MAP2 (primary)	5 µg/mL	Abcam
Goat Anti-Rabbit IgG H&L conjugated to AlexaFluor 488 (secondary)	4 µg/mL	Abcam

Optimization of Micro Particles

In preliminary experiments FITC labeled micro particles (Sigma-Aldrich) were used to develop a phagocytosis assay. Micro particles were optimized for the recognition by RAW 264.7 macrophages by incubation with 5% normal mouse serum (Thermo-Fisher Scientific) in DMEM. Once particles were optimized they were placed onto polarized macrophages at a ratio of 2:1 (beads:macrophages).

CFSE staining of Neuro-2a Cells

Neuro-2a cells were harvested from growing flasks after they reached 70% confluency. Cells were counted using a hemocytometer and 3×10^6 cells were placed into 15 mL centrifuge tubes. Neuro-2a cells were resuspended in PBS and 0.5 µM of staining solution was added to the cell suspension and allowed to incubate at room temperature

(RT). Cells were then washed twice with 5% complete growth medium and incubated at 37°C. All wash steps were spun at 1700 revolutions per minute for 5 minutes at 4°C. Once the cells were washed they were placed on top of polarized macrophages at a ratio of 2:1 (Neuro-2a cells:macrophages).

Phagocytosis Assay

Micro-particles, CFSE stained Neuro-2a, or Anti-CRT cells were placed on top of a polarized monolayer of macrophages at a ratio of 2:1 with fresh 10% growth medium. Cells underwent phagocytosis assay at 5% CO₂ and a temperature of 37°C in a water-jacketed incubator. After phagocytosis micro particles or stained cells were washed five times using 4°C PBS to stop the uptake of either the micro-particles or Neur-2a cells. Macrophages were then removed from growth flasks using cell scrapers and collected into 15 mL centrifuge tubes. Cells were then prepared for cell surface antigen staining of macrophages to be detected by flow cytometry.

Flow Cytometry

Cell Surface Antigen Staining

Once the cells were collected after the phagocytosis they were washed once with 1% Bovine Serum Albumin (BSA). Following incubation and wash steps, cells were centrifuged at 1200 revolutions per minute for 5 minutes at 4 °C and the supernatants were removed. Cells were then blocked for nonspecific binding by incubation for 20 minutes with 3% BSA at RT. Cells were then washed to remove any excess blocking solution with 1% BSA. Following the washing, cells were incubated at RT for 30 minutes

with F4/80 primary antibody conjugated with eFluor 660 (ebioscience). After incubation cells were washed with 1% BSA once and then resuspended in 4°C PBS and filtered through falcon tubes to be analyzed by flow cytometry on an Accuri C6 Flow Cytometer (BD Biosciences).

Intracellular Staining

Cells were grown to approximately 70% confluency, harvested from growth flasks using trypsin, and resuspended in 1X PBS. Cells were then counted using a hemocytometer and approximately 3.0×10^6 cells were added to 1.5 mL microcentrifuge tubes. Following incubation and wash steps, cells were centrifuged at 1200 revolutions per minute for 5 minutes at 4 °C and the supernatants were removed. Cells were then fixed using fixation buffer (Biolegend) in the dark for 20 minutes at RT. Following fixation cells were washed twice using 1X intracellular staining perm wash buffer (Biolegend). Next, cells were stained for Calreticulin with Anti-CRT antibody conjugated with Alexa Fluro 488 (abcam). Antibodies and cells were incubated at RT for 20 minutes followed by washing with 1X intracellular staining perm wash buffer. Finally, stained cells were resuspended with 4°C PBS and filtered through falcon tubes and to be analyzed by flow cytometry on an Accuri C6 Flow Cytometer (BD Biosciences) that recorded 50,000 events.

Table 2: Summary of Primary Antibody and Isotype Control Concentrations used for Flow Cytometry Analysis in the Current Study.

Antibody	Concentration	Company
Anti-F4/80 conjugated to eFluro 660	10 µg/mL	ThermoFisher
Rat IgG2a K Isotype Control conjugated to eFluro 660	10 µg/mL	ThermoFisher

Anti-Calreticulin conjugated to Alexa Fluro 488	10 µg/mL	Abcam
Rabbit IgG Isotype Conrol conjugated to Alexa Fluro 488	10 µg/mL	Abcam

Statistical Significance

Statistical significance was determined using a One-Way ANOVA using SigmaPlot 12.0; all experiments were completed on separate days in triplicates.

Results

F4/80 Receptor is a Universal Murine Macrophage Marker

RAW264.7 murine macrophages were treated with various cytokines to promote phenotypic changes: IFN- γ and LPS to achieve M1 phenotype, IL-1-0 to reach M2 phenotype and untreated control macrophages (M0). Macrophages were then stained for the murine cell surface receptor F4/80 and isotype controls to calculate the positive differences in binding efficiency. M2 and M0 macrophages showed the highest difference in binding, with M1 macrophages showing a slight decrease (Fig. 3). These results allow the use of F4/80 as a common murine macrophage marker.

M1 Polarized Macrophages are More Efficient at Clearing Foreign Materials

Following 18 hour cytokine treatment for M1 (IFN- γ / LPS) and M2 (IL-10) polarization, M1 macrophages phagocytized more FITC latex beads compared to control M0 and M2 macrophages, while M2 macrophages exhibited similar amounts of phagocytosis compared to control M0 macrophages. (Fig. 4, Fig. 5). Similarly, when N2a cells stained with CFSE were co-cultured with macrophages, M1 macrophages phagocytized more N2a cells and M0 and M2 macrophages phagocytized similar amounts (Fig. 6, Fig. 7).

Phagocytosis of N2a Cells is Decreased When Prominent “Eat Me” Signal is Blocked

When calreticulin is blocked on N2a cells the amount of overall phagocytosis was decreased significantly in control, M1, and M2 macrophages (Fig. 13, Fig. 14, Fig. 15). M1 macrophages still phagocytized a greater amount than either control and M2 macrophages. M2 and M0 control macrophages phagocytized similar amounts of N2a cells that had their “eat me” signal blocked. Continuing the understanding that calreticulin is a major signal that is involved in tumor clearance.

SOCS1 Peptide Mimetic has a Positive Affect on Phagocytosis Ability of M1 Macrophages

SOCS1 peptide mimetic increases the amount of phagocytosis of CFSE labeled N2a cells when compared to M1 macrophages without SOCS peptide mimetic treatment (Fig. 7B Fig. 9, Fig. 11). When SOCS1 peptide mimetic was added to control M0 macrophages and M2 macrophages the amount of phagocytosis of CFSE labeled N2a cells was not increased (Fig. 8, Fig. 10, Fig. 11). M1 macrophages that were treated with SOCS1 peptide mimetic also had the ability to increase phagocytosis of N2a cells that had their “eat me” signals blocked (Fig. 17). Again SOCS1 peptide mimetic had no effect on the phagocytic ability of M0 or M2 macrophages when targets had their “eat me” signals blocked (Fig. 16, Fig. 17).

SOCS3 Peptide Mimetic Increases Phagocytosis of Early Apoptotic N2a Cells by M2 Macrophages.

M2 and M0 control macrophages were treated with SOCS3 peptide mimetic and co-cultured with anti-CRT N2a cells; phagocytosis was increased compared to no SOCS

peptide mimetic treatment (Fig. 16, Fig.18). M1 macrophages treated with SOCS3 peptide showed a slight increase compared to macrophages that were treated with no SOCS peptide mimetic (Fig. 17).

Figures

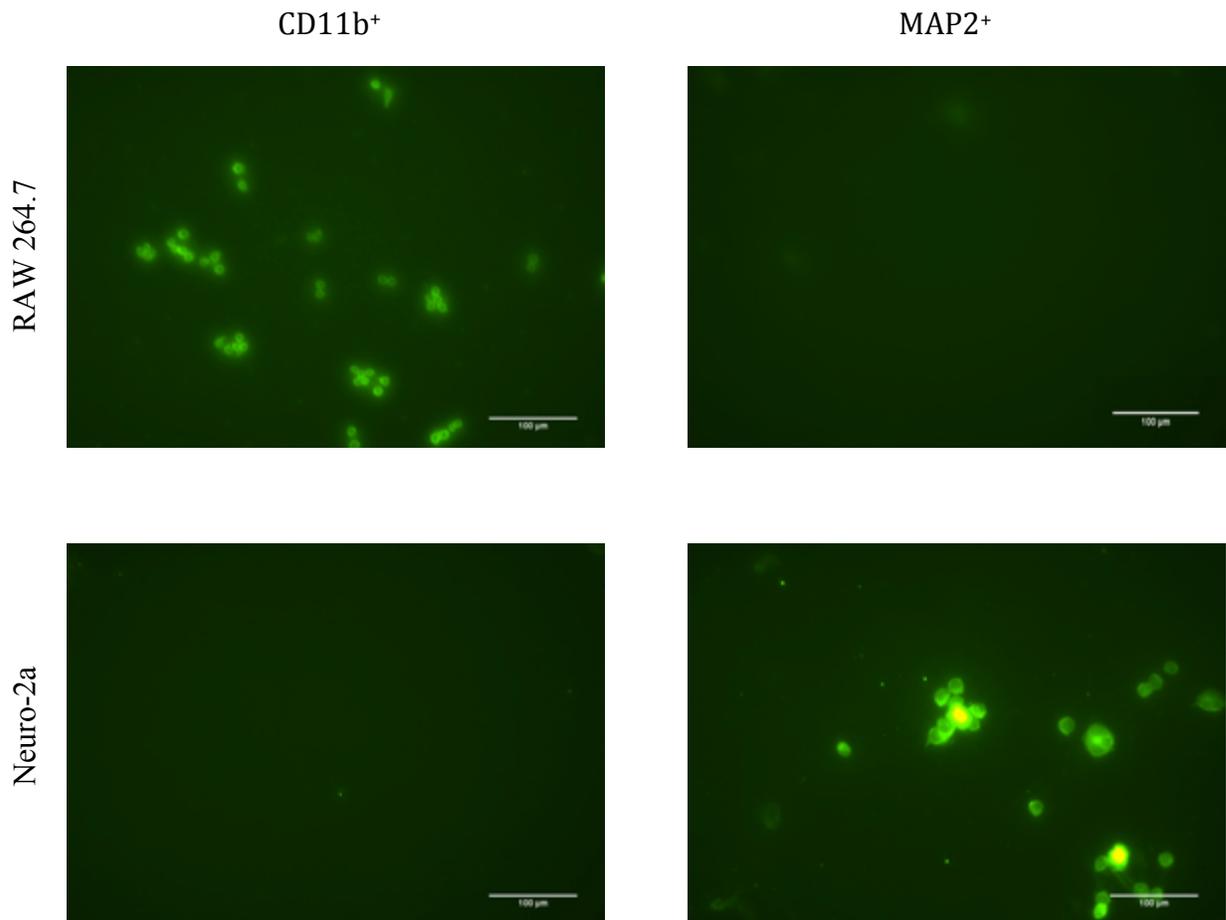


Figure 1: Immunofluorescent images confirming authenticity of Cell Lines.

RAW264.7 and Neuro-2a cell lines were stained for the macrophage cell surface marker CD11b and the intracellular neuronal protein MAP2.

Cell Viability of Treated Macrophages (18 hours)

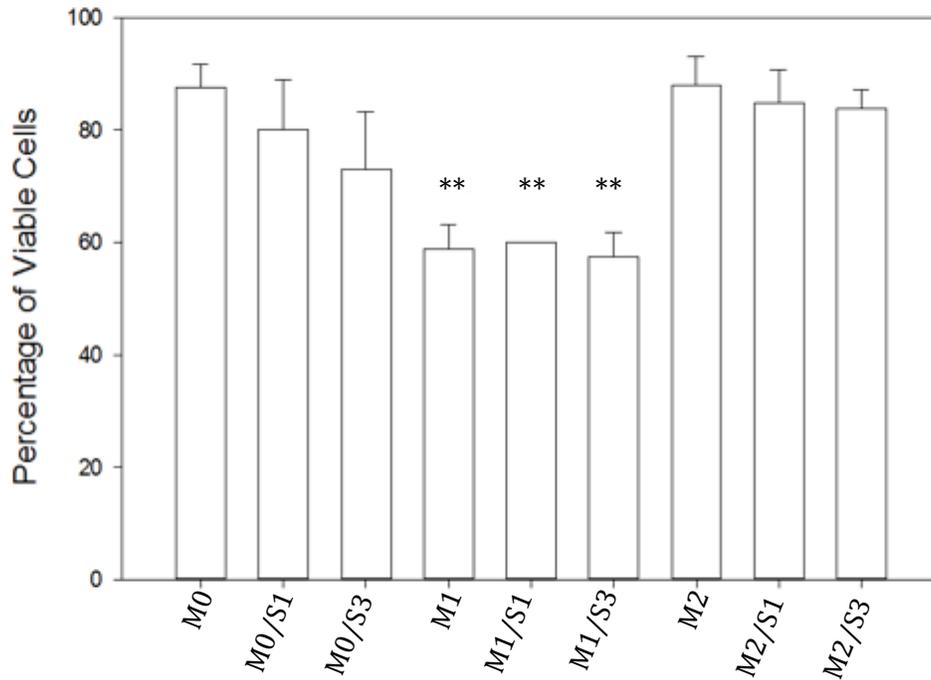


Figure 2: Percentage of viable RAW264.7 macrophages after 18-hour polarization treatment. M1 polarized macrophages showed a decrease in viability following IFN- γ /LPS treatment. SOCS1/3 peptide mimetics had no effect on the viability of treated or untreated cells. *represents a statistical significant difference compared to unpolarized macrophages (M0). **= $p < 0.001$.

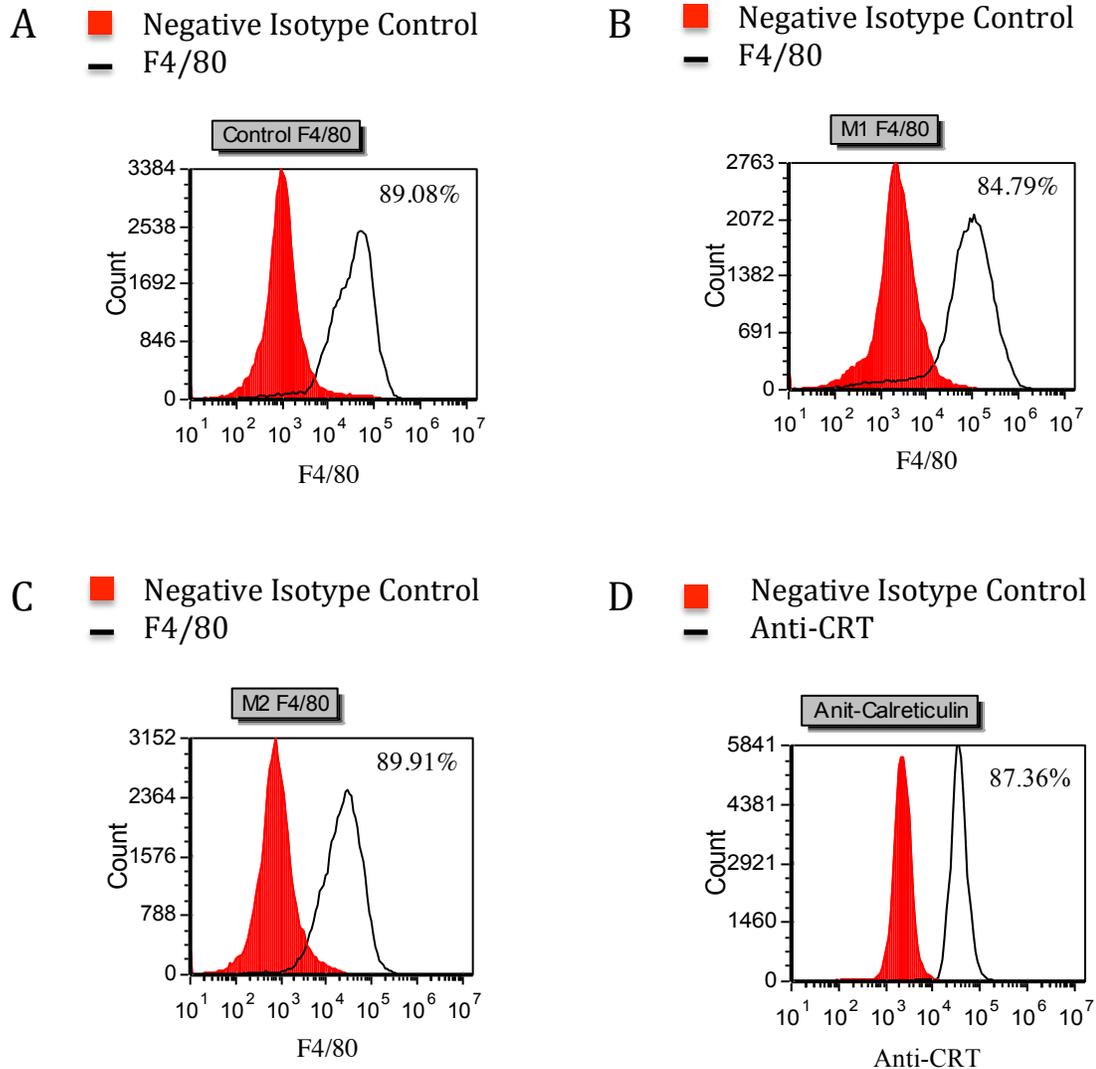


Figure 3: Flow cytometry analysis of F4/80 and anti-CRT expression on respective cell lines. F4/80 is expressed equally across all three polarization states M0(A), M1(B), and M2(C) of RAW264.7 macrophages. Anti-CRT antibody has a high affinity for binding to Neuro-2a cells(D). Red: negative isotype control (A-D); black: eFluor® 660 conjugated anti-mouse F4/80 (A,B, & C) and Anti-Calreticulin conjugated to Alexa Fluoro 488 (D). Experiments were completed in triplicates with one histogram being presented.

F4/80-FITC Expression in Polarized Macrophages (18 hours)

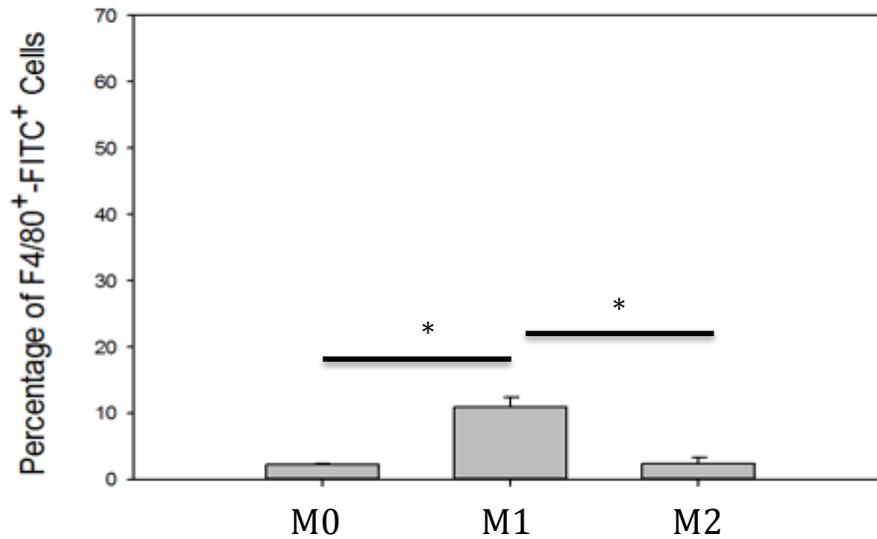


Figure 4: Phagocytosis of FITC labeled latex beads by RAW264.7 macrophages.

M1 macrophages display a greater ability to phagocytose latex beads compared to both M0 and M2 macrophages. M0 and M2 macrophages show a similar ability to phagocytose latex beads. *=p<0.05

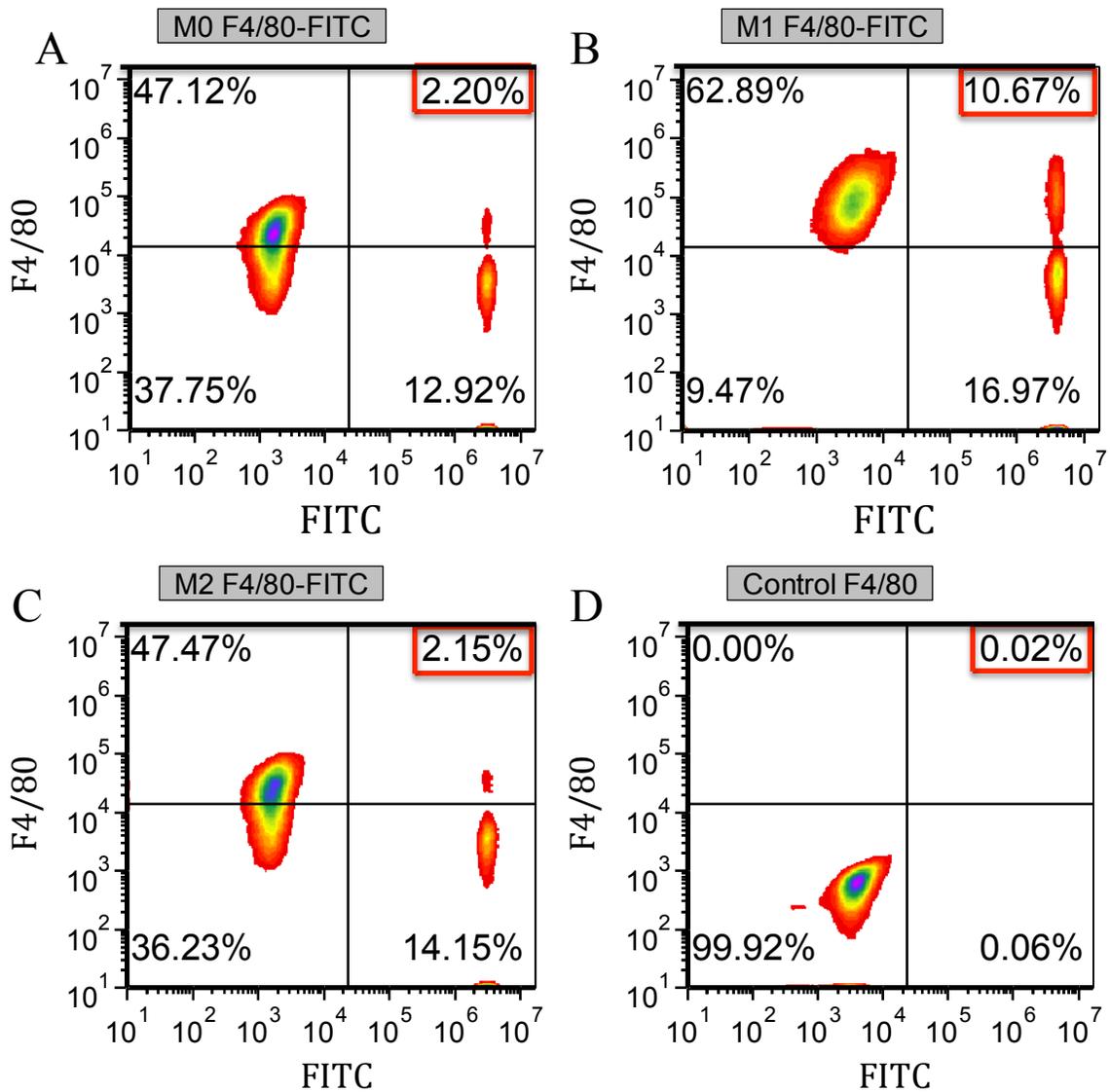


Figure 5: Flow cytometry data, represented as quadrant graphs, of the phagocytosis of latex beads by RAW264.7 macrophages. Percentage of phagocytosis is found in the upper right quadrant. M1 (B) polarized macrophages show a greater ability to phagocytose latex beads compared to both M0 (A) and M2 (C), while M2 and M0 show a similar pattern of phagocytosis. Quadrants were drawn with respect to unstained unpolarized macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

F4/80-CFSE Expression in Polarized Macrophages (18 hours)

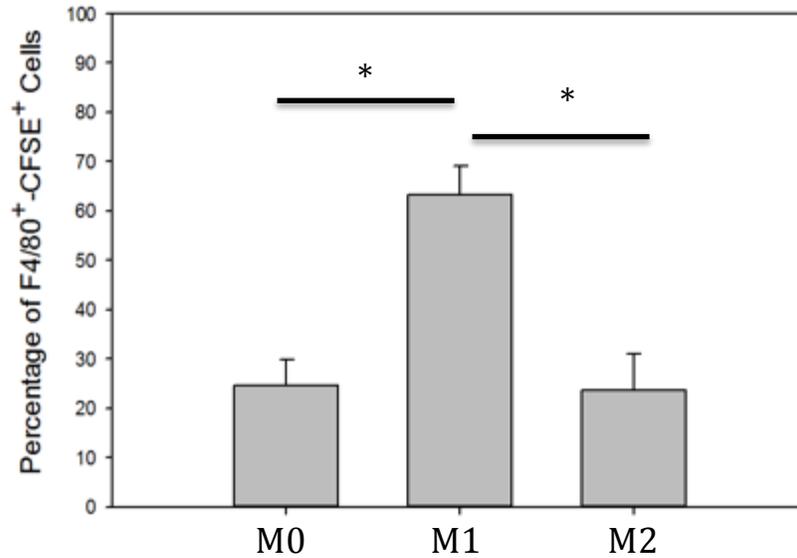


Figure 6: Phagocytosis of CFSE labeled Neuro-2a (N2a) cells by RAW264.7 macrophages. M1 polarized macrophages phagocytose more N2a cells than both M0 and M2 macrophages. There was no observed difference in the ability of M0 or M2 macrophages to phagocytose N2a cells. *=p<0.05

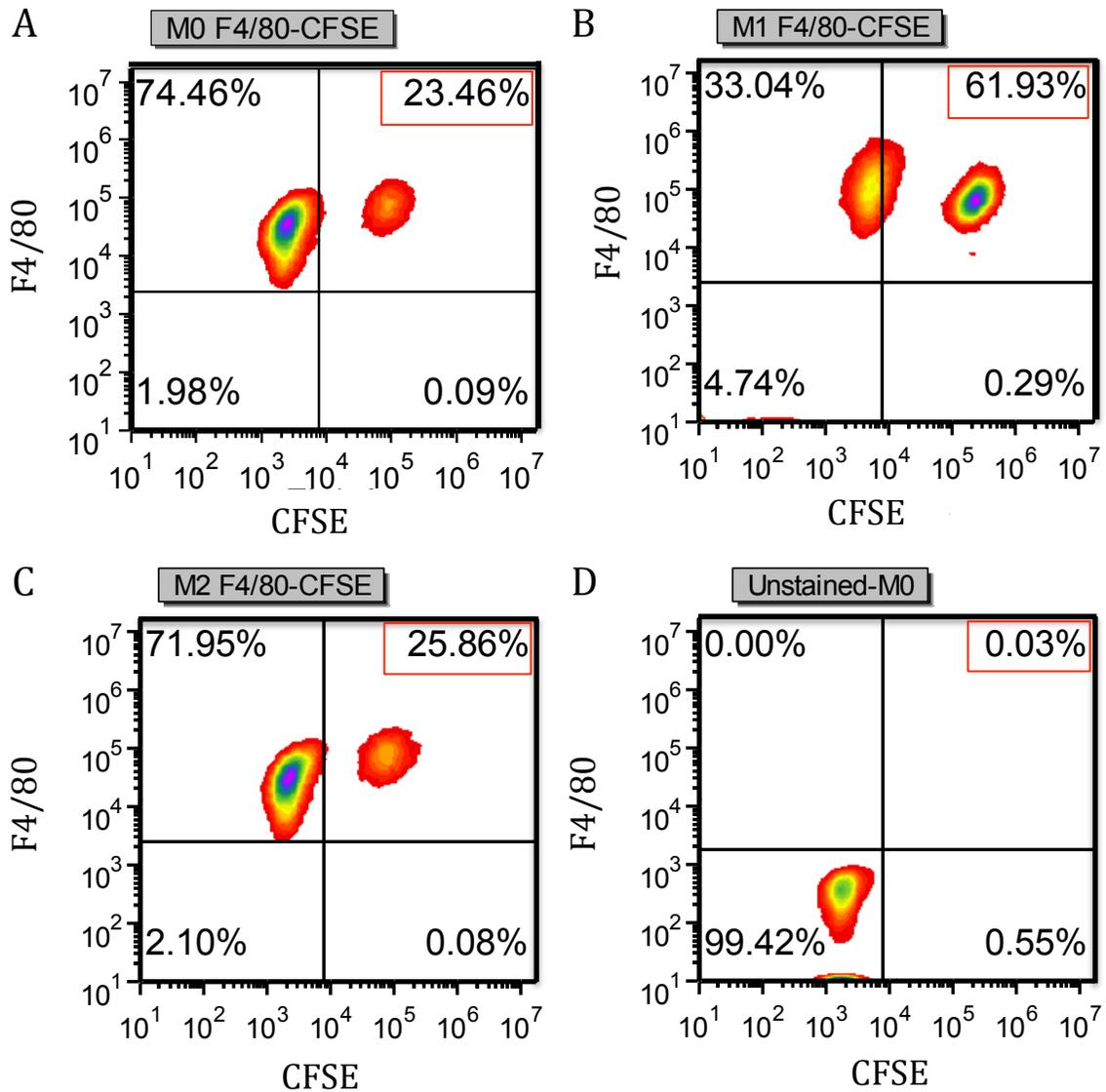


Figure 7: Quadrant graphs representing the ability of RAW264.7 macrophages to phagocytose Neuro-2a (N2a) cells. M1 polarized macrophages (B) were able to phagocytose labeled N2a cells more successfully than both M0 (A) and M2 (C), these results were statistically significant. M0 and M2 were not different in their ability to phagocytose the labeled N2a cells. Quadrants were drawn with respect to unstained unpolarized macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

Unpolarized Macrophage Phagocytosis After Treatment
with SOCS1/3 Peptide Mimetic (18 hours)

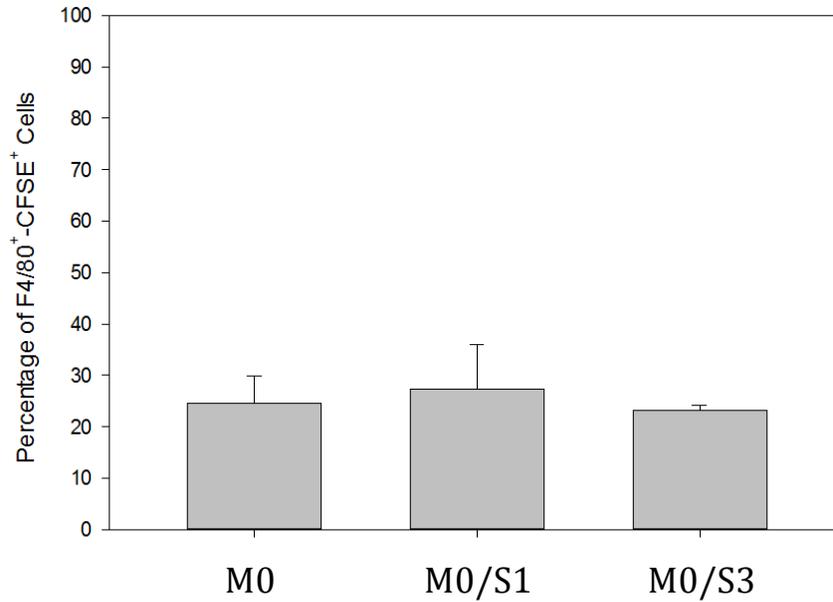


Figure 8: Effects of SOCS1 (S1) and SOCS3 (S3) peptide mimetic on the phagocytosis of Neuro-2a cells by unpolarized (M0) RAW264.7 macrophages. There was no statistical difference in the phagocytosis of Neuro2a cells by M0 macrophages when treated with SOCS1 or SOCS3.

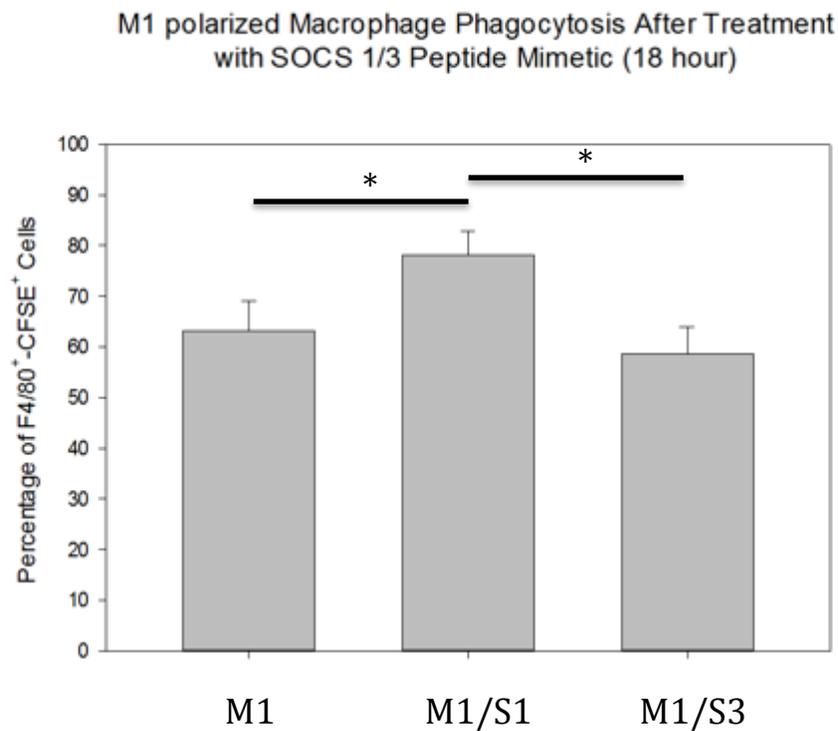


Figure 9: Effects of SOCS1 (S1) and SOCS3 (S3) peptide mimetic on the phagocytosis of Neuro-2a (N2a) cells by M1 polarized RAW264.7 macrophages. M1 macrophages were treated with SOCS1 peptide mimetic and there was a statistical increase in the amount of phagocytosis observed. However, M1 macrophages that were treated with SOCS3 peptide mimetic showed no change in the amount of phagocytosis. *=p<0.05.

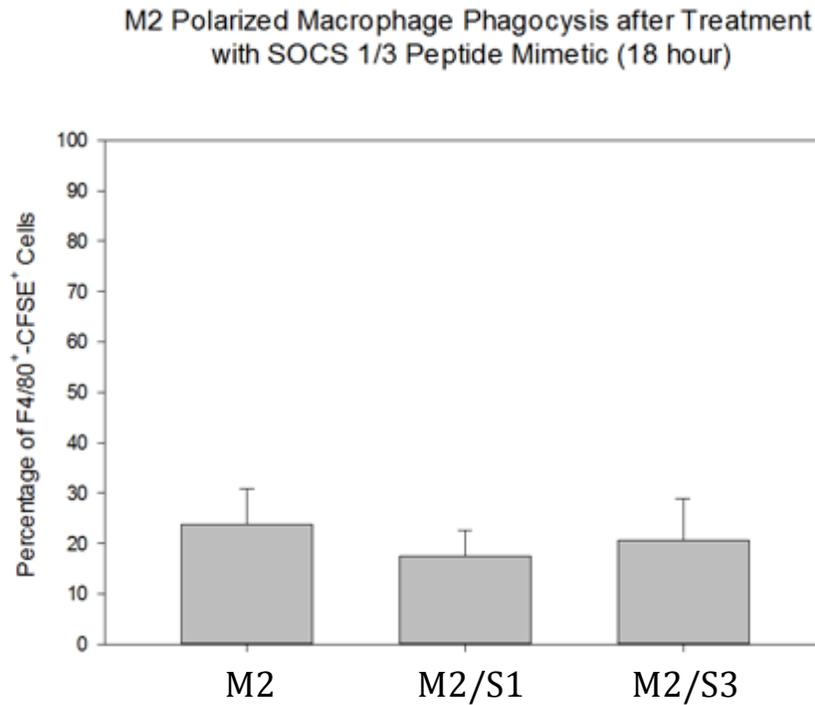


Figure 10: Effects of SOCS1 (S1) and SOCS3 (S3) peptide mimetic on the phagocytosis of Neuro-2a (N2a) cells by M2 polarized RAW264.7 macrophages. Treatment with SOCS1 or SOCS3 peptide mimetic showed no statistical difference on the ability of M2 polarized macrophages to phagocytose N2a cells.

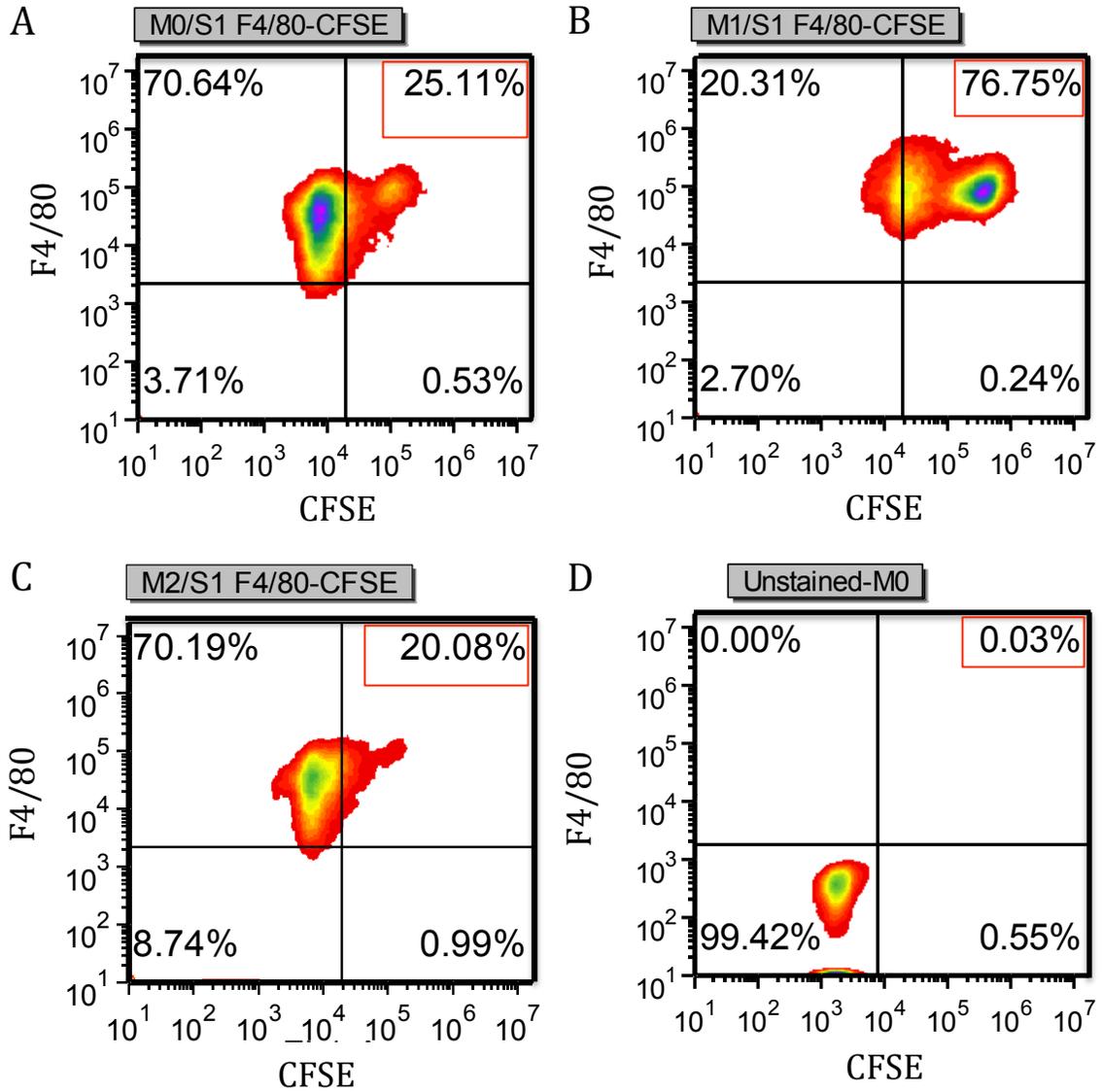


Figure 11: Quadrant graphs representing the effects of SOCS1 (S1) peptide mimetic on the ability of RAW264.7 macrophages to phagocytose Neuro-2a (N2a) cells. M1 polarized macrophages (B) showed an increase ability to phagocytose N2a cells compared to both M0 (A) and M2 (C) macrophages. SOCS1 was able to increase M0 macrophages phagocytosis compared to M2 macrophages but it was not statistically significant. Quadrants were drawn with respect to unstained unpolarized macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

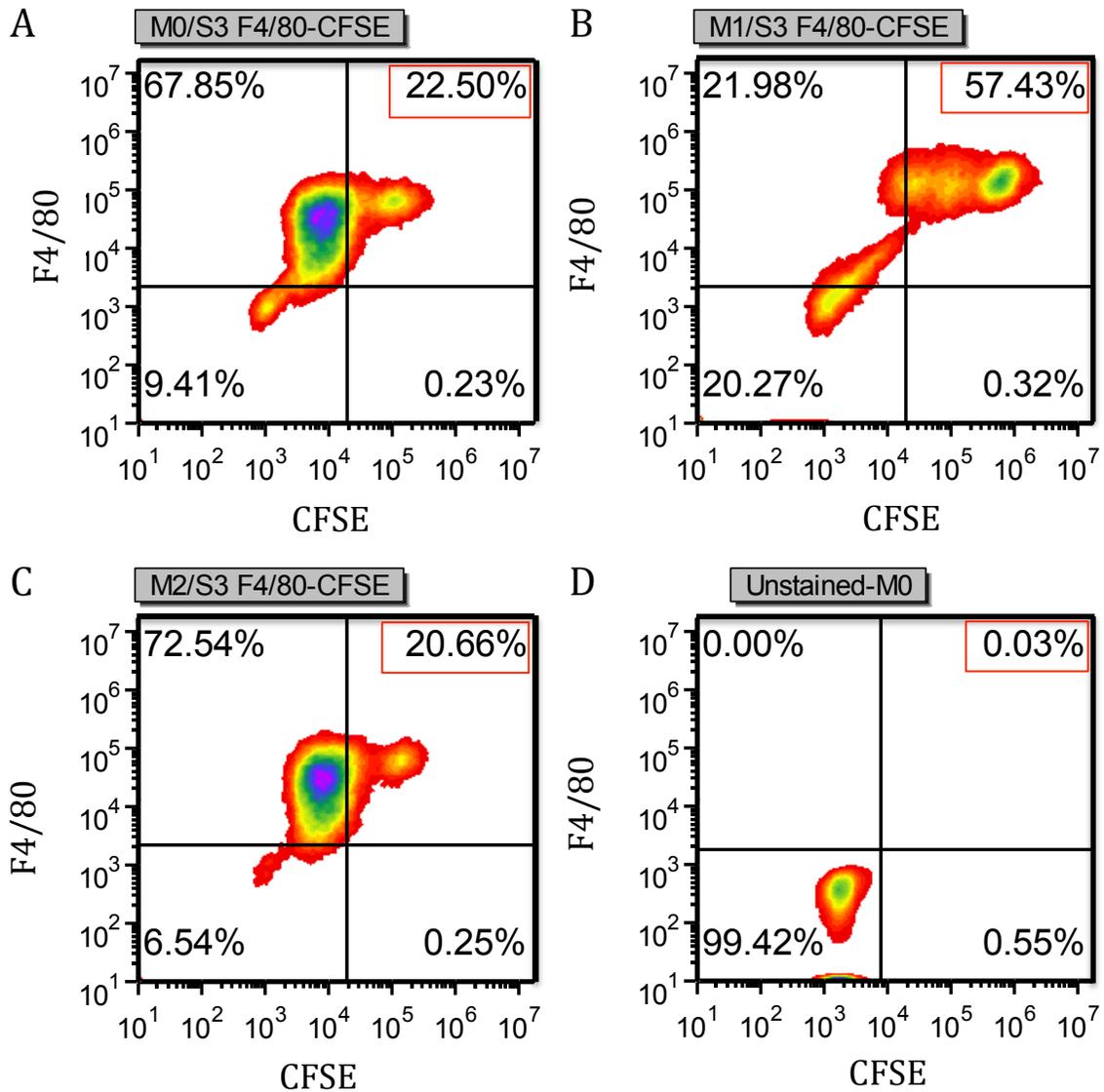


Figure 12: Quadrant graphs representing the effects of SOCS3 (S3) peptide mimetic on the ability of RAW264.7 macrophages to phagocytose Neuro-2a (N2a) cells. M1 polarized macrophages (B) had the ability to phagocytose more N2a cells compared to either M0 (A) or M2 (C) polarized macrophages. M0 macrophages showed a slight increase in phagocytosis but it was not statistically significant compared to M2 macrophages. Quadrants were drawn with respect to unstained unpolarized macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

F4/80-Anti-CRT Expression in Polarized Macrophages (18 hours)

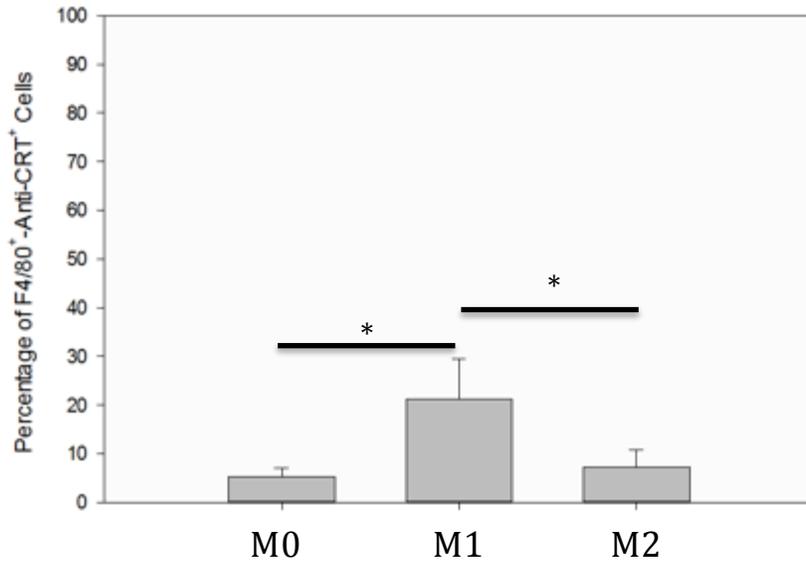


Figure 13: Phagocytosis of Anti-CRT Neuro-2a (N2a) cell by RAW264.7 macrophages. M1 polarized macrophages were able to phagocytose more N2a cells compared to both M0 and M2 macrophages. Also, there was no statistical difference between M0 and M2 cells ability to phagocytize N2a cells. *=p<0.05

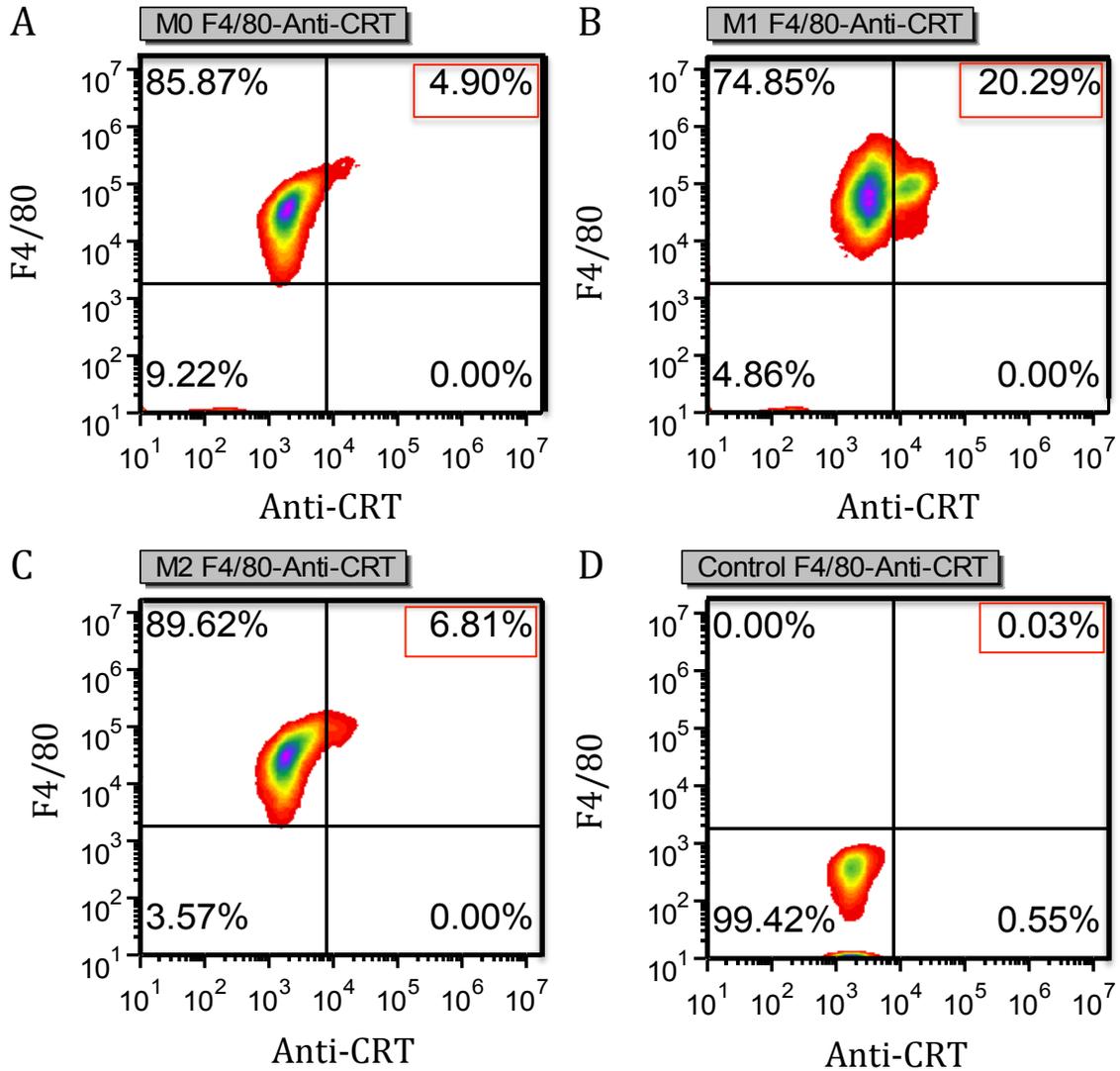


Figure 14: Quadrant graphs representing phagocytosis of anti-CRT Neuro-2a (N2a) cells by RAW264.7 macrophages. M1 polarized macrophages (B) showed an increase in the amount of phagocytosis compared to M0 (A) and M2 (C). M2 macrophages showed a greater percentage of phagocytosed N2a cells compared to M0 but it was not statistically significant. Quadrants were drawn with respect to unstained unpolarized macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

Comparison of Phagocytosis When Blocked with Anti-Calreticulin

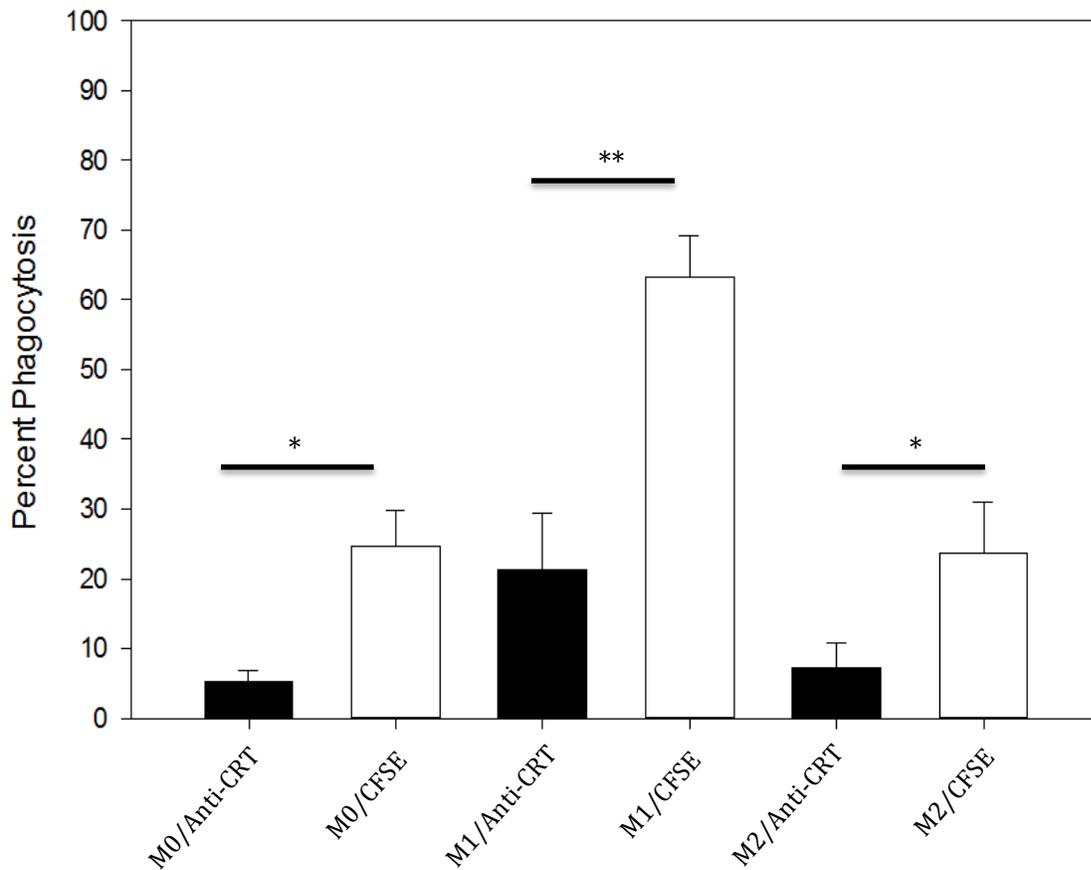


Figure 15: Anti-CRT affects the ability of RAW264.7 macrophages to phagocytize Neuro-2a (N2a) cells. N2a cells treated with anti-CRT decrease the amount of phagocytosis by RAW264.7 macrophages, leading to a greater survival rate of N2a cells. All three phenotypes (M0, M1, & M2) of macrophages showed a decrease in phagocytosis when N2a had calreticulin blocked. *= $p < 0.05$; **= $p < 0.001$.

**Effects of SOCS 1/3 Peptide Mimetics and
Anti-Calreticulin Antibody on
Phagocytosis of Malignant Cells
by Unpolarized Macrophages**

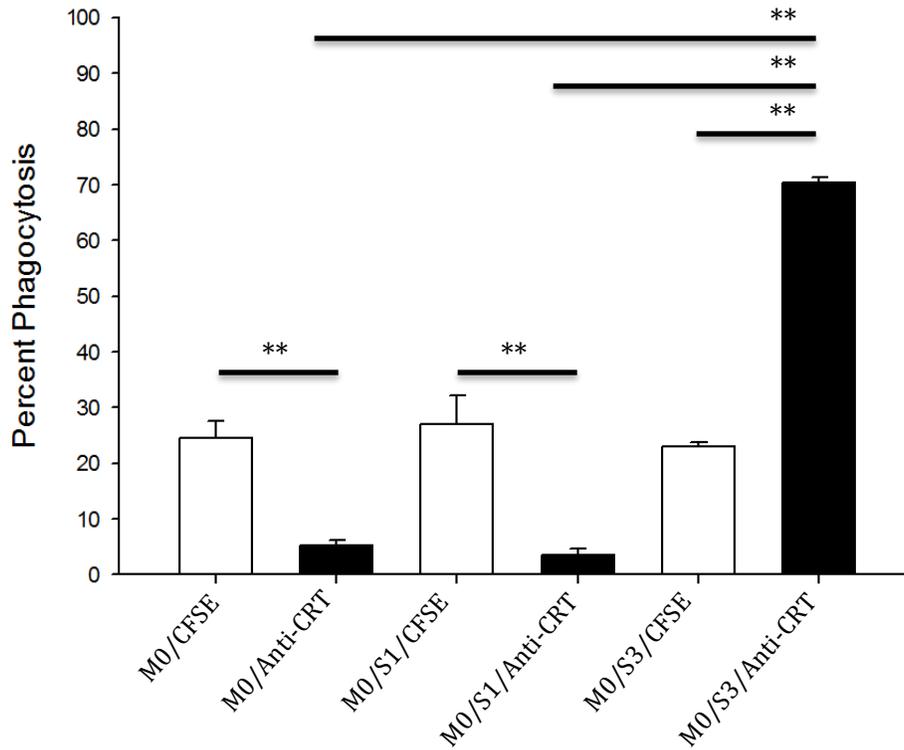


Figure 16: Effects of SOCS peptide mimetics on phagocytosis of Nuero-2a (N2a) cells stained with either CFSE or anti-CRT by unpolarized RAW264.7 macrophages. N2a cells blocked with anti-CRT showed a decrease in phagocytosis when treated with SOCS 1 peptide mimetic and with no SOCS peptide mimetic treatment. However, SOCS3 peptide mimetic increased phagocytosis of N2a cells blocked with anti-CRT. **= $p < 0.001$.

Effects of SOCS 1/3 Peptide Mimetics and Anti-Calreticulin Antibody on Phagocytosis of Malignant Cells by M1 polarized Macrophages

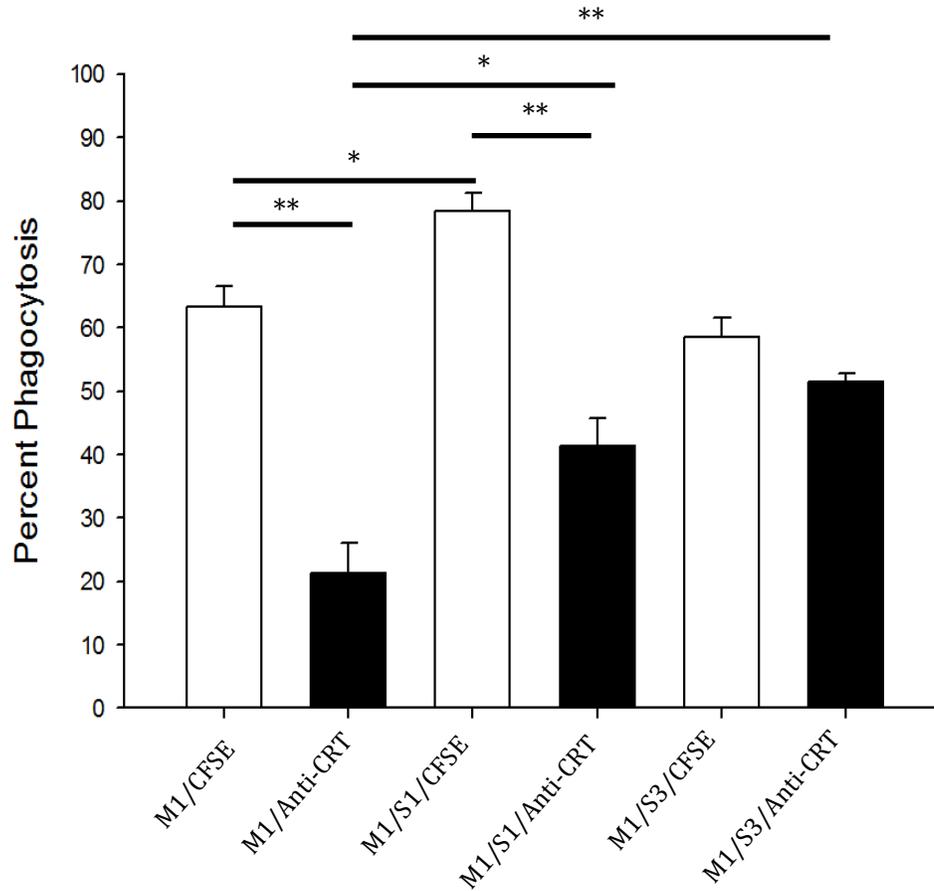


Figure 17: Effects of SOCS peptide mimetics on phagocytosis of Neuro-2a (N2a) cells stained with either CFSE or anti-CRT by M1 polarized RAW264.7 macrophages. M1 polarized macrophages, with SOCS1 peptide mimetic and without, showed a decrease in phagocytosis of anti-CRT N2a cells. Treatment with SOCS3 peptide mimetic showed an insignificant decrease in phagocytosis. *= $p < 0.05$; **= $p < 0.001$

Effects of SOCS 1/3 Peptide Mimetics and Anti-Calreticulin Antibody on Phagocytosis of Malignant Cells by M2 polarized Macrophages

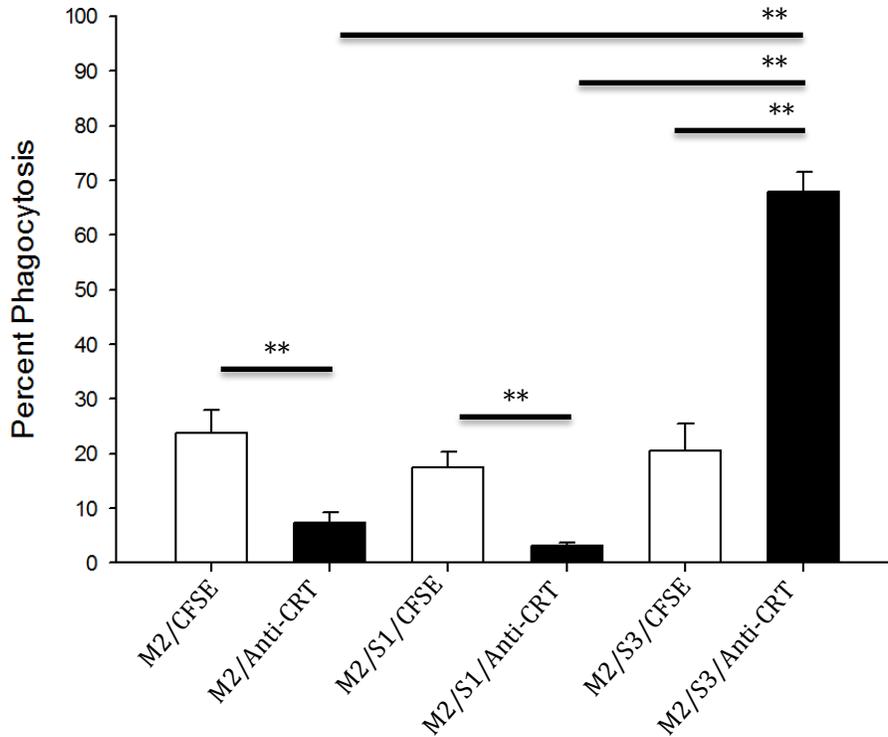


Figure 18: Effects of SOCS peptide mimetics on phagocytosis of Neuro-2a (N2a) cells stained with either CFSE or anti-CRT by M2 polarized RAW264.7 macrophages. M2 polarized macrophages, with SOCS1 peptide mimetic treatment and without, showed a decrease ability to phagocytize N2a cells with anti-CRT treatment. SOCS3 peptide mimetic increased the amount of phagocytosed anti-CRT treated N2a cells. * $p < 0.05$.

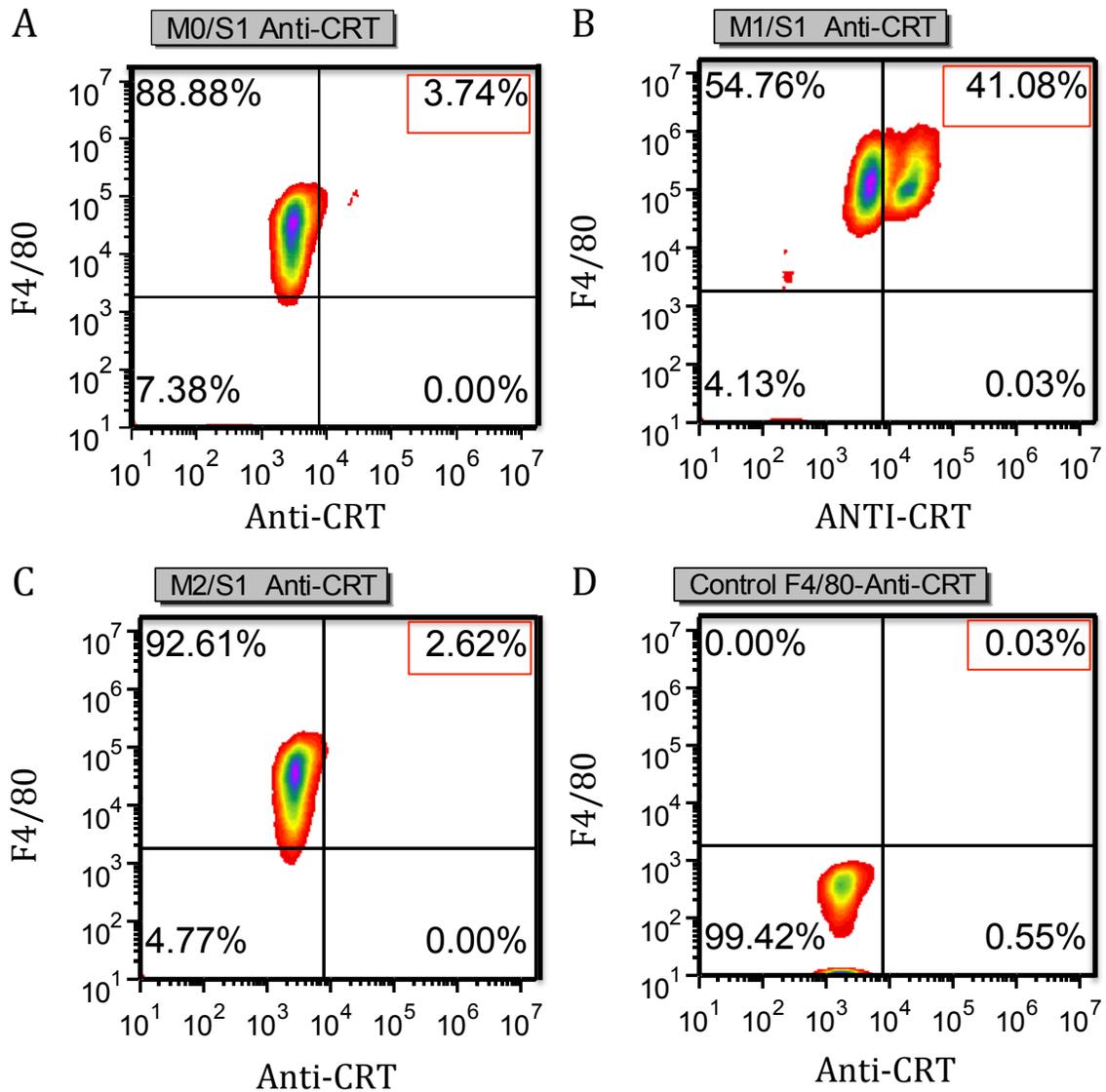


Figure 19: Quadrant Graphs representing effects of SOCS1 peptide mimetic on phagocytosis of anti-CRT Neuro-2a (N2a) cells by RAW264.7 macrophages. M1 polarized macrophages (B) exhibit a greater amount of phagocytosis when compared to M0 (A) and M2(C). M0 macrophages showed a greater amount of phagocytosis but it was not significantly significant. Quadrants were drawn with respect to unstained unpolarized macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

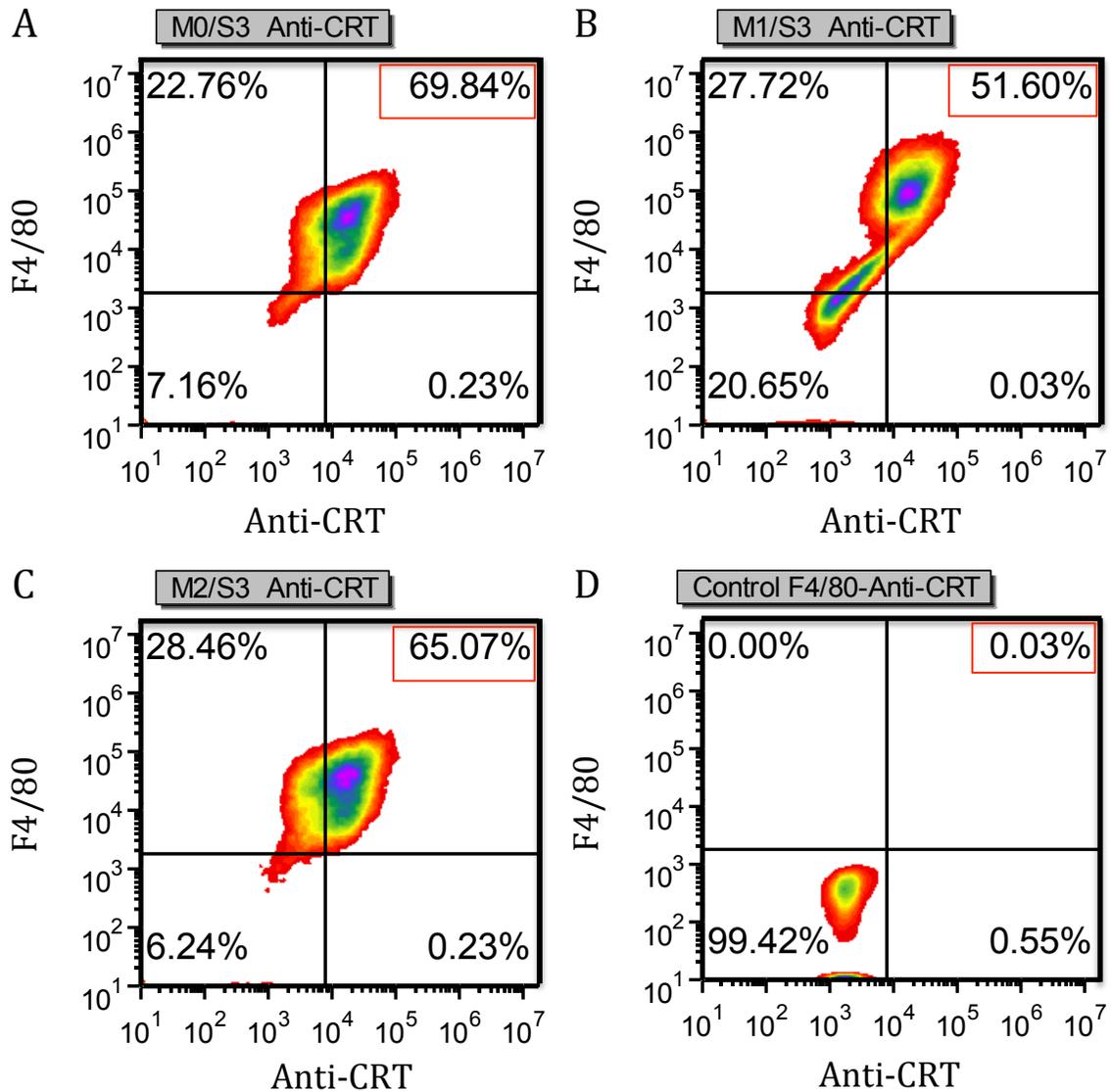


Figure 20: Quadrant Graphs representing effects of SOCS3 peptide mimetic on phagocytosis of anti-CRT Neuro-2a (N2a) cells by RAW264.7 macrophages. M0 macrophages (A) and M2 polarized macrophages (C) showed an increase amount of phagocytosis when treated with SOCS3 peptide mimetic. By contrast M1 polarized macrophages (B) show a decreased amount of phagocytosis compared to both M0 and M2 macrophages when treated with SOCS3 peptide mimetics.

Quadrants were drawn with respect to unstained unpolarized macrophages (D).

Experiments were completed in triplicates with one quadrant graph being presented.

Discussion

This study was focused on determining whether treatment of pro-inflammatory M1 RAW264.7 macrophages with SOCS3 peptide mimetic resulted in an anti-inflammatory phenotype leading to a decreased phagocytosis of malignant cells while treatment of anti-inflammatory M2 macrophages with SOCS1 peptide mimetic increased phagocytosis. Blocking the “eat me” signal of the Neuro-2a target cells should decrease phagocytosis by the RAW264.7 macrophages.

When CFSE labeled N2a cells were added to polarized macrophages, pro-inflammatory M1 macrophages showed a significant increase in the percentage of phagocytosed malignant cells, whereas M0 and M2 macrophages did not differ in their ability to phagocytose the target malignant cells. These results suggest that M1 macrophages play a key role in the clearance of the malignant cells, while M2 macrophages act more closely to control M0 macrophages in the clearance of viable cells. When calreticulin “eat me” signaling was blocked with anti-CRT in each of the three macrophage phenotypes, significant decreases in phagocytosis occurred while M1 macrophages phagocytized significantly higher percentages of target cells than did M0 or M2 macrophages. This observation demonstrates that CRT is a prominent “eat me” signal present on N2a cells.

SOCS1 or SOCS3 peptide mimetic were added to adherent RAW264.7 macrophages polarized to either the pro-inflammatory M1 or anti-inflammatory M2

phenotype. At 18 hours after polarization, M1 treatment caused a significant decrease in cell viability compared to other treatment groups. The most likely explanation for the decrease in cell viability is that upon treatment with M1 polarizing substance, LPS and IFN- γ , M1 macrophages secrete nitric oxide (NO), which is toxic to cells (Seminara *et al.*, 2007). It is worth noting that the addition of either SOCS1 or SOCS3 peptide mimetic did not negatively affect the viability of RAW264.7 macrophages. Control M0, M1, and M2 macrophages were all stained for the cell surface receptor F4/80 and analyzed by flow cytometry. All three subtypes of macrophages have equal expression of the F4/80 receptor supporting its use as a macrophage marker in this study.

While SOCS1 peptide mimetic significantly increased the percentage of malignant cells phagocytosed by M1 polarized macrophages, it did not enhance the percentage of target cells phagocytosed by M2 polarized macrophages. This observation indicates the addition of SOCS1 peptide mimetic may induce increased levels of receptors and cytokines involved in the phagocytic process but cannot cause a phenotypic switch from anti-inflammatory to pro-inflammatory behavior by M2 macrophages. These results were seen when target cells were stained with CFSE (unblocked) or anti-CRT. When LPS is used as a polarization agent in macrophages cellular SOCS1 expression is increased, so with the addition of the SOCS1 peptide mimetic will cause an over expression of the SOCS1 protein (Nakagawa *et al.*, 2002). This over expression enhances the phagocytic potential of M1 macrophages permitting them to clear significantly more target cells.

SOCS3 peptide mimetic had a positive affect on the phagocytic ability of M2 polarized macrophages when target cells were treated with anti-CRT. One explanation

for this observation is that this increase in phagocytosis occurs because blocking of CRT, an endoplasmic reticulum protein, involves disrupting the cell membrane. This disruption in the cell membrane will cause cells to express the apoptotic signal PS which M2c cells recognize and phagocytose. Another possible explanation is that M2 macrophages polarized with IL-10 have an increased amount of the tumor associated macrophage (TAM) receptor merTK, an intracellular negative feedback regulator that inhibits the macrophage inflammatory response and functions in the clearance of apoptotic cells (Zizzo *et al.* 2012). Graham *et al.* 2006 suggest that the high levels of merTK expressed on tumor infiltrating macrophages may increase their efficiency at clearing apoptotic melanoma cells. The addition of SOCS3 peptide mimetic to the macrophages could have increased their expression of merTK resulting in the increased phagocytosis since merTK expression is activated along with SOCS3 expression (Zhang *et al.*, 2016).

Overall the present study shows the importance of macrophage phenotype in cytokine signaling as it pertains to clearance of malignancy. Taken together the current results suggest a potential pathway for using SOCS peptide mimetics to increase phagocytosis of malignant cells by murine macrophages in a phenotypic specific manner. M1 polarized cells treated along with SOCS1 peptide mimetic significantly increased the phagocytosis of target cells by manipulating the release of cytokines. Interestingly, the addition of SOCS3 peptide mimetic to macrophages polarized to the M2c phenotype showed a remarkably significant increase in the percentage of phagocytosis of target cells with their “eat me” signal blocked. One potential explanation for this occurrence is that the treatment of target cells with a blocking antibody to calreticulin causes the cells to become pre-apoptotic.

Future Studies

Based on the present study, there are many opportunities for future studies using the current protocols. The current study utilized a Neuro-2a cell line as a target cell and RAW264.7 murine macrophages as phagocytes. Logically the next step would be to compare the current results with the phagocytic ability of J774A.1 murine macrophages. It would be beneficial to expand the study to include a primary human macrophage as well to compare phagocytosis across species. Another avenue to explore would be to use different target cells. Other malignancies that have been shown to express high levels CRT include breast, brain, and bladder cancer.

This study used macrophages that were polarized using Il-10 to the M2c phenotype; they are known for apoptotic cell clearance. M2c macrophages along with SOCS3 peptide mimetic treatment resulted in a significant increase in the clearance of target cells that had CRT blocked. To gain a better understanding of the spectrum of the M2 phenotype, polarization treatments of IL-4 and IL-13 should be used to analyze their phagocytic ability. Along with examining the other phenotypes the expression of the TAM merTK receptor should be studied because it has been linked to have an increased expression in Il-10 stimulated macrophages. These macrophages are known for their clearance of cells that express apoptotic signals on the cell surface. Since M2c macrophages are known for clearing apoptotic cells, target cells with CRT blocked need to be studied to see if they have an increased expression of these signals.

SOCS proteins' major role in the immune system is to regulate the production and release of cytokines into the cellular microenvironment by immune cells. The cytokines that are released or inhibited will control how the immune cell and surrounding cells react to foreign materials. The present study shows a significant increase in the phagocytic ability of M1 macrophages treated with SOCS1 peptide mimetic. Since TNF- α , a polarizing M1 cytokine, functions in an autocrine manner the supernatant fluids of cells from this experiment should be analyzed for TNF- α . The continued production of TNF- α would maintain the M1 state at the site of inflammation.

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