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

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

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Tahirah M. Madkhali ENTITLED The 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

Madkhali, Tahirah M. M.S. Department of Microbiology and Immunology, Wright State University, 2019. The Effects of SOCS1 and SOCS3 Peptide Mimetics on Macrophage Phagocytosis of Malignant Cells.

Macrophages are essential phagocytic cells involved in both innate and adaptive immune systems and play vital roles in the host defense and inflammation. Macrophages have a remarkably high capacity to clear unnecessary cellular materials in interstitial environment through a process called “phagocytosis”, which is affected by many factors including suppressors of cytokine signaling (SOCS). SOCSs are a group of intracellular proteins that downregulate the cytokine signals involved in various JAK/STAT pathways through a negative feedback loop. This study focuses on investigating the effects of SOCS1 and SOCS3 on the phagocytic ability of RAW 264.7 macrophages polarized into M2a with IL-4/IL-13 and into M2c with IL-10 to clear fluorescently marked Neuro-2a malignant cells. Calreticulin (CRT) is one of the cellular signals, exposed on the surface of malignant cells, that play vital roles in controlling the phagocytosis process mediate by phagocytes including macrophages. Treatment of M2a (IL-4/IL-13) and M2c macrophages with SOCS1 peptide mimetics had no significant effects on M2a and M2c-mediated phagocytosis of CFSE-stained N2a cells compared with M2a (IL-4/IL-13) and M2c cells without SOCS1 peptide mimetic treatment, while it slightly increased the M2a (IL-4/IL-13), but not M2c, macrophage-mediated phagocytosis of N2a cells that had their calreticulin receptors blocked. When N2a cells were blocked with anti-CRT antibody, M2a and M2c-mediated phagocytosis was decreased. Interestingly, treatment of M2c with SOCS3

peptide mimetics coincided with blockage of CRT signals on N2a cells caused a significant increase in the phagocytosis of N2a cells mediated by M2c polarized macrophages.

These data help to get a deeper understanding of macrophage functions in cleaning the microenvironment from apoptotic and malignant cells and even wound healing. Overall, this study sheds more light on how SOCS1/ SOCS3 can be used along with manipulation of “eat-me” signals exposed on malignant and apoptotic cells to increase the clearance rate of malignant cells and decrease the tumor survival.

LIST OF ABBREVIATIONS

Arg-I = Arginase-I

ATCC = American type culture collection

ATP = Adenosine triphosphate

BSA = Bovine serum albumin

CCL2 = The chemokine (C-C motif) ligand 2

CD36 = Cluster of differentiation marker 36

CD47 = Cluster of differentiation marker 47

CFSE = Carboxyfluorescein succinimidyl ester

CIS = Cytokine-inducible Src homology 2 (SH2)-containing protein

CRT = Calreticulin

CXCL10 = C-X-C motif chemokine 10

DMEM = Dulbecco’s Modified Eagle's Medium

FBS = Fetal bovine serum

GH = Growth hormone

IC= Immune complexes

IFN-γ = Interferon gamma

IL-10 = Interleukin-10

IL-13 = Interleukin-13

IL-4 = Interleukin-4

iNOS = Inducible nitric oxide synthase

IRF = interferon-responsive factor

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

M-CSF = Macrophage colony-stimulating factor

merTK = Tumor associated macrophage receptor tyrosine kinase

N2a = Nuero-2a cell line

NB = Neuroblastoma

NO = nitric oxide

PAMPs = Pathogen-associated molecular patterns

PBMcs = from peripheral-blood mononuclear cells

PBS = Phosphate buffered saline

PRL = prolactin

PS = Phosphatidylserine

PSRs = phosphatidylserine receptors

RAW264.7 = Macrophage cell line

ROS = reactive oxygen species

RT = Room temperature

S1 = Suppressor of cytokine signaling 1

S3 = Suppressor of cytokine signaling 3

SH2 = Src homology 2

SOCS1 = Suppressor of cytokine signaling 1

SOCS3 = Suppressor of cytokine signaling 3

STAT = Signal transducers and activators

TGF-B = Transforming growth factor beta

Th1 = T helper cell 1

Th 2 = T helper cell 2

TLR = Toll like receptors

TNF = Tumor necrosis factor

TNF-α = Tumor necrosis factor alpha

TPO = Thrombopoietin

TSP1 = anionic thrombospondin 1

UV = Ultra-violet

μg = microgram

μL = microliter

μM = micromolar

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INTRODUCTION

Capan's studies (Thesis, Wright State University, 2017) examined the roles of suppressors of cytokine signaling SOCS1 and SOCS3 peptide mimetics on the phagocytosis of fluorescently labeled malignant neuroblastoma target cell (Neuro-2a) by RAW264.7 murine macrophages. The 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 RAW 264.7 macrophages were treated with SOCS1 peptide mimetic. An increase in phagocytosis was observed, but SOCS3 peptide mimetic had no effect on phagocytosis. Neither SOCS1 nor SOCS3 peptide mimetics showed a significant effect on the phagocytic ability of the IL-10-polaraized M2c (anti-inflammatory) 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 M2c 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 was observed when compared to unblocked target cells.

The present study is a continuation of Capan's study in which SOCS1 and SOCS3 peptide mimetics were examined to determine if they had a similar effect on M2a macrophages polarized with either IL-4 or IL-13. M1 macrophages are considered as inflammatory phagocytes involved in the phagocytosis of bacteria or

foreign particles, while M2 macrophages are considered to be anti-phagocytic and involved in efferocytosis or apoptosis of tissue cells.

LITERATURE REVIEW

1.1 Macrophage polarization and functions

Macrophages are essential phagocytic cells involved in both innate and adaptive immune systems and play vital roles in the host defense and inflammation such as clearance of cellular debris formed during tissue remodeling and removal of apoptotic cells (Mosser and Edwards, 2008). Macrophages are found almost in all tissues, and they arise from peripheral-blood mononuclear cells (PBMcs), which originate from myeloid progenitor cell in the bone marrow (Mosser and Edwards, 2008). Recent studies have reported that tissue- resident macrophages in the embryo are derived from yolk sac and fetal live precursors and then developed into resident state throughout adulthood (Gordon and Plüddemann, 2018). Under steady state or in response to inflammation, monocytes translocate from blood into tissues to restore the tissue-specific macrophages of the bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells), spleen and peritoneum (Mosser and Edwards, 2008). Macrophages have extraordinary plasticity; thus, they efficiently respond to signals within their environment and adjust their phenotype (Mosser and Edwards, 2008). Both innate and adaptive immune systems can strongly alter the macrophage physiology in a way that make the macrophages more sensitive to pathogens and inflammation and limit macrophage- mediated production of cytokines that could harm the immune system (Mosser and Edwards, 2008). Macrophages can be polarized into classical (M1) or

alternative (M2) macrophages, which are mirroring the T helper cells (Th1 and Th2) polarization, respectively (Wang et al. 2014). Macrophages can be polarized into M1 phenotype by exposure to lipopolysaccharide (LPS) and interferon- γ (IFN-γ) or tumor necrosis factor (TNF), or can be polarized into M2 macrophages, which are further classified into three subsets; M2a, activated by exposure to IL-4 or IL-13; M2b, activated by immune complexes (IC) and toll-like receptors (TLRs) or IL-1R agonists; and M2c, activated by IL-10 and glucocorticoid hormones (Mantovani et al. 2002; Oishi et al. 2016). The hallmarks of M1 phenotype are high expression of pro-inflammatory cytokines including IL-12, IL-23, and tumor necrosis factor-α (TNF-α) as well as production of ROSs and high nitric oxide (NO) (Oishi et al. 2016). However, M2 phenotype is characterized by production of IL-10 and IL-1 receptor antagonist (IL-1Ra) as well as high expression of scavenger, mannose and galactose receptors (Oishi et al. 2016). In addition, M1 cells are known to have antibacterial and anti-tumor effects as they release cytokines that suppress the nearby cells proliferation and destroy the contiguous tissue, while M2a and M2b function as immunoregulators and drive M2 phenotype responses through releasing cytokines that enhance the contiguous cell proliferation, and M2c macrophages are involved in immunosuppression and tissue remodeling (Mantovani et al. 2002). The imbalanced dynamic changes of M1-M2 macrophage polarization is related to multiple diseases or inflammatory responses; M1 macrophages are associated with enhancing and preserving inflammation, while M2 macrophages are implicated in persistence of chronic inflammation (Wang et al. 2014). Different factors collaborate to regulate the macrophage polarization involving inflammatory modulators, signaling molecules, and transcription factors. In particular, Th1and Th2 cells play roles in driving macrophage polarization depending on interferon-responsive factor (IRF) and signal

transducers and activators of transcription (STATs) signaling pathways (Wang et al. 2014). The M1 macrophage phenotype is activated by the IRF/STAT1 signaling pathway mediated by IFN- γ and TLR, while the M2 phenotype is activated by the activation of IRF/STAT6 pathway mediated by IL-4 and IL-13 (Wang et al. 2014). Thus, it is vital to identify the molecules involved in macrophage polarization and their interactions, which could help in understanding the diseases progression and finding new macrophage-dependent therapeutic strategies.

1.2 Controlling the macrophage phagocytosis

Macrophages are the professional phagocytes of immune system. In particular, they have a remarkably high capacity to clear unnecessary cellular materials in interstitial environment through a process called “phagocytosis” (Savill, 1997). Phagocytes are able to recognize and ingest all membrane-attached products resulting from apoptosis (i.e., programmed cell death of cells) and apoptotic bodies released from dying cells (Savill, 1997). The ability of phagocytes to speedily and efficiently clear the apoptotic cells helps to minimize tissue injury that could be caused by harmful materials released from dying cells and also protects the immune system from destructive auto-immune responses to apoptotic cell antigens (Savill, 1997). The uptake of viable or apoptotic cells is dependent on the existence of “eat-me” and/ or lack of “don’t-eat-me” signals (Gordon and Plüddemann, 2018). The cellular surfaces of apoptotic cells are different from viable cells as the cells undergoing apoptosis express particular markers of disposal including “eat-me” signals that are developed after multiple modifications in the plasma membrane elements, such as carbohydrate changes, exposure of phosphatidylserine (PS), existence of anionic thrombospondin 1(TSP1) binding sites (Savill, 1997). Phagocytes employ various classes of receptors to initiate phagocytosis of apoptotic cells and even cancer cells, including phagocyte

lectins, phosphatidylserine receptors (PSRs), thrombospondin receptors, CD36 and the $\alpha_v\beta_3$integrin, macrophage 61D3 antigen, scavenger receptors, and ATP-binding cassette superfamily of membrane transporters (Savill, 1997).

Calreticulin (CRT) is one of the cellular signals that play vital roles in controlling the phagocytosis process. This signal has been at the forefront of medical research into prediction of cancer condition and it has been investigated towards advancing fundamental knowledge of its characteristics and functions and its contribution to controlling tumor progression (Raghavan et al. 2013). CRT is defined as an endoplasmic reticulum- resident chaperon. CRT is constantly exposed on the cancer cell surface, when stimulated during the early phase of apoptosis by apoptotic stimuli, such as UV irradiation and anthracycline. CRT acts as “eat-me” signal that attracts macrophages and promotes the phagocytosis of the cells it is expressed on (Chao et. al., 2012; Martins et al. 2014; Fricker et al. 2012). Another signal that is defined to have inhibitory effects on the immune system during tumor growth is cluster of differentiation (CD47), which is a receptor expressed on the surface of some cells and works as anti-phagocytic “don’t-eat me” signal that inhibits the phagocytosis of apoptotic cells (Chao et. al., 2012). The tumor cells can escape the immune system through up-regulating the expression of CD47 to neutralize CRT signal resulting in continuing cancer growth (Chao et. al., 2012). CRT and CD47 along with phosphatidylserine (PS), another “eat-me” signal, work together to clear tumors and other pathogens from cellular microenvironment (Raghavan et al. 2013). It has been shown that CRT and CD47 are both expressed on living cells, but CD47 is expressed at a higher rate leading to offset the CRT signal (Raghavan et. al., 2013). However, CRT is predominantly expressed in neuroblastoma patients (Hsu et. al., 2004). In steady state, CRT and PS are expressed at the same levels on the surface of cells

undergoing programmed cell death. However, blocking or manipulating one of these signals will result in up-regulating the expression the other signal to make it detectible and phagocytosed by phagocytic cells (Krysko and Vandenabeele, 2008). Currently, the modulation of these signals expressed on cancer cells is under investigation and is advancing approach to increase the tumor clearance rate.

1.3 Suppressors of cytokine signaling (SOCSs)

Cytokines are substances secreted by particular types of immune cells and they are well known to be involved in the pathophysiology of different diseases, including their functions as modulators of the proliferation and differentiation of multiple types of cells including macrophages (Zhou et al. 2017). These suppressors of cytokine signaling (SOCS) cascades are activated as a result of stimulated interaction of cytokines with their corresponding cellular receptors, which in turn leads to stimulation of intracellular molecules, such as Janus kinases (JAKs) and STATs (Zhou et al. 2017). Macrophages are defined as target cells of certain cytokines and thus may be involved in many diseases' pathogenesis. Cytokines and intracellular signaling cascades stimulate the polarization of macrophages into M1or M2 phenotypes in response to invading pathogens, injuries, or cancer onset (Zhou et al. 2017). Suppressors of cytokine signaling (SOCS) are a group of intracellular proteins that downregulate the cytokine signals through a negative feedback loop (Zhou et al. 2017). There are eight classes of SOCS family: SOCS1 to SOCS7 and cytokine-inducible Src homology 2 (SH2)-containing protein (CIS) (Zhou et al. 2017). Recently, SOCS proteins have been identified to function as a molecular switch that regulates the immune initiation and inhibition as well as the polarization of macrophages into M1or M2 phenotypes (Zhou et al. 2017). SOCS1 and SOCS 3 are the most widely identified members of SOCS family to have crucial roles in

directing M1 and M2 macrophage polarization (Wilson, 2014). Thus, these molecules are at the forefront of research into investigation new macrophage-based therapeutic strategies. JAK-STAT pathway is the most studied signaling cascade that is regulated by SOCS1 and SOCS3 (Wilson, 2014). Upon cytokines activation, the JAK-STAT pathway is stimulated leading to induction of SOCS1and SOCS3. SOCS1and SOCS3 can down-regulate the cytokine signaling cascade by different ways: SOCS1directly binds to key phosphorylated tyrosine residues on JAK and blocks the catalytic function, while SOCS3 inhibits the JAK function by binding to the JAK-proximal sites on the cytokine receptors (Zhou et al. 2017).

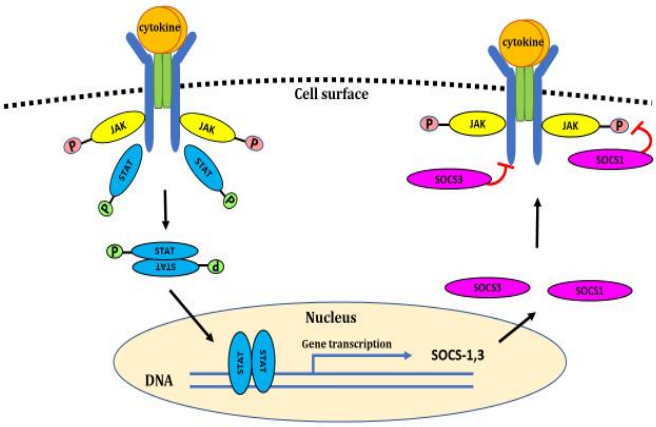

Figure1: JAK-STAT signaling pathway. The cytokine binds to its specific receptor and activates the STAT molecules. The activated STAT molecules bind to DNA and initiate the expression of SOCS1 or SOCS3 proteins, which in turn suppress the cytokine signaling pathway. Adapted from Sujin Kang, Toshio Tanaka, Tadamitsu Kishimoto (2015).

1.4 SOCS1

SOCS1expression is activated and can block the signaling pathway in the presence of growth hormones (GH), interferons, IL-4, IL-6, thrombopoietin (TPO), and prolactin (PRL) (Kile and Alexander, 2001). SOCS1 regulates M1 phenotype by

inhibiting the INF-γ -induced JAK2/STAT1 pathway and toll like receptor (TLR) / nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling pathway through penetration of the stimulation loop of JAKs using its kinase inhibitory region (KIR) domain. This downregulation is correlated with attenuated LPS, IL-4, and INF-γ in some diseases (Zhou et al. 2017). SOCS1 not only regulates the M1 phenotype, but it can also regulate the M2 macrophage polarization (Wilson, 2014). SOCS1 expression is strongly increased with M2 macrophage polarization and it plays important roles in driving the M2 functional features including a high arginase I (Arg-I) with a low inducible nitric oxide synthase (iNOS) expression (Wilson, 2014). It has been clarified that a higher SOCS1/SOCS3 ratio is a potential marker for M2 phenotype, while a higher SOCS3/ SOCS1 ratio is associated with the expression of the M1 phenotype (Wilson, 2014). Recent studies have confirmed that SOCS-1gene is vital for living after birth. Mice with deleted SOCS-1 gene were born with obvious normal health, but after 10 days, those mice were remarkably smaller than wild-type mice, failed to survive, and passed away within 3 weeks (Naka et al. 1998; Starr et al. 1998; Zhou et al. 2017).

1.5 SOCS3

SOCS3 is generally known to function as an inhibitor of STAT3, which is the fundamental regulatory molecule of immune homeostasis and disease pathogenesis. SOCS3 can be induced by various cytokines and growth hormones including IL-1β, IL-2, IL-4, and macrophage colony-stimulating factor (M-CSF) (Zhou et al. 2017). SOCS3 protein plays an important role in macrophage-mediated phagocytosis as it is able to repress the pro-inflammatory M1phenotype, which in turn inhibits the macrophage inflammatory responses (Qin et al. 2012). Recent studies investigated the effects of SOCS3 on the inflammatory properties by knocking out the SOCS3 gene

(Qin et al. 2012). SOCS3-deficient macrophage exhibited greater levels of pro-inflammatory M1 phenotype markers, including IL-1β, IL-6, IL-12, IL-23, iNOS, CCL2, CXCL10, compared to wild type mice (Qin et al. 2012). In addition, SOCS3 has been shown to have a regulatory effect on another molecule called "Mer receptor tyrosine kinase (MerTK)", an intracellular regulator that inhibits the inflammatory responses mediated by the interactions between TLRs expressed on macrophages and DCs and pathogen-associated molecular patterns (PAMPS) exposed by attacking pathogens or even malignant cells, which in turn initiate the innate immune response (Zhang et al. 2016). Graham et al. (2006) suggest that the increased expression of MerTK on malignant cells penetrating macrophages might enhance the macrophage capability to clear apoptotic melanoma cells. Since the expression of MerTK on macrophages is associated with SOCS3 expression, the treatment of macrophages with SOCS3 could increase the expression of MerTK on macrophages, which consequently could enhance the macrophage phagocytosis (Zhang et al. 2016).

The current study focuses on the sophisticated roles of SOCS1 andSOCS3 peptide mimetics on the inflammatory characteristics of RAW 264.7 murine macrophages. The primary inflammatory response of macrophage is detecting, engulfing and killing pathogens and apoptotic cells through phagocytosis process mediated by M2a and M2c Macrophage. SOCS proteins are strong regulators of target cell phagocytosis as they can repress particular intracellular signaling pathways and following cytokine production, so those proteins are able to restrict innate and adaptive responses.

HYPOTHESIS

The hypothesis of this study is that anti-inflammatory M2a macrophages treated with SOCS1 protein will not display elevated phagocytosis of the neuro-2a cells while IL-4 or IL-13 polarized macrophages treated with SOCS3 peptide mimetic will show enhanced increases in the percentage of neuro-2a cells phagocytosed. These increases may be less than that induced by IL-10 polarized cells treated with SOCS3 peptide mimetic because IL-10 is an autocrine for M2c cells. If this hypothesis is shown to be correct, both M2a and M2c macrophages induce apoptosis of malignant neuro-2a cells.

MATERIALS AND METHODS

2.1 Cell lines

RAW 264.7 murine macrophage cell line (ATCC TIB-71) is an Abelson leukaemia virus transformed cell line. RAW264.7 cells are adherent and macrophage-like cells obtained from adult male BALB/c mice. RAW264.7 macrophage-like were grown in 25 cm^2 or 75cm^2 vented cap cell culture flasks and incubated at 5% CO_2 with a temperature of 37°C using a water jacketed incubator. Cells were cultured in media contained Dulbecco's Modified Eagle's Medium (DMEM) and 10% fetal bovine serum (FBS). Cell subcultures were done in vented cap flasks at a ratio of 1:5 until reaching 70% confluency

The Neuro-2a murine neuroblast cell line (ATCC-CCL-131) is an adherent neuroblastoma cell line produced in a strain A mouse. Cells were cultured in 75 cm^2 vented cap flasks at 37°C and 5% CO_2 in a water jacket incubator. Cells were cultured in a media containing Eagle's Medium (DMEM) and 10% fetal bovine serum (FBS). Cell subcultures were completed in vented cap flasks at a ratio of 1:10 until reaching 80-90 % confluency. Eagle's Medium (DMEM), fetal bovine serum (FBS), and vented cap flasks were purchased from ThermoFisher Scientific.

2.2 Polarization treatment

RAW 264.7 macrophages were grown in 75 cm^2 vented cap flasks until reaching a confluency of 70%. Cells then were removed from the flasks by using a

cell scraper. The cell suspension was transferred into 15 ml conical tubes. Cells were centrifuged at 1700 revolutions per minute (4°C) for 5 minutes. Supernatants were removed using vacuum flask, while cell pellets were re-suspended in 1ml of complete growth medium. Cells were counted and aliquoted into 25 cm^2 culture flasks containing 10% growth media and polarization treatment at seeding density of 1-2×10^6 cells per flask. Cells were allowed to become adherent to flasks for 18 hours. RAW 264.7 cells were treated with IL-4 and IL-13 to reach M2a phenotype with and without SOCS1/3 and treated with IL-13 to reach M2c phenotype with and without SOCS1/3. IL-4, IL-13, and IL-10 were used at concentration of 20 ng/mL (Biolegend) and SOCS1/3 were used at concentration of 35 μM (Genscript). Cells were then prepared for phagocytosis assay.

2.3 CFSE staining of Neuro-2a Cells

Neuro-2a cells were harvested from culture flasks upon reaching 80-90% confluency by using trypsin. Cells were counted on hemocytometer, and 3×10^6 cells were aliquoted into each 15 mL conical tube. Cells were then re-suspended in Phosphate-buffered saline (PBS) containing 0.5 μM of staining solution (1 mL the final volume), and then cells were incubated at room temperature (RT) for 20 minutes. Cells were then washed with 10% complete growth medium and centrifuged at 1700 revolutions per minute (rpm) for 5 minutes at 4°C. Finally, cells were re-suspended in 1 mL of 10% growth media and added to the polarized macrophages at 2:1 ratio.

2.4 Phagocytosis assay

CFSE-stained N2a cells or anti-CRT blocked N2a cells were added into flasks of polarized macrophages at a ratio of 2:1 with 4 mL new 10% of growth media. Cells were then incubated with macrophages for one hour at 37°C and 5% CO_2 to allow the phagocytosis process to occur. Cells were then washed five times with 1X cold PBS to stop the uptake of N2a cells. Cells then were scrapped with final PBS wash and added to 15 mL conical tube and prepared for cell surface antigen staining of macrophages to be detected by flow cytometry.

2.5 Flow cytometry

Cell surface antigen staining

After the phagocytosis assay, the cells were collected, centrifuged, supernatant was removed, and cell pellet was re-suspended with 500 µL of 1% Bovine Serum Albumin (BSA) and transferred into micro-centrifuge tube. Cells were centrifuged, supernatant was removed, and then cells were treated with 500 µL of 3% BSA to prepare for the antibody staining. Then, cells were washed with 500 µL of 1% BSA, centrifuged, and supernatant was removed. Cells were stained with an appropriate amount of F4/80 primary antibody conjugated with eFluor 660 (ebioscience) and antibody staining solution (ASS) at 100 µL final volume and incubated for 30 minutes at RT in dark. Cells were then washed with 300 µL of 1% BSA. Finally, cells were re-suspended with 500 µL of cold PBS and filtered through Falcon tubes and analyzed by flow cytometry on an Accuri C6 Flow Cytometer (BD Biosciences).

Intracellular staining

Neuro 2a cells were grown until reached 80-90% confluency. Cells were harvested from the flask using trypsin and re-suspended in 1X PBS. Cells were then

counted on hemocytometer and 3×10^6 cells per tube were placed into micro-centrifuge tubes with PBS at 500 µL final volume. Cells were centrifuged at 1200 rpm for 5 minutes at 4°C and the supernatants were removed. Cells were then re-suspended with 500 µL of fixation buffer (Biolegend) for 20 minutes in the dark at RT. Cells were centrifuged and the supernatants were removed. Cells were washed twice with 500 µL of 1X permeabilization buffer (Biolegend). After centrifuging and removing supernatants, cells were stained for Calreticulin (CRT) with an appropriate amount of anti-CRT antibody conjugated with Alexa Fluro 488 (Abcam) and incubated at RT for 30 minutes in the dark. Cells were then washed with 500 µL of permeabilization buffer. Finally, cells were re-suspended in 1 mL of fresh 10% growth media and added on top of RAW cells with 4 mL fresh 10% media to undergo phagocytosis assay and then analyzed by flow cytometry on an Accuri C6 Flow Cytometer (BD Biosciences**).**

Table 1: List of antibodies and isotypes and their concentrations used in flow cytometry analysis

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	ThermoFisher
Rabbit IgG Isotype Control conjugated to Alexa Fluro 488	10 µg/mL	ThermoFisher

2.6 Statistical Significance

Statistical significance was calculated using One-Way ANOVA - SigmaPlot 12.0; all experiments were completed in triplicates.

RESULTS

M2a (both treated by IL-4/IL-13) and M2c displayed similar abilities to clear the malignant cells

Following 18-hour treatment with IL-4/IL-13 for M2a and with IL-10 for M2c phenotype, M2a (both polarized with IL-4/IL-13) and M2c macrophages showed similar amount of phagocytosis of CFSE-stained N2a cells (Fig. 2-7).

M2a (polarized with I-4/IL-13) and M2c-mediated phagocytosis of N2a cells is decreased when Calreticulin receptor, the “eat-me” signal is blocked

When calreticulin (CRT) receptors on neuro (N2a) cells are blocked, the amount of phagocytosed N2a cells is significantly decreased in M2a (both polarized with IL-4/IL-13) and M2c macrophages compared to M2a and M2c cells co-cultured with unblocked N2a cells. (Fig.9 -12).

SOCS1 peptide mimetic has no significant effects on phagocytosis ability of M2a and M2c macrophages

SOCS1 peptide mimetic does not significantly affect the M2a (IL-4/IL-13) and M2c cells-mediated phagocytosis of CFSE-stained N2a cells compared with M2a and M2c cells without SOCS1 peptide mimetic treatment (Fig. 2– 7), while it slightly increases the M2a (IL-4/IL-13), but not M2c, macrophage-mediated phagocytosis of N2a cells that had their calreticulin (CRT) receptors blocked (Fig. 9-13).

SOCS3 peptide mimetic significantly increases the phagocytosis rate of M2c macrophages, but not M2a macrophages

M2a (IL-4/IL-13) macrophages treated with SOCS3 peptide mimetics display no significant increases in their phagocytosis of either CFSE-stained N2a cells or N2a cells blocked with anti-CRT antibody (Fig.9-14). However, M2c macrophages treated with SOCS3 peptide mimetics show a significant increase in their phagocytosis of N2a cells that had their “eat-me” signal blocked with anti-CRT antibody (Fig.9-14).

FIGURES

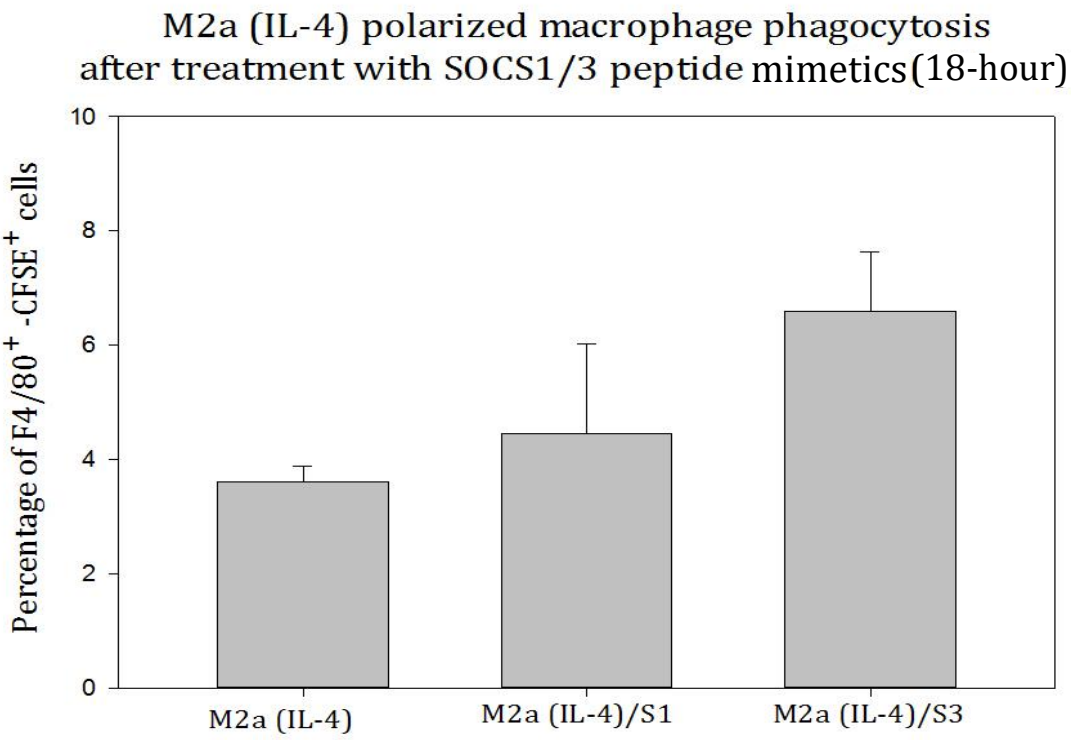

Figure 2: Phagocytosis of CFSE-stained Neuro-2a (N2a) cells by RAW264.7 Macrophages polarized with IL-4 into M2a macrophages with SOCS1/SOCS3. M2a cells treated with SOCS1 and SOCS3 phagocytose similar amount of CFSE-stained N2a cells compared with M2a cells without SOCS1/SOCS3. There was no significant statistical difference in the phagocytosis rate of M2a cells before and after treatment with SOCS1/3.

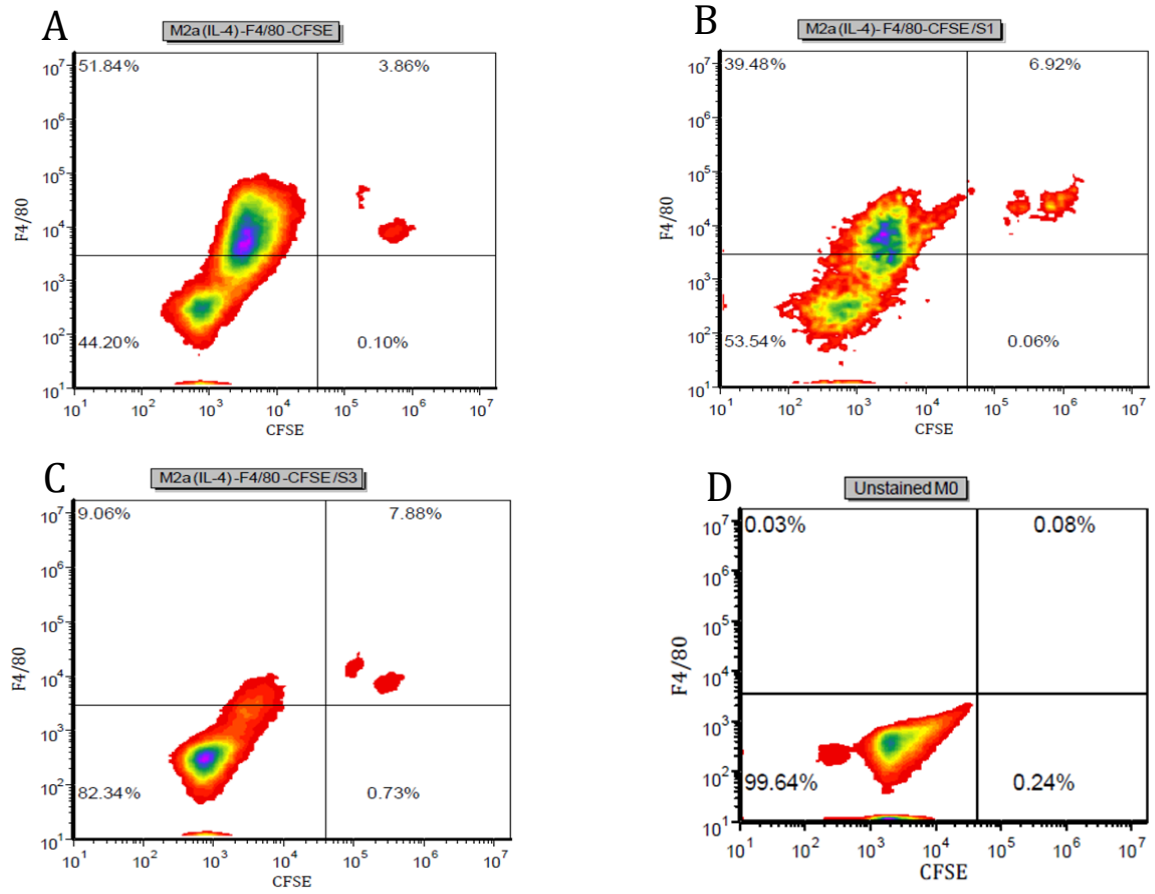

Figure 3: Quadrant graphs represent the phagocytosis rate of RAW264.7 macrophages polarized into M2a (IL-4) phenotype. M2a cells treated with either SOCS1 (B) or SOCS3 (C) phagocytosed higher but not significant amount of CFSE-stained N2a cells compared with M2a Cells not treated with SOCS1/3 (A) there was no significant difference in the phagocytic ability between SOCS1-treated M2a cells (B) and SOCS3-treated M2a cells (C). Quadrants were represented compared to unstained M0 macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

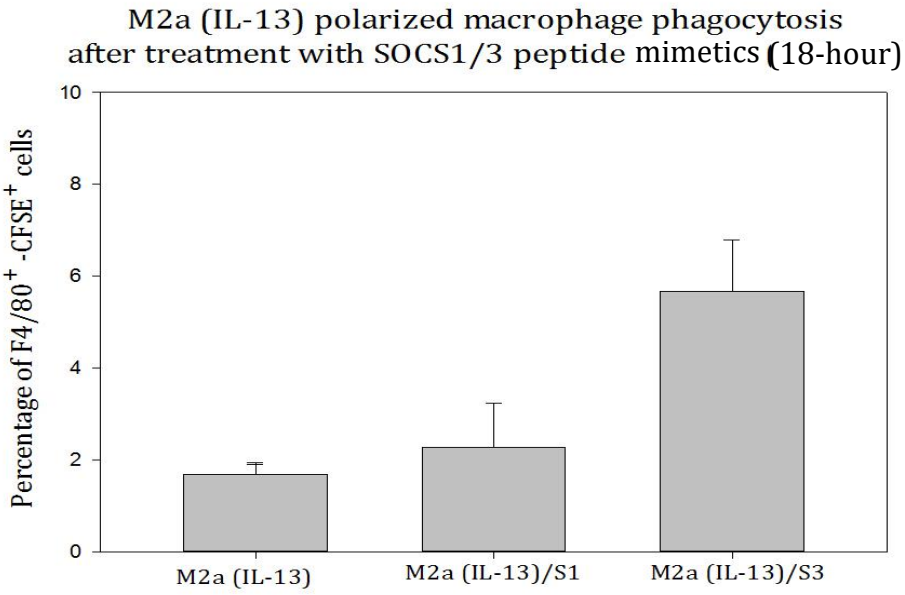

Figure 4: Phagocytosis of CFSE-stained Neuro-2a (N2a) cells by RAW264.7 Macrophages polarized with IL-13 into M2a macrophages with SOCS1/SOCS3. M2a cells treated with SOCS1 phagocytose similar amount of CFSE-stained N2a cells compared with M2a cells without SOCS1/SOCS3. M2a macrophages treated with SOCS3 show a slight increase in their phagocytic ability compared with M2a cells without SOCS1/3. There was no significant statistical difference in the phagocytosis rate of M2a cells before and after treatment with SOCS1/3.

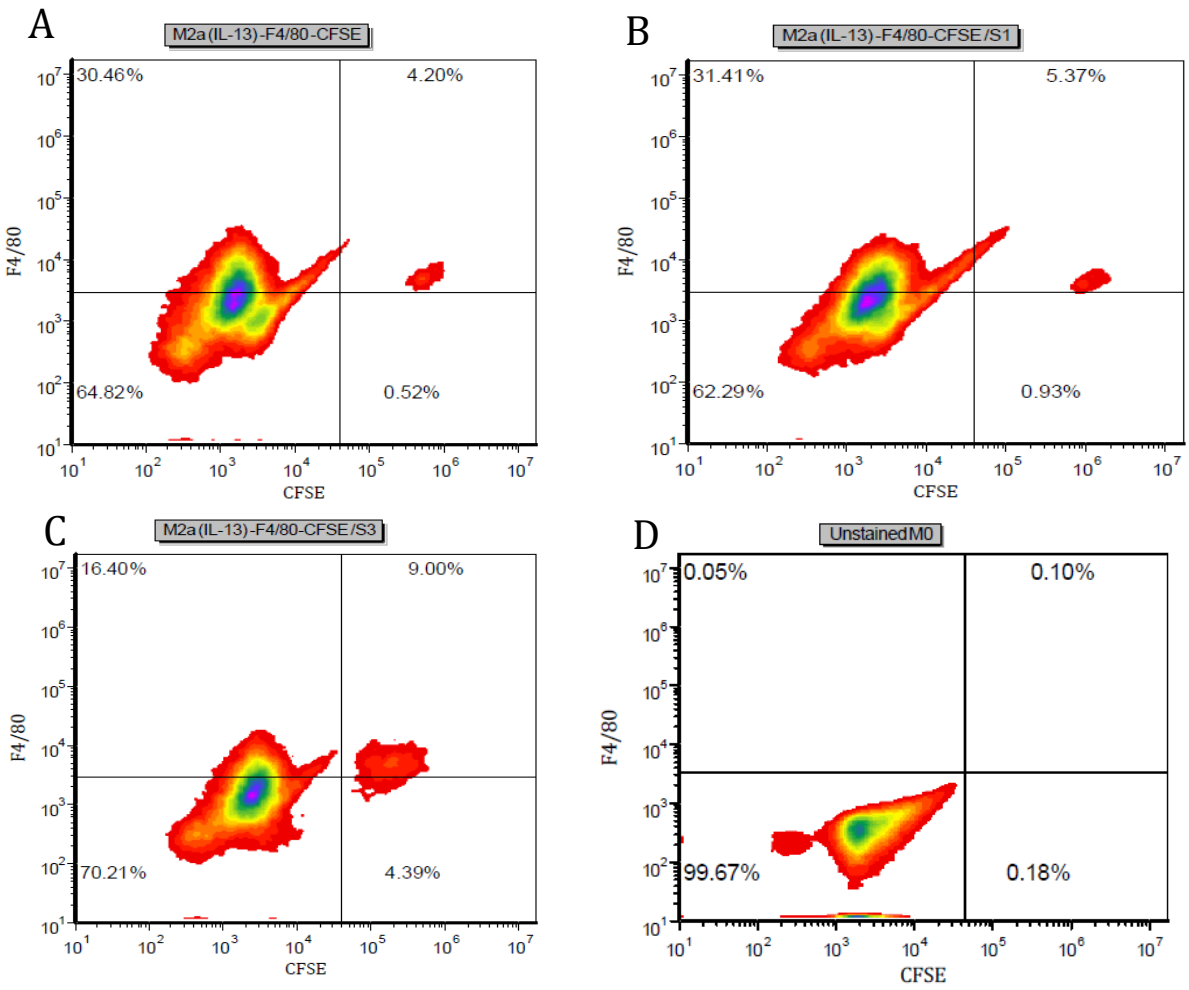

Figure 5: Quadrant graphs represent the phagocytosis rate of RAW264.7 macrophages polarized into M2a (IL-13) phenotype. M2a cells treated with either SOCS1 (B) or SOCS3 (C) phagocytosed higher amount of CFSE-stained N2a cells compared with M2a Cells not treated with SOCS1/3 (A) but not statistically significant. There was no significant difference in the phagocytic ability between SOCS1-treated M2a cells (B) and SOCS3-treated M2a cells (C). Quadrants were represented compared to unstained M0 macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

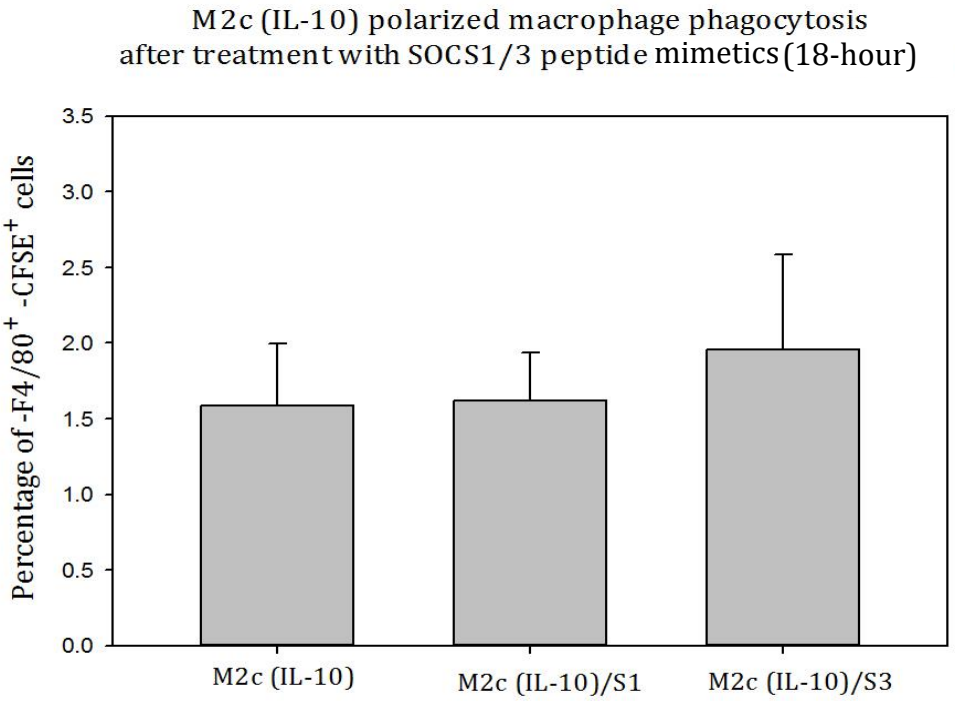

Figure 6: Phagocytosis of CFSE-stained Neuro-2a (N2a) cells by RAW264.7 Macrophages polarized with IL-10 into M2c macrophages with SOCS1/SOCS3. M2c cells treated with either SOCS1 or SOCS3 peptide mimetics phagocytosed similar amount of CFSE-stained N2a cells compared to M2c cells without SOCS1/SOCS3. There were no significant statistical differences in the phagocytosis rate of M2a cells before and after treatment with SOCS1/3.

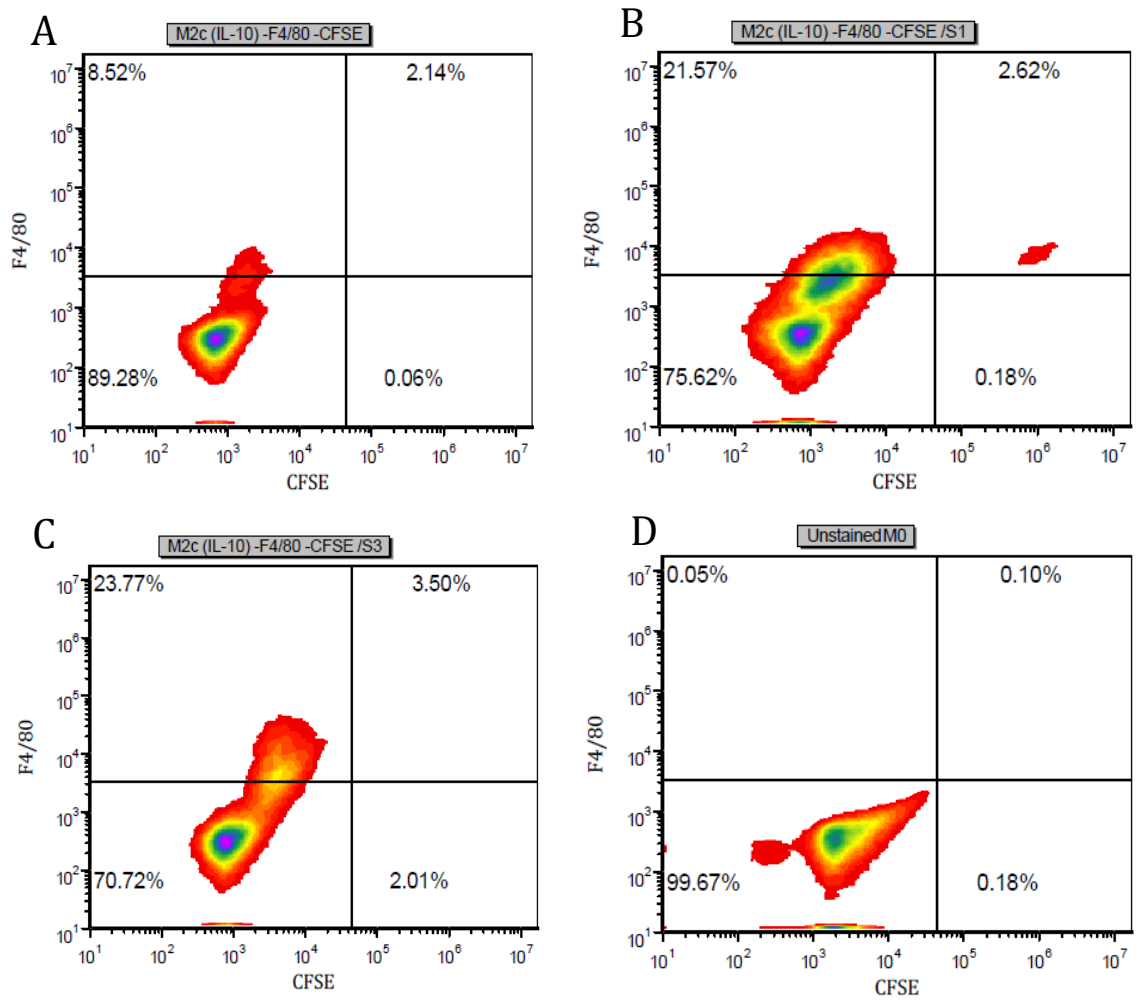

Figure 7: Quadrant graphs represent the phagocytosis rate of RAW264.7 macrophages polarized into M2c (IL-10) phenotype. M2c cells treated with either SOCS1(B) or SOCS3 (C) peptide mimetics phagocytosed similar amount of CFSE-stained N2a cells compared with M2c cells not treated with SOCS1/3 (A) and. There were no significant statistical differences in the phagocytic ability among SOCS1-treated M2c cells, SOCS3-treated M2c, and M2c cells without SOCS1/3. Quadrants were represented compared to unstained M0 macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

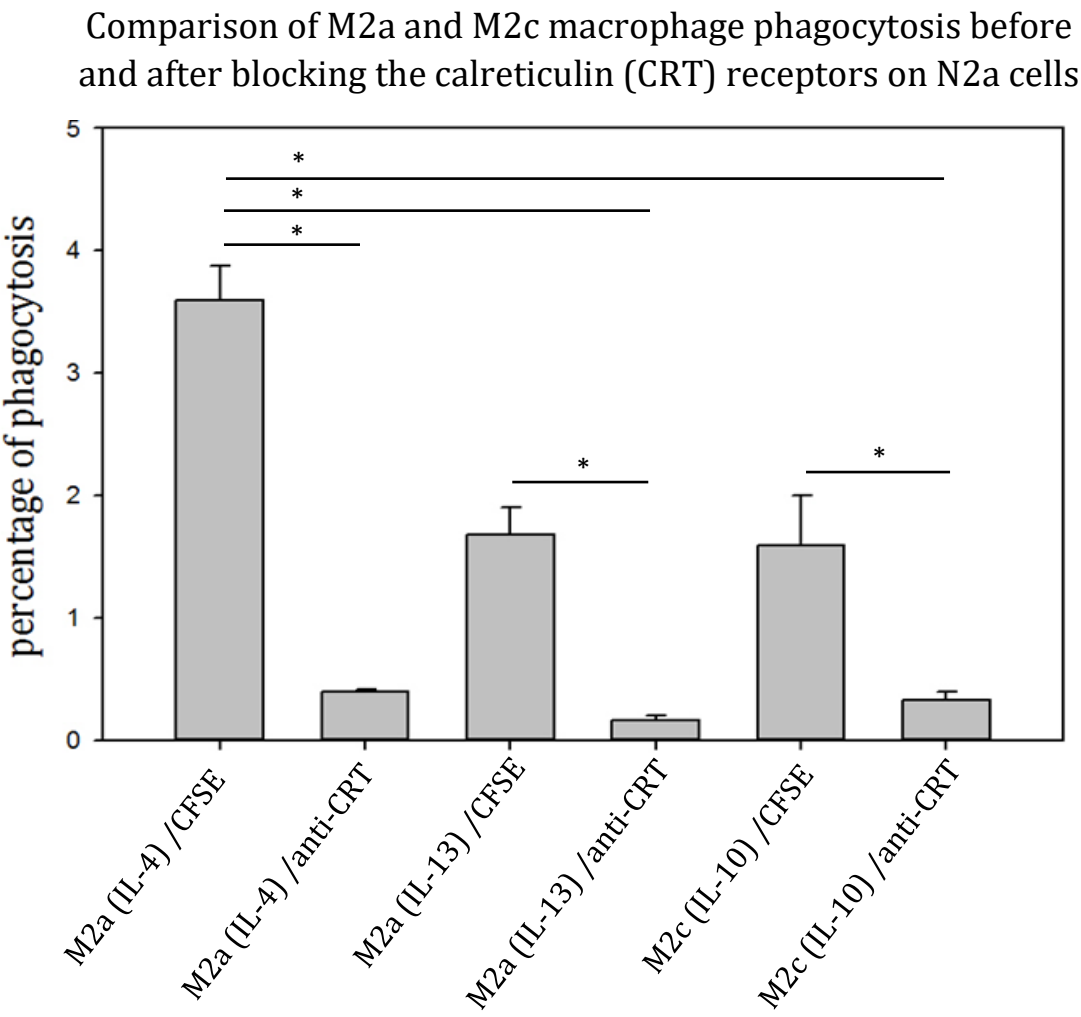

Figure 8: The effects of anti-CRT antibody on the phagocytic abilities of M2a and M2c polarized RAW264.7 macrophages. Blockage of calreticulin receptors on N2a cells significantly decreased the phagocytosis rate of N2a cells, showing higher survival rates of N2a cells. All M2a and M2c polarized macrophages displayed reduced phagocytic activates when co-cultured with N2a cells that had their calreticulin blocked. *=$p<0.05$

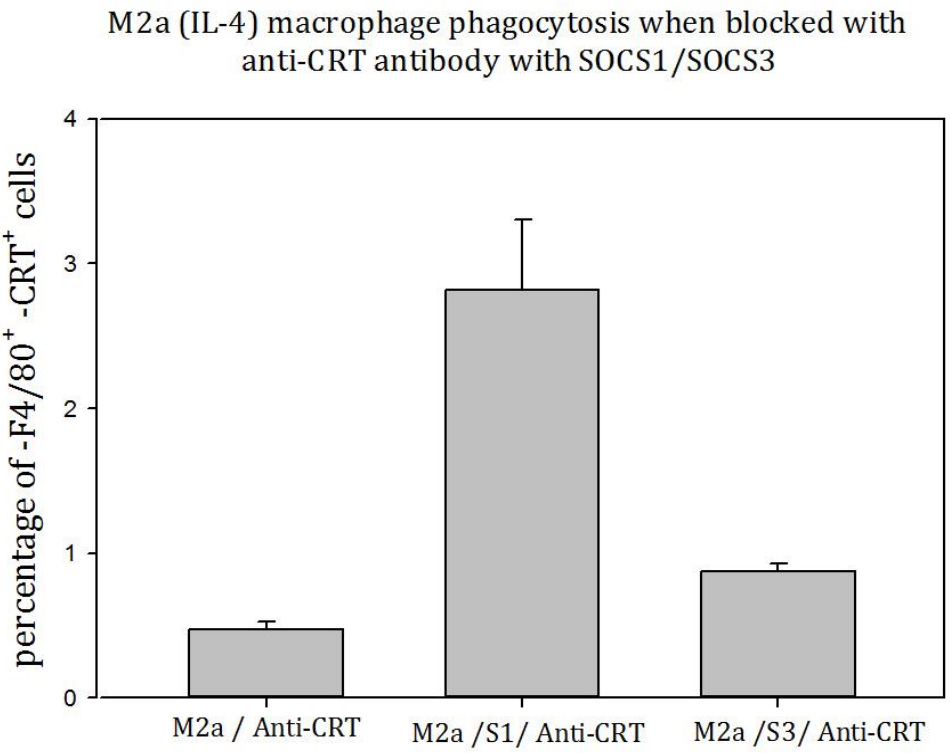

Figure 9: M2a (IL-4) polarized macrophages-mediated phagocytosis of N2a cells blocked with anti-CRT antibody before and after treatment with SOCS1/SOCS3. M2a cells treated with SOCS1 showed a non-significant increase in the phagocytosis rate compared to M2a cells not treated with SOCS1/3 and M2a macrophages treated with SOCS3. There were no observed differences in the phagocytic abilities between SOCS3-treated M2a cells and non-treated M2a cells.

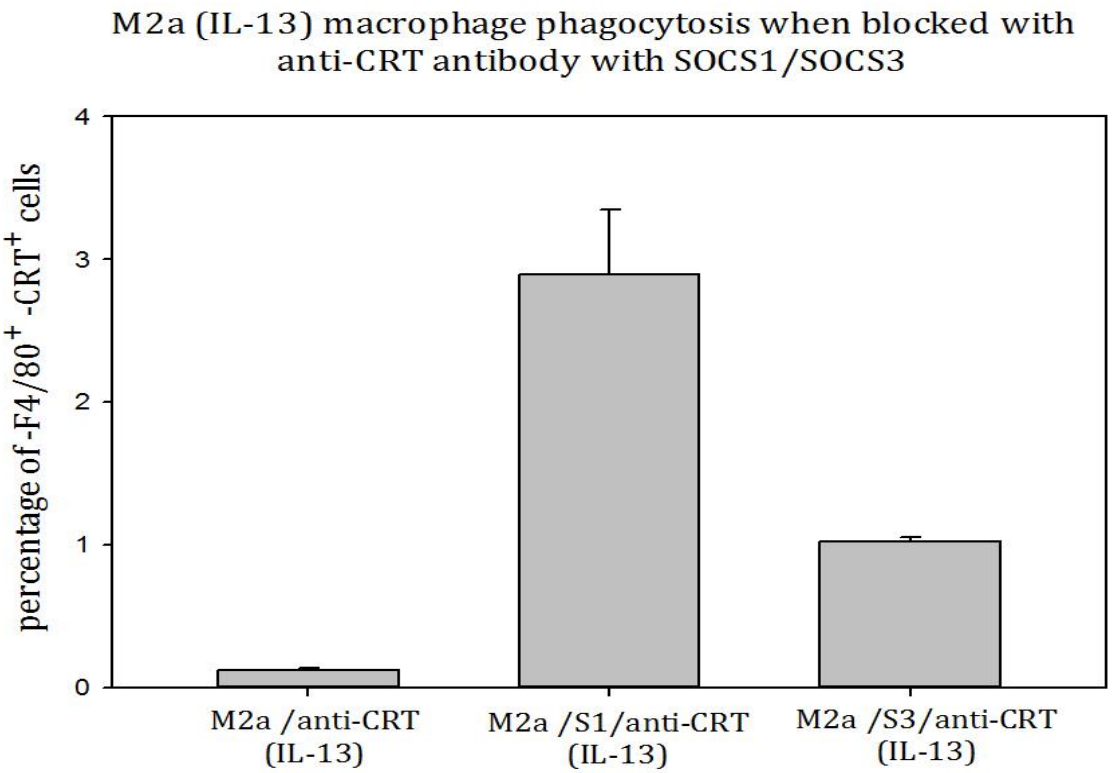

Figure 10: M2a (IL-13) polarized macrophages-mediated phagocytosis of N2a cells blocked with anti-CRT antibody before and after treatment with SOCS1/SOCS3. M2a cells treated with SOCS1 displayed non-significant higher phagocytosis rate compared to M2a cells without SOCS1/3 and M2a macrophages treated with SOCS3. There were no observed differences in the phagocytic abilities between SOCS3-treated M2a cells and non-treated M2a cells.

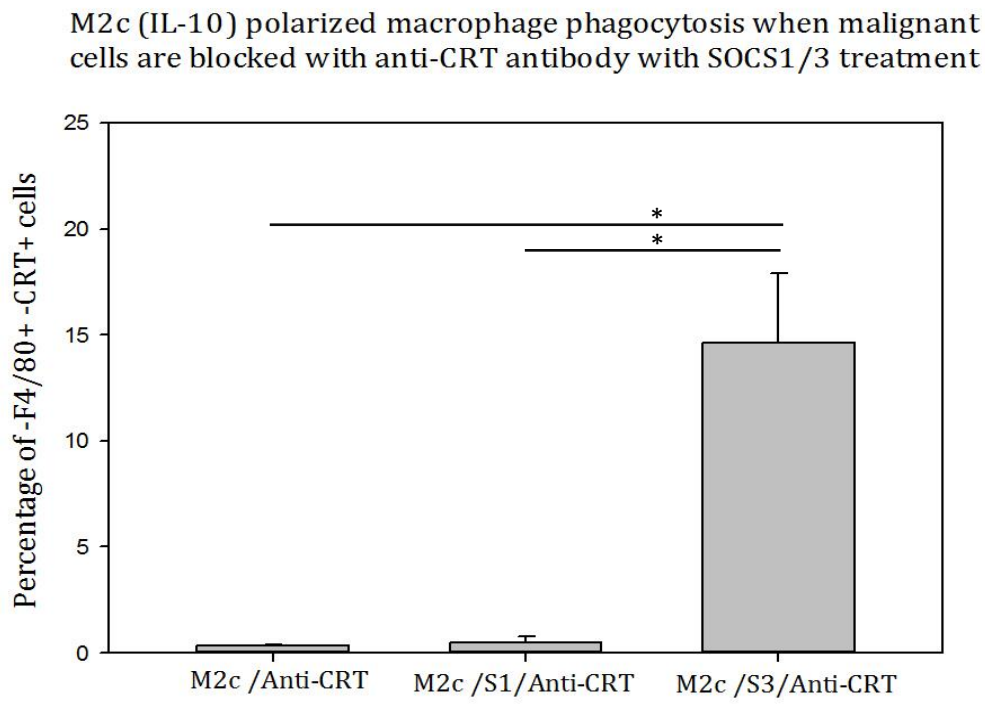

Figure 11: M2c (IL-10) polarized macrophages-mediated phagocytosis of N2a cells blocked with anti-CRT antibody before and after treatment with SOCS1/SOCS3. M2c cells treated with either SOCS3 displayed a significant increase in their phagocytosis rate compared to M2a cells not treated with SOCS1/3 and M2c treated with SOCS1. *=$p<0.05$

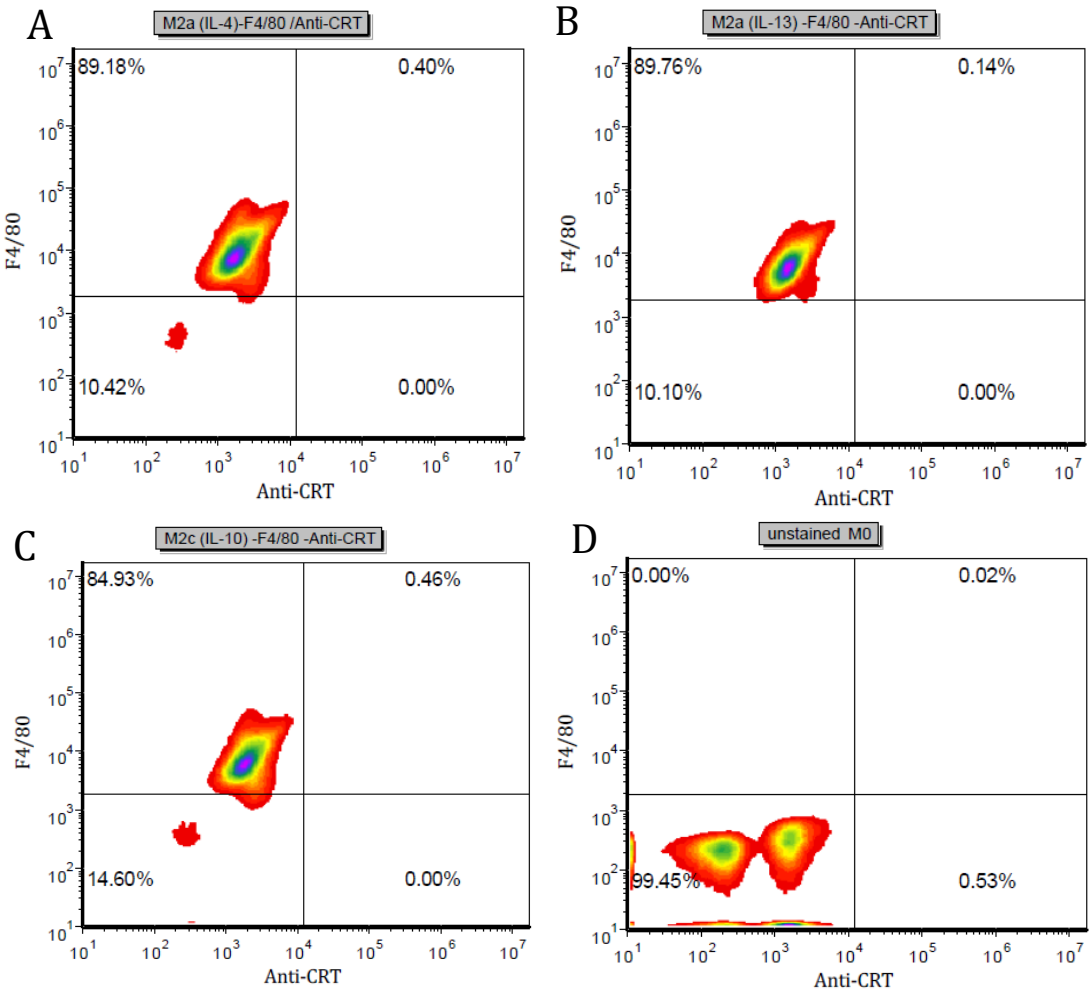

Figure 12: Quadrant graphs represent the RAW264.7 macrophages phagocytosis rate of Anti-CRT Neuro-2a (N2a) cells. M2a (IL-4) polarized macrophages (A), M2a (IL-13) polarized macrophages (B) and M2c (IL-10) polarized macrophages (C) displayed no significant differences in their phagocytosis of N2a cells that had their "eat-me" signal blocked with anti-CRT antibody. Quadrants were represented compared to unstained M0 macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

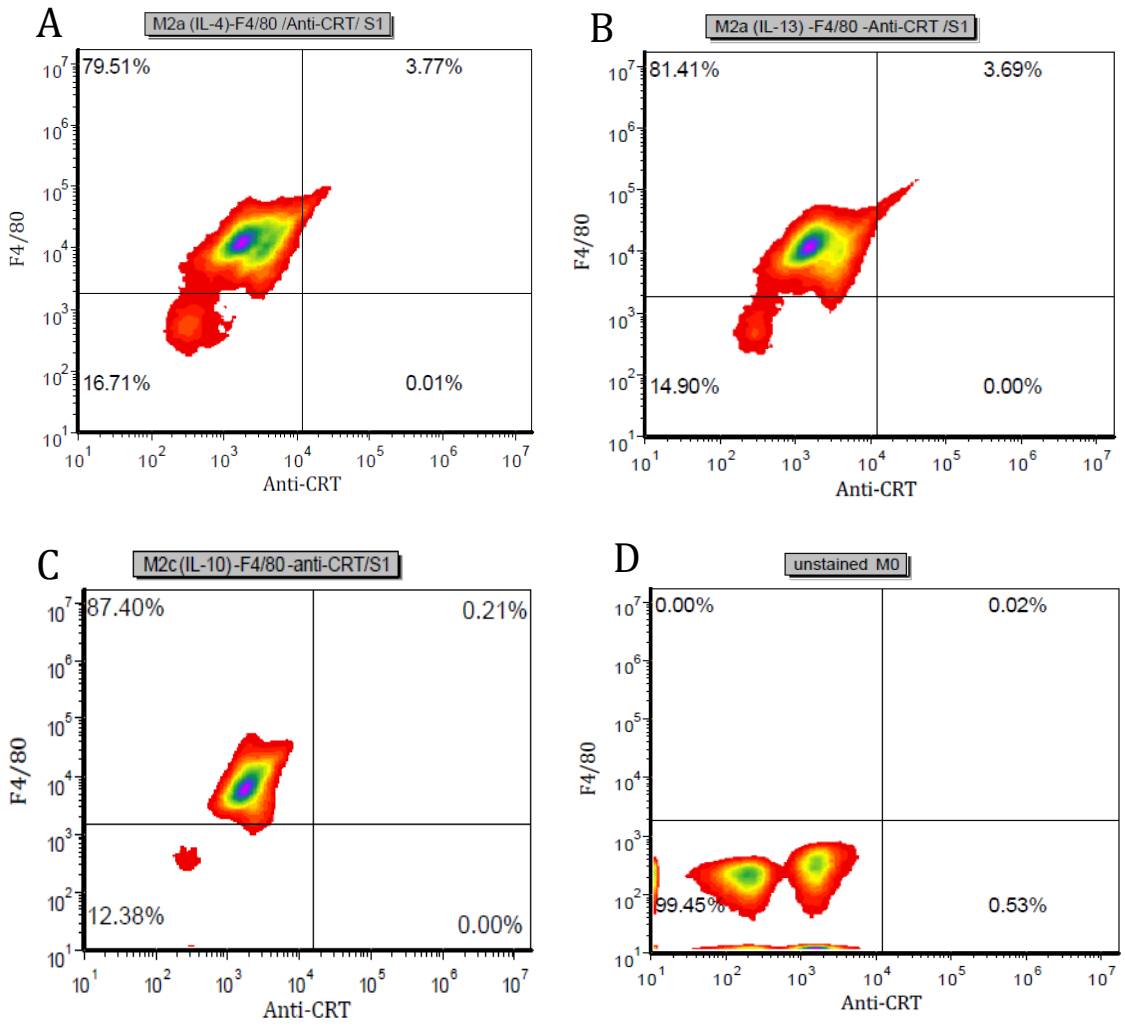

Figure 13: Quadrant graphs represent the effect of SOCS1 peptide mimetics on RAW264.7 macrophages phagocytosis of Anti-CRT Neuro-2a (N2a) cells. M2a (IL-4) polarized macrophages (A) and M2a (IL-13) polarized macrophages (B) phagocytosed more amount of anti-CRT-N2a cells than M2c (IL-10) polarized macrophages (C) but not statistically significant. There was no a significant difference in the phagocytosis rate of N2a cells between M2a (IL-4) (A) and M2a (IL-13) (B) polarized macrophages. Quadrants were represented compared to unstained M0 macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

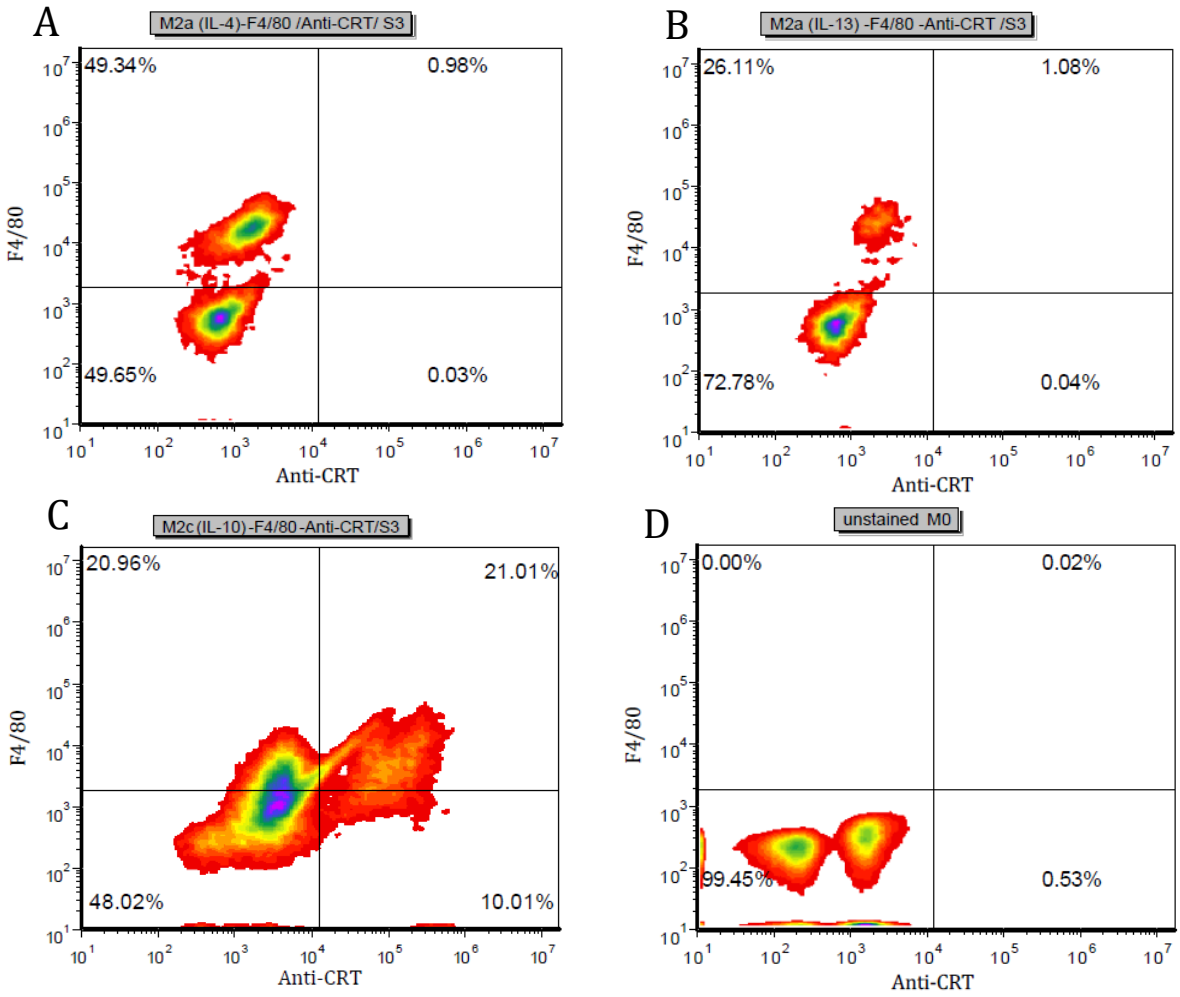

Figure 14: Quadrant graphs represent the effect of SOCS3 peptide mimetics on RAW264.7 macrophages phagocytosis of Anti-CRT Neuro-2a (N2a) cells. M2c (IL-10) polarized macrophages (C) significantly phagocytosed more amount of anti-CRT-N2a cells than M2a (IL-10) (A) and M2a (IL-13) (B) polarized macrophages. There was no a significant difference in the phagocytosis rate of N2a cells between M2a (IL-4) (A) and M2a (IL-13) (B) polarized macrophages. Quadrants were represented compared to unstained M0 macrophages (D). Experiments were completed in triplicates with one quadrant graph being presented.

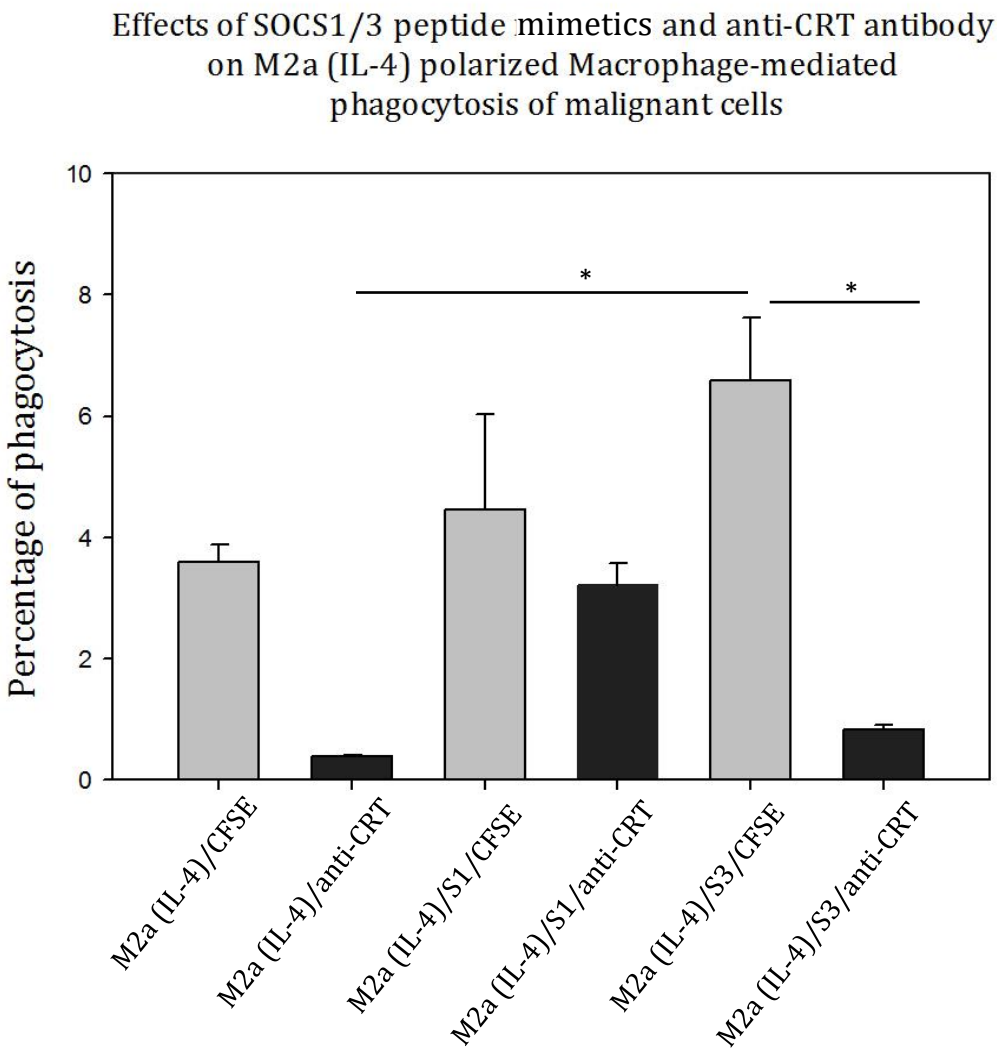

Figure 15: The effects of SOCS1/3 peptide mimetics on M2a (IL-4) polarized RAW264.7 macrophage-mediated phagocytosis of either CFSE-stained or anti-CRT-blocked N2a cells. Blockage of CRT receptors on N2a cells caused a decrease in the phagocytosis rate of M2a(IL-4) treated with SOCS1/SOCS3 and M2a macrophages without SOCS peptide mimetic treatment. *=$p<0.05$

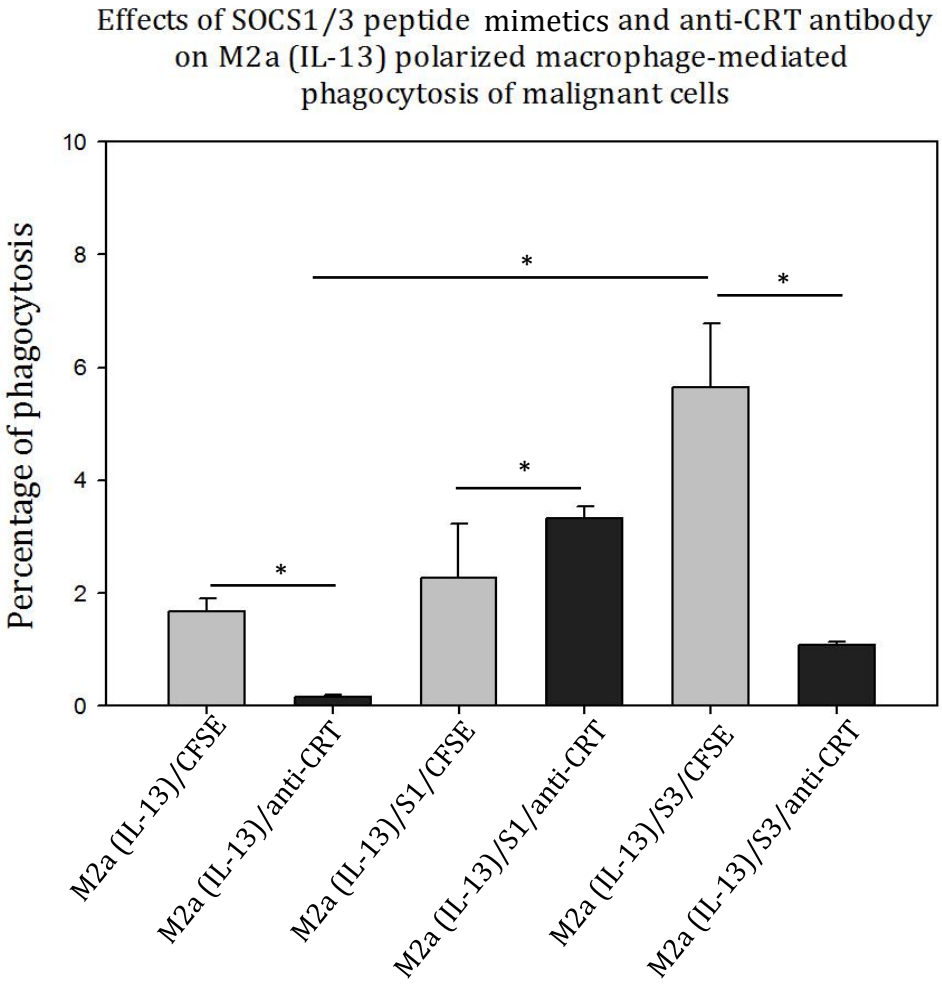

Figure 16: The effects of SOCS1/3 peptide mimetics on M2a (IL-13) polarized RAW264.7 macrophage-mediated phagocytosis of either CFSE-stained or anti-CRT-blocked N2a cells. Blockage of CRT receptors on N2a cells caused a decrease in the phagocytosis rate of M2a(IL-13) treated with SOCS3 and M2a macrophages without SOCS peptide mimetic treatment, while it caused a slight increase in the phagocytosis mediated by M2a(IL-13) treated with SOCS1 peptide mimetics. *=$p<0.05$

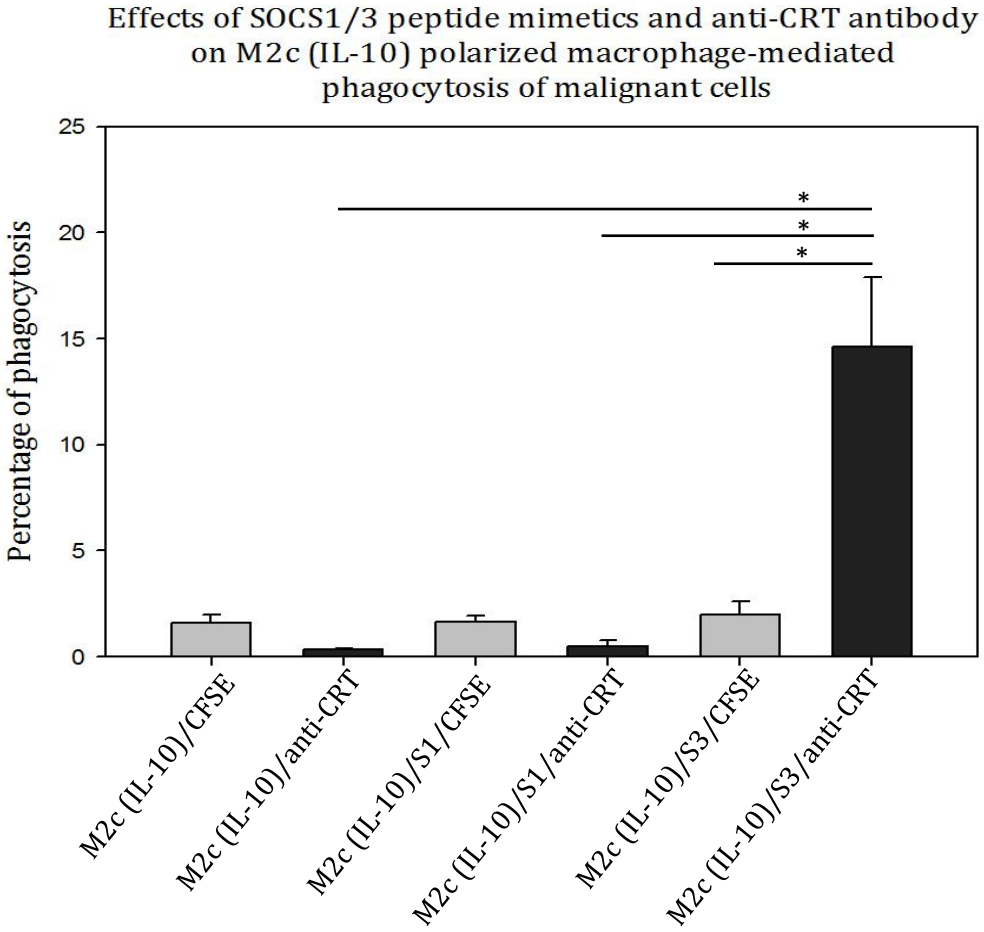

Figure 17: The effects of SOCS1/3 peptide mimetics on M2c (IL-10) polarized RAW264.7 macrophage-mediated phagocytosis of either CFSE-stained or anti-CRT-blocked N2a cells .Blockage of CRT receptors on N2a cells caused a significant increase in the phagocytosis mediated by M2c (IL-10) treated with SOCS3, while it decreased phagocytosis mediated by M2c(IL-10) treated with SOCS1 peptide mimetics and M2c without SOCS1/3 treatment. *=$p<0.05$

DISSCUTION

The current study aims to investigate whether treatment of anti-inflammatory M2a and M2c polarized macrophages with SOCS3 peptide mimetics could result in increased phagocytosis rate of malignant cells. Blockage of calreticulin (CRT) receptors, the “eat-me” signal that induce phagocytosis of malignant cells, should reduce the ability of RAW264.7 macrophages to phagocytose neuro-2a cells.

When CFSE-stained N2a cells were co-cultured with M2a and M2c polarized macrophages, M2a (both polarized with IL-4/IL-13) and M2c macrophages showed similar amount of phagocytosis of CFSE-stained N2a cells. M2a and M2c phagocytic abilities were also similar to the phagocytic ability of M0 unpolarized macrophages (data not shown) as investigated by Capan (2017). These data indicate that M2a and M2c polarized macrophages are regulatory effectors in the clearance of malignant cells.

Upon blockage of the “eat-me” signal, calreticulin (CRT) receptor, on the target N2a cells using anti-CRT antibody, the phagocytic abilities of M2a (both polarized with IL-4/IL-13) and M2c polarized macrophages were significantly decreased compared with the phagocytosis rates of M2a and M2c polarized macrophages co-cultured with CFSE-labeled N2a cells. This investigation indicates that calreticulin receptor is a crucial “eat-me” signal exposed on N2a cells and plays a key role in the clearance of malignant cells.

RAW264.7 macrophages polarized into M2a (with IL-4 or IL-13) and M2c (with IL-10) were treated with SOCS1/ SOCS3 peptide mimetics for 18 hours. The addition of SOCS1 peptide mimetic did not significantly affect the phagocytic ability of M2a macrophage polarized with either IL-4 or IL-13 and M2c macrophages. M2a (IL-4/IL-13) polarized macrophages treated with SOCS1 showed a non-significant slight increase in their phagocytosis of CFSE- stained N2a cells. These observations could be attributed to the upregulation of cytokines and receptors involved in M2a-mediated phagocytosis without switching the anti-inflammatory phenotype into pro-inflammatory phenotype. Similarly, M2c treated with SOCS1 peptide mimetics displayed no difference in their phagocytic ability compared with M2c macrophages without SOCS1 treatment.

SOCS3 treatment had a positive impact on the phagocytic ability of RAW264.7 macrophages polarized into M2a and M2c phenotypes. The addition of SOCS3 peptide mimetics into M2a (IL-4 or IL-13) and M2c macrophages caused these polarized cells to phagocytose more target CFSE-stained N2a cells compared to M2a and M2c macrophages without SOCS3. Similar to SOCS1, this observation could be due to activation of particular cytokines and receptor expression that may be involved in phagocytic process. Interestingly, SOCS3 peptide mimetics-treatment of M2c polarized macrophages with blockage of calreticulin receptor, the “eat-me” signal, on N2a cells remarkably enhanced M2c polarized macrophages ability to phagocytose much more target anti-CRT-N2a cells. This result indicates that the increase in the SOCS3-treated M2c macrophage phagocytosis of anti-CRT- N2a cells is due to the blockage of calreticulin receptor before they are exposed on the surface of N2a cells, which in turn causes a disturbance in the cellular membrane composition. The absence of CRT results in up-regulating the expression of the other

phagocytic signal PS, which makes the malignant cells apoptotic and detectible by phagocytic SOCS3-treated M2c macrophages. Another clarification is that the enhanced increase in the SOCS3-treated M2c macrophages could be attributed to the up-regulated expression of tumor associated macrophage receptor merTK, an apoptotic intracellular inhibitor that down-regulates macrophage inflammatory responses in a negative feedback loop and play a key role in the apoptosis of malignant cells (Zizzo et al. 2012). Zhang et al., (2016) have found that merTK expression is up-regulated by SOCS3 peptide mimetics, and this increase in the expression of merTK leads to enhancing macrophage-mediated phagocytosis.

Overall, the current study illustrates that polarization of macrophages into different phenotypes with various functions play a key role in the regulation of inflammatory responses including phagocytosis of pathogens, apoptotic cells or malignant cells through activation or inhibition of cytokine signaling pathways. All the current results indicate that SOCS peptide mimetics are crucial effectors that can be used to enhance the phagocytic abilities of macrophages with particular phenotypes to efficiently clear malignancies. Treatment of M2a polarized macrophages with SOCS3 caused those macrophages to phagocytose more N2a cells through increasing cytokines involved in phagocytic process. Remarkably, treatment of M2c polarized macrophages with SOCS3 caused an enhanced increase in their phagocytosis of N2a cells that had their calreticulin receptors blocked. This result could be attributed to upregulation of the expression of PS signal or tumor associated macrophage receptor merTK, which make malignant cells become apoptotic cells and targeted to phagocytes.

FUTURE DIRECTION

The current study provides many various directions for future study depending on the protocol used in the present study. This study included RAW264.7 macrophages as phagocytes and Neuro-2a cells as target cells. The logical direction in the future is investigating the phagocytic abilities of other types of macrophages such as J774A.1 murine macrophage. Other target cells should be tested including cells obtained from other types of malignancies, including lung, brain, breast, and pancreatic cancers.

In this study, RAW264.7 macrophages were polarized with IL-4 or IL-13 into M2a phenotype and with IL-10 into M2c phenotype. SOCS3 peptide mimetic treatment of M2a and M2c characterized the phagocytic abilities of these phenotypes. Only M2c polarized macrophages showed an enhanced increase in their phagocytic ability when they co-cultured with Neuro-2a cells that had their calreticulin receptors blocked. To get more understanding of M2c phenotype characterization and its ability to clear malignant cells, macrophages should be polarized using glucocorticoid hormones as these hormones have been reported to be polarizing agents that drive M2c phenotype.

Recent studies have shown that SOCS3-treatment of M2c polarized macrophages is associated with high expression of tumor associated macrophage receptor merTK. Thus, measuring the expression of merTK on M2c macrophages along with other macrophage phenotypes is another avenue that would be

beneficial to explore the molecular basis of this receptor and its contribution in the clearance of malignant cells.

Polarization of macrophages switches them into various phenotypes with distinct properties and functions. Cytokine profile is one of the properties that is affected with polarization treatment of macrophages along with using SOCS peptide mimetics, which in turn lead to inhibition or activation of cytokines. Any alterations in the cytokine profiles could affect the whole microenvironment, including the immune cells and their capabilities to detect, respond and react with harmful foreign materials. Since SOCS3-treated M2c macrophages possess high ability to phagocytose malignant cells, their cytokines should be measured to understand their roles and contributions in clearance of pathogens, apoptotic cells, malignant cells, and other foreign molecules.

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