MACROPHAGE INFLAMMASOME ACTIVATION IN BREAST CANCER

by

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ABSTRACT

JULIA ROBERSON. Macrophage Inflammasome Activation in Breast Cancer.
(Under the direction of DR. DIDIER DREAU)

Breast cancer progression is promoted by local inflammation. Indeed, inflammatory cytokines and immune infiltration are independent prognosis markers of breast cancer progression. However, the mechanisms underlying the interplay between inflammasome, pro-inflammatory cytokines and tumor progression remain to be fully understood. Our early observations highlighted a potential role of inflammasome activation in cancer-associated macrophages in both local inflammation and tumor growth. Here, we specifically assessed macrophage NLRP3 inflammasome activation, pro-inflammatory cytokine secretions and phagocytosis triggered by tumor cells. Through *in vitro* assessments, our data indicates that activation of macrophages by tumor cell secretions was associated with promotion of inflammasome especially NLPR3 inflammasome activation, and pro-inflammatory IL-1ß and IL-18 cytokine secretions. Indeed, following incubation with tumor secretions, macrophage phenotype and phagocytic activities were significantly altered. Moreover, incubation with the NLRP3 inflammasome specific inhibitor, MCC950 reduced both the expression and secretion of pro-inflammatory cytokines with some effects on phenotype and phagocytosis of inflammasome-activated macrophages. Taken together our data suggest that breast tumor cell secretions promote inflammasome activation and secretion of pro-inflammatory cytokines in macrophages *in vitro* and that inflammasome activation promotes a pro-tumorigenic phenotype in macrophages favoring breast cancer growth and metastasis. Future investigations will further those observations and assess the potential of targeting those pathways in macrophages to prevent breast cancer progression.

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CHAPTER 1: INTRODUCTION

Clinical, Therapeutic, and Biological Approaches to Breast Cancer

Although female breast cancer mortality has decreased over 40% since its peak in 1989, incidence rates have been gradually increasing by 0.5% per year since the mid-2000's and breast cancer remains the second leading cause of cancer-related death in females.[1] Triple negative breast cancer (TNBC) account for 15-20% of all breast cancers and is characterized by the absence of estrogen receptor (ER) and progesterone receptor (PR) expression in addition to the absence of human epidermal growth factor 2 receptor (HER2) expression.[2] Patients with TNBC present breast tumors that have more aggressive progression, associated with earlier recurrence, and worse survival rate with an overall 5-year relative survival rate of 77%.[1, 3] Currently, there is no targeted treatment against TNBC and surgery in combination with chemotherapy (anthracyclines and taxanes) remains the primary systemic treatment, although resistance and recurrence is common among a significant portion of patients that undergo chemotherapy.[4] Recently there have been advancements in targeted molecular therapies against TNBC including inhibition of poly (ADP-ribose) polymerases (PARP), immune checkpoints (PD-1/PD-L1), signaling kinases (serine/threonine or tyrosine type), and others, although none have proven enough efficiency to be considered a primary therapeutic for treating TNBC.[5] Therefore, there is a need for further investigations toward the discovery of novel and more efficient therapeutic approaches to prevent TNBC progression.

The Tumor Microenvironment and Tumor-Associated Macrophages

The tumor micro-environment (TME) is comprised of an extracellular matrix (ECM) and non-malignant cells including fibroblasts, endothelial, and immune cells associated with the tumor mass.[6] In TNBC, the TME has been linked to tumor promoting activities such as induction of proliferation, angiogenesis, inhibition of apoptosis, immune system suppression, evasion of immune surveillance, and immune cell recruitment.[7] [8] Macrophages are the most abundant innate immune cell found in the TME and a high infiltration of macrophages in human tumors is associated with a poor clinical outcome.[9]

Macrophages are myeloid cells that are highly heterogenous and plastic in phenotype and activity.[10] As key players of the innate and adaptive immune responses, major functions of macrophages include phagocytosis, antigen presentation, and immunomodulation to defend the body against invasive pathogens and damaged/dying cells.[11] In particular, macrophages can recognize pattern motifs associated with either pathogens (PAMPs) or cell damage (DAMPs) through their pattern-recognition receptors (PRRs) and trigger an innate immune response by promoting local inflammation through secretions of pro- and anti-inflammatory cytokines.[12] Additionally, phagocytosis of cells, debris and foreign particles, and subsequent antigen presentation by macrophages through the recognition by the major histocompatibility complex class II (MHC II) are crucial mechanisms by which macrophages contribute to and activate the adaptive immune response.[11] However, macrophages recruited the tumor microenvironment are influenced by cytokines, growth factors, and other environmental cues and polarized into tumor-associated macrophages (TAMs).[13] TAMs modulate multiple aspects favoring tumor progression including growth, angiogenesis, extracellular matrix remodeling as well as anti-tumor adaptive immune response.[9]

Macrophage Phenotype Within Tumors

Macrophage polarization into distinct phenotypes including tumor-associated macrophages is influenced by their developmental origin, tissue of residence, and microenvironmental cues.[14] While macrophage activation and phenotype falls on a spectrum, the two extreme ends of this spectrum include: classically activated (also known as M1) macrophages and alternatively activated (M2) macrophages.[9] M1 macrophages are induced by cytokines such as interferon gamma or the presence of lipopolysaccharide (LPS) from gram-negative bacteria and exhibit pro-inflammatory anti-tumoral activities, whereas M2 macrophages are activated by cytokines including IL-4/IL-10/IL-13 and give rise to anti-inflammatory macrophages that exhibit pro-tumoral activities.[9] It is important to note that macrophages are not limited to either only the M1 or M2 phenotypes but often express mixed phenotypes depending on the disease and disease stage.[15] In particular, the subtype of M2d macrophages is induced by TLR ligands and A2 adenosine receptor agonists or by IL-6 and contributes to tumor growth and metastasis and thus, are often referred to as tumor-associated macrophages (TAMs).[12] Additionally, M2d macrophages secrete high levels of IL-10, TGF-β, and VEGF and low levels of IL-12, TNF-a, and IL-1β, respectively.[12]

Macrophages are plastic and can rapidly switch from one phenotype to another depending on the microenvironment and pathological conditions during various phases of disease progression.[16-18] For example, during the early stages of tumorigenesis NF-kB activation in M1 macrophages is important for cancer-related inflammation, while in the later stages of

tumorigenesis macrophages are polarized to M2-like macrophages with low NF-kB activity and exhibit immunosuppressive activities.[15] These studies highlight the highly dynamic cell plasticity and complex multifactorial processes of macrophage polarization.

Table 1: Key stimuli and markers of M1 and M2 macrophage subtypes (adapted from[19, 20]).

Phenotype	Stimuli	Metabolic Enzymes	Secreted Markers	Surface Markers	Function	Phagocytic Activity
M1	LPS+ IFN-γ, GM-CSF	iNOS	Cytokines: IFN-γ, IL-1β, IL-6, IL-8, IL-12, IL-23, IL-10 (low) IL-18, TNF-α	CD80, CD86, MHCII	Pro-inflammatory, stimulation of Th1 immune response, Phagocytosis, tissue damage	↑
M2a	IL-4/ IL-13	Arg-1	Cytokines: IL-10, TGF-β	CD206, CD163	Anti-inflammatory, tissue remodeling, wound healing	↓
M2b	IC, TLR ligands/IL-1Ra	Arg-1	Cytokines: IL-10, IL-1β, IL-6, TNF-α	CD163	Tumor progression, stimulation of Th2 immune response, immunoregulation	↓
M2c	Glucocorticoids/IL-10 (TGF-B1?)	Arg-1	Cytokines: IL-10, TGF-β	CD163	Tissue remodeling, immunosuppression, phagocytosis of apoptotic bodies	↓
M2d	TLR ligands	Arg-1	IL-10, VEGF	CD206, CD163	Angiogenesis, Tumor progression	↓

Abbr.: Arg (Arginase); CD (Cell determinant); GM-CSF (Granulocyte-macrophage colony-stimulating factor); IC (Immune complex); IFN (Interferon); IL (Interleukin); iNOS (Inducible nitric oxide synthase); LPS (Lipopolysaccharide); MHC (major histocompatibility complex); TGF (transforming growth factor) TNF (tumor necrosis factor); TLR (toll-like receptor); VEGF (Vascular endothelial growth factor)

Macrophage Activities

Macrophages are plastic and differentially activated in multiple subtypes by cytokines present within the microenvironment (see Table 1). Overall, M1 macrophages are pro-inflammatory and secrete the cytokines TNF-α, IL-1β, IFN-γ, IL-6, IL-12, and IL-23. Additionally, M1 macrophages express cell surface markers MHC-class II, CD80, CD86 and exhibit higher iNOS enzyme cytoplasmic concentrations.[10, 21] In contrast, alternatively activated or M2 macrophages generally display anti-inflammatory phenotype and secrete the cytokines IL-10, IL-4, IL-13, TGF-β, IL-8, and EGF. M2 macrophages also express specific cell surface markers including CD206, CD163, PD-L1, and have higher Arginase-1 enzyme cytoplasmic concentrations.[10, 22] Within the tumor microenvironment, the M2 macrophage subtype prevails and favors tumorigenesis.[18, 23 24]

Macrophages are the major phagocytic population within tumors.[25, 26] Phagocytosis by macrophages can also impact anti-tumor immunity by redirecting antigens away from circulating dendritic cells, reduce the presence of DAMPs, and suppressing tissue-resident macrophage activation in response to apoptotic cell phagocytosis.[27, 28] Engulfment of cancer cells by macrophages can override anti- tumor functions of macrophages via upregulation of PD-L1 and also inhibition of macrophage functions including phagocytosis, cytokine production and inflammasome activation via inducing expression of TIM3, TIM4, and PD-1, and PD-L1 by macrophages.[29-33] Overall, macrophage phagocytic activities are increased and decreased in M1 and M2 subtypes, respectively.[22]

Clinically, high intratumor levels of CD163 expressing TAMs in breast cancer patients were associated with immune suppression and predictive for reduced survival.[34] Also, in human breast cancer, tumor cells synthesize colony-stimulating factor 1 (CSF-1) that stimulates

macrophages to produce epidermal growth factor (EGF) and results in activation of tumor cell migration.[35, 36] Vascular endothelial growth factor (VEGF) is produced by multiple cell types including macrophages in both human and mouse mammary tumors and VEGF over-expression is associated with increased vascularization and tumor aggressiveness.[37] In hepatocellular carcinoma, macrophages suppress cytotoxic T-cell responses through IL-10 production and PD-L1 cell surface expression.[38] Furthermore, in murine mammary tumor xenografts immune responses were also suppressed through increased PGE2 and TGF-β secretions.[39]

Inflammation and Cancer

While targeted immune responses can hinder and suppress tumor growth, inflammatory cells, and the pro-inflammatory cytokines they produce have been shown to promote breast tumor development, resistance to apoptosis, angiogenesis, and metastasis.[40] Elevated concentrations of inflammatory cytokines are associated with poor prognosis in breast cancer.[41] Beside promoting the recruitment of immune cells to sites of infection and tissue damage, inflammatory cytokines also stimulate cell growth, differentiation, and migration.[42] Indeed, sustained cytokine secretions lead to a persistent inflammation state which promotes cancer growth.[43] In particular, elevated concentrations of cytokines belonging to the IL-1 superfamily have been measured in multiple cancers including breast cancer and have been associated with generally bad prognosis.[44, 45]

IL1 and Cancer Growth

IL-1 is the first of a family of pro-inflammatory cytokines that play a crucial role in local and systemic inflammation.[46] There are seven IL-1 family ligands that possess pro-inflammatory activity: IL-1α, IL-1β, IL-18, IL-33, IL-36 -α, -β, -γ.[47] These cytokines bind to different receptors including IL-1RI, IL-18Ra, ST2, and IL1Rp2.[47] The IL-1 superfamily members preeminently

participate in the generation of an inflammatory microenvironment mainly through the signaling by the pro-inflammatory IL-1β modulated by the secreted receptor agonist IL-1RA and the IL-18 modulated by secreted binding protein IL-18BP, respectively.[44, 48]

There are multiple mechanisms by which the IL-1 family of cytokines contribute to tumor progression including promoting angiogenesis, recruitment of myeloid cells in tumors, induction of inflammatory cytokine expression and secretion, and lymphoid cell polarization.[49] In breast cancer, IL-1β and IL-1R have been shown to be related to tumorigenesis and it has been proposed that this occurs through IL-1β induced production of pro-inflammatory cytokine IL-6.[50] Moreover, IL-1 secreted by TAMs has been shown to increase COX-2 production in human breast cancer cells and also activate neutrophils to dampen CD8-mediated anti-tumor response thus contributing to cancer progression.[51, 52] IL-1 production also induces the secretion of other cytokines, chemokines including IL-8, and TGF- ß.[44]

NLRP3 Inflammasome and Pro-inflammatory Cytokines

Inflammasomes: Among the mechanisms involved in the generation of an inflammatory microenvironment, the role of inflammasome activation has been characterized more recently.[53, 54] Inflammasomes mediate pro-inflammatory cytokine secretion and play a critical role in immune responses. Inflammasomes are intracellular, pattern recognition receptors, which respond to signaling by pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). NLR- family inflammasomes encompass a family of protein complexes that primarily form structures combining one nucleotide-binding and oligomerization domain-like (NOD) receptor, a scaffolding protein and pro-caspase 1 (or pro-caspase 11).[55] In the case of canonical inflammasomes, activation by either PAMP or DAMP signaling triggers the formation of a complex between the three proteins leading to activation of caspase 1, which in turn cleaves

pro-IL-1ß and pro-IL-18 into their ready for secretion mature forms (Fig 1).[56] Secreted IL-1ß and IL-18 support and sustain a pro-inflammatory local environment through their signaling and pyrogenic activities. Additionally, inflammasome activation promote the cleavage of gasdermin D resulting in membrane pore formation that if sustained lead to cell death through pyroptosis.[54] In particular, in macrophage the formation of gasdermin D pores has been shown to be transient and not associated with pyroptosis.[57] First characterized in immune cells, mainly macrophages during

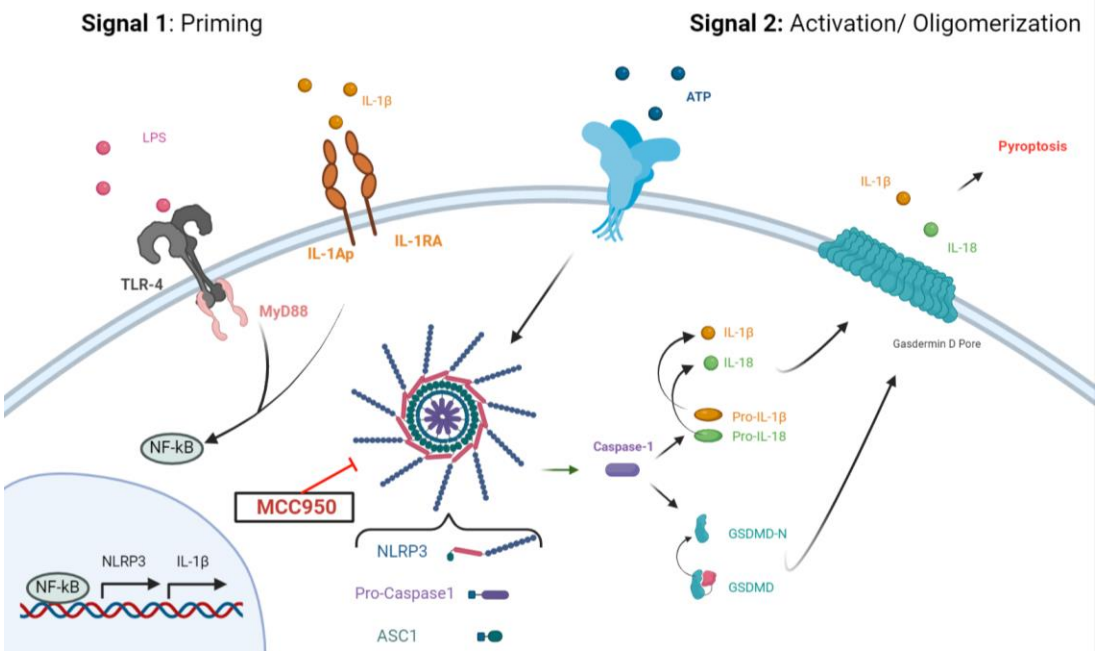

Figure 1. NLRP3 Inflammasome activation. NLRP3 inflammasome activation generally involves two signals: a priming signal e.g., LPS and TLR and an oligomerization signal e.g., ATP and P2XR7. As with other inflammasomes, NLRP3 inflammasome activation leads to cleavage and secretion of IL-1ß and IL-18 mature pro-inflammatory cytokines.

bacterial infections, inflammasomes include multiple cytoplasmic receptors e.g., NLRP1 to NLRP16 but also AIM2 with either activating or inhibiting effects on inflammation.[58] NLRP3 inflammasomes remains the most extensively studied.[53] Beside macrophage during bacterial infection, other cells including epithelial cells and tumor of epithelial origin have been shown to express and have functional inflammasomes.[59] Indeed, NLRP3 in particular (but also AIM2)

contribute to local inflammatory response in multiple cancers including colorectal and pancreatic cancer.[60, 61]

NLRP3 Inflammasome Activation

The signaling through inflammasome has been detailed mostly for the NLRP3 inflammasome activation as a two-signal process (Fig 1).[62] During the priming step, activation of pattern recognition receptors such as toll-like receptors (TLRs) or nod-like receptors (NLRs) by inflammatory stimuli cause these receptors to engage and induce nuclear translocation of NF-kB.[63] NF-kB activation then triggers transcription of multiple genes including NLRP3, ASC1, pro-IL-1ß, pro-IL-18. During the activation, NLRP3 inflammasome complex assemble induced by pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) such as lysosomal damage, potassium and calcium fluxes, reactive oxygen species (ROS), mitochondrial injury, and protein aggregates.[62] The assembled NLRP3 inflammasome complex trigger the autocleavage of pro-caspase-1 into active caspase-1, which through it enzymatic activity cleaves pro-inflammatory pro-cytokines IL-1ß and IL-18 into their mature ready-to-secrete form.[53, 58] Active caspase-1 also activates gasdermin D, a pro-pyric factor that forms pores in the cell membrane facilitating the secretion of IL-1ß and IL-18.[54]

Inflammasomes and Cancer

Overproduction of inflammasome effector molecules or constitutive activation of inflammasomes promote progression of several metabolic diseases and a variety human malignancy including colon, colorectal, breast, gastric, lung, and skin cancers.[64] In cancer progression, roles of inflammasomes including NLRP3 inflammasome are multiple. For example, in crosstalk between macrophages and colorectal cancer cells (CRC) NLRP3 activation supports

CRC migration and metastases to the liver through signaling by IL-1ß and other cytokines as demonstrated by *in vitro* blocking of NLRP3 signaling suppressing CRC cell migration.[61] Furthermore, patients with colorectal tumors displaying high NLRP3 expression had a poor prognosis.[61] Similarly, in lung adenocarcinoma and small-cell lung cancer, NLRP3 overexpression is associated with more advanced cancers.[65] In the pancreatic ductal adenocarcinoma orthotopic mouse model, NLRP3 inflammasome expression was upregulated leading to increased platelet activation and aggregation which promoted pancreatic tumor growth.[60] The pro-tumoral role of NLRP3 inflammasome in breast cancer has also been investigated.[66-68] Inflammasome activation and elevated IL-1ß expression were detected in breast tumor tissues.[67] Indeed, in breast cancer, IL-1ß secreted following NLRP3 inflammasome activation is associated with enhanced angiogenesis, tumor invasiveness, and metastasis.[48] The pro-inflammatory-rich tumor microenvironment also promoted the infiltration of myeloid cells and TAMs which in turn maintained the local inflammation favoring breast cancer progression.[68]

Inhibitor of inflammasomes

Recent evidence suggest that inflammatory diseases including cancer can be treated more effectively by targeting the NLRP3 inflammasome. [69] The NLRP3 inflammasome inhibitor, MCC950, has been demonstrated to promote improvement in several pre-clinical models including neuro-inflammatory diseases, colitis, and head and neck squamous cell carcinoma.[70-72] The molecular mechanism of MCC950 involves binding to NLRP3 at the NACHT domain preventing NLRP3 conformational change and oligomerization, ultimately blocking the release of IL-1β induced by NLRP3 activators such as ATP and monosodium urate crystals (MSU).[73, 74] While other direct inhibitors of the NLRP3 inflammasome exist such as CY-09, OLT1177, 3-4-

methylenedioxy-β-nitrostyrene, and Oridonin, thus far MCC950 is considered to be one of the most selective inhibitors of the NLRP3 inflammasome.[75-77]

RATIONALE, EXPERIMENTAL APPROACH, and SPECIFIC AIMS

Rationale, Innovation, Potential Benefits

Tumor development and progression is associated with the ability of cancer cells to subvert inflammatory signaling molecules to potentiate cell invasion, proliferation, and migration.[78] Inflammasomes, especially NLRP3 have been studied for their role in promoting inflammation during various infections, diseases and more recently cancers especially colitis-associated colorectal cancer.[61] Macrophages are the most abundant innate immune cell found in the tumor microenvironment and elevated infiltration of tumor-associated macrophages is associated with tumor growth, invasion, and metastasis.[79, 80] Our previous work confirms the presence of activable NLRP3 inflammasomes in macrophages, however, the role of macrophage inflammasome activation in the progression of breast cancer remains poorly understood. Our results further the understanding of the effects of inflammasome activation on the tumor-promoting activities of macrophages.

It is unclear whether crosstalk between tumor cells and macrophage inflammasome activation promote breast tumor growth and metastasis. Our research aimed to further our understanding of the interplay between tumor cells and macrophages and their implications on tumor development. Besides providing a better understanding of the role of inflammasome activation and tumor promoting activities of macrophages, the innovation of this research is in the assessment of NLRP3 inflammasome antagonist MCC950 in reducing secretion of pro-

inflammatory IL-1ß and IL-18 cytokines involved in tumor-promoting inflammation and in reducing the pro-tumorigenic activities of tumor-associated macrophages.

The goal of this research is to further our understanding of the role of inflammasome activation in breast cancer progression and whether there is a link between tumor cells and macrophage crosstalk in inflammasome-driven inflammation and tumorigenesis. Specifically, this research aims to investigate the effect of NLRP3 inflammasome activation on tumor cell proliferation, macrophage polarization, and phagocytic activity within the breast cancer microenvironment. Gaining further insight into this may contribute to the development of novel immunotherapeutic approaches targeted at inflammasome activation for treating and preventing breast cancer progression.

Experimental Approach

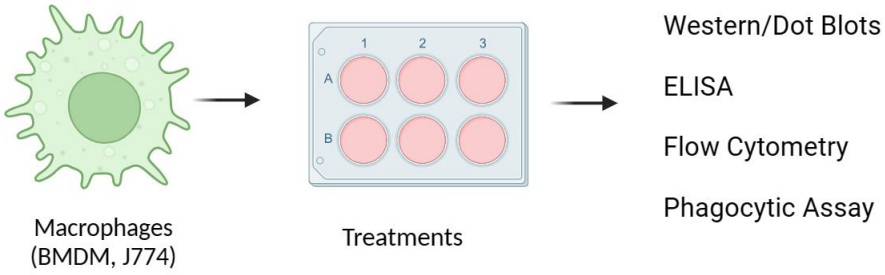

Figure 2. Schematic of the experimental approach used.

While *in vivo* models are more relevant especially when assessing metastasis, the numerous compounding variables render any detailed investigations of cell-cell interactions within the tumor microenvironment challenging. In contrast, *in vitro* cell models allow assessments of specific and multiple interactions between cell types, extracellular matrices, and soluble factors mimicking specific aspects of the tumor microenvironment and enabling the control of multiple variables.[81]

In addition to our previous work,[82-86] *in vitro* mammalian cell models have provided numerous demonstrations of the appropriateness and relevance of *in vitro* models to study cell-cell interactions including cell phenotypes, secretions, proliferations, and/or apoptosis following alterations of one or more parameter(s).[87-89]

Hypothesis

We hypothesize that breast tumor cell secretomes activate NLRP3 inflammasome in macrophages resulting in both increased pro-inflammatory secretions and macrophage polarization toward an immunosuppressive phenotype thereby generating a pro-tumorigenic microenvironment favoring breast tumor growth.

Specific Aims

In Specific Aim 1, we determined the macrophage inflammasome activation as defined by NLRP3, ASC1 and CASP1 protein expression generated by known activators and by mammary tumor cell secretomes. Furthermore, we characterized macrophage inflammasome functional activities by measuring the secretions of IL-1ß and IL-18 pro-inflammatory cytokines and assessing the effect of inflammasome inhibitors on these activities. Aim 1 hypothesis is that the breast tumor microenvironment stimulates inflammasome activation in macrophages and promotes a pro-inflammatory response by macrophages.

In Specific Aim 2, we determined the alterations in macrophage phenotype and phagocytic activity in the presence of known activators and tumor cell secretomes. We characterized macrophage phenotype by measuring the expression of markers associated with the M2 macrophage immunosuppressive phenotype. Phagocytic activity of macrophages was also assessed in the presence of known activators and tumor cell secretomes. Furthermore, we assessed

the effect of inflammasome inhibitors on macrophage phenotype and phagocytosis activities. Aim 2 hypothesis is that the breast tumor microenvironment promotes less phagocytic and M2-like phenotype macrophages.

CHAPTER 2: METHODOLOGY.

Media and cell cultures

Cells were grown and cultured in DMEM supplemented with antibiotic, antifungal and 10% FBS (Atlanta biologic, Atlanta, GA). J774 (obtained from ATCC, Manassas, VA) and BMDM (isolated and differentiated as previously)[90] cells were incubated in FBS-free media (0% FBS) for 3 hours and then treated with negative control (FBS-free media), positive control (LPS 5ug/ml + ATP 5mM) and 4T1 conditioned media. Cells were then harvested for flow-cytometry and immunohistochemistry analysis. Both cell lysates and supernatants were collected and stored at -20°C until use in Western dot blots and cytokine measurements.

Flow-cytometry

Flow cytometry analyses were conducted as previously.[82] Briefly, post-treatment cells were collected and fixed using buffered formalin (Boston Biologics) and stored at 4°C until use. For the detection intracellular proteins (e.g., NLRP3, ASC1) cells were permeabilized using 0.1% saponin. Cells were then incubated in blocking buffer (1% BSA in TBST (0.05% Tween 20 in Tris-buffered saline (TBS)) and incubated next with cocktail of primary antibodies against NLRP3, ASC1, Active caspase 1, CD206, MHC-Class II (all obtained from R&D Systems or Santa Cruz Biotechnology). After incubation, cells were washed with TBST and incubated with specie-specific secondary antibody conjugated with different fluorophores (e.g., FITC or 488; Texas Red or 590; APC or 633). Following additional PBS washes, cells were diluted in PBS and stored until ran on a BD Fortessa flow-cytometer (BD Biosciences). Controls included cells incubated with secondary antibody only. Changes in percent of cells expressing the protein of interest were recorded and presented.

Immunoblot Analyses

Western blots/dot blots: Following treatment, macrophage cell lysates and macrophage supernatants were combined with SDS (0.25%) in PBS (150mM), and loaded onto a nitrocellulose membrane (Millipore, 45 μm pores) using a dot-blot apparatus (Thermo Fisher). After washing in TBST (0.05% Tween 20 in Tris-buffered saline (TBS)), membranes were assessed for protein loading using Ponceau reversible protein stain (Sigma) and then blocked in 5% milk TBS-Tween 20 buffer (Boston Biologicals). After blocking, dot-blots were incubated with primary antibodies against cleaved (active form) caspase-1, ASC1, NLRP3, active IL-1β, IL-18, CD206, CD163, EGF, iNOS, Arginase, IL-10, PD-PDL1 (all obtained from R&D Systems or Santa Cruz Biotechnology) overnight at 4°C under gentle rocking. Following removal of primary antibodies and washes in TBST buffer, blots were incubated with species-specific secondary horse-radish peroxidase (HRP) conjugated antibody for 1 hour in similar conditions. After secondary antibody removal and multiple TBST buffer washes, membranes were incubated with an electrochemiluminescence (ECL) substrate (Biorad) and detection using the MP Bioimager (Bio-Rad) to visualize presence of proteins of interest. Protein signal was quantified using Image J and the Protein Array Analyzer plugin. Protein expressions were normalized to protein loading (Ponceau staining) and control conditions.

Phagocytosis Assays

Macrophages were grown to confluency in 96-well tissue culture plates and incubated for 6 hours with either negative control (media alone), positive control (LPS 5ug/ml + ATP 5mM) conditions and in the presence of 4T1 conditioned media along with dsRED fluorescent polymer beads (1mm in diameter; Ex/Em:542/612nm ThermoFisher) and the nuclear dye Hoechst 33342 (Ex/Em:350/461nm; Tocris). After repeated washes to remove non-phagocytosed beads, cells were

briefly (30-45 min) incubated with media. Beads (red) and nuclei (blue) fluorescence were measured using ID5 (Molecular Devices) system and phagocytosis index i.e., the fluorescence bead to cell (i.e., Hoechst) ratios derived. Additionally, collected cells were fixed and ran through the Fortessa flow cytometer and proportions of cells having engulfed one or more beads to cells that did not engulf any bead ratios determined.

Tumor Cell Proliferation Assays

Effects of IL-1β, IL-18 on 4T1 tumor cell proliferation were assayed using MTT assays. Briefly, 4T1 cells were cultured in control conditions (FBS free media only) or supplemented with IL-1β, IL-18 or the combination IL-1β and IL-18. After 44.5 hours, Hoechst 33342 (Tocris) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) were added to cell media and cells incubated for an additional 3.5 hrs. Increase in Hoechst 33342 fluorescence associated with increase in cell number was determined by ID5 fluorescence determination (arbitrary unit (AU). In addition, the reduction of MTT (yellow) to formazan crystals (purple) a measure the cell metabolic activity was assayed following cell lysis and solubilization of the formazan crystals as previously. Data collected were normalized to control conditions.

Statistical Analysis

All experiments included controls and utilized independent repeats ($n \geq 3$). Graphs were generated and statistical analyses were conducted using Prism 10.0 (GraphPad). Measurements are presented as Mean ± SEM and differences between groups were analyzed using one-way or two-way ANOVAs and Fisher's LSD test unless noted otherwise with a $p<0.05$ significance threshold.

CHAPTER 3: RESULTS.

1- 4T1 Tumor Cell Secretions Promote Macrophage NLRP3 Complex Protein Expression

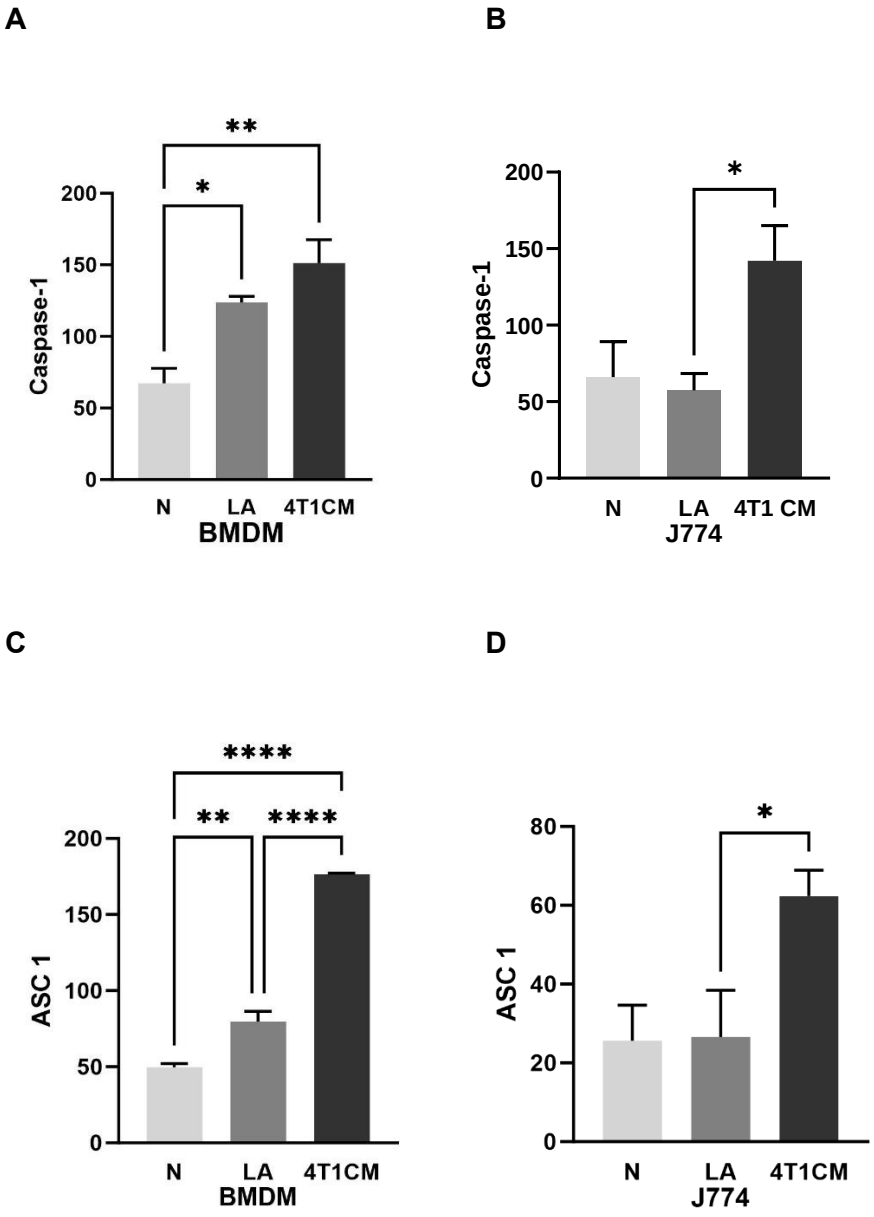

Figure 3. 4T1CM promotes expression of NLRP3 inflammasome proteins in macrophages. BMDM and J774 cells were incubated in culture media alone (N) or supplemented with the inflammasome activator LPS and ATP (LA), or with 4T1 tumor cell conditioned media (4T1CM). Following cell harvesting, BMDM and J774 cell expressions of active Caspase 1 **(A,B)** and ASC1 (**C,D**) were determined by Western/Dot blots. Data normalized to protein loading and presented as mean±SEM. Differences between treatments were tested using one-way ANOVA and Tukey post-hoc tests with *P<0.05, **p<0.01 and *** p<0.001.

First, we determined the expression of NLRP3 protein components and NLRP3 inflammasome activities of bone marrow derived macrophages (BMDMs) and J774 macrophages. BMDM and J774 cells were treated with a combination of LPS and ATP, a known inflammasome activator and 4T1 tumor cell conditioned media (4T1 CM) and the expression of NLRP3 inflammasome proteins were determined. Regardless of treatment, all macrophages expressed the NLRP3 protein (not shown). All macrophages also expressed active caspase 1 regardless of treatment, however, there was a significant increase in expression following treatment with the combination LPS and ATP and a significant two-fold increase following treatment with 4T1 CM in BMDM cells ($p<0.05$, Fig 3A). J774 cell expression of active caspase also resulted in a significant two-fold increase following incubation with 4T1 CM ($p<0.05$, Fig 3B).

ASC1 expression was significantly increased following treatment with the LPS and ATP combination and increased roughly by 3.5-fold with 4T1CM in BMDM ($p<0.05$, Fig 3C). In contrast, J774 cell ASC1 expression was unaffected by LA treatment and increased significantly only following incubation with 4T1CM ($p<0.05$, Fig 3D).

2- 4T1 Tumor Cell Secretions Promote Macrophage Proinflammatory Cytokine Secretions

Next, we assessed secretions of pro-inflammatory cytokines IL-1β and IL-18 by inflammasome-activated BMDM and J774 cells. Both the LPS and ATP combination and 4T1 CM treatments significantly promoted the secretion of the pro-inflammatory IL-1β cytokine by BMDM and J774 cells ($p<0.05$, Fig 4AB). Similarly, LPS and ATP combination and 4T1 CM significantly promoted the secretion of IL-18 by both BMDM and J774 cells ($p<0.05$, Fig 4)

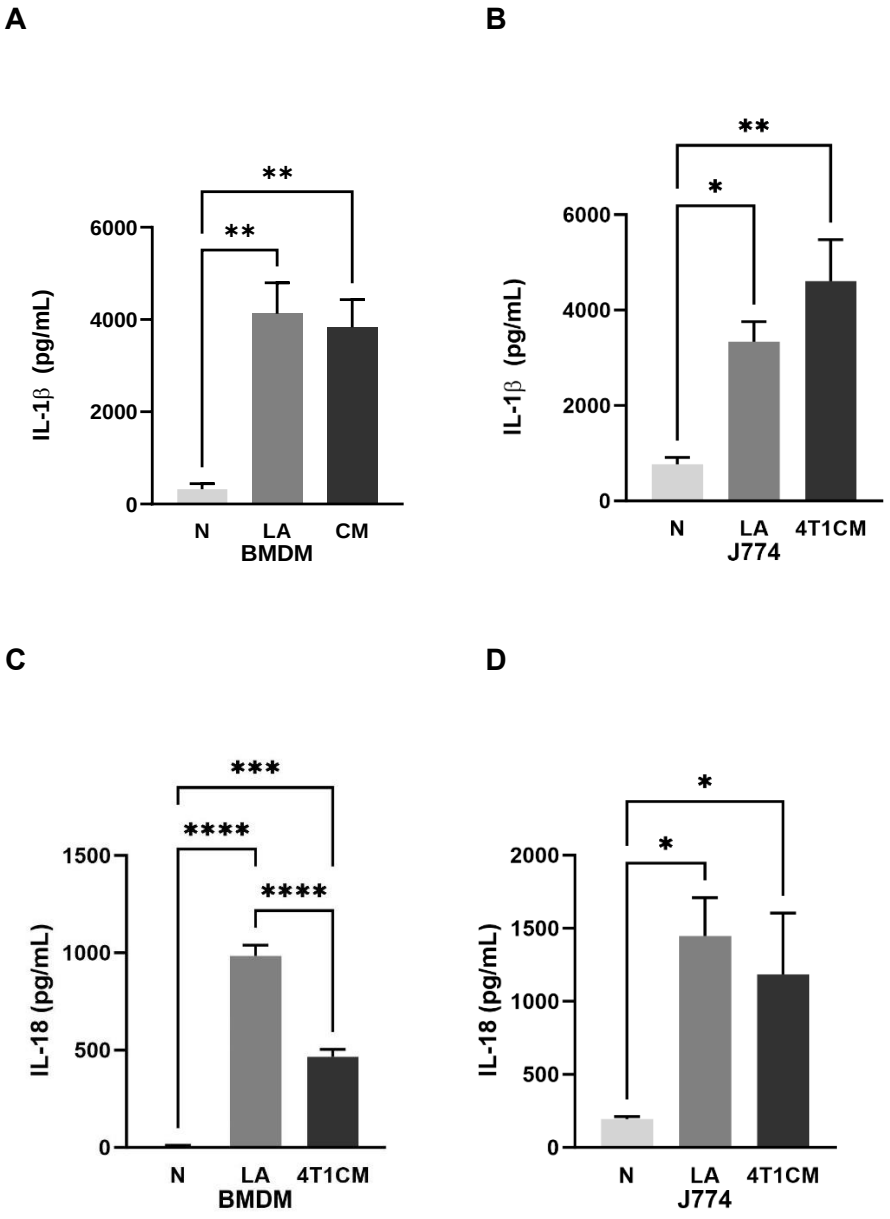

Figure 4. ***4T1 CM promote IL-1β and IL-18 pro-inflammatory cytokine secretions by macrophages.*** BMDM and J774 cells were incubated in culture media alone (N) or media supplemented with LPS and ATP (LA), or with 4T1 tumor cell conditioned media (4T1CM). BMDM and J774 cell supernatants were obtained and concentrations of IL-1β (**A,B**) and IL-18 **(C,D)** determined by ELISAs (expressed as mean ± SEM in pg/mL). Differences between treatments were tested using one-way ANOVA and Fisher's LSD with ***p<0.001, **p<0.01, *p<0.05.

3- 4T1 Secretome Promoted a M2-like Macrophage Phenotype

We then assessed whether NLRP3 inflammasome activation led to the generation of M2-like pro-tumorigenic macrophages. Expression of markers associated with the M2 macrophage

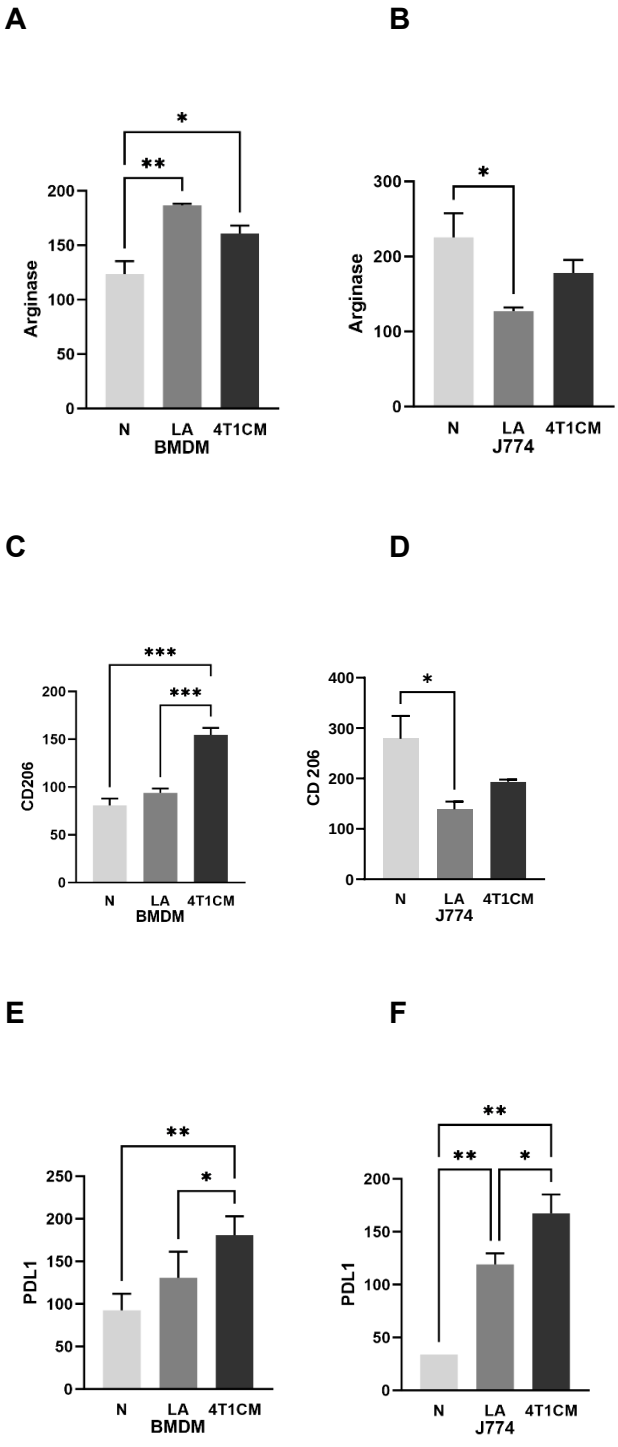

Figure 5. ***4T1 CM-driven Macrophage NLRP3 Inflammasome Activation is associated with a Pro-tumorigenic Macrophage Phenotype.*** BMDM and J774 cells were incubated in culture media alone (N) or supplemented with the inflammasome activator LPS and ATP (LA) or with 4T1 tumor cell conditioned media (4T1CM). Following cell collection and lysis, BMDM and J774 cell expressions of Arginase **(A, B)** CD206 **(C, D)** and PDL1 **(E, F)** were determined by Western/Dot blots. Data normalized to protein loading and presented as mean±SEM. Differences between treatments were tested using one-way ANOVA and Tukey post-hoc tests with *P<0.05, **p<0.01 and *** p<0.001.

phenotype [19] were assessed following treatment with the inflammasome activation combination LPS and ATP and 4T1 conditioned media. Both inflammasome activating LPS and ATP combination and 4T1 conditioned media significantly increased arginase production in BMDM cells ($p<0.05$, Fig 5A). In contrast, J774 cell arginase expression decreased following both LPS and ATP combination and 4T1 conditioned media treatment (Fig 5B).

The expression of M2 phenotype marker CD206 was also determined. In BMDM, CD206 expression remained unchanged following LPS and ATP treatment compared to control conditions (Fig 5C). However, following treatment with 4T1 conditioned media, BMDM's displayed significantly increased expression of CD206 ($p<0.001$, Fig 5C). In contrast, J774 macrophages displayed a significant decrease in CD206 expression ($p<0.05$, Fig 5D) following LPS and ATP inflammasome activation treatment. The expression of the M2 marker programmed death ligand-1 (PDL1), assessed following LPS and ATP combination and 4T1 conditioned media treatments, was significantly increased in both BMDM and J774 macrophages ($p<0.01$, Fig 5EF).

Beside cell surface expression, some markers associated with M2 macrophage phenotype including CD163 and CD206 are shed.[91, 92] Therefore, we also assessed BMDM and J774 macrophage supernatants for soluble/shed CD163 (sCD163) and CD206 (sCD206). There was a significant increase in shed CD163 levels following both LPS and ATP combination and 4T1 conditioned media treatment in BMDM's ($p<0.01$, $p<0.001$ Fig 6A). Expression of shed CD163 also significantly increased following treatment with inflammasome activating LPS and ATP combination in J774 macrophages ($p<0.05$, Fig 6B). In contrast, following incubation with 4T1 conditioned media, J774 cell sCD163 expression significantly decreased ($p<0.05$, Fig 6B).

Following incubation with 4T1 conditioned media but not in the presence of the LPS-ATP cocktail, BMDM sCD206 expression was significantly increased ($p<0.0001$, Fig 6C). In contrast,

J774 sCD206 expression remained similar to control conditions following either LA or 4T1CM treatments (ns, Fig 6D).

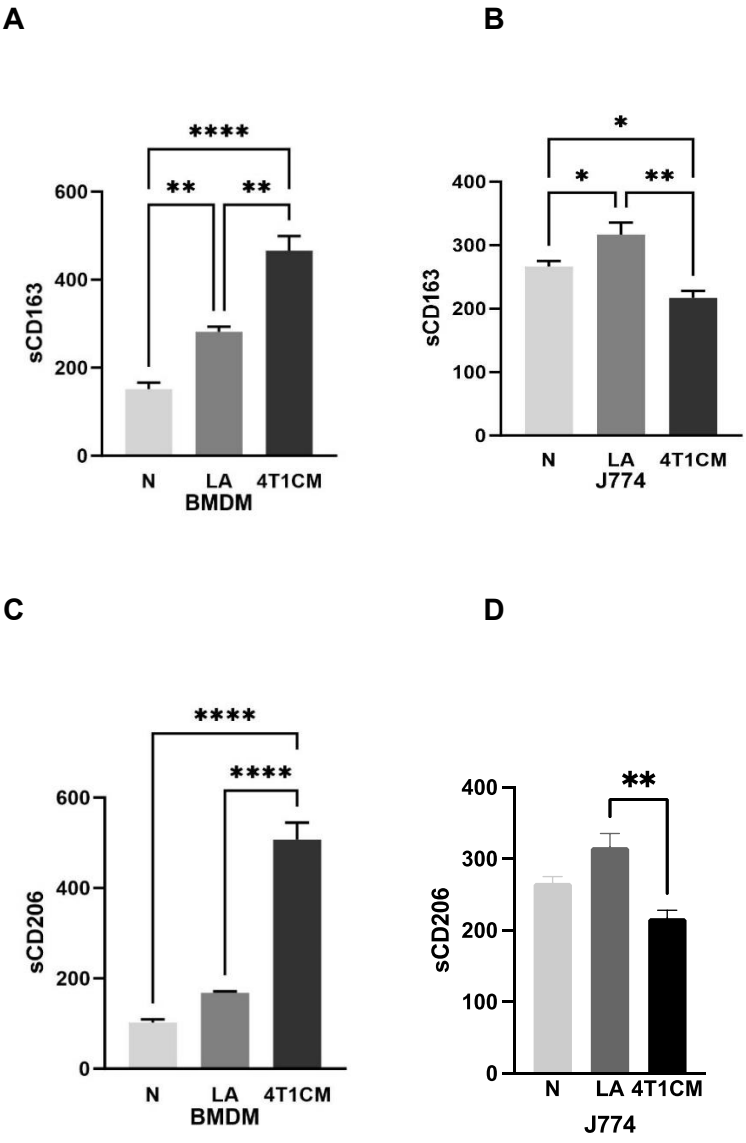

Figure 6. ***Shed CD163 and CD206 increased following Macrophage Inflammasome Activation.*** BMDM and J774 cells were incubated in culture media, media supplemented with the LPS and ATP combination, or with 4T1 tumor cell conditioned media. Cell supernatants were obtained and shed **(A)** sCD163 and **(B)** sCD206 were determined following immunoblots. Treatment groups were compared through one-way ANOVA and Fisher's LSD with ***p<0.001, **p<0.01, *p<0.05.

4- 4T1 Secretome Triggered Decreases in Macrophage Phagocytic Activities

M2 macrophages display decreased phagocytic activities.[93] [94] Thus, we also determined the phagocytic activities of J774 and BMDM cells through measurements of the engulfing of 1mm dsRed fluorescent beads. Treatments with both inflammasome activator LPS and ATP cocktail and 4T1 conditioned media led to significantly reduced bead phagocytosis by BMDM and J774 cells ($P<0.001$, Fig 7AB). In addition, as determined by flow-cytometry, the ratio of cells that engulfed beads to cells that did not was significantly lower when J774 cells were incubated with either 4T1CM alone or combined with ATP ($P<0.01$, Fig 7C).

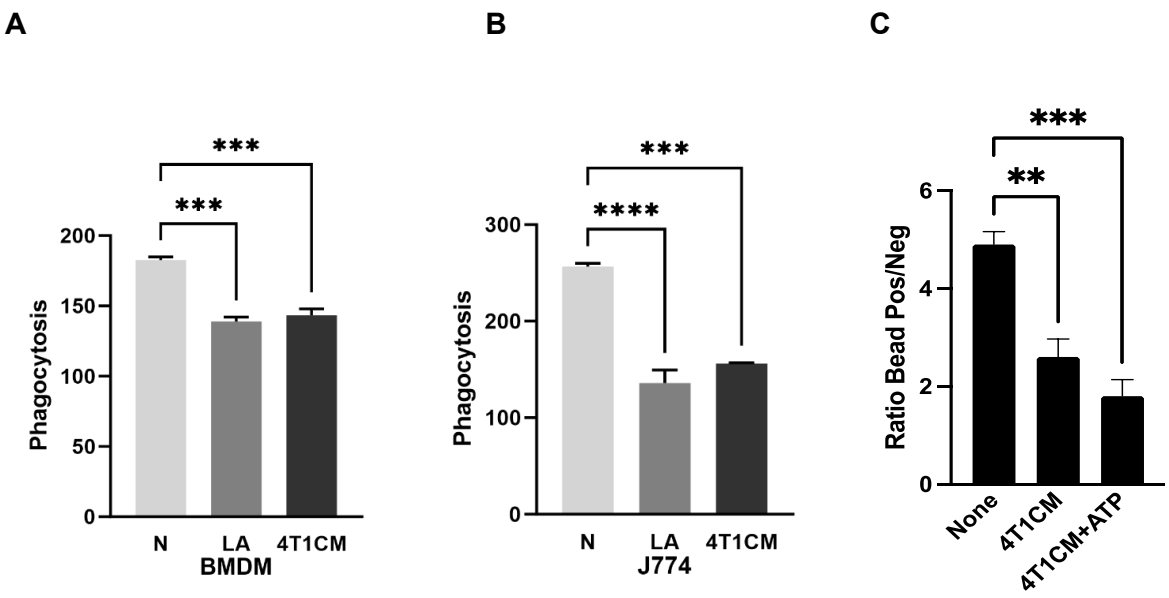

Figure 7. ***4T1 secretomes promote reduced macrophage phagocytic activities.*** Phagocytic activities as determined by 1mm fluorescent bead engulfing by BMDM **(A)** J774 **(B, C)** cells determined by fluorescence measurements and normalized cell numbers determined through Hoechst-stain nuclear fluorescence were significantly decreased following both LPS+ATP combination and 4T1 CM treatment. (**C**) Moreover, by flow-cytometry, the ratio J774 macrophages that engulfed beads to J774 macrophages that did not engulf bead was significantly decreased following 4T1 CM and 4T1CM+ATP treatments. Treatment groups were compared through one-way ANOVA and post-hoc tests with ***$p<0.001$, ****$p<0.0001$.

5- Pro-inflammatory IL-1β and IL-18 Cytokines Activate 4T1 Tumor Cell Proliferation

Multiple stroma secretions present within a pro-inflammatory microenvironment promote tumor growth.[95-97] Thus, we assessed whether specifically increasing concentrations of pro-inflammatory cytokines IL-1β and IL-18 individually or combined promoted 4T1 tumor cell proliferation (Fig 8). Both IL-1β and IL-18 increasing concentrations led to increases in 4T1 cell proliferation (Fig 8A). In addition, 4T1 proliferation was dose-dependent and increased with IL-1β concentration (Fig 8B, p=0.0004). In contrast, combinations of IL-1β: IL-18 (1:1) were not associated with significant proliferation (Fig 8A).

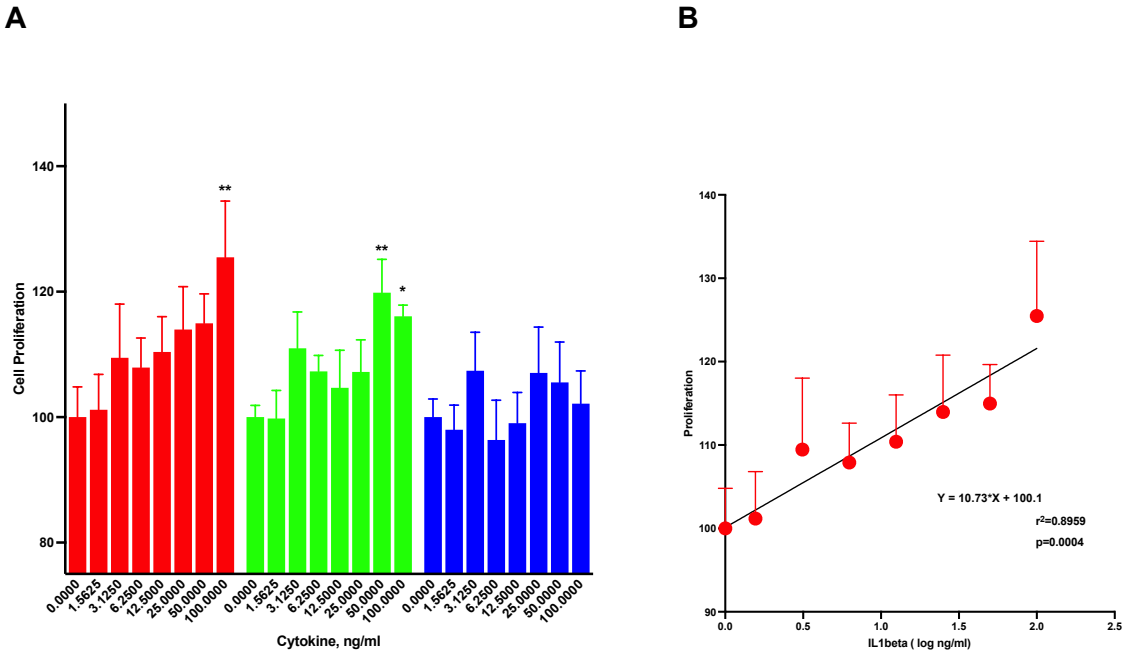

Figure 8. IL-1β dose-dependently promoted 4T1 mammary tumor cell proliferation in vitro. Briefly, 4T1 cells were incubated 48hrs with increasing concentrations (0-100 ng/ml) of IL-1β (red), IL-18 (green), and 1:1 of IL-1β: IL-18 (blue) and (**A**) proliferation determined using nuclear intercalating stain Hoechst. Difference between treatment tested by ANOVA and post-hoc tests (**p<0.01 and *p<0.05). (**B**) IL-1β increasing concentrations correlated with an increase in 4T1 cell proliferation (r^2=08959; p=0.0004).

6- MCC950 Altered Pro-inflammatory IL-1β and IL-18 Secretions in Inflammasome-Activated Macrophages

To determine whether NLRP3 inflammasome specifically was involved in the inflammasome activation triggered by LA and 4T1CM, we assessed whether the NLRP3 specific inhibitor MCC950 [74, 98] prevented inflammasome-associated pro-inflammatory cytokine secretions by both BMDM and J774 macrophages (Fig 9).

IL-1β secretions by both BMDM and J774 cells following LPS and ATP cocktail incubation were significantly decreased when also treated with MCC950 ($p<0.01$, Fig 9AB). The decrease was marginal or not observed in BMDM and J744 cells supplemented with 4T1 conditioned media and treated with MCC950, respectively (Fig 9AB). The effects of MCC950 treatment of IL-18 secretions following incubation with either LPS and ATP combination or 4T1CM were limited (Fig 9CD) although IL-18 secretions by BMDM significantly increased in the presence of LA and MCC950 treatments ($P<0.01$, Fig 9C).

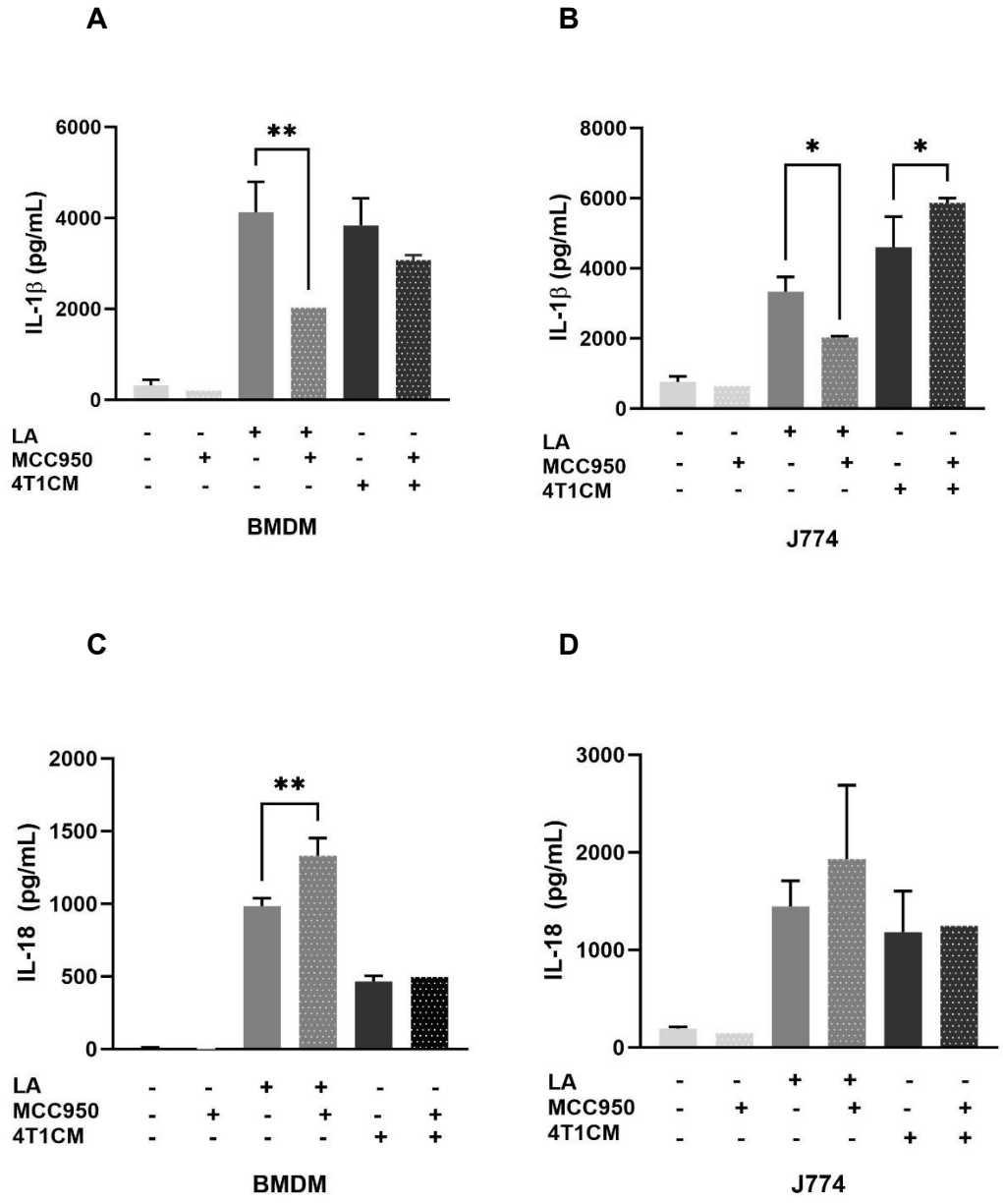

Figure 9. ***The NLRP3-Specific Inflammasome Inhibitor, MCC950 reduced IL-1β secretions by macrophages treated with LPS and ATP.*** BMDM and J774 cells were incubated in culture media alone (N) or media supplemented with LPS and ATP (LA), or with 4T1 tumor cell conditioned media (4T1CM) and treated with and without inflammasome inhibitor MCC950. Cell supernatants were obtained and IL-1β **(A, B)** and IL-18 **(C, D)** secretions (pg/mL, average SEM) were determined by ELISAs. treatment groups were compared through one-way ANOVA and Fisher's LSD with ***p<0.001, **p<0.01, *p<0.05.

7- MCC950 Blunts the Pro-tumorigenic Phenotype of Inflammasome-activated Macrophages

Inflammasome activated BMDM cells through incubation with 4T1 tumor cell conditioned media display increased arginase and CD206 expressions that were blunted with the addition of MCC950 ($P<0.05$; Fig 10AB).

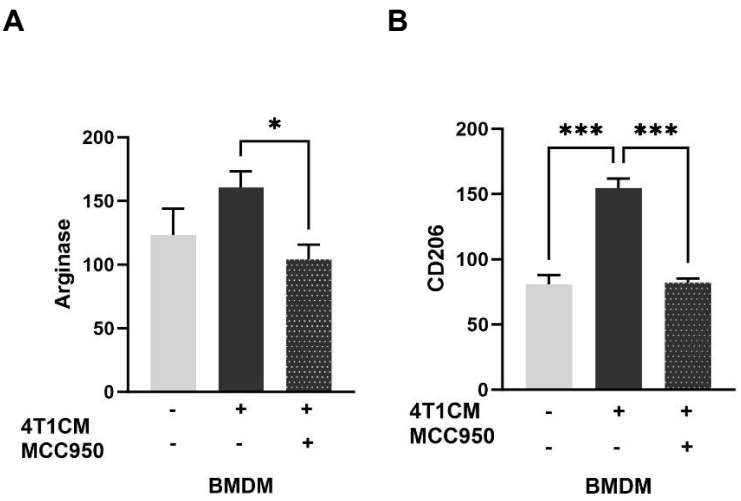

Figure 10. The NLRP3 inflammasome inhibitor *MCC950 limits the pro-tumorigenic phenotype of BMDM cells.* BMDM cells incubated with media alone, LPS+ATP, or secretomes from 4T1 tumor cells were treated with and without the NLRP3 inflammasome inhibitor MCC950 and expressions of Arginase **(A)** and CD 206 **(B)** were determined by Western/dot blots and compared by one-way ANOVA and Tukey post-hoc tests with **$p<0.001$ and *$p<0.05$.

8- MCC950 Treatment Restores Phagocytic Activity in Inflammasome-Activated Macrophages

As shown earlier, inflammasome-activated BMDM and J774 cells had reduced phagocytosis (see Fig 7 above) and thus a reduced phagocytic index. When cells were inflammasome activated in similar conditions, that is, either in the presence of ATP alone, LPS+

ATP cocktail and 4T1CM + ATP or 4T1CM + LPS+ ATP cocktail and then the NLRP3 inflammasome inhibitor MCC950 was added, phagocytosis was restored (P<0.05; Fig 11AB).

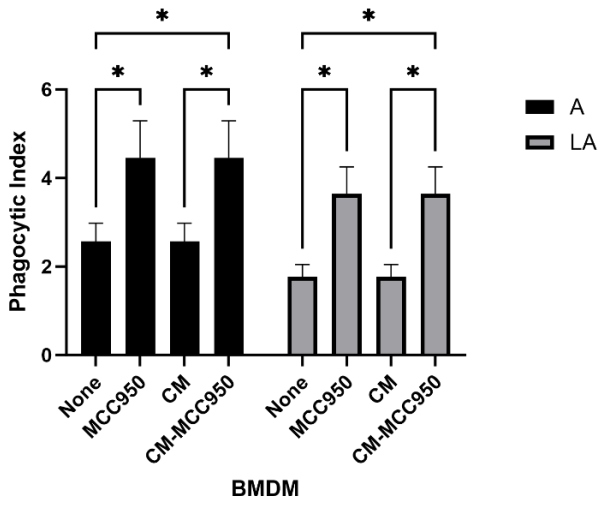

Figure 11. ***MCC950 restores phagocytic activity in NLRP3 inflammasome activated in BMDM macrophages.*** Briefly, BMDMs were incubated with 4T1 tumor cell conditioned media + ATP or 4T1 tumor cell conditioned media + ATP+LPS and treated with and without MCC950. The phagocytosis of fluorescent beads was determined after a 6hr incubation by normalizing the bead fluorescence (red) to the nuclei present (Hoechst stained) measured using the ID5 plate reader (Molecular Devices). Differences between treatments were tested by one-way ANOVA and Tukey post-hoc test with *p<0.05, **p<0.01.

CHAPTER 4: DISCUSSION.

Breast cancer progression is favored by a pro-inflammatory, pro-tumorigenic microenvironment,[99, 100] and recently the role of macrophage NLRP3 inflammasome activation has been implicated in the generation of a tumor microenvironment that promotes tumor growth.[60, 67] Tumor-associated macrophages are a prognostic indicator of invasive disease and poor overall survival in breast cancer and the presence of TAMs with activated NLRP3 inflammasome have been associated with primary tumor growth and metastases.[48, 101, 102] However, the mechanisms associated with macrophage polarization and activities promoting a pro-inflammatory tumorigenic microenvironment are not fully understood. Here, we assessed the hypothesis that that breast tumor cell secretomes activate NLRP3 inflammasome in macrophages resulting in both increased pro-inflammatory secretions and macrophage polarization toward an immunosuppressive phenotype thereby generating a pro-tumorigenic microenvironment favoring breast tumor growth. Using *in vitro* model systems, the effects of the combination LPS and ATP, a known inflammasome activator, and of 4T1 tumor cell secretome were assessed on both BMDMs and J774 macrophage phenotype and activities. In addition, the effects of the specific NLRP3 inflammasome inhibitor MCC950 with and without LPS+ATP or 4T1 conditioned media on macrophage inflammasome activation and polarization were assessed *in vitro.* Our results demonstrate that (1) 4T1 tumor cell secretomes promote macrophage inflammasome activation and pro-inflammatory secretions; in turn, (2) secreted IL1ß and IL18 pro-inflammatory cytokines promote breast tumor cell proliferation. Moreover, (3) tumor cell secretions promote polarization of macrophages toward an immunosuppressive and tumor-promoting phenotype and critically (4) a marked decrease in phagocytic activities. Importantly, (5) the specific NLRP3 inflammasome inhibitor MCC950

drastically prevented the phenotype alterations and pro-inflammatory secretions albeit to a lesser extent, and phagocytosis decrease triggered by the 4T1 secretome.

Assessment of cell-cell interactions of multiple cell types is challenging *in vivo* as the signaling interplays between stroma cells, tumor cells, and the extracellular matrix including components within the TME is complex.[103] Thus, *in vitro* cell models are routinely used to mimic specific aspects of the tumor microenvironment including multiple interactions between varying cell types, soluble factors, and components within extracellular matrices.[81] Here, we utilized the *in vitro* models to mimic the interactions of macrophages with tumor cells using. Both primary macrophages i.e., BMDMs and J774 macrophages along with syngeneic 4T1 mammary murine tumor cells were assessed to ascertain the role of inflammasome activation in macrophage phenotype, cytokine secretion, and phagocytosis activities. We also verified the effects of pro-inflammatory cytokine in tumor cell proliferation and demonstrated the role of NLRP3 inflammasome specifically.

First, congruent with our previous work, our data indicate that macrophage NLRP3 inflammasome activation is triggered not only by the inflammasome activating cocktail LPS+ATP, but also by 4T1 mammary tumor cell secretions.[90] NLRP3 inflammasome activation results in macrophage secretion of pro-inflammatory cytokines IL-1β and IL-18.[67] Our results demonstrate that macrophage IL-1β and IL-18 secretions were triggered by 4T1 tumor secretomes. Indeed, conditioned media from tumor cells including 4T1 tumor cells, increased IL-1β secretions in BMDMs *in vitro*.[104, 105] Within the TME, as breast cancer cells secrete only IL-1β low concentrations,[104] activated macrophages are the main source of pro-inflammatory cytokine secretions. Our data support a role for tumor secretomes in triggering macrophage NLRP3

activation leading to subsequent secretion of pro-inflammatory cytokines that contribute to a pro-inflammatory tumor-microenvironment including through direct tumor proliferation activation.

Second, our results highlight that both IL-1β and IL-18 individually and dose-dependently promoted the proliferation of 4T1 tumor cells. Indeed, IL-1β has been demonstrated to promote tumor growth and the capacity of cells to metastasize in a pre-clinical model for invasive breast cancer.[106, 107] Moreover, pro-inflammatory cytokine secretions have been shown to promote tumor growth and metastasis in other cancers including carcinogen-induced sarcoma and liver cancer.[108, 109] In contrast, lowered IL-18 expression is associated with an increase in in several colorectal cancers and ovarian cancer.[110-112] Interestingly, the combination of both IL-1ß and IL-18 at a ratio 1:1 was not associated with similar promotion of cell proliferation indicative of additional regulatory levels including those associated with receptor subtype expression, as well as IL-18BP and IL-1RA neutralizing mechanisms. [113, 114]

Third, our data provide evidence that inflammasome activation triggered by 4T1 CM promotes a pro-tumorigenic macrophage phenotype as highlighted by an increase in expression of cell surface proteins CD206, PD-L1, arginase and shed molecules sCD206 and sCD163 associated with the M2 macrophage subtype.[115] Both CD206 and CD163 expressed onto the plasma membrane of macrophages are shed through a TNF-α activated TACE-related shedding process and increases in sCD206 and sCD163 associated with M2 macrophage polarization.[92, 116 117] Our data confirm the polarization toward the M2 macrophage phenotype associated with tumor growth as demonstrated previously.[118, 119]

Fourth, our data show that incubation with the tumor secretomes also decreases both BMDM and J774 macrophage phagocytic activities as did the canonical inflammasome activator i.e., the LPS and ATP cocktail. This decrease is consistent with similar phagocytic activity

decrease recorded with M2 macrophages.[120] These results further support the critical role of the interplay between tumor cell secretions and macrophages in the macrophage phenotype polarization toward a pro-tumorigenic phenotype that favor tumor progression.

Fifth, our data notably highlight the involvement of the NLRP3 inflammasome specifically in tumor secretome driven macrophage activities. Indeed, when incubating tumor secretome activated macrophages with the specific NLRP3 inflammasome inhibitor MCC950,[98] pro-inflammatory cytokine secretions, phenotype alterations, and phagocytic activities were to a large extent similar to those observed in control conditions. Our observations support a key role of NLRP3 inflammasome in macrophages as demonstrated by decrease in IL-1β secretion activated by the cocktail LPS + ATP as highlighted previously.[71, 72] As the effects of 4T1 secretomes were not as potently inhibited by the NLRP3 inflammasome specific MCC950 treatment, 4T1CM may activate other member(s) of the inflammasome family.[70] Indeed, although the NLRP3 inflammasome remains the inflammasome mainly activated in cancer, other inflammasomes including AIM2, NLRP1 and NLRP4 have been implicated.[121-123] Additionally, other mechanisms including the involvement of TLRs or RIPK3 signaling also promote the secretion of IL-1β and IL-18 pro-inflammatory cytokines.[124] Interestingly, following MCC950 treatment macrophage phagocytic activity was markedly increased. This observation is in line with the key role of NLRP3 inflammasome activation in gearing macrophages toward a M2 phenotype.[115]

Overall, our data indicate that macrophage incubation with 4T1 tumor cell conditioned media promotes NLRP3 inflammasome activation, the subsequent secretion of pro-inflammatory cytokines IL-1β and IL-18 which dose-dependently promote tumor cell proliferation. Additionally, 4T1 tumor cell secretome treatment leads macrophages toward a pro-tumorigenic macrophage phenotype characterized by increased expression of proteins associated with the M2 phenotype

and critically decreased phagocytic activity.[24] Furthermore, our results show that in tumor secretome activated macrophages, treatments with NLRP3 inflammasome specific inhibitor MCC950 reduced blunted expressions of the M2 protein markers arginase and CD206 and rescued phagocytic activities supporting the involvement of NLRP3 inflammasome in tumor secretome activated macrophage pro-tumorigenic phenotype.

Future investigations will further our understanding of the crosstalk between tumor cells and macrophages. In light of the support for NLRP3 involvement in the generation of pro-tumorigenic macrophages, the NLPR3 targetable mechanisms in especially macrophages will be assessed. Additionally, the role of other inflammasomes including AIM2, NLRP1 and NLRP4 in macrophages but also in other stroma cells including fibroblasts and endothelial cells will be delineated as the entire TME participates in the promotion of breast cancer.

PRESENTATIONS

Acosta S., Roberson J., Dréau D., 2021. Inflammation, Inflammasome and breast cancer progression. Undergraduate Research Conference (Charlotte, NC, April 17, 2021) Poster presentation *(2022 URC Virtual*, OUR fellow).

Azar J., Roberson J., Dréau D., 2022. NLRP3 Inflammasome activation, stemness and breast cancer progression**.** Undergraduate Research Conference (Charlotte, NC, April 21, 2022) Oral presentation *(2022 URC Virtual).*

Reddy B., Roberson J., Dréau D., 2022. NLRP3 Inflammasome activation and PDL1 expression by macrophages and mammary tumor cells**.** Undergraduate Research Conference (Charlotte, NC, April 21, 2022) Oral presentation *(2022 URC Virtual).*

Almasian R., Roberson J., Dréau D., 2023. NLRP3 Inflammasome Activation of IL-25 Secretion by Macrophages Promotes a Pro-Tumorigenic Environment**.** Undergraduate Research Conference (Charlotte, NC, April 25, 2023) Oral presentation.

Moure K., Roberson J., Dréau D., 2023. NLRP3 Activation and Phagocytic Activity of Breast Tumor Macrophages**.** Undergraduate Research Conference (Charlotte, NC, April 21, 2022) Oral presentation.

Acosta S., Roberson J., Dréau D., 2023. Inflammasomes and Macrophage Phagocytosis in Breast Cancer Progression. Summer Symposium UNC Charlotte (Charlotte, NC, August 4th, 2023) Poster presentation.

Acosta S., Roberson J., Dréau D., 2023. Inflammasomes and Macrophage Phagocytosis in Breast Cancer Progression. [SNCURCS] State of North Carolina Undergraduate Research & Creativity Symposium (Wingate, NC, November 11th, 2023) Poster presentation.

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