FIBROBLAST-DRIVEN FIBROSIS AND INFLAMMATION IN BREAST CANCER PROGRESSION

by

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ABSTRACT

COURTNEY SAMUELS. Fibroblast-driven Fibrosis and Inflammation in Breast Cancer Progression. (Under the direction of DR. DIDIER DREAU)

Breast cancer progression is promoted by both local inflammation and fibrosis. Indeed, accumulation of fibrous collagen is an independent prognosis marker of breast cancer. However, the mechanisms underlying the interplay between fibroblasts, inflammation and tumor progression remain unclear. Our early observations highlighted a potential role of inflammasome activation in cancer associated fibroblasts in both local inflammation and fibrous collagen accumulation. Thus, in the L929 *in vitro* fibroblast model, we investigated whether inflammasome activation led to a cancer- associated fibroblast phenotype and whether such phenotype promoted pro- inflammatory cytokine release and/or fibrous collagen secretions. Our data indicate that activation of fibroblasts was associated with promotion of inflammasome especially NLPR3 inflammasome activation. Moreover, inflammasome activation was associated with a cancer- associated fibroblast (CAF) phenotype as demonstrated by increases in alpha smooth actin and vimentin expression. In addition, incubation with the NF-kB inhibitor MG132 underlined the critical role of NF-kB in the secretion of fibrous collagen. Our observations regarding the potential of the gasdermin inhibitor Disulfiram (DS) to alone prevent inflammasome driven activities were unconclusive. Interestingly, the NLRP3 inflammasome specific inhibitor, MCC950 altered both the expression and secretion of pro-inflammatory cytokines. With regard to fibrosis and collagen accumulation, blocking the NF-KB or the inflammasome activation but not the gasdermin D cleavage drastically altered the CAF phenotype in particular with regard to collagen subtype secreted and cytokines produced. Moreover, combination of MG132 and MCC950 reverted CAF phenotype and limited both pro-inflammatory cytokine secretion as well as fibrous collagen secretions. Taken together our data

suggest that L929 fibroblasts model CAFs *in vitro* and that fibrosis is in part triggered by NLRP3 inflammasome activation, and also actively involve the NF-kB signaling pathway. Future investigations will aim to further those observations and possibility target those pathways in stroma cells to prevent/limit breast cancer progression.

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INTRODUCTION

Clinical, Therapeutic, and Biological Approaches to Breast Cancer

While breast cancer mortality has decreased by 40% over the past 30 years, breast cancer remains the 2nd leading cause of cancer-related death amongst women.[1] In particular, triple negative breast cancer (TNBC) remains especially deadly with a 5-year survival rate of 77%.[2] Currently, there are no targeted treatments for TNBC and the standard-of-care therapy remains the systemic administration of combinations of anti-mitotic drugs.[3] Recently, cancer immunotherapy, especially the promotion of adaptive immune responses has shown some success and effectiveness in TNBC patients.[3,4] One in 8 women will develop invasive breast cancer in her lifetime and, despite advances in treatment, breast cancer remains the second leading cause of cancer-related deaths among U.S. women.[1,2,30] While systemic treatments of early American Joint Committee on Cancer (AJCC) stages i.e., stage 0, I and II in particular, through estrogen modulators demonstrate success in preventing breast cancer progression, many breast cancers progress toward more detrimental lethal stages. Current standard-of-care for TNBC includes surgery in combination with chemotherapy (anthracyclines and taxanes) and, more recently, checkpoint inhibitors (e.g., Atezolizumab).[2,3] Additionally, poly-adenosine diphosphate-ribose polymerase inhibitors (PARPis) are indicated for patients with breast cancer with BRCA mutations (20% of TNBC patients).[3] However, those therapeutic approaches still lead to ~50% recurrence with short progression-free survival (3-4 months).[4,31] Therefore, the development of novel and more efficient therapeutic approaches to prevent TNBC progression is critical.

Biologically, breast cancer originates from mutations in the epithelial cells lining the ducts and acini [32]. Progression through in situ (Stage 0), local (Stage I) and regional (Stage II) growth, invading lymph nodes (Stage III) and metastasis (Stage IV) is associated with dynamic cancer cell

alterations including mutations but also phenotypical changes that facilitate cancer spread through lymph and blood vessels. Hallmarks of the progression of normal epithelial cells to malignant cancerous cells have been summarized and updated to encompass microenvironmental cues.[33] The hallmarks include resisting cell death, sustaining proliferative signaling, and activating invasion and metastasis.[33] Indeed, beside cancer cells, the microenvironment is a key promotor of breast cancer progression.[20]

The Tumor Microenvironment, Fibrosis, and Cancer-Associated Fibroblasts

While the relative proportions and organization of tumor microenvironment (TME) components and tumor cells are dynamic regardless of the cancer type or subtype, somewhat similar TME features are observed in all cancers including breast cancers.[48] The accumulation of extracellular matrix (ECM) proteins, mostly fibrous collagens, mainly collagen I secreted chiefly by fibroblasts leads to fibrosis, which is an independent prognosis of breast cancer.[48] Within the TME, local inflammation promotes phenotypically different activated fibroblasts, i.e., cancer-associated fibroblasts (CAFs).[49] CAFs actively participate in tumor initiation, progression, and malignancy, and dramatically increase the metastatic dispersion of breast cancer cells.[49] As key TME contributors, CAFs remodel the ECM through ECM protein deposition and the secretions of enzyme cleaving the matrix proteins i.e., matrix metalloproteinases (MMPs). Alpha-smooth muscle actin (α-SMA) crucial for tissue fibrogenesis and vimentin (Vim) both a cellular intermediate filament protein and a secreted ECM proteins are both recognized CAF markers.[50,51] Moreover, α-SMA and Vim increased expressions are associated with tumor progression and metastasis.[52] Local inflammation also promotes the activation of both normal and cancer-associated fibroblasts to produce more collagen.[52,53] Moreover, fibroblasts in particular CAF-like

fibroblasts and other stroma cells, in part through inflammasome activation, participate in the generation of the pro-inflammatory environment. While numerous ECM proteins are secreted, fibrous collagens especially collagen I constitute a significant amount of the ECM protein secreted with the most abundant being COL1 fibrous collagen.

Fibrosis, the mechanism of stromal hardening, is triggered by a wide variety of stimuli and is mediated in part by fibroblasts. In normal conditions, local inflammation promotes the activation of fibroblasts to become myofibroblasts, which more actively produce collagen.[21] In addition to being a marker for CAFs, vimentin is also a marker of fibrosis. Myofibroblasts as collagen-producing cells are key mediators of fibrosis. This is significant because excessive collagen production can lead to the development of fibrosis. Moreover, inflammation and fibrosis are intertwined in favoring the generation of a pro-tumorigenic microenvironment.

Inflammation

The immune system plays a key role in the progression of breast cancer.[34] In particular, inflammation participates in the development and progression of cancer; serving as an enabling hallmark contributing to tumor growth, resistance to apoptosis, angiogenesis, invasion and metastasis.[33] As highlighted by the poor prognosis of patients with inflammatory breast cancer, inflammation is a major contributor to breast tumorigenesis.[5] In particular, innate immune response mechanisms during cancer progression including those triggered in macrophages such as inflammasome activation have been assessed.[6,7] Moreover, inflammation generated by mainly immune cells, including macrophages in part through inflammasome activation, also triggers a pro-inflammatory and pro-fibrotic microenvironment that favor cancer progression.

Inflammasomes are cytoplasmic multi-protein complexes recently uncovered that in response to various stressors trigger the generation of a pro-inflammatory environment, especially through the secretion of the pro-inflammatory IL-1β and IL-18 cytokines.[28] Until recently, inflammasome research has primarily focused on their contributions to the development of other pathologies such as infection, sepsis, and neurological diseases.[8-12]

Macrophages are specialized cells that phagocytes foreign materials and cellular debris including dead cells.[7] In addition, macrophages are potent antigen presenting cells. Macrophages also promote local inflammation through the secretion of cytokines.[7] These cells originate as monocytes in the hematopoietic centers i.e., bone marrow mainly in adults. They participate in both innate immune responses and in the adaptative immune responses.[12] In particular, they serve as first line of defense especially against bacterial infection. During innate immune response, macrophages' recognition through their pattern-recognition receptors (PRRs) of pattern motifs associated with either pathogens (PAMPs) or cell damage (DAMPs) triggers an innate immune response.[12] Additionally, macrophages also participate in the adaptative immune responses mainly through antigen presentation, opsonization and an effector role as phagocytic of tagged antigen and associated materials including cells.[7]

The macrophage innate response to bacterial pathogen has been extensively investigated and the presence of specialized intracytoplasmic protein complex sensing and responding to stress, in particular by promoting the maturation of pro-inflammatory cytokines has been demonstrated.[43] In cancer, the presence and cellular distribution and role of inflammasome remains to be fully investigated. Nevertheless, beside immune cells especially macrophages, inflammasome have been identified in multiple tumor cells and stroma cells including endothelial cells and fibroblasts.[28] Whether the cancer associated fibroblasts (1) express specific inflammasomes, (2)

have functional inflammasome i.e., leading to Caspase 1 expression and pro-inflammatory cytokine IL-1β and IL-18 secretions remains to be determined. Moreover, whether inflammasome activation triggers changes in ECM protein secretions remains to be determined.

Inflammasomes are major contributors of inflammation and, as such, a promising therapeutic target.[39] Inflammasomes are intracellular, pattern recognition receptors which respond to signaling by damage associated molecular patterns (adenosine triphosphate, heat shock proteins, cytoplasmic dsDNA) and pathogen associated molecule patterns (lipopolysaccharide).[6,40] NLR-family inflammasomes are multiprotein complexes which consist of a nucleotide-binding and oligomerization domain-like (NOD) receptor, an adaptor protein and pro-caspase-1 (or pro-caspase-11).[6] Upon activation, these proteins oligomerize and, through caspase activity, cleave pro-inflammatory IL-1β and IL-18 cytokines into their active forms.[40] Additionally, inflammasome activation can lead to an inflammatory form of cell death called pyroptosis through the cleavage of gasdermin D resulting in membrane pore formation.[26] The most extensively studied NLR is the NLRP3 inflammasome. Another key component in this activation is the NF-kB pathway.

The NLRP3 Inflammasome

Effects of inflammasomes, especially of the NLRP3 inflammasomes, in cancer progression are emerging.[13-18] Inflammation is associated with activation of cellular inflammasome complexes. Commonly found in immune cells, inflammasomes are multimeric cytoplasmic complexes that trigger an innate inflammatory pathway resulting in the secretion of pro-inflammatory cytokines. Activation of the NLRP3 inflammasome in absence of genetic alterations occurs as a 2-step path.[40] The first hit occurs via PAMP or DAMP signaling resulting in the autophosphorylation of a toll-

like receptor (TLR), which stimulates expression of pro-IL-1β and NLRP3 by nuclear translocation of NF-κB.[40] The diversity of stimuli leading to NLRP3 inflammasome oligomerization suggests that the NLRP3 sensor protein does not directly interact with the stimuli.[26] Mechanistically, following priming by PAMPs, cytokines or ligands for TLRs leads to nuclear factor-kB (NF-κB) transcriptional upregulation of NLRP3, and of pro-inflammatory cytokines including pro-IL-1β.[32,41] NLRP3 inflammasome activation occurs formation of the multimeric NLRP3-ASC1 (ASC: apoptosis-associated speck like protein containing a caspase recruitment domain also called PYCARD) complex, the activation of caspase-1, the maturation of pro-IL-1β and pro-IL-18, and the secretion of pro-inflammatory IL-1β and IL-18 cytokines.[41]

Elevated concentrations of the inflammatory marker interleukin (IL)-6 is also associated with increased mortality and recurrence of breast cancer.[35] The key roles of the IL-1 cytokine superfamily in inflammation including tumor inflammation have been detailed.[36] Indeed, IL-1β along with IL1RA and IL-1 receptors (IL-1R and IL-1RAP) have been assessed as targets in multiple conditions (Figure 1).[37]

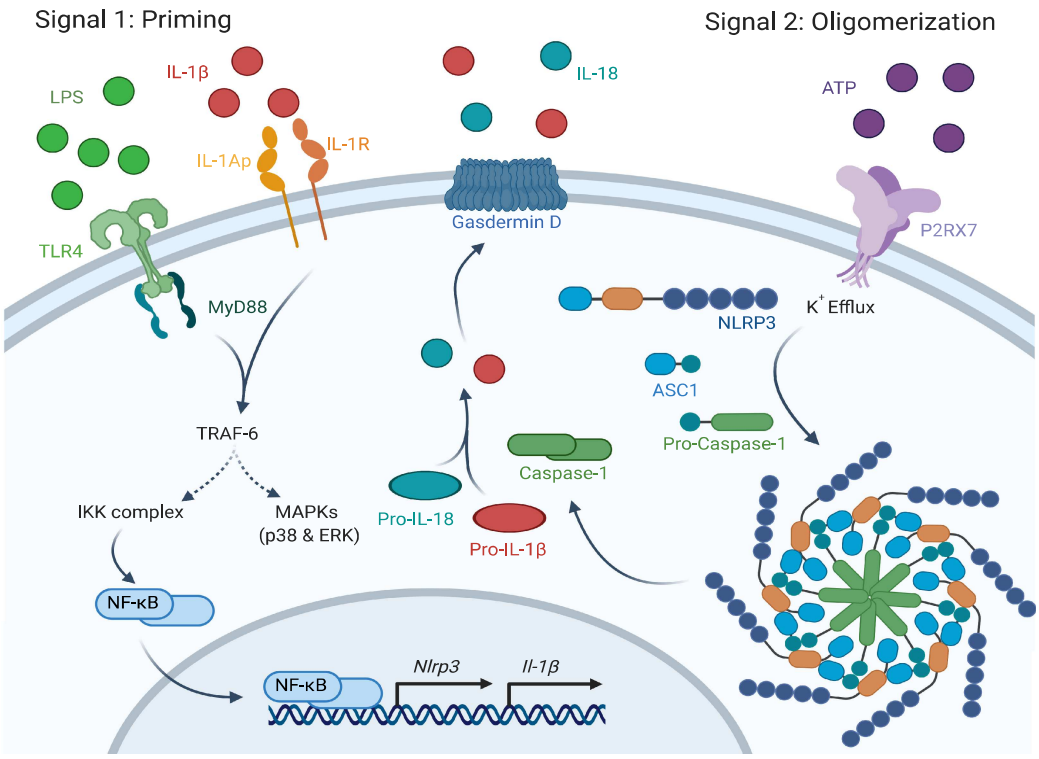


Figure 1: NLRP3 Inflammasome activation. NLRP3 inflammasome activation generally involves two signals: a priming signal e.g., LPS and TLR and an oligomerization signal e.g., ATP. As with other inflammasomes, NLRP3 inflammasome activation leads to cleavage and secretion of IL-1ß and IL-18 mature pro-inflammatory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

Among the mechanisms associated with the generation of IL-1β secretions, the role of the inflammasome activation is gaining more interest.[38] Administration of the NLRP3 inflammasome-specific inhibitor, MCC950, led to promising improvements in preclinical models of neurological pathologies, colitis and, importantly, in head and neck squamous cell carcinoma.[19,24,25] Besides the NLRP3 inflammasome inhibitor, MCC950, other molecules have been shown to prevent inflammasome activation.[26] Whether targeting inflammasome activation along with current clinical treatments leads to significant therapeutic improvements is unknown.

Currently, four mechanisms of NLRP3 inflammasome oligomerization have been identified, though not fully understood: potassium efflux, calcium flux, reactive oxygen species (ROS) and lysosomal degradation. Potassium efflux occurs in response to ATP stimulus and

occurs in tandem with sodium influx.[26] Purogenic P2X7-ATP interaction drives the recruitment of pannexin-1 and hemi-channel formation responsible for potassium efflux.[40] Furthermore, NEK7 (NIMA-related kinase 7) binds directly to NLRP3, facilitating oligomerization following potassium efflux.[26] Endoplasmic reticulum derived calcium flux is another mediator of NLRP3 inflammasome oligomerization. Consequently, pharmacological inhibition of inositol 1,4,5-triphosphate (IP3) receptor prevented NLRP3 activation.[26,40] Furthermore, exogeneous HSP70 has been demonstrated to prime the inflammasome in a CD14/TLR4 -, calcium-flux dependent manner resulting in the secretion of TNF-α, IL-1β, and IL-6.[43] Finally, lysosomal dysfunction activates NLRP3 activation via cathepsin B.[40] Particulates such as silica and alum lead to lysosome destabilization and the release of cathepsin B leading to NLRP3 inflammasome activation.[26,40]

Of interest, NLRP3 activation has been demonstrated in various cancers mainly colorectal cancer, head and neck squamous cell carcinoma, lung cancer, and endometrial cancer.[72,73,74] Experimentally, mice lacking inflammasome receptors: NLRP1b, NLRP3, AIM2 and NLRC4 are hyper-susceptible to colitis-associated cancer by azoxymethane and dextran sulfate sodium.[44] In contrast, dense presence of macrophages with strong NLRP3 expression in colorectal cancer resulted in faster colorectal cancer migration and NLRP3 deficiency reduced visible liver metastasis *in vivo*.[17]

The role of inflammasome activation in breast cancer has also been investigated. For example, caspase-1 and NLRP3 knockout mice implanted with EO771 and PyT8 mammary tumors experienced reduced tumor growth and less lung metastasis.[44,45] IL-1β, a product of NLRP3 inflammasome activation, is associated with angiogenesis, tumor invasiveness and metastasis in breast cancer.[36,40,46] Additionally, administration of the chemical IL-1β inhibitor Anakinra, a IL1RA analog, reduced tumor growth, hindlimb metastasis and micro-vessel formation in MDA-

MB-231 tumors in an *in vivo* pre-clinical murine model.[47] Interestingly, IL-18 leads to the downregulation of claudins in breast cancer, promoting migration.[44] Direct causation between NLRP3 inflammasome-derived IL-1β and IL-18 and tumor aggressiveness has yet to be established.

The NF-kB Pathway

The NF-κB pathway encompasses a family of inducible transcription factors. These transcription factors regulate a large array of genes including key genes that are associated with immune and inflammatory responses.[41] NF-kB participates in intercellular communication within complex networks of cytokines, chemokines, growth factors, inflammatory mediators and matrix remodeling enzymes (Figure 2).

Mechanistically, the NF-κB pathway can be activated through two main, i.e., canonical and non-canonical, pathways. The NF-kB canonical pathway is initiated in response to specific signals ligands of cytokine receptors, PAMPs, damage-associated molecular patterns (DAMPs), and PRRs. Multiple proteasome inhibitors, such as MG132 have been tested for blockage of specific aspects involved in the NF-κB pathway (Figure 2).[59] In contrast, the non-canonical pathway has been described in cell responses to stimuli groups.[59]

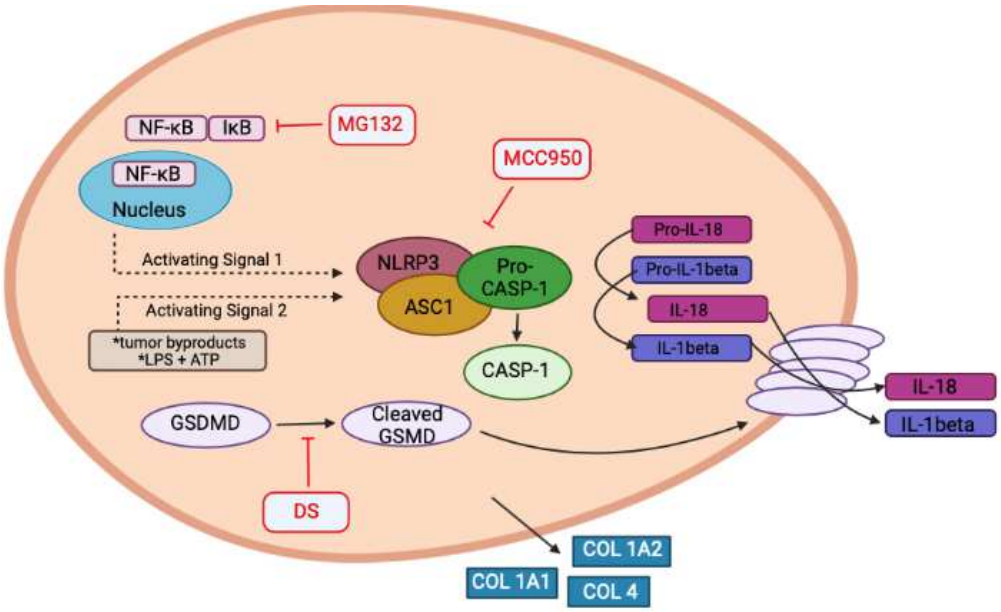


Figure 2: NLPR3 & NF-κB signaling pathway and inhibitors. NF-kB transcription factor promotes expression of pro-cytokine proteins, proteins of the NLRP3 inflammasome complex and gasdermin D. Known inhibitors include the IkB degradation inhibitor MG132 that prevents NF-kB nuclear translocation, the NLRP3 specific inhibitor MCC950 and the inhibitor of gasdermin D cleavage Disulfiram (DS) and thus the formation of transient membrane pores allowing the release of IL-1ß. (see text for details).

Physiologically, inflammation is a protective response to infectious agents and tissue damages. NF-κB is a crucial central mediator inducing the expression of pro-inflammatory molecules in most cells including immune cells. NF-κB activities increase the production of inflammatory cytokines but also regulate cell proliferation, cell differentiation and apoptosis.[42] Blockage and inhibition of NF-κB pathways affect production and protein signaling, which has the potential to encourage breast cancer progression.[59]

RATIONALE, EXPERIMENTAL APPROACH, HYPOTHESIS, and SPECIFIC AIMS

Rationale and Innovation

The role of inflammasome-driven inflammation on the progression of breast cancer remains poorly understood. Much research on inflammasomes and cancer has focused on their contradictory roles in colitis-associated colorectal cancer.[14] Our results further the understanding of the function of NLRP3 inflammasome-induced inflammation in CAFs within breast cancer. Our data supporting a role of inflammasome activated CAFs also confirm the association of fibrosis and breast cancer with a dimer clinical prognosis,[55] a demonstrated increased cancer cell migration and metastasis,[55] and the observation that a pro-inflammatory microenvironment promotes CAF to secrete more fibrous collagen I.[56] Whether preventing inflammasome activation and thus, the secretion of IL-1beta and IL-18 pro-inflammatory cytokines limit/prevent fibroblast collagen secretion is unknown.[41]

Within breast tumors, a significant proportion of stromal cells are fibroblasts that promote tumor progression in part through active remodeling of the tumor matrix.[57] In addition, our preliminary data suggests that activated fibroblasts (1) are responsive to known inflammasome activators, (2) secrete pro-inflammatory cytokines and (3) importantly secrete higher concentrations of fibrous collagens. It is unclear whether the interplay between fibroblasts and breast tumor cells furthers or sustains the local inflammasome-derived inflammation. our research aimed to further our understanding of the crosstalk between tumor cells and stromal fibroblasts and their implications on tumor development. Besides providing a better understanding of the role of fibrosis and of inflammasome activation in breast tumor progression, the innovation of this research is in the assessment as proof-of-principle of the NLRP3 inflammasome antagonist MCC950 alone or in combination with the NF-kB inhibitor MG132 in reducing two markers of

breast cancer progression: IL-β and IL-18 pro-inflammatory cytokine and fibrous collagen secretions.

The long-term goal of this research is to further our knowledge of the role of inflammasome activation in breast cancer progression including angiogenesis, extracellular matrix remodeling, epithelial-mesenchymal transition as well as during and following immunotherapeutic approaches. The present research specifically investigates the effect of NLRP3 inflammasome activation on fibroblasts, signaling, activity and phenotypes within the breast cancer microenvironment. To demonstrate a shift in fibroblast phenotype and in activity especially collagen secretions, *in vitro* assays treating fibroblasts with tumor conditioned media and/or known inflammasome activators were conducted. Along with evidence in other cancers,[27] our preliminary work demonstrates that fibroblasts express NLRP3 inflammasome, the adaptor protein, ASC1 and Caspase 1. Moreover, activated fibroblasts also secreted pro-inflammatory cytokines and collagens confirming recent demonstration of the presence of functional inflammasome in fibroblasts.[28,29]

Hypothesis

The main hypothesis tested is that fibroblast inflammasome activation, a feature of cancer-associated fibroblasts, leads to increased fibrous collagen secretion through NF-kB and/or NLRP3 dependent pathways thereby generating a pro-tumorigenic pro-fibrotic microenvironment favoring breast tumor growth.

Experimental approach

The signaling interplays between tumor cells stroma cells and ECM components within the tumor microenvironment are extremely complex.[20] Consequently, detailed signaling investigations

in *in vivo* models are challenging. Therefore, *in vitro* cell models have been developed. In particular, the *in vitro* culture of the cell type of interest permits *at minima* the investigation of signaling and responses to drug targeting specific signaling pathways. For example, tumor cells but also stroma cell have been cultured in vitro and the role of the MAPK, NF-kB pathway in cell proliferation and protein expression demonstrated.[52]

Specific Aims

In Specific Aim 1, we hypothesized that the breast tumor microenvironment supports NLRP3 inflammasome activation and promotes a cancer-associated fibroblast phenotype. We assessed the role of NLRP3 inflammasome activation of cancer associated fibroblasts. Specifically, we characterized the phenotype of fibroblasts following NLRP3 inflammasome activation by known NLRP3 activators and breast tumor cell and macrophage secretomes. Further, the NLRP3 inflammasome-specific inhibitor, MCC950, was used to ascertain that CAF phenotype is NLRP3-dependent.

In Specific Aim 2, we hypothesized that NLRP3 inflammasome activation in fibroblasts promotes a pro-fibrotic environment favoring breast cancer progression. We assessed the NLRP3 inflammasome activation in fibroblasts, the secretion of pro-inflammatory cytokines and of fibrous collagen I and determined whether NLRP3 inflammasome activation in fibroblasts favors pro-inflammatory and pro-fibrotic microenvironments. Further, effect of the NLRP3 and/or NF-kB pathway inhibition in fibroblast on pro-inflammatory cytokines and fibrous collagen were determined.

METHODS

Cells and Culture Conditions

The L929 murine fibroblast cells (obtained from ATCC, Manassas, VA) was used as a model for fibroblast cells. L929 cells were routinely grown and cultured in DMEM supplemented with gentamycin, amphotericin B, and 10% FBS. The aggressive triple negative murine mammary cancer 4T1 and the murine macrophage J774 (both obtained from ATCC, Manassas, VA) were cultured using the same media. The 4T1 cells have been shown to mimic late stages of human breast cancer.[48] The J774 macrophage have functional NLRP3 inflammasome.[40] Lastly, 4T1 and J774 cells are syngeneic to L929 cells. Briefly, to obtain conditioned media (CM), 4T1 and J774 cells were starved and cultured in media free of FBS for 48 hrs. The culture media were collected, filtered (0.2μm pore filter, Fisher Scientific) and stored (-20°C).

Fibroblast Activation

To assess the effects of fibroblast activation, in particular of the inflammasome, on the TME, L929 cells were seeded at concentrations ranging from 0.25-0.50 $\times$ 10^6 cells/well - depending on the tissue culture vessels used - in 10% FBS DMEM media.

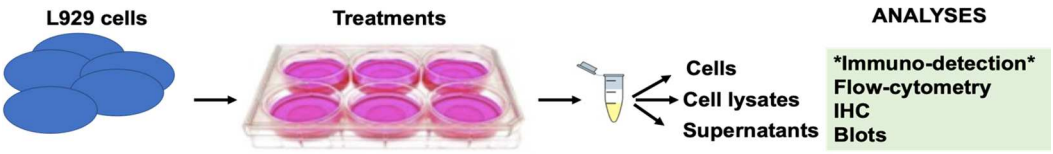


Figure 3: Experimental approach. Using the murine L929 fibroblasts *in vitro* model system inflammasome presence, effects of activation by canonical LPS + ATP stimulus or the presence of 4T1 mammary tumor or J774 macrophage secretomes, together with inhibition by MG132 proteasome inhibitor, MCC950 NLRP3 inhibitor and DS Gasdermin D inhibitor targeting NF-kB, NLRP3 and/or Gasdermin D, respectively on CAF, NLRP3 inflammasome and pro-inflammatory and collagen secretions were determined (N > 3 independent repeats).

Following a starving step (~6hrs incubation with FBS-free media i.e., 0% FBS), cells were incubated with either negative control (0% media alone i.e., control conditions), positive control (0% media + LPS 5µg/mL + ATP 5mM), 4T1CM or J774CM at increasing concentrations (1X to 3X) for 0 to 48 hrs. LPS and ATP were added 6 and 1 hour(s) prior to the collection of cells and supernatant, respectively.[13] Following treatment, cells were harvested by scraping and prepared for cell lysates and histochemistry experiments (flow cytometry and Western/dot blots (WB). Additionally, supernatants were collected. Overtime experiments were conducted with L929 fibroblasts and protein expression was much higher 24hrs post-treatment initiation especially compared to 6hr and 48hrs (See Supplemental Fig 1S). Thus, the present analysis focused on protein expression following a 24hrs exposure.

Fibroblast inhibition

In additional experiments, L929 cells were also investigated for their sensitivity to known inhibitors of key pathways namely, the NF-kB, NLRP3 and GSDMD pathways using MG132, MCC950 and DS, respectively. The proteasome inhibitor MG132 through limitation of IkB degradation favors the stabilization of cytoplasmic IkB-NF-kB complex preventing the nuclear translocation of NF-κB and thus the activity the NF-kB transcription factor.[59] The inhibitor of the NLRP3 inflammasome MCC950 has been shown to prevent the activation and activity of the NLRP3 both *in vitro* and in preclinical *in vivo* murine models.[7] The activation of GSDMD associated with GSDMD cleavage and the formation of GSDMD transient membrane pores that allows IL-1beta release,[47] can be inhibited by Disulfiram (DS), a drug used clinically to treat alcoholism.[60]

Preliminary experiments were conducted to optimize the concentrations of the inhibitors tested individually. Optimal concentrations, i.e., concentrations that were not associated with cell death/apoptosis/detachment/death were determined for each inhibitor alone or in activation conditions. Thus MG132 (0.5 μM), MCC950 (10 nM) and DS (0.5 μM) were used unless noted. The inhibitors were used alone or in combination with the activators (see above). Following treatment, cells were harvested by scraping and prepared for cell lysates and histochemistry experiments (flow cytometry and Western blots (WB)). Additionally, supernatants were collected. Changes in secretion and expression of proteins within cells were assessed using semi-quantitative dot-blots on supernatants and cell lysates, respectively.

Flow-cytometry

L929 fibroblasts with or without LPS + ATP treatment were harvested and fixed in buffered paraformaldehyde and evaluated for protein expression using flow-cytometry as previously. Briefly, fixed cells were permeabilized, and non-specific antigen blocked using incubation buffer (BSA (1%) PBS and Tween 20 (0.01%) in PBS (150 mM). Cells were then incubated in incubation buffer supplemented with a cocktail of primary antibody to NLRP3, ASC1 and CASP1 raised in rat, rabbit and goat, respectively in blocking buffer. After repeated washes, L929 cells were incubated with the cocktail of following secondary antibodies: fluorescent anti-rat-488; anti-rabbit-555 and anti-goat-647. Following additional washes in incubation buffer, cells were run onto a Fortessa flow-cytometer (BD Biosciences). Expression of cells positive for NLRP3, ASC1 and CASP1 (in %) and their mean fluorescence intensity (MFI) determined based on BD Diva software.

Western Blots and Dot Blots

Western blots (dot-blots) were employed as previously to detect expression of various inflammatory cytokines, collagens, and other factors important to the TME.[65] Following treatment, L929 cells and supernatants were collected. Cell pellets were lysed in T-PER (ThermoFisher) supplemented with an EDTA-free protease inhibitor cocktail (Roche) and mixed with Pro-Prep (2:1; Intron Biotechnology) for the analysis of protein expressions. Following sonication, cell lysates (10μL) and supernatants (50μL) were mixed with SDS (0.25 % final) in PBS (15 mM) to a final volume (62μL) were loaded onto a nitrocellulose membrane (Millipore, 0.45 μm pores) using a dot-blot apparatus. After washes in TBST (TBS Boston scientific) + 0.1 Tween 20 (Sigma),

membranes were stained using the Ponceau reversible protein stain (Biorad) to determine protein load. All protein expressions were normalized to protein load prior to analyses.

Following rapid Ponceau de-staining in water, membranes were blocked in TBST+5% fat-free milk proteins. Then, membranes were immunodetected as described previously.[65] Cell lysates were investigated for protein expressions including CAF markers (vimentin, MMP14 and alpha-SMA) and proteins associated with NF-kB, NLRP3, GSDMD pathways along with some secreted proteins including IL-1beta and IL-18. Expressions of other related proteins such as NF-kB, pNF-kB, Caspase 1, ASC1 and NLRP3 were also assessed. Cell supernatants were assessed mainly for CAF markers (vimentin, MMP14), secreted pro-inflammatory cytokines (IL-1beta, IL-18), and collagen (1A1, 1A2 and 4A3) subtypes.

Following incubation with appropriate secondary HRP-conjugated antibodies, protein expression proportional to chemiluminescence was measured using a ChemiDoc system (Biorad). The expression of each protein tested was semi-quantified using the Protein Array Analyzer macro and the image analysis software ImageJ (NIH). Expression was normalized to loading control and to control conditions.

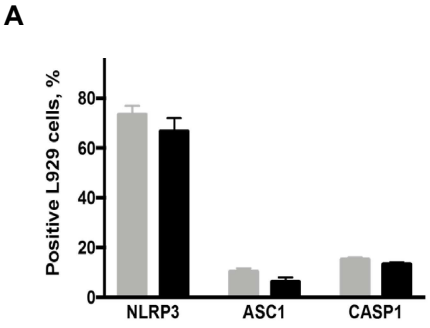
Experimental Rigor and Statistical Analyses

All experiments included controls and utilized multiple independent repeats ($n \geq 3$). Measurements are presented as Mean ± SEM and differences between treatment groups were determined by one-way or two-way ANOVA analyses and appropriate post-hoc tests as warranted with an *a priori* $p<0.05$ significance threshold.

RESULTS

1- LPS-ATP activated L929 fibroblasts express the NLRP3 inflammasome proteins

Expression of inflammasome proteins was altered upon activation with the canonical inflammasome activator LPS/ATP with alterations in cells expression of NLRP3, ASC1 and active Caspase 1 (Fig 4A). Moreover, mean fluorescence intensity that evaluates the expression per cell was increased upon treatments (Fig 4B). Additionally, protein assessment through western dot blots (Fig 4C) confirmed the presence and a significant decrease in ASC1 expression after a 24hr exposure to treatment ($P<0.05$).



B

	Treatment	
	None	LPS + ATP
NLRP3	11789±526	15660±699***
ASC1	34874±300	45019±387***
Casp1	31685±281	41704±370***

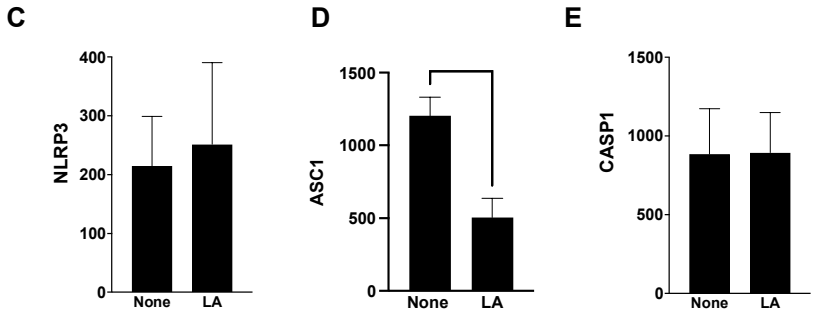


Figure 4. ***L929 fibroblasts express NLRP3 inflammasome complex proteins.*** Briefly, L929 cells were incubated in culture media or in media supplemented with the inflammasome activator LPS and ATP. Following cell harvesting and intra-cytoplasmic immunostaining of the NLRP3, ASC1 and CASP1 inflammasome proteins using specific antibodies, the percent of positive cells (**A**) and their mean fluorescence intensity (**B**) were determined by flow-cytometry. Using cell lysates, NLRP3 (**C**), ASC1 (**D**) and active Caspase 1 (**E**) expressions (AU, average ±SEM) were determined by Western/Dot blots. Independent experiments and n≥3, One-Way ANOVA, Tukey post-hoc test with *p<0.05, **p<0.01 and ***p<0.001.

2- LPS/ATP activation promotes pro-inflammatory IL-18 secretion by L929 fibroblasts

Further, activated L929 fibroblasts had significantly increased secretions of IL-1beta and IL-18 pro-inflammatory cytokines (Fig 5, $P<0.05$) highlighting the presence of fully functional inflammasomes.

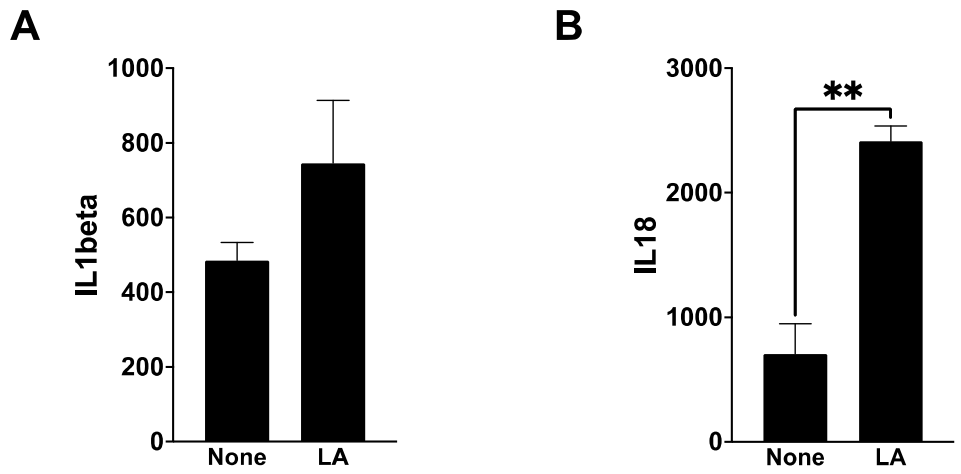


Figure 5. LPS-ATP activated L929 fibroblasts has enhanced IL1beta and IL18 secretions. Briefly, L929 cells were incubated in culture media or in media supplemented with the inflammasome activator LPS and ATP. Following cell culture supernatant collection, IL-1beta and IL-18 secretions (AU, average ±SEM) were determined by Western/Dot blots. Independent experiments and n≥3, One-Way ANOVA, Tukey post-hoc test with *p<0.05, **p<0.01 and ***p<0.001.

3- Following LPS/ATP activation, L929 fibroblasts secrete higher concentrations of the fibrous 1A2 collagen subtype.

Moreover, LPS + ATP activated L929 fibroblasts notably secreted more fibrous collagen 1A2 whereas fibrous collagen 1A1 and globular collagen 4A3 secretions were unchanged (Fig 6, $P<0.05$). In addition, the COL 1A1/COL 4A3 ratio but not the COL 1A1/Col 4A3 ratio secreted by L929 fibroblasts decreased in the presence of LPS + ATP (Fig 6DF, $p<0.05$). Lastly, the COL1A2/COL1A1 ratio increased upon LPS + ATP treatment (Fig 6E, $p<0.001$).

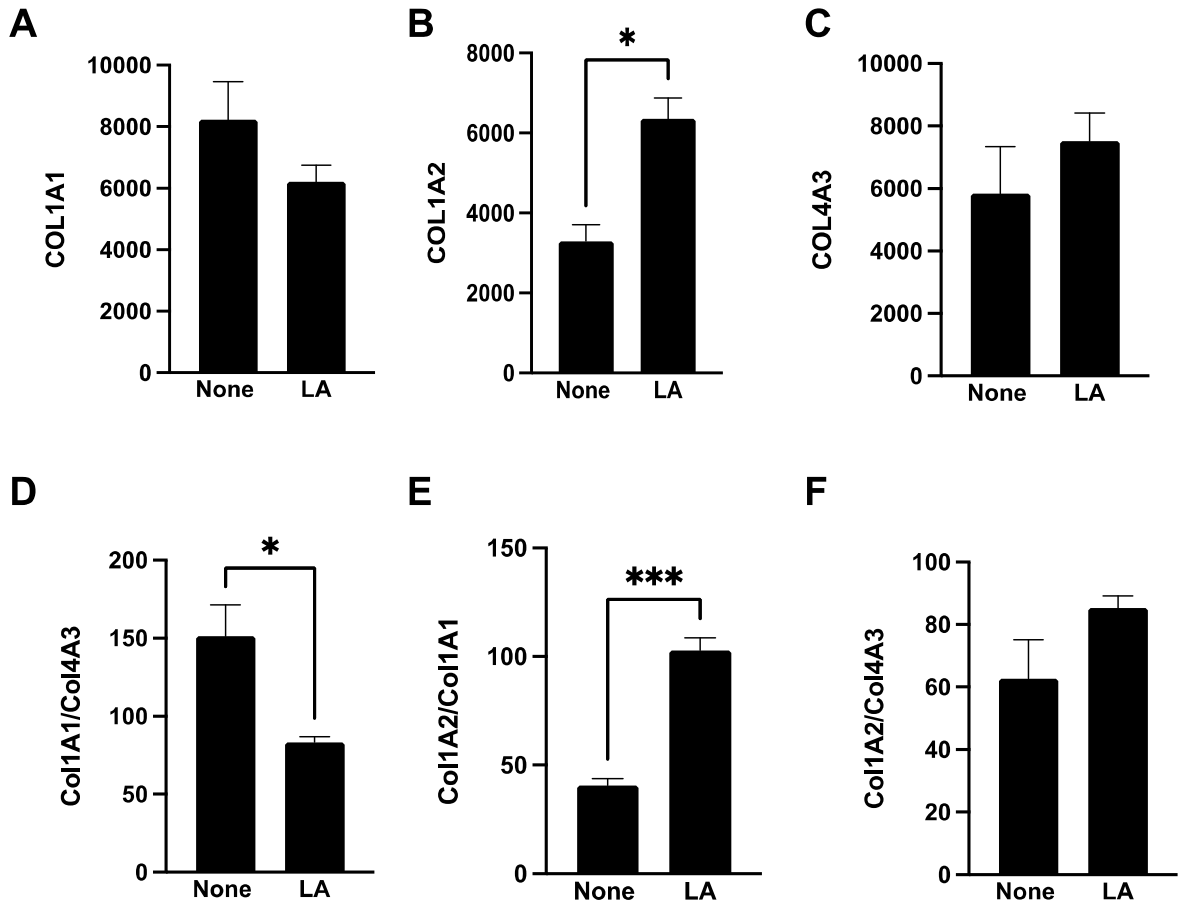


Figure 6. LPS-ATP activated L929 fibroblasts secreted more fibrous collagen. Briefly, L929 cells were incubated in culture media or in media supplemented with the inflammasome activator LPS and ATP. Following L929 cell culture supernatant collection, fibrous collagen 1A1 (**A**) and 1A2 (**B**) and non-fibrous collagen 4A3 (**C**) secretions (AU, average ±SEM) were determined by Western/Dot blots. Collagen 1A1 to collagen 4A3 (**D**) Collagen 1A2 to collagen 1A1 (**E**) and Collagen 1A2 to collagen 4A3 (**F**) ratio were calculated. Ratio Independent experiments and n≥3, One-Way ANOVA, Tukey post-hoc test with *p<0.05, **p<0.01 and ***p<0.001.

4- L929 fibroblasts display a CAF phenotype in the presence of LPS/ATP, 4T1 CM and J774 CM

Cancer associated fibroblasts (CAFs) actively participate in the tumor growth and metastasis.[51] We assessed whether LPS + ATP treatment but also secretions from 4T1 mammary tumor (4T1CM) cells and from J774 macrophages (J774CM) modulated key phenotype markers of CAFs

in L929 fibroblasts. Both alpha smooth muscle actin (aSMA) expression after 48hrs and vimentin secretion after 24hrs of LPS + ATP treatments were significantly increased compared to unstimulated L929 cells (Fig 7AB, $p<0.01$). The expression of MMP14, another marker of CAF phenotype also tended to increase upon activation with LPS + ATP (Fig 7C, ns). Interestingly, the incubation of L929 fibroblasts with 4T1 tumor secretome, but not with J774 secretome also drastically increased the CAF marker vimentin secretion (Fig7D, $p<0.001$).

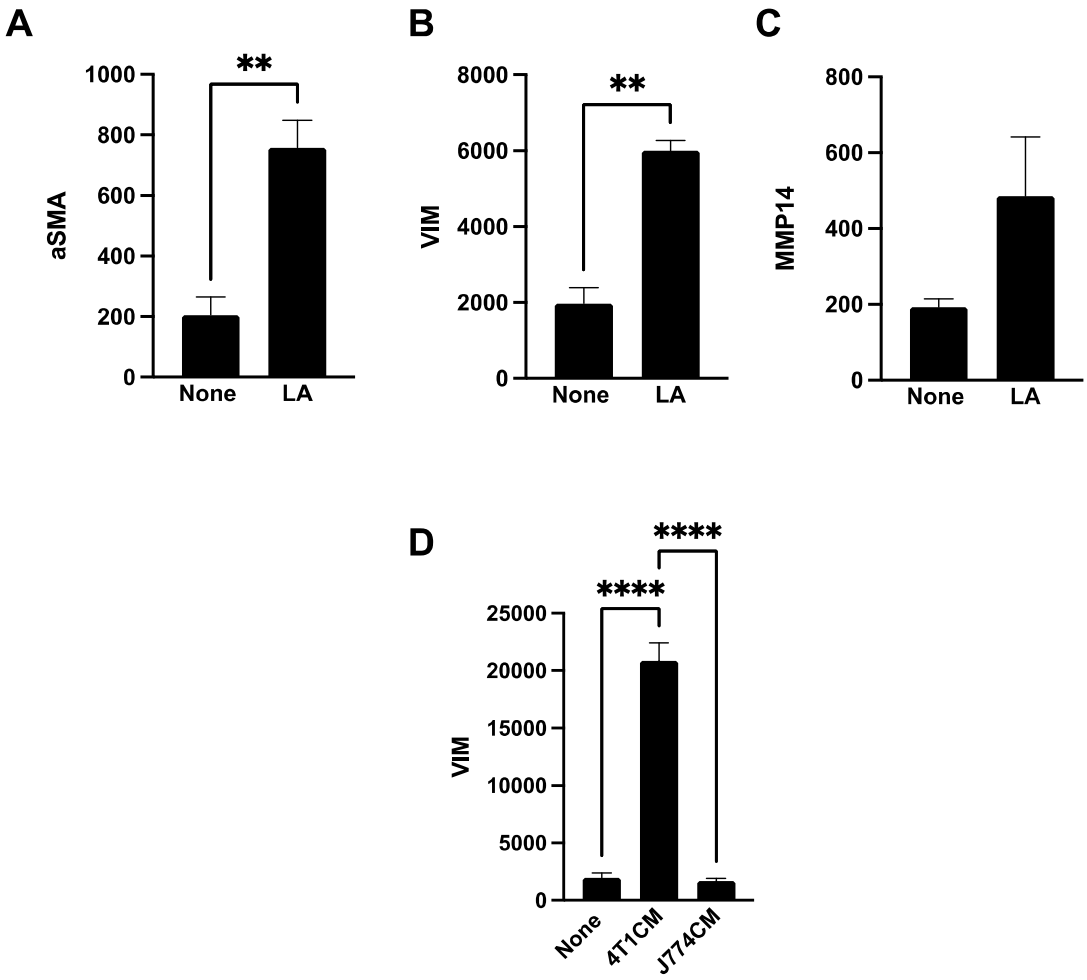


Figure 7. Activated L929 fibroblasts display a CAF phenotype. Briefly, L929 cells were incubated in culture media or in media supplemented with the inflammasome activator LPS and ATP or secretomes from 4T1 mammary tumor cells (4T1CM) or J774 macrophage (J774) and both cell lysates and supernatants obtained. Alpha smooth actin (aSMA) expression (**A**) and vimentin (**B**, **D**) and MMP14 (**C**) secretions (AU, average ±SEM) were determined by Western/Dot blots. Independent experiments and $n\geq3$, One-Way ANOVA, Tukey post-hoc test with *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

5- Following incubation with 4T1CM and J774CM, L929 fibroblasts had decreased NLRP3 protein expression and reduced pro-inflammatory cytokine secretions

To assess whether L929 fibroblasts inflammasome activation response, the expression and function of NLRP3 inflammasome in the presence of secretomes from 4T1 mammary tumor cells (4T1CM) or J774 macrophage (J774) were determined.

NLRP3 inflammasome protein NLRP3, ASC1 and Casp1 expression were significantly decreased in the presence of the secretome of either 4T1 mammary tumor cells (4T1CM) or J774 macrophage (J774) (Fig 8, $p<0.01$).

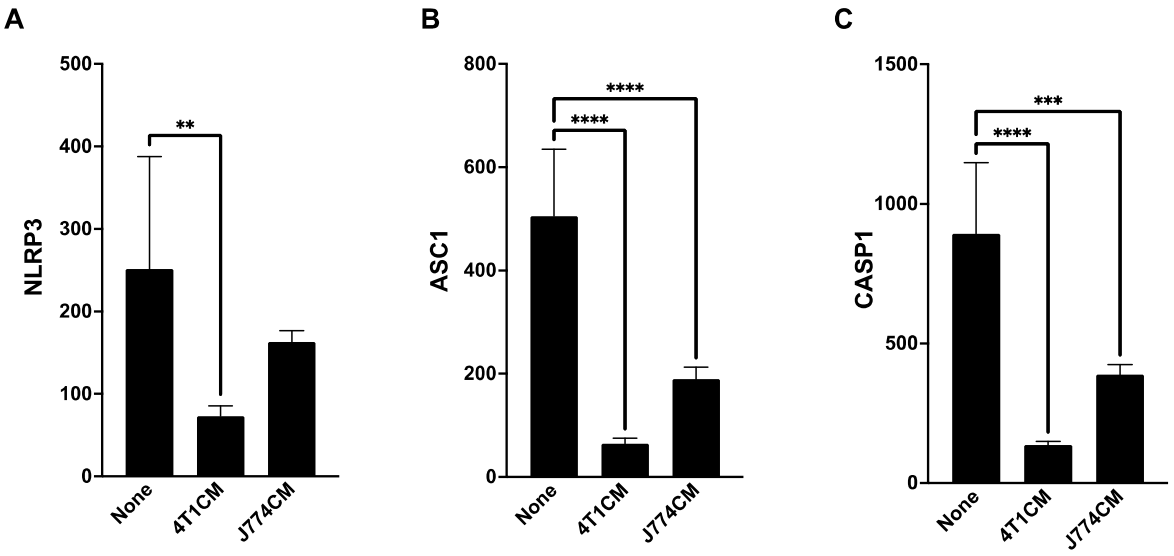


Figure 8. Following incubation with 4T1CM or J774CM, L929 fibroblasts expressed less of the NLRP3 inflammasome proteins. Briefly, L929 cells were incubated with secretomes from 4T1 mammary tumor cells (4T1CM) or J774 macrophage (J774CM) for 24hrs and the expressions (AU, average ±SEM) of NLRP3 (**A**), ASC1(**B**) and Casp1 (**C**) in lysate were determined by western dot-blots. Independent experiments and n≥3, One-Way ANOVA, Tukey post-hoc test with *p<0.05, **p<0.01 and ***p<0.001.

Moreover, while IL-1beta was not significantly decreased 24hr post-incubation with either secretomes from 4T1 mammary tumor cells (4T1CM) or J774 macrophage (J774), Il-18 secretions were markedly decreased in the presence of either (Fig 9, $p<0.001$).

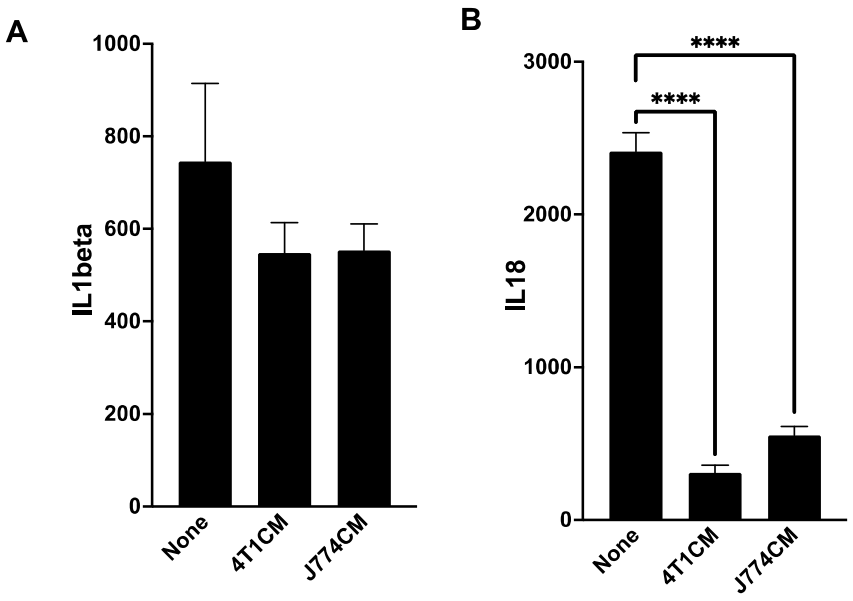


Figure 9. Following incubation with 4T1CM or J774 CM, L929 fibroblasts secreted IL-18 pro-inflammatory cytokine. Briefly, L929 cells were incubated with secretomes from 4T1 mammary tumor cells (4T1CM) or J774 macrophage (J774CM) for 24hrs and the expressions (AU, average ±SEM) of IL-1beta (**A**), IL-18(**B**) in supernatants were determined by western dot-blots. Independent experiments and n≥3, One-Way ANOVA, Tukey post-hoc test with ***p<0.001.

6- Following incubation with 4T1CM and J774CM, L929 fibroblasts had decreased NLRP3 protein expression and reduced pro-inflammatory cytokine secretions

Fibroblasts actively participate in the production of extra-cellular matrix components especially collagens.[75,76] Thus, fibrous 1A1 and 1A2 and globular 4A3 collagen secretions were assessed following a 24hr incubation with secretomes from 4T1 mammary tumor cells (4T1CM) or J774 macrophage (J774) (Fig 10).

Notably, collagen 1A1, 1A2 and 4A3 secretions were significantly decreased in the presence of the secretome of either 4T1 mammary tumor cells (4T1CM) or J774 macrophage (J774CM) (Fig 10ABC, $p<0.001$). Furthermore, col1A1 and col1A2 to globular col4A3 ratios were decreased and increased, respectively, in the presence of the secretomes (Fig 10DF, $p<0.05$). Interestingly,

fibrous collagen 1A1 and 1A2 were differentially expressed as the collagen 1A2 to collagen 1A2 ratio was markedly increased in the presence of the secretome of either 4T1 mammary tumor cells (4T1CM) or J774 macrophage (J774CM) (Fig 10E, $p<0.001$).

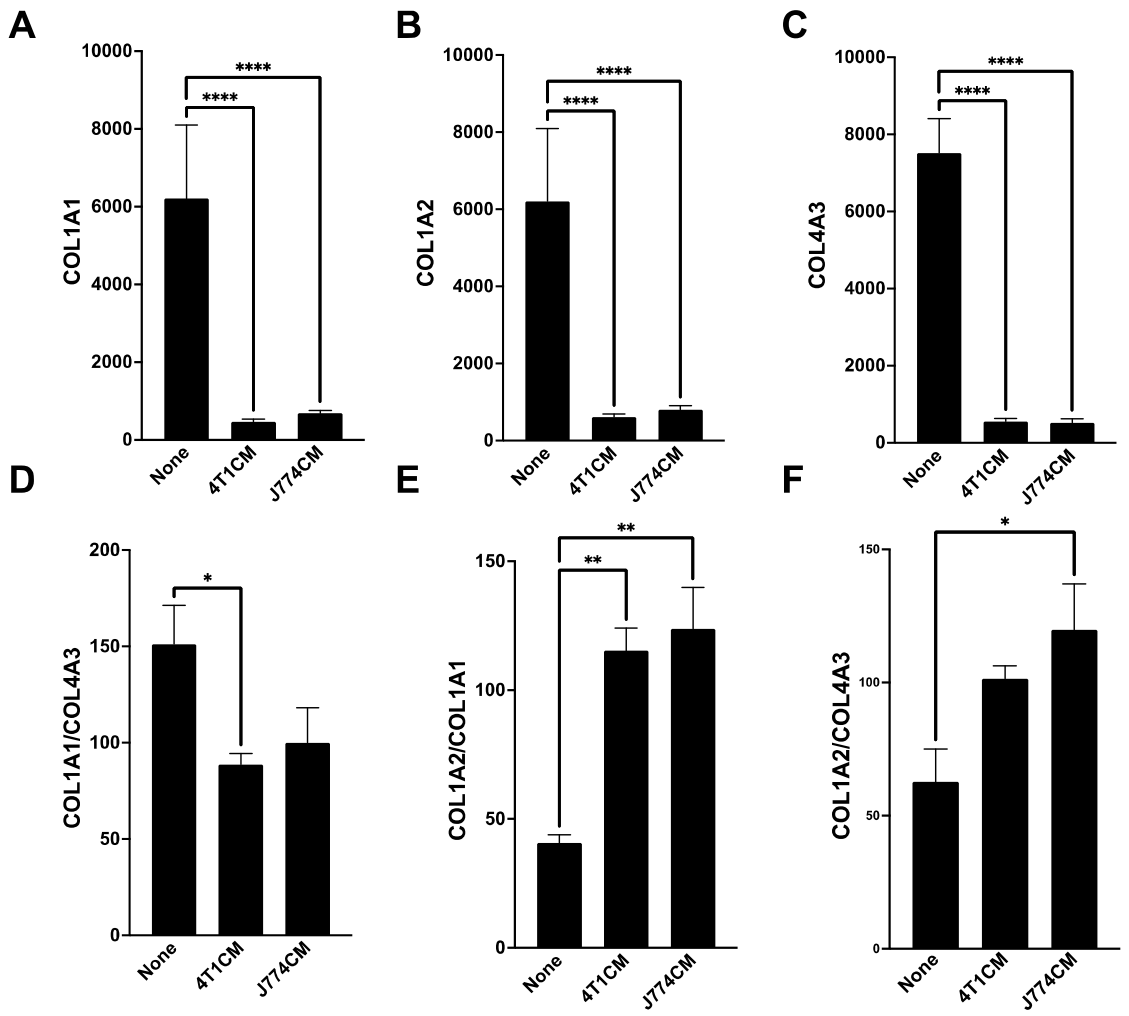


Figure 10. Following incubation with 4T1CM or J774CM, L929 fibroblasts secreted markedly less collagens. Briefly, L929 cells were incubated in culture media or with secretomes from 4T1 mammary tumor cells (4T1CM) or J774 macrophage (J774CM) for 24hrs. Following L929 cell culture supernatant collection, fibrous collagen 1A1 (**A**) and 1A2 (**B**) and non-fibrous collagen 4A3 (**C**) secretions (AU, average ±SEM) were determined by Western/Dot blots. Collagen 1A1 to collagen 4A3 (**D**) Collagen 1A2 to collagen 1A1 (**E**) and Collagen 1A2 to collagen 4A3 (**F**) ratio were calculated. Independent experiments and $n\geq3$, One-Way ANOVA, Tukey post-hoc test with *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

7- The NF-kB pathway critically promotes L929 fibroblast CAF phenotype, NLRP3 protein expression, pro-inflammatory cytokine and collagen secretions

First, we confirmed that at the concentration used in L929 cells, MG132 inhibited IkB degradation, thereby limiting the nuclear translocation of transcription factor NF-kB. As shown Fig 11, 0.5mM of MG132 drastically limited the proteasome degradation of IkB, thus preventing NF-kB nuclear translocation and its promoting transcription activity.

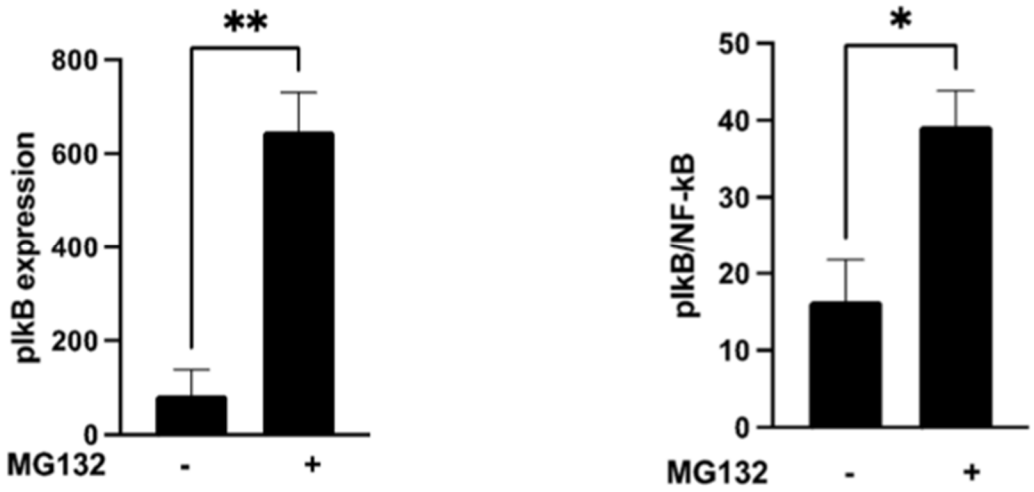


Fig 11. MG132 (05mM) proteasome inhibition significantly increased IkB expression in L929 fibroblasts. Briefly, L929 cells were incubated in culture media with or without MG132 (0.5mM) for 24hrs and cell lysates obtained. Lysates were assessed for phosphor-IkB (**A**), NF-kB (p65) expression (AU, average ±SEM) by Western/Dot blots. The ratio pIk/NF-kB was calculated (**B**). Independent experiments and n≥3, One-Way ANOVA, Tukey post-hoc test with *p<0.05, **p<0.01 and ***p<0.001

The L929 CAF phenotype observed following both LPS + ATP treatments was inhibited by MG132 treatment. Remarkably, VIM expression was fully inhibited and similar to control conditions (Fig 12, $p<0.001$) highlighting that inhibiting the NF-kB signaling pathway prevents the generation of a CAF phenotype in L929 fibroblasts.

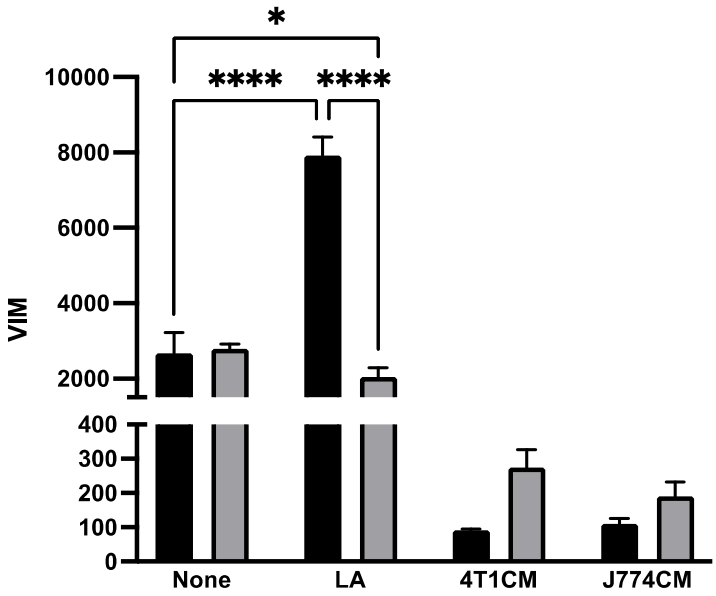


Fig 12. MG132 inhibits CAF markers expression VIM. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated with the NF-kB inhibitor MG132 (gray bars) or not (black bars) and vimentin (VIM) secretion (AU, average ±SEM) was determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Sidak post-hoc test with ** $p<0.01$.

NLRP3 inflammasome complex protein expression triggered by the canonical LPS+ATP activator and secretomes from tumor cells (4T1CM) or macrophages (J774CM) was altered following incubation with the NF-kB inhibitor MG132 (Fig 13). Whereas in the absence of activator, MG132 had no effects on either NLRP3, ASC1 or Casp1 protein expression, in the presence of LPS + ATP activation, MG132 treatment led to increases in NLRP3, ASC1 and Casp1 protein expression (Fig 13, $p<0.01$). Moreover, in the presence of 4T1CM activation, MG132 treatment led to a significant increase in NLRP3 expression (Fig 13A, $p<0.05$) but not in ASC1 or Casp1. In the presence of J774CM, MG132 treatment led to a significant decrease in Casp1 expression (Fig 13C, $p<0.01$). Indeed, NPRP3, ASC1 and Casp1 expressions were increased 3-, 2- and 1.5-fold, respectively, in L929 LA treated cells contrasting with LA alone especially for ASC1 (2.5-fold decrease vs. 3-fold increase following LA+MG132 treatment).

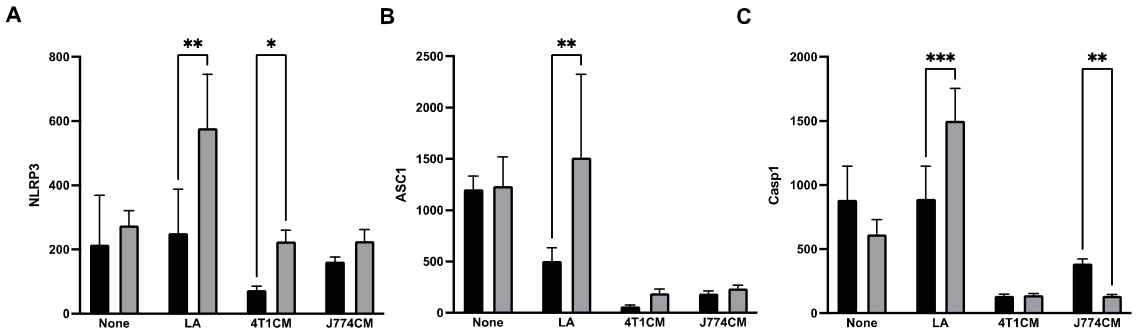


Fig 13. MG132 modulated NLRP3 protein expression. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated with MG132 (gray bars) or not (black bars) and NLRP3 complex protein i.e., NLRP3 (**A**), ASC1 (**B**) and Casp1 (**C**) expression (AU, average ±SEM) determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Sidak post-hoc test with *p<0.05, **p<0.01 and ***p<0.001.

Additionally, MG132 treatments decreased IL1beta and IL-18 secretions triggered by the secretomes of 4T1 tumor cells and J774 macrophages (Fig 14, p<0.05). MG132 inhibitor treatment blunted increases in IL-1beta or IL-18 secretion (see Fig 5., above) triggered by LA in L929 fibroblasts (Fig 14, ns).

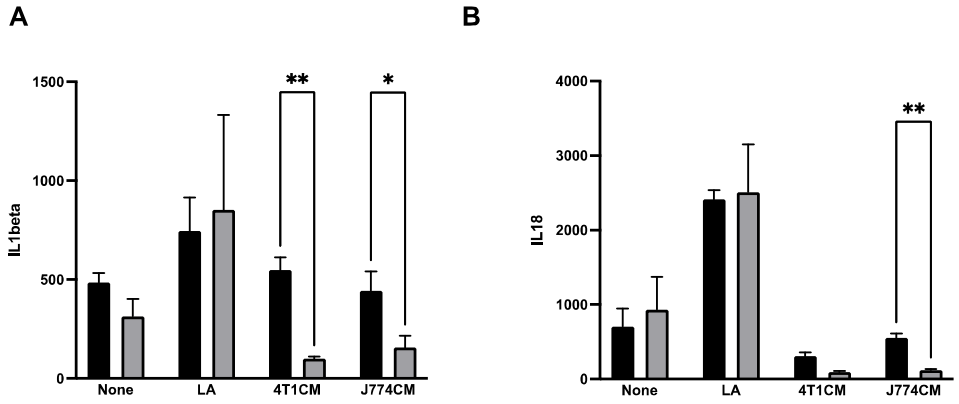


Fig 14. MG132 prevented pro-inflammatory cytokine secretion in activated L929 fibroblasts. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated with MG132 (gray bars) or not (black bars) and IL-1beta (**A**) and IL-18 (**B**) pro-inflammatory cytokine secretion (AU, average ±SEM) determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Sidak post-hoc test with *p<0.05, and **p<0.01.

MG132 treatment also tended to decrease fibrous and globular collagen secretions by L929 fibroblasts activated by 4T1 and J774 macrophage secretomes (Fig 15). Notably, Col1A2 fibrous collagen secretions were enhanced in the presence of both LPS + ATP and MG132 inhibitor (Fig 15B, $p<0.05$).

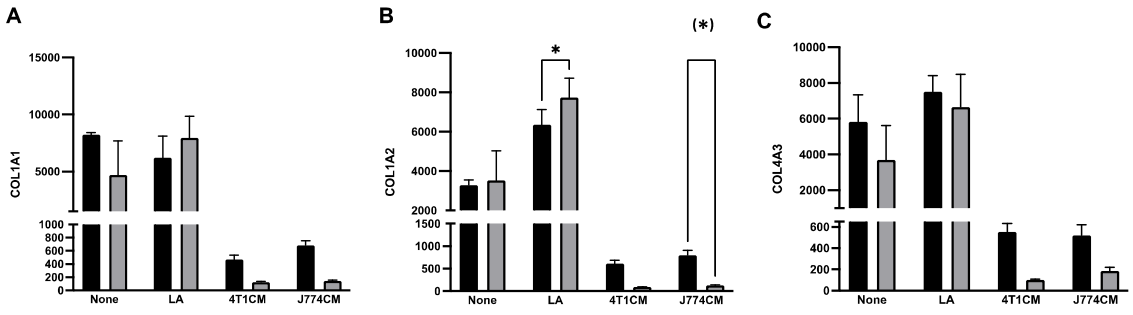


Fig 15. MG132 tended to decrease collagen expression in L929 fibroblasts activated by tumor and macrophage secretomes. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated with MG132 (gray bars) or not (black bars) and fibrous collagen 1A1 (**A**) 1A2 (B) and globular Col4A3 (**C**) secretion (AU, average ±SEM) were determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Sidak post-hoc test with (*) $p<0.1$ and *$p<0.05$.

8- The Inflammasome inhibitor MCC950 prevented vimentin secretion by L929 fibroblasts

NLRP3 inflammasome inhibitor MCC950 treatment of non-activated and L929 fibroblasts incubated with 4T1 and J774 secretomes had no effect on the expression of the key CAF marker vimentin (Fig 16, ns) contrasting with the 10-fold increase observed following incubation with 4T1CM (Fig 7D). However, MCC950 treatment enhanced LPS + ATP activation of the CAF phenotype as Vimentin secretions increased (Fig 16, $p<0.001$).

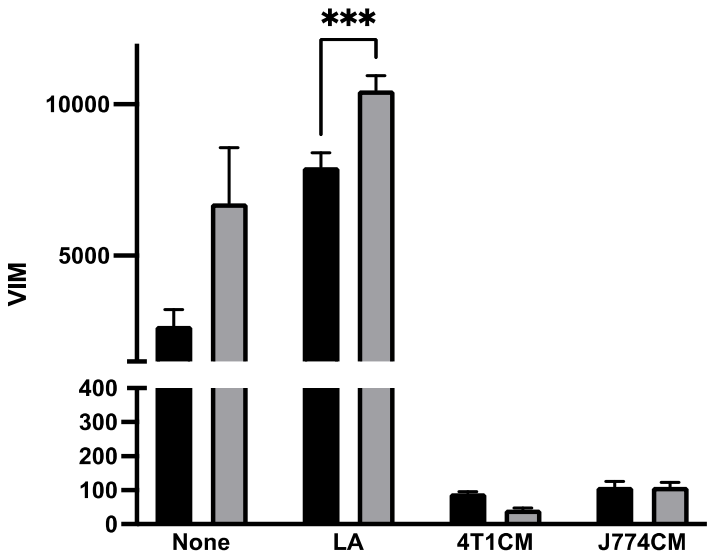


Fig 16. The NLRP3 inflammasome inhibitor MCC950 inhibited vimentin secretion in L929 fibroblasts. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated with the inflammasome inhibitor MCC950 (gray bars) or not (black bars) and vimentin (VIM) secretion (AU, average ±SEM) were determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Sidak post-hoc test with (*) $p<0.1$ and *$p<0.05$.

9- A 24hr incubation with the NLRP3 inhibitor MCC950 differentially modulated the expressions of NLRP3 complex proteins and pro-inflammatory cytokine secretions

Twenty-four hours treatment with MCC950 of L929 fibroblasts activated through incubation with LPS and ATP, led to significant increases in NLRP3 protein components (Fig 17, $p<0.001$). In contrast, treatment with MCC950 of L929 cells activated with 4T1 tumor cells and J774 macrophage had no significant effects on the expression of NLRP3 protein components (Fig 17, ns).

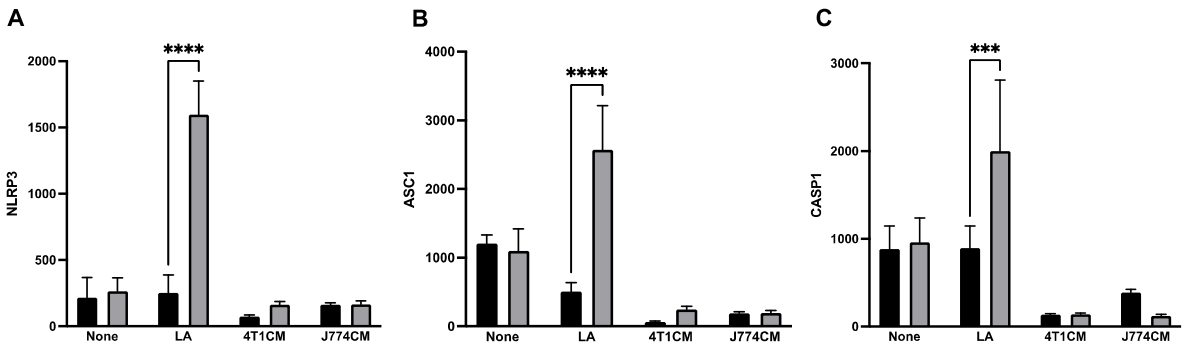


Fig 17. In L929 fibroblasts activated by LPS and ATP, the NLRP3 inflammasome inhibitor MCC950 promoted an increased NLRP3 inflammasome protein expression. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated 24hrs with the inflammasome inhibitor MCC950 (gray bars) or not (black bars) and expression (AU, average ±SEM) of NLRP3 (**A**), ASC1 (**B**) and Casp1 (**C**) were determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Tukey post-hoc test with (*) $p<0.1$ and *$p<0.05$.

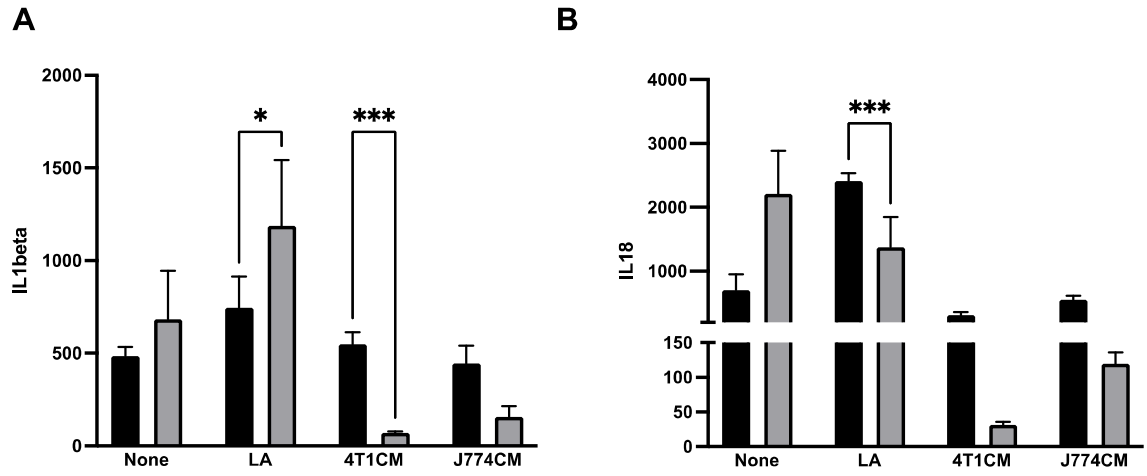


Fig 18. The NLRP3 inflammasome inhibitor MCC950 modulated activated L929 fibroblast pro-inflammatory cytokine secretions. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated 24hrs with the inflammasome inhibitor MCC950 (gray bars) or not (black bars) and secretions (AU, average ±SEM) of IL-1beta (**A**), and IL-18 (**B**) pro-inflammatory cytokines were determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Sidak post-hoc test with *$p<0.05$ and ***$p<0.001$.

As the expression of NLRP3 inflammasome components was increased when LPS + ATP and MCC950 were combined, Il-1beta secretion increased whereas IL-18 decreased contrasting with the LA-triggered IL-18 increase (see Fig 5) (Fig 18, $p<0.05$). L929 fibroblasts incubated with either secretome from tumors (4T1CM) or macrophages (J774CM) and treated with MCC950 tended to secrete less IL-1beta and IL-18. IL1beta secretion was significantly decreased (Fig 18A, $p<0.001$) supporting the role of NLRP3 activation in the secretions of IL-1beta and IL-18pro-inflammatory cytokines by fibroblasts.

10- Following 4T1CM and J774CM, NLRP3 inhibitor MCC950 treatment decreased collagen secretions

Following combination treatment: activation of L929 through combination of LPS and ATP and NLRP3 inflammasome inhibitor MCC950 was associated with increases in secretions of Col1A1 (**A**) and Col1A2 (**B**) fibrous, and Col4A3 (**C**) globular collagen (Fig 19, $p<0.001$). In contrast, 4T1 and J774 secretomes combined with MCC950 tended to decrease collagen secretion (Fig 19, ns).

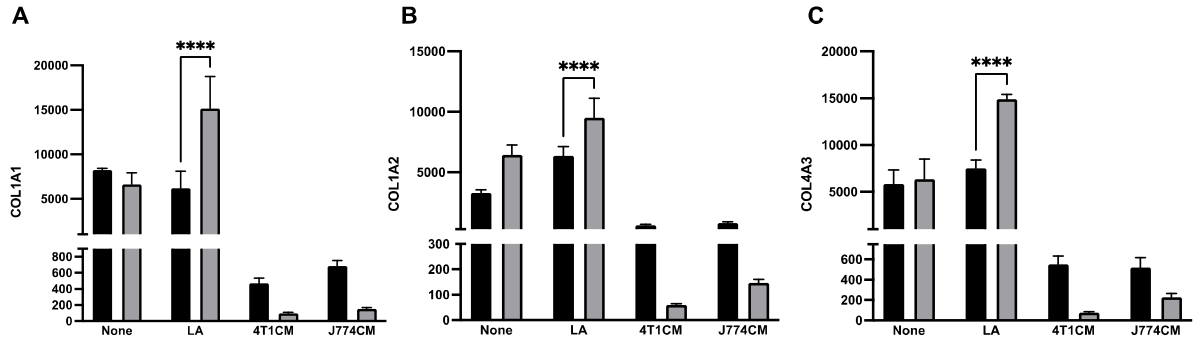


Fig 19. The NLRP3 inflammasome inhibitor MCC950 increased LPS/ATP activated L929 fibroblast collagen secretions. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated 24hrs with the inflammasome inhibitor MCC950 (gray bars) or not (black bars) and secretions of Col1A1 (**A**) and Col1A2 (**B**) fibrous, and Col4A3 (**C**) globular collagen secretions (AU, average ±SEM) as determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Sidak post-hoc test with *p<0.05 and ***p<0.001.

11- The gasdermin D inhibitor Disulfiram promotes L929 fibroblast vimentin secretion

Gasdermin D has been shown to form transient membrane pores facilitating mature IL-1beta secretion.[77] The gasdermin D inhibitor disulfiram (DS) treatment of L929 fibroblasts incubated with LPS + ATP enhanced activation of the CAF phenotype as vimentin secretions were increased further (Fig 20, $p<0.001$). In contrast, DS treatment had no effects on vimentin expression of L929 fibroblasts incubated with either 4T1 and J774 secretomes (Fig 20, ns).

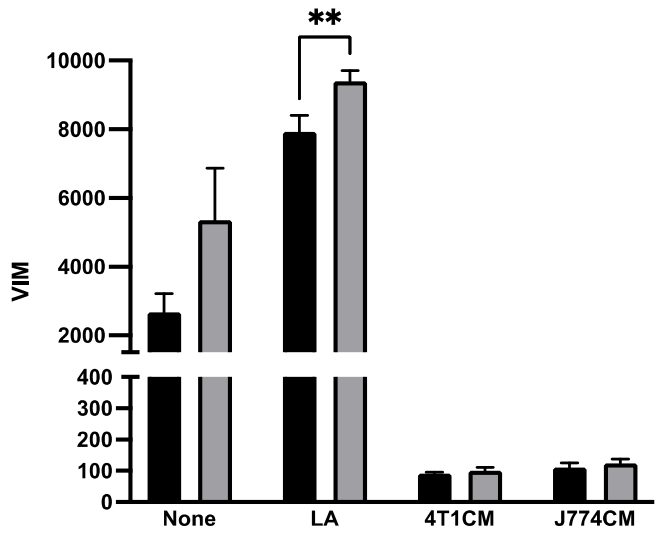


Fig 20. The Gasdermin inhibitor DS promoted vimentin secretion by L929 fibroblasts activated by LPS and ATP. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated with the gasdermin D inhibitor DS (gray bars) or not (black bars) and vimentin (VIM) secretion (AU, average ±SEM) was determined by Western/dot blots. Independent experiments and $n\geq3$, Two-way ANOVA, Sidak post-hoc test with ** $p<0.01$.

12- The gasdermin D inhibitor DS promotes NLRP3 complex protein expression and modulate pro-inflammatory cytokine secretions

Treatment with DS of L929 fibroblasts activated through incubation with LPS and ATP or 4T1 tumor secretome led to significant increases in NLRP3 inflammasome protein components including NLRP3, ASC1 (Fig 21, $p<0.01$). In contrast, treatment with DS of L929 cells activated with the J774 macrophage secretome led to a decreased expression of active Caspase 1 (Fig 21C, $p<0.05$).

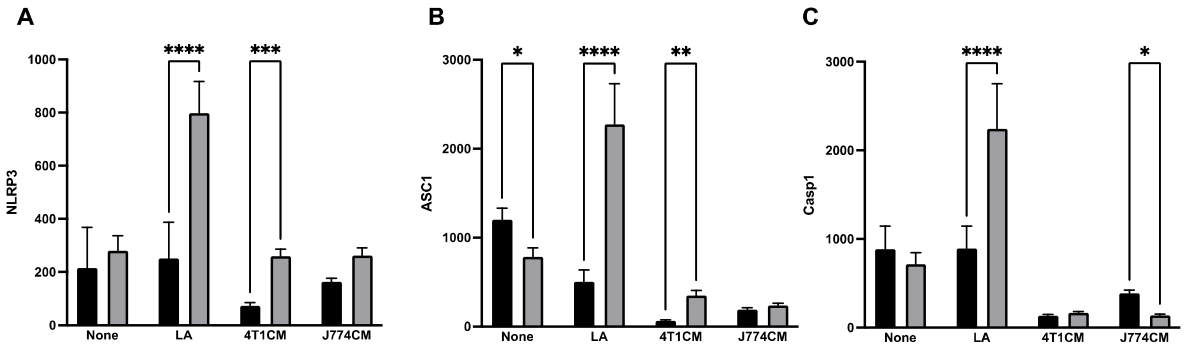


Fig 21. DS treatment of activated L929 fibroblasts mainly promoted increases NLRP3 inflammasome protein expression. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated 24hrs with the gasdermin D inhibitor DS (gray bars) or not (black bars) and expression (AU, average ±SEM) of NLRP3 (**A**), ASC1 (**B**) and Casp1 (**C**) were determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Sidak post-hoc test with (*) $p<0.1$ and *$p<0.05$.

DS treatment alone promoted IL-18 but not IL-1beta secretions (Fig 22B, $p<0.001$). L929 fibroblast activated by LPS + ATP also secreted more IL-1beta when incubated in the presence of DS (Fig 22A, $p<0.01$).

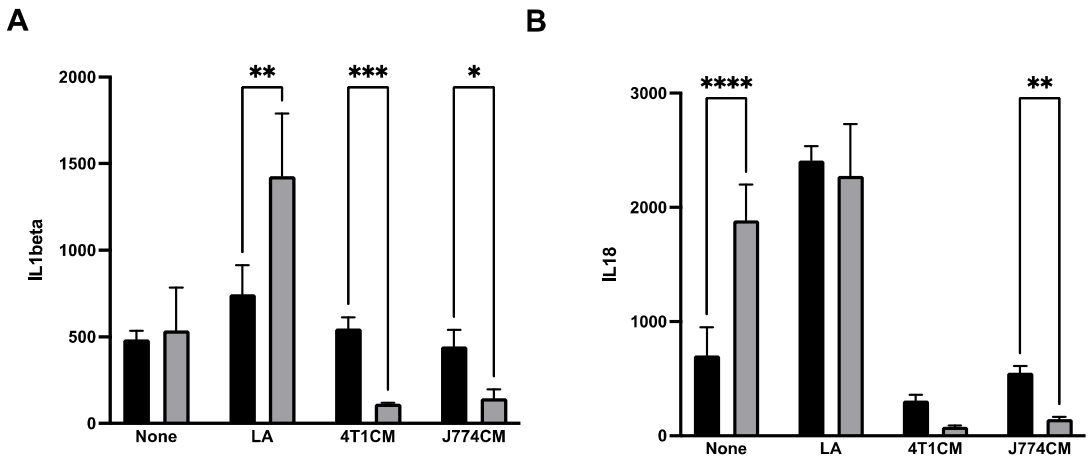


Fig 22. DS treatment modulated L929 fibroblast pro-inflammatory cytokine secretions. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated 24hrs with the gasdermin D inhibitor DS (gray bars) or not (black bars) and secretions (AU, average ±SEM) of IL-1beta (**A**), and IL-18 (**B**) pro-inflammatory cytokines were determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Sidak post-hoc test with *p<0.05, **p<0.01 and ***p<0.001.

In contrast, DS addition to either 4T1 or J774 secretomes significantly decreased Il-1beta secretions (Fig 22, $p<0.05$). The combination J774 secretome and DS led to a decrease in IL-18 secretions (Fig 22B, $p<0.01$).

13- The gasdermin D inhibitor DS decreases collagen 1A2 secretions by L929 fibroblasts

Activation of L929 fibroblasts through the canonical inflammasome activator LPS + ATP treatment combined with DS treatment led to increases in 1A1, 1A2 and 4A3 collagen secretions (Fig 23, $p<0.05$). In contrast, 4T1 and J774 secretomes combined with DS tended to decrease collagen secretions (Fig 23, ns). In particular, collagen 1A2 secretions were significantly decreased in the presence of the combination DS with either 4T1 and J774 secretomes (Fig 23B, $p<0.05$).

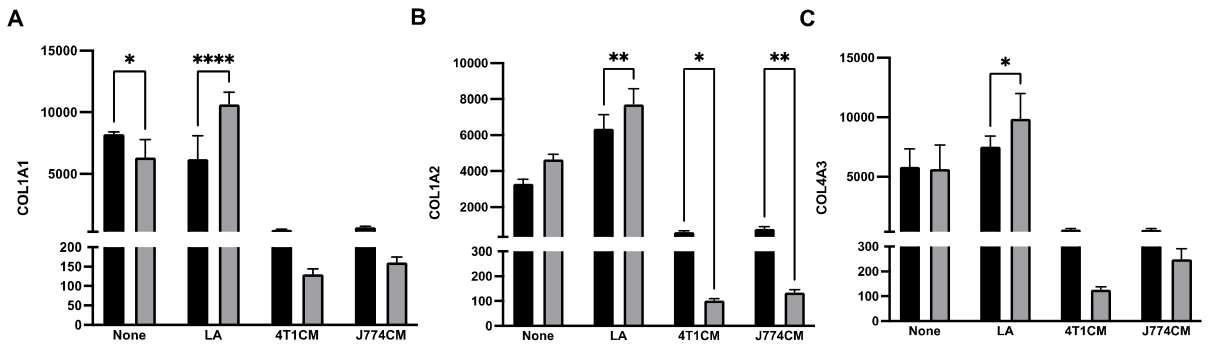


Fig 23. The gasdermin D inhibitor DS modulated L929 fibroblast collagen secretions. L929 fibroblasts untreated (None) or activated with either LPS + ATP combination (LA), secretomes from 4T1 tumor cells (4T1CM) or J774 macrophages (J774CM) were incubated 24hrs with the inflammasome inhibitor MCC950 (gray bars) or not (black bars) and secretions of Col1A1 (**A**) and Col1A2 (**B**) fibrous, and Col4A3 (**C**) globular collagen secretions (AU, average ±SEM) as determined by Western/dot blots. Independent experiments and n≥3, Two-way ANOVA, Sidak post-hoc test with *p<0.05, **p<0.01and ***p<0.001.

14- Combination of MG132, MCC950 and DS and CAF L929 fibroblast phenotype

As MG132, MCC950 and DS are purported to target distinct pathways associated CAF phenotype, inflammasome activation and pro-inflammatory cytokine secretion, the effects drug combinations were assessed first on CAF phenotype. In the absence of activation MG132, MCC950 and DS alone or in combination had no significant effects on L929 fibroblast vimentin secretions (Fig 24A, ns).

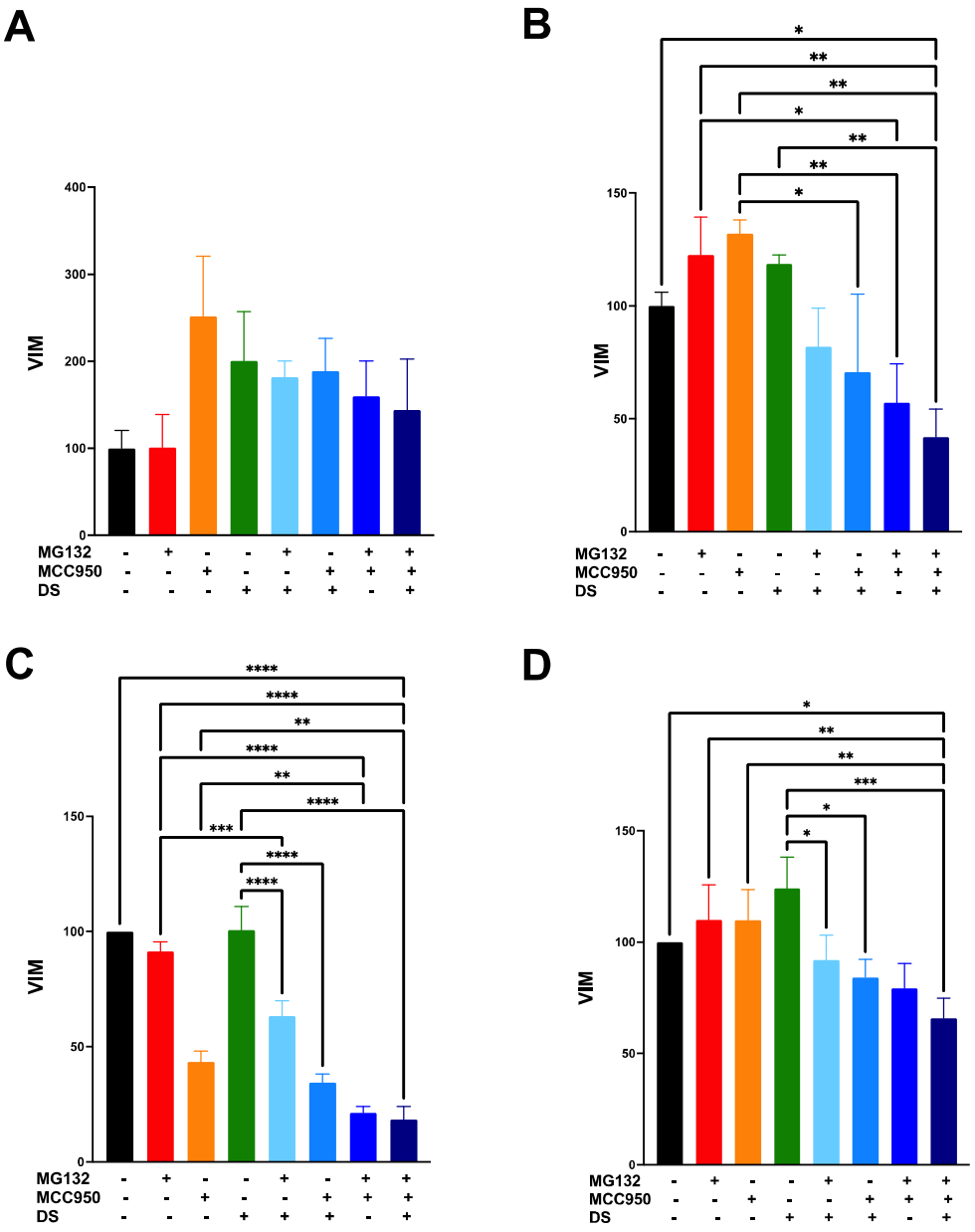


Fig 24. Effects of combination of MG132, MCC950 and L929 fibroblast vimentin protein secretions. L929 fibroblasts untreated (**A**) or activated with either LPS + ATP combination (**B**), secretomes from 4T1 tumor cells (4T1CM) (**C**) or J774 macrophages (J774CM) (**D**) were incubated 24hrs with combinations of MG132, MCC950 and DS and vimentin (VIM) secretion (AU, average ±SEM) determined by Western/dot blots. Independent experiments and n≥3, One-way ANOVA, Tukey post-hoc test with *p<0.05, **p<0.01and ***p<0.001.

The vimentin secretions of LPS/ATP (Fig 24B) and J774 macrophage secretome (Fig 24D) activated L929 fibroblasts were not affected by either MG132, MCC950 or DS individually. However, in particular drug combination with MCC950 were associated with significant decrease in vimentin secretions (Fig 24, $p<0.05$). The combination of the three inhibitors led to decreased vimentin expression by 60% and 35% from L929 cells incubated with LPS/ATP or J774CM alone (Fig 24, $p<0.001$). Notably, the vimentin expression of L929 fibroblasts incubated with tumor 4T1 secretome (4T1CM Fig 24C) was decreased through incubation with MCC950 alone. Moreover, combination of either MG132 or DS with MCC950 led to at least 65% decrease in vimentin secretion (Fig 24C, $p<0.01$). The combination of the three inhibitors led to decreased vimentin expression by 80% from L929 cells incubated with 4T1CM alone (Fig 24C, $p<0.001$).

15- Combination of MG132, MCC950 and DS and L929 fibroblast Il-18 pro-inflammatory cytokine secretions

Inflammasome activation leads to pro-inflammatory secretions including of IL-1beta and IL-18. Observations made here at 24hrs indicated changes mainly in IL-18 secretions (see above). Therefore, we assessed whether combination of the inhibitors altered IL-18 secretions by L929 fibroblasts. Individually, in the absence of activation both MCC950 and DS significantly promoted IL-18 secretion by L929 fibroblasts (Fig 25A). In contrast combinations of the inhibitors blunted the increase observed with MCC950 or DS alone to a secretion comparable to control conditions (Fig 25A).

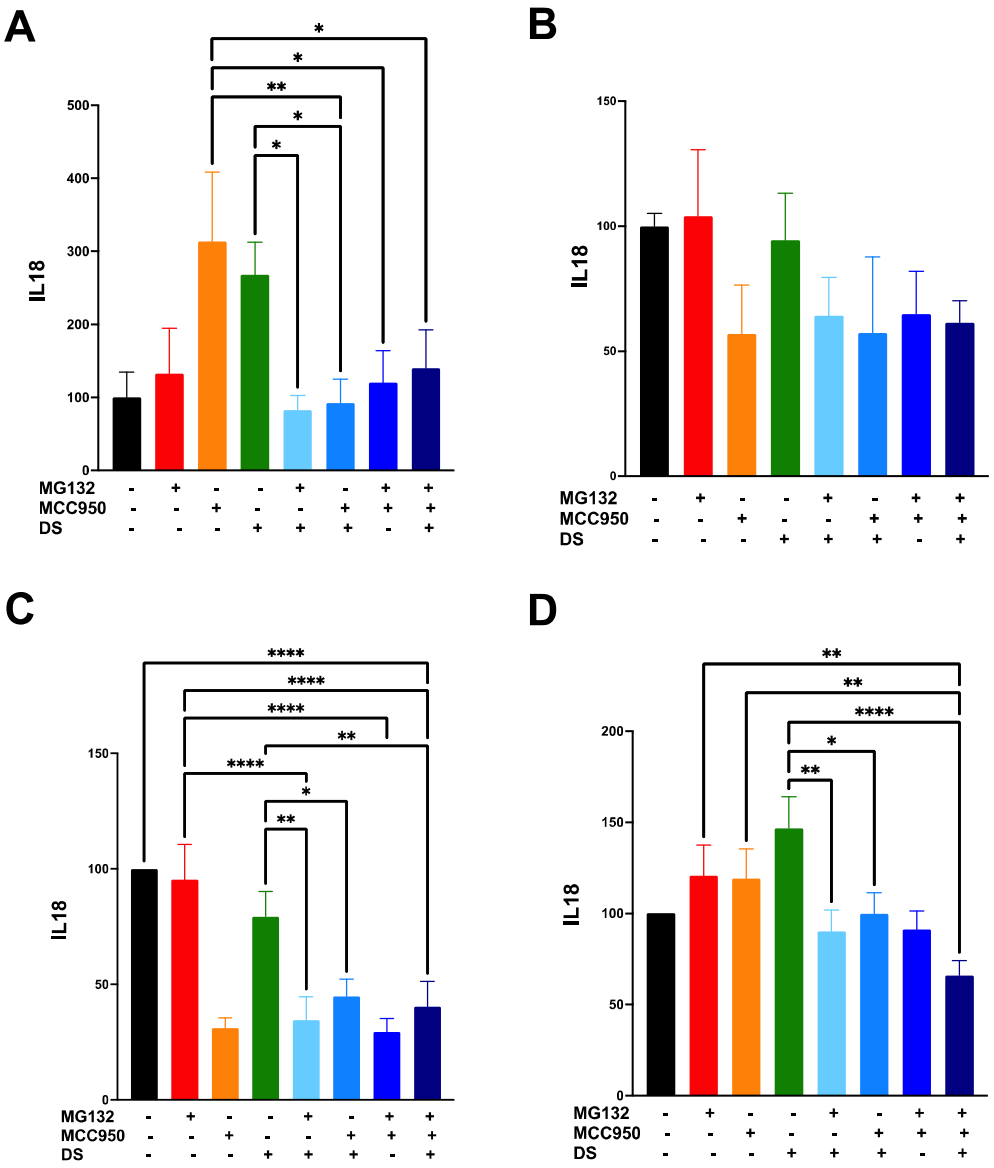


Fig 25. Effects of combinations of MG132, MCC950 on L929 fibroblast IL-18 secretions. L929 fibroblasts untreated (**A**) or activated with either LPS + ATP combination (**B**), secretomes from 4T1 tumor cells (4T1CM) (**C**) or J774 macrophages (J774CM) (**D**) were incubated 24hrs with combinations of MG132, MCC950 and DS and IL-18 secretion (AU, average ±SEM) determined by Western/dot blots. Independent experiments and n≥3, One-way ANOVA, Tukey post-hoc test with *p<0.05, **p<0.01and ***p<0.001.

In LPS +ATP treated L929 fibroblasts, neither inhibitor individually or in combination led to difference in Il-18 secretions (Fig 25B, ns). Interestingly, 4T1 tumor secretome-driven L929 cell

IL-18 secretions were decreased by MCC950 alone as with dual combinations of either MG132, MCC950 or DS and the triple inhibitor combination (Fig 25C, $p<0.05$). J774 tumor secretome-driven L929 cell IL-18 secretions tended to increase in the presence of individual inhibitors notably DS (Fig 25D). The triple combination MG132, MCC950 and DS decreased IL18 secretion by 35% compared to J774CM alone (Fig 25D).

16- Combination of MG132, MCC950 and DS and L929 fibroblast Col1A2 secretions

Collagen production including fibrous and globular collagens is a hallmark of fibroblast activities. Here, in particular fibrous collagen 1A2 secretion was modulated depending on the activator tested (see above). Therefore, we assessed whether combinations of MG132, MCC950 and DS altered activated L929 fibroblast Col1A2 secretions (Fig 26). In the absence of activation, MG132, MCC950 and DS alone or in combination had only limited effects on L929 fibroblast Col1A2 secretions (Fig 26A).

Following activation with LPS and ATP, MCC950 alone triggered an increase in COL1A2 secretions of L929 fibroblasts (Fig 26B). With regard to inhibitors combinations, only the triple combination and the MG132 + MCC950 combination decreased LPS-ATP-driven L929 cell Col1A2 secretions by ~30% and 20%, respectively (Fig 26B). Similar observations were made in J774 macrophage secretome driven L929 fibroblast secretions of Col1A2 (Fig 26D) with only a modest decrease in Col1A2 secretions even in the presence of the triple inhibitor combination (Fig 26D).

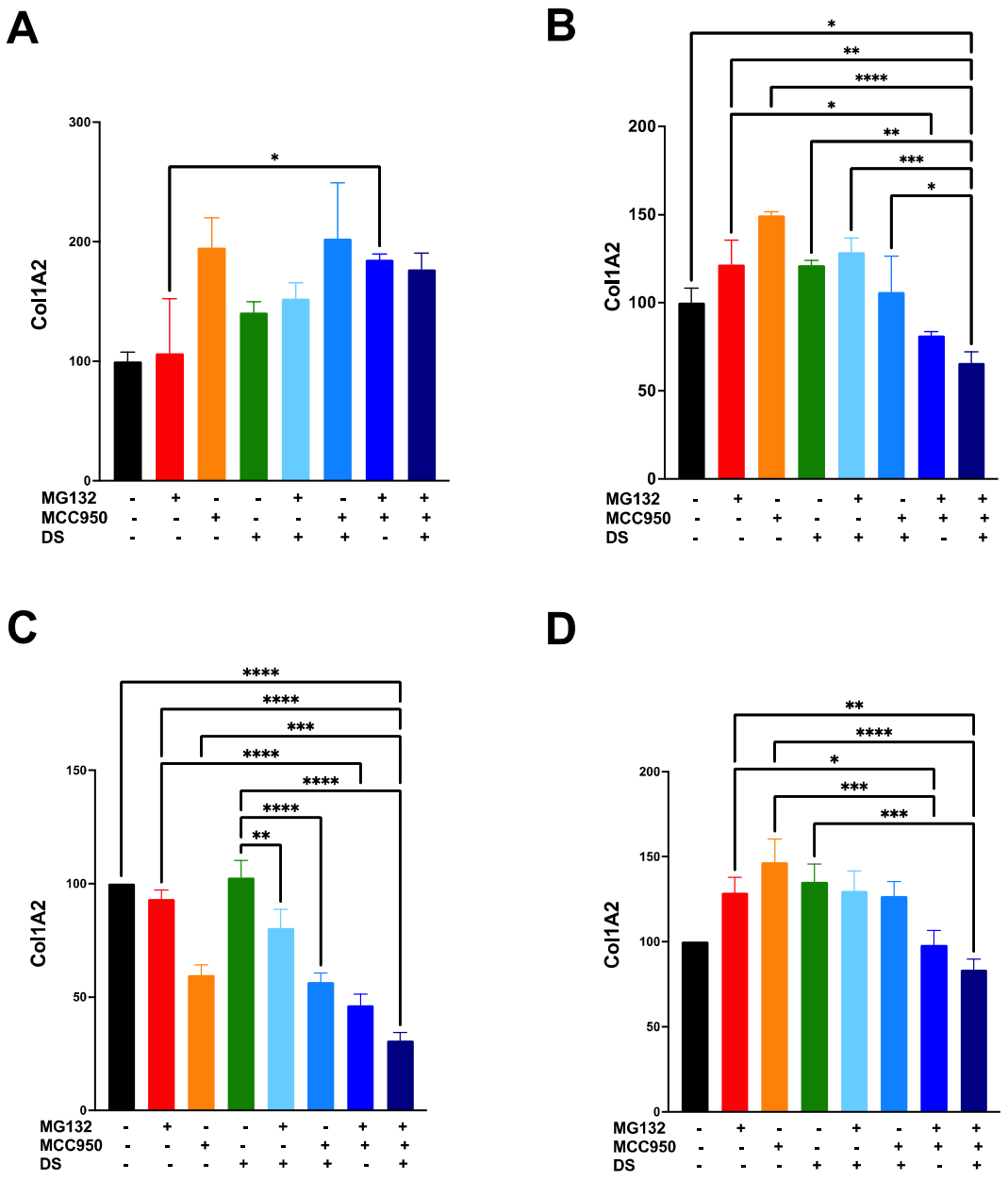


Fig 26. Effects of combination of MG132, MCC950 onL929 fibroblast collagen 1A2 secretions. L929 fibroblasts untreated (**A**) or activated with either LPS + ATP combination (**B**), secretomes from 4T1 tumor cells (4T1CM) (**C**) or J774 macrophages (J774CM) (**D**) were incubated 24hrs with combinations of MG132, MCC950 and DS and col1A2 secretion (AU, average ±SEM) determined by Western/dot blots. Independent experiments and n≥3, One-way ANOVA, Tukey post-hoc test with *p<0.05, **p<0.01and ***p<0.001.

In contrast, 4T1 tumor secretome driven L929 fibroblast Col1A2 secretions were decreased in the presence of the NLRP3 inflammasome inhibitor MCC950 alone whereas other inhibitors had not effects (Fig 26C). Moreover, dual inhibitor combinations including MCC950 and the triple inhibitor combination decreased Col1A2 secretions by L929 cell activated be 4T1CM up to 70% (Fig 26C).

DISCUSSION

Breast cancer progression and especially the development of metastasis associated with all breast cancer associated death biologically rely no only on aberrant protein expression and activity of tumor cells but also on alteration of TME components.[20] Above all, signaling molecules concentrations, matrix composition and density as well as stroma cells activities actively modulated cancer progression.[28] In particular, CAF have been implicated in breast cancer progression in part through their secretions of fibrous collagens and the generation of a fibrotic tumor environment.[20] Indeed, fibrosis is an independent marker of breast cancer progression. As inflammation and fibrosis are intertwined, CAF also significantly participate in modulating local immune responses specifically inflammation.[35] Recent research demonstrated the role of pro-inflammatory cytokines especially of the IL1 superfamily in cancer progression.[45] Moreover, innate immune response specifically the activation of the NLRP3 inflammasome have been implicated in the secretion of those pro-inflammatory cytokines within the tumor microenvironment by immune cells as well as tumor cell and other stroma cell lineages including fibroblasts.[50] Here, using the L929 fibroblast model and NF-kB, NLRP3 and GSDMD inhibitors, we tested the hypothesis that fibroblast inflammasome activation, a feature of cancer-associated fibroblasts, leads to increased fibrous collagen secretion through NF-kB dependent pathways thereby generating a pro-tumorigenic pro-fibrotic microenvironment favoring breast tumor growth.

Our results (summarized in Table 1) indicate that (1) L929 fibroblasts have functional NLRP3 inflammasomes leading to pro-inflammatory secretions (2) activation of L929 fibroblasts generated fibroblast with CAF phenotype and (3) secretions of collagen subtypes are differentially altered following L929 activation. Moreover, mechanistically, NF-kB and NLRP3 pathways

critically participate in the CAF phenotype expression and functional inflammasome activities of fibroblasts *in vitro*.

As the signaling interplays between tumor cells stroma cells and ECM components within the tumor microenvironment are extremely complex,[20] assessment of alterations in multiple cell type signaling changes within the TME *in vivo* is challenging. Consequently, *in vitro* cell models are routinely used to mimic signaling and responses to activation and/or to inhibition of specific signaling pathway targets. The experimental design consisting of L929 fibroblast cells used here mimics, for example, tumor cells but also stroma cell have been cultured in vitro and the role of the MAPK, NF-kB pathway in cell proliferation and protein expression demonstrated.[52] L929 cells have been used extensively to assessed the fibrotic potential of environmental substances. [66]

To assess the role of the NF-κB pathway in the development of a pro-tumorigenic microenvironment, the L929 murine fibroblast *in vitro* model was used as previously stated.[15] L929 fibroblasts have functioning inflammasomes (specifically, the NLRP3 inflammasome), and this inflammasome can be activated using common inducers (i.e., LPS and ATP).[7] The NLRP3 inflammasome is one of the most studied in innate immune cells. This cytoplasmic protein complex is known to mediate caspase-1 activation, which then allows for the maturation and secretion of pro-inflammatory cytokines and collagens.[7]

Our data support the presence of active inflammasome i.e., NLRP3 inflammasome in fibroblast through protein expression as determined by flow-cytometry and western blot. That result confirm observation of NLRP3 inflammasome presence and activities in fibroblast-like synoviocytes,[67] adventitial fibroblasts,[68] gingival fibroblasts,[69] hepatic fibroblasts[70] as well as dermal fibroblasts.[71] Moreover, alteration in NLRP3 protein expression observed here were generally associated with changes in pro-inflammatory cytokine IL-1beta and Il-18 secretions.

Indeed, the NLRP3 inflammasome activation leads to activation of caspase 1, which in turn cleaves pro-IL-1beta and pro-IL-18 precursors into the to-be-secreted mature IL-1beta and IL-1-18, respectively.[9] While the secretions of IL-1beta through gasdermin D transient cell membrane pore has been shown, here observations regarding DS effects on both IL-1beta and IL-18 secretions were unconclusive and will require further analyses possibly using additional in vitro models and other approaches.[51] Interestingly, L929 fibroblasts at least in the presence of 4T1CM developed a CAF phenotype that was blunted by both NF-kB and NLRP3 inhibitors, respectively. The commonly used LPS + ATP activator generated L929 with a CAF phenotype. However, inhibitors of NF-kB, NLRP3 were not associated with marked alterations in particular for IL-1beta and Il-18 secretions possibly because LPS and ATP stimulation may activate other inflammasomes and cellular pathways.[51] Lastly, as preliminary observations suggest alterations in IL-1RA and IL-18 binding protein should also be assessed as they will modulate available IL-1beta and IL-18 within the TME.

Table 1. L929 fibroblast activation and CAF, NLRP3, IL-1beta, IL-18 and collagen subtype protein expression

		LA	4T1CM	J774CM
CAF phenotype[(a)]	VIM	++	++++	=
	SMA	++	NA	NA
	MMP14	(+)	NA	NA
NLRP3 inflammasome[(a)]	NLRP3	=	----	----
	ASC1	--	----	----
	Casp1	=	----	----
Pro-inflammatory cytokines[(b)]	IL-1beta	(+)	(-)	(-)
	IL-18	+++	----	----
Fibrosis & Collagens[(c)]	COL1A1	=	----	----
	COL1A2	++	----	----
	COL4A3	=	----	----
	1A2/1A1	+++	++++	++++

[(a)]Vimentin and CAF phenotype blocked by inhibition of NLRP3 inflammasome by MCC950, alone or combined with MG132 and or DS.

[(b)]IL18 secretion and NLRP3 activation blocked by the NLRP3 inflammasome inhibitor MCC950, alone or combined with MG132 and or DS.

[(c)]Fibrous Col 1A2 secretion promoted by 4T1CM mainly blocked by the NLRP3 inflammasome inhibitor MCC950 alone or combined with MG132 and or DS.

Our results also highlight effects of activation on especially the increase in fibrous collagen secretions consistent with the role of fibrosis in breast cancer progression.[61] Interestingly, not only is the fibrous collagen COL1 secretion increased but the specific subtypes are differentially increased compared to globular collagen. In particular, the COL1A2/COL1A1 ratio is markedly increased following activation. This observation concurs with *in silico* observations that change in COL1A1 and COL1A2 are associated with differential breast cancer patient overall survival outcomes (Fig 2S). Whether intervention that trigger differential collagen subtype expressions may participate in preventing breast cancer progression is intriguing and may warrant further research.

Taken together, our observations in the L929 in vitro fibroblast model provide a better understanding of the role of fibroblasts within the TME namely that activation through the tumor microenvironment i.e., tumor cells' secretome participate in the generation of CAFs, that activation also trigger NLRP3 inflammasome and increased secretion of pro-inflammatory cytokines IL-1beta and IL-18 and participate in fibroblast collagen secretions. Whether targeting CAF inflammasome activities especially pro-inflammatory and collagen subtype secretions would benefit and limit breast cancer progression may warrant further investigation.

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Supplemental Material

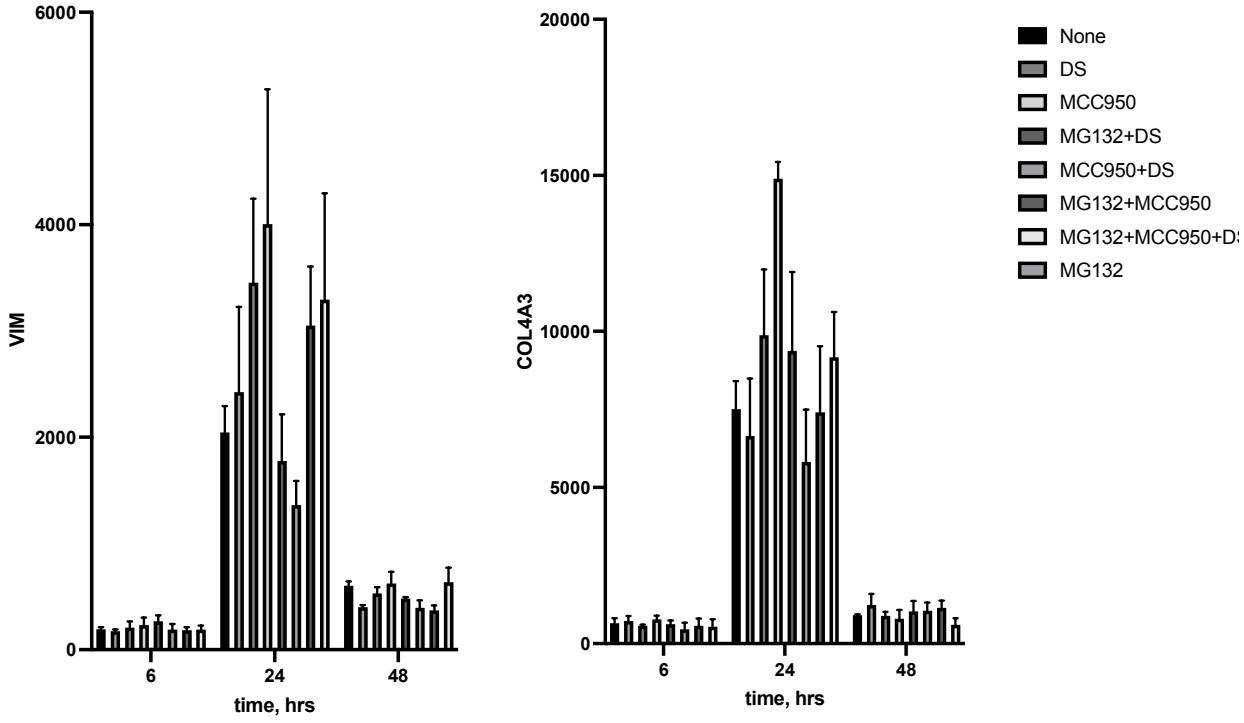

Figure 1S. Protein expression over time was assessed highlighting optimal expression detected 24hrs post treatment. Expression of intracellular (lysates) VIM (**A**) and extracellular (supernatants) COL4A3 (B) were determined using western/dot blots quantified post- ECL chemiluminescence analyses. As shown, protein expression 24hrs post-treatment were markely higher and thus used throughout the present study unless noted.

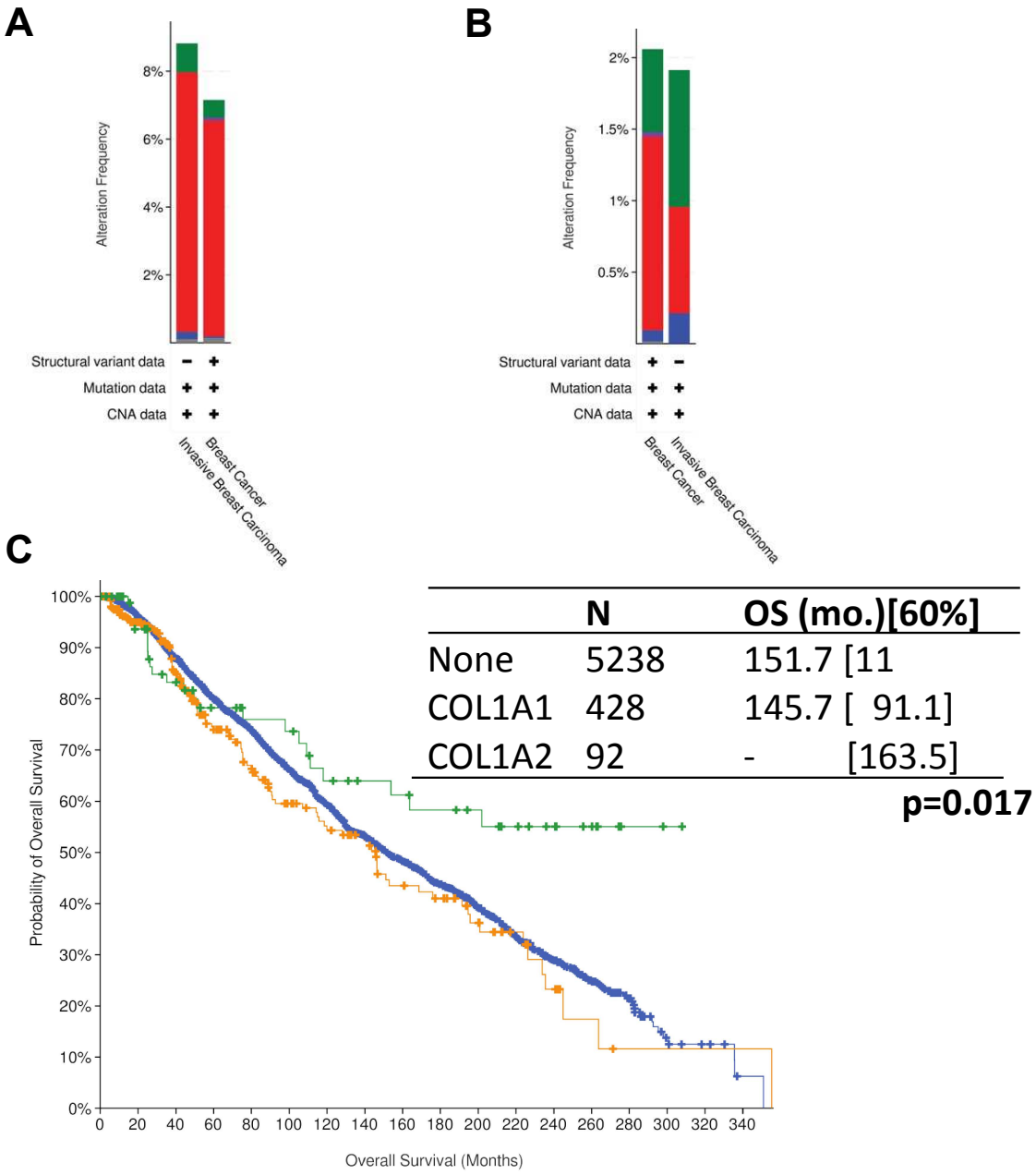

Figure 2S: Genetic alterations in Col1A1 and COL1A2 and breast cancer patient overall survival. Briefly, COL1A1 and COL1A2 genetic alterations in breast cancer patients were identified through *in silico* investigations using CBioportal.org. Most genetic alterations in COL1A1 (**A**) and COL1A2 (**B**) were amplifications (red) and with mutations second (green). Overall survival of patients with mutation in COL1A1 (orange) or COL1A2 (green) differed from patients without either of those two genetic alterations (blue) Log-rank p = 0.017) with increased survival associated with alterations in COL1A2.