INFLAMMASOME-DRIVEN IL-1β AND IL-18 CYTOKINE SIGNALING BETWEEN MACROPHAGES AND CANCER CELLS WITHIN THE BREAST TUMOR MICROENVIRONMENT

by

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ABSTRACT

DEEPIKA SURYAPRAKASH. Inflammasome-driven IL-1β and IL-18 cytokine signaling between macrophages and cancer cells within the breast tumor microenvironment (Under the direction of DR. DIDIER DRÉAU)

Breast cancer especially triple-negative breast cancer remains deadly, despite detection, monitoring and therapeutic advances. Indeed, aggressive breast cancers metastasize to tissues including the brain, bones, and lungs. Metastasis is favored by a pro-inflammatory tumor microenvironment regulated in part by tumor-associated macrophages. Macrophages especially secrete the pro-inflammatory cytokines IL-1β and IL-18 mainly following the activation of inflammasomes. The role of the inflammasome-driven pro-inflammatory IL-1ß and Il-18 cytokines in the cross-talk between macrophages and tumor cells within the breast tumor microenvironment remains to be elucidated. Here, we used *in vitro* models to assess the role of inflammasome in the production of IL-1β, IL-18 and the subsequent effects of IL-1β and IL-18 on both 4T1 mammary tumor growth and J774, RAW and bone marrow derived macrophages. Our *in vitro* results demonstrate that (1) inflammasome proteins are expressed and co-localized in 4T1 tumor cells and in J774 macrophages; (2) conditioned media from inflammasome-activated 4T1 cells promoted macrophage secretions of inflammasome-related cytokines, (3) conditioned media from inflammasome-activated macrophages promoted 4T1 cell growth. Furthermore, (4) recombinant IL-18 reduced J774 cell growth and, dose-dependently, 4T1 cell growth. Finally, IL-18BP abrogated IL-18-driven J774 growth inhibition. Taken together, these data suggest that inflammasome modulation, especially through the disruption of the IL-18/IL-18BP signaling, may have potential therapeutic benefit for breast cancer patients.

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CHAPTER 1: INTRODUCTION

1.1 Inflammasomes

Inflammasomes are cytoplasmic protein complexes and a component of the innate immune system. Upon dysregulation by stressors and/or in numerous pathological states, inflammasome assembly and activities are markedly increased [1]. Inflammasomes are implicated in several inflammatory disease modalities including rheumatoid arthritis, systemic lupus erythematosus and Sjogren's syndrome [2].

While inflammasomes can form extended protein complex, the base structure is formed by the assembly of three proteins – typically a receptor, an adaptor and a caspase. Discovered a decade ago [3], several types of inflammasomes usually named based on their receptor have been defined [4]. Tschopp et al. first described the activation of the inflammasome machinery as the molecular basis leading to an increased production of the pro-inflammatory cytokine (interleukin-) IL-1β and its causal relationship to patient fever syndromes [5].

The inflammasomes types have been reviewed recently [1, 5, 6]. Inflammasomes most thoroughly investigated include the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) subsets NLRP3, NLRP1, and NLRC4 [1, 7], all sensors of cellular stress [2], and involved in the regulation of immune responses and cell death pathways [7]. The AIM2 (absent in melanoma) inflammasome is a different inflammasome that contributes to defense against bacterial and viral DNA [8]. The composition and function of inflammasomes, in particular, of NLRP1 (i.e, NALP1) and NLRP3 inflammasomes, have been investigated [3]. To date, the NLRP3 inflammasomes remain the best-characterized inflammasomes [8].

NLRP3 inflammasomes are multi-protein complexes with the following components:

- The NLRP3 (NOD-like receptor protein 3) which binds inflammation inducers;
- The ASC-1 (apoptosis-associated speck-like protein 1) adaptor, which contains a CARD (caspase recruitment domain). It is also called PYCARD and brings the receptor and caspase together; and
- The caspase-1 which cleaves the immature pre-proteins i.e., pro-IL-18 and pro-IL-1β into their mature active forms

The NLRP3 inflammasome is activated by a range of stimuli reviewed elsewhere [8]. The stimuli are diverse and include LPS and toxins; danger-associated molecular patterns (DAMPs) like ATP, asbestos and silica [8]; and oxidative stress and related ROS (reactive oxygen species) [9]. Depending on the nature of the activating signal, the composition of the inflammasomes may vary [1]. Upon activation, the receptor-adaptor protein complex is assembled and ASC-1 recruits the caspase, mainly caspase 1 (although caspase 11 can also be recruited), which is activated upon binding to ASC. Caspase-1 then cleaves the pro-IL-18 and pro-IL-1β into their active, mature forms IL-18 and IL-1β (Fig 1).

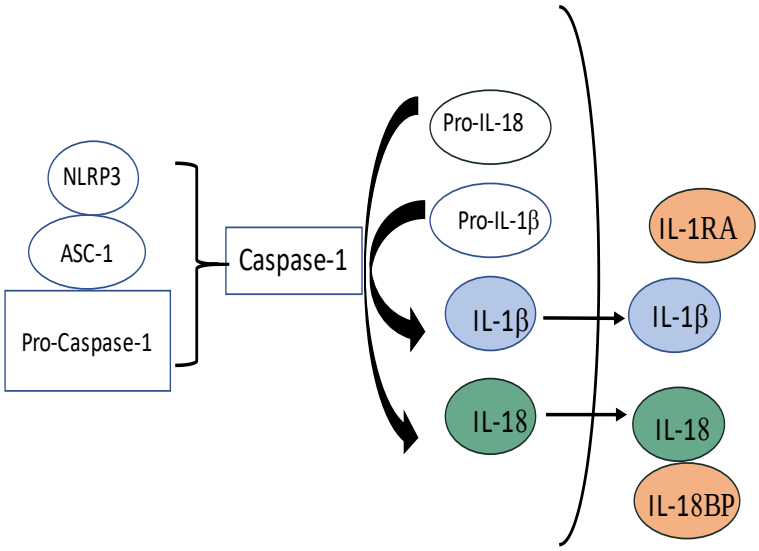

Fig 1. Schematic of the NLRP3 inflammasome, its secretions and their interactions

1.2 Inflammasomes and Cancer

The establishment and progression of cancer are associated with several hallmarks, summarized earlier by Hanahan and Weinberg [10, 11]. While the accumulation of critical genetic lesions is essential to tumorigenesis, other factors critically modulate cancer progression [1]. Indeed, extrinsic factors, especially inflammation and the infiltration of inflammatory cells, are key to the development and the malignant nature of cancer [1]. In particular, the high levels of pro-inflammatory cytokines of the IL-1 super-family like IL-18 and IL-1β have been associated with immune-suppressive tumor microenvironment in cancer patients [1].

Tumors, often referred to as "wounds that never heal" [12], are sites of injury with constant recruitment of immune cells, particularly macrophages. The number and type of immune cells as well as the nature of the cytokines secreted participate in the generation of an inflammatory microenvironment that is associated with a worse prognosis [13]. Among the consequences of the local inflammation within tumor are immunosuppressive signaling, cell signaling triggering changes in immune cell phenotypes [14]. In particular, the alteration of macrophage phenotype from anti-tumorigenic (M1) to pro-tumorigenic (M2) within the tumor microenvironment has been detailed [14, 15]. These macrophages, through signaling from tumor cells, "get educated" to secrete pro-inflammatory signaling molecules favoring tumor progression [14].

Both tumor cells and especially macrophages secrete multiple signaling molecules, including pro-inflammatory cytokines leading to a pro-tumor microenvironment [14]. Tumor-associated macrophages (TAMs) promote tumor progression and metastasis by secreting pro-inflammatory cytokines, including IL-1ß and IL-18 [16]. While multiple

pathways are involved in pro-inflammatory cytokine production, the activation of inflammasomes specifically stimulates the secretion of active pro-inflammatory cytokines [3]. Inflammasomes are especially active in macrophages, and NLRP3 inflammasomes are key to the secretion of the pro-inflammatory cytokines (interleukin-) IL-18 and IL-1β [8].

Inflammasome activation is critically initiated following the engagement of toll-like receptors (TLRs), key elements of the innate immune response pivotal in the detection of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) [1] including signals like LPS and ATP, respectively. NLRs / inflammasomes sense and respond to PAMPs and DAMPs serving as sentinels for a large number of cellular distresses including infections and other cell insults [1].

Along with the production of pro-inflammatory cytokines IL-18 and IL-1β, the activation of inflammasomes can trigger a specific cell death mechanism, i.e., pyroptosis [6]. While pyroptosis is commonly observed as a host response against bacteria [6], pyroptosis also occurs in hematopoietic cells, stromal cells, and in cells of the central nervous system [17]. The mechanisms of pyroptosis and of production of pro-inflammatory cytokines have been correlated to poor prognosis for cancer patients [18]. The control of these pro-inflammatory pathways is essential to preventing chronic inflammation [1]. The inflammasome-driven regulation of programmed cell death, differentiation and proliferation [1, 18] is key to tumor progression. Epidemiological and clinical data support the crucial role of inflammasome in tumor development [1, 6]. Indeed, NLRP3 inflammasomes are associated with increased susceptibility to melanoma [19], and poor survival rate for colorectal cancer and myeloma [20, 21].

1.3 Inflammation and Breast Cancer

Breast cancer is the second most commonly diagnosed cancer in women, second only to skin cancer [22]. The American Cancer Society estimates that 266,120 new cases of invasive breast cancer will be diagnosed in women and about 40,920 women will die from breast cancer in 2018 [22]. The high mortality rate of breast cancer is directly correlated with its invasive nature and the presence of metastases in critical tissues such as bones, brain and lungs [23].

In MCF-7 breast cancer cells, inflammasomes facilitate the maturation and subsequent secretion of IL-18 and IL-1β upon multiple stimulations including adipocyte-derived leptin [16, 24]. The NLRP3 inflammasome stimulates dendritic cells to secrete IL-1β [53], and has been shown to play a pro-tumorigenic role, by increasing lymphangiogenesis and, thus promoting lung metastasis [25].

On the other hand, AIM2 inflammasomes suppressed human breast cancer cell proliferation and tumor growth through repression of NF-kB promoter activity in an immunocompromised mouse model [16, 26]. Thus, different inflammasomes appear to play varying roles in cancer progression, with IL-1β being a promoter of cancer progression [16]. Increased serum levels of IL-1β have been associated with higher rate of recurrence of breast cancer, and are critical for tumor growth, invasiveness and angiogenesis [27, 28]. In contrast, polymorphisms of the IL-18 gene have been associated with increased breast cancer risk [16]. Additionally, human mesenchymal stem cells (hUMSCs) derived from the human umbilical cord overexpressing IL-18 were shown to suppress proliferation and migration of MCF-7 breast cancer cells when co-cultured *in vitro* [29]. The contradictory

roles of IL-18 likely depend on the context and cell types that generate IL-18 [16]. Whether IL-18 plays promote or hinder breast cancer progression remains to be clearly delineated.

1.4 The Interleukins 18 and 1β, and their regulators

Both IL-1β and IL-18 pro-inflammatory cytokines belong to the interleukin-1 superfamily, which includes IL-1α, IL-33 and IL-36 as well [30]. The IL-1 superfamily also encompasses the antagonists IL-1RA, IL-37 and IL-38 each of which promotes anti-inflammatory activities [30]. Following the binding of these IL-1 superfamily ligands to their respective tyrosine kinase cell surface receptors, the recruitment of a co-receptor initiates intracellular signaling. IL-1 family cytokines participate in potent inflammatory responses and their activities are tightly controlled at multiple levels including protein production, processing and maturation [30].

Interleukin 1ß:

IL-1ß effects in the tumor microenvironment are pleiotropic [31]. Myeloid cells secrete the pro-inflammatory cytokine IL-1β [32]. IL-1β promotes tumorigenesis and tumor invasiveness in melanoma, prostate and mammary gland tumor models [27, 28]. Indeed, through interaction with NF-κB, IL-1ß leads to the production of FGF2 (fibroblast growth factor 2), pro-angiogenic VEGF (vascular endothelial growth factor), TNF-α (tumor necrosis factor alpha) and TGF-β (transforming growth factor beta) [28, 33]. IL-1ß also induces migration and tube formation via activation of the MAPK pathway and the upregulation of VEGF production and secretion [28]. Secreted IL-1ß supports local inflammation, enhances inflammatory cell infiltration, and promotes adhesion molecule expression on endothelial cells and leukocytes thereby favoring angiogenesis [14]. The

proximity of myeloid cells to endothelial cells lining blood vessels plays a critical role in angiogenesis [14].

The activity, receptor binding and post-receptor signaling of IL-1ß are inhibited by naturally occurring inhibitor IL-1RA [30] (Fig A). IL-1RA is a receptor antagonist that, through competitive binding to the IL-1 receptor, prevents IL-1R dimerization, and thus abrogates IL-1ß signaling [30]. Both preclinical and clinical investigations demonstrated that a higher IL-1β/IL-1RA ratio shifted the IL-1 system towards inflammation [32]. These evidences are the basis for IL-1RA clinical trials [32, 34]

Interleukin-18:

On the other hand, the roles of IL-18 are not as well characterized. The effects of Il-18 are context dependent and vary depending on the pathology including cancer types [35]. Originally defined as an inducer of the critical regulatory cytokine interferon (IFN)-γ [36], IL-18 is associated with pro-inflammatory conditions [30]. IL-18 is central to several inflammatory pathologies including atherosclerosis and psoriasis [37].

Multiple studies have described the inhibitory role of IL-18 in cancer [35, 38, 39]. Contrasting with IL-1β, IL-18 enhances the immune checkpoints against the dissemination of colon cancer cells [38]. Additionally, in a mouse model, intra-tumoral delivery of IL-18 enhanced IFN-γ production and caused regression of CT26 liver tumors [39]. Interestingly, the downstream signaling pathways of IL-18 and IL-1β likely differ. Indeed, in adenocarcinoma cells, IL-18, unlike IL-1β, does not activate the key transcription factor NF-κB [40]. NF-κB is required for regulation of several genes including TNF-α [40] and IFN-γ [37].

Like other members of the IL-1 superfamily, the activity, receptor binding and post-receptor signaling of IL-18 are regulated by naturally occurring inhibitors [30]. Isolated in 1999 [41], IL-18BP is a soluble decoy receptor sharing structural homology with the IL-1 receptor family [42]. The human IL-18BP is expressed as four separate isoforms (a, b, c, d). IL-18BPa and IL-18BPc have the highest binding affinity to IL-18 – over 90% at a two-fold molar excess of IL-18 [43]. IL-18 binding protein (IL-18BP) strongly binds to and, thereby, neutralizes IL-18 signaling (Fig 1.). It has been demonstrated that IFN-γ upregulates the expression of IL-18BP [44]. Therefore, IL-18BP through its binding to IL-18 regulates the IL-18 / IFN-γ / IL-18BP feedback loop [42]. An imbalance in the ratio of IL-18 to its binding protein has been associated with increased disease severity [30, 42]. Overall, the roles of IL-18 and IL-18BP during breast cancer progression remain to be fully elucidated.

1.5 Interferon-γ

IFN-γ has both growth promoting and inhibiting activities, and the former is usually suppressed by STAT1 [45]. In addition, IL-18 is a strong upregulator of IFN-γ [36]. Upon binding the heterodimeric IFN-γ receptor (IFNGR) IFN-γ signals via the phosphorylation of the JAK1 and JAK2 tyrosine kinases [46]. This, in turn, allows the translocation of the STAT1 homodimer transcription factor to the nucleus, leading to targeted gene expression regulation [46]. In Ba/F3 lymphocytes, IFN-γ promoted DNA synthesis, i.e., cell proliferation, when STAT1 was inhibited, suggesting that the growth-inhibiting role of IFN-γ is triggered by the STAT1 signaling pathway [45].

Within the tumor microenvironment, mainly NK cells, innate-like γδ T lymphocytes and Th1 lymphocytes produce IFN-γ [47]. The anti-tumor and cytotoxic effects of IFN-γ have

been reviewed extensively [15, 45, 46]. IFN-γ enhances tumor cell immunogenicity [46]. For example, mutations in a subset of melanoma and lung adenocarcinoma cell lines were associated with inactivation of IFN-γ pathway components [15], suggesting that tumors develop IFN-γ insensitivity-like mechanisms to evade immuno-surveillance [46].

Independent of STAT1 signaling, IFN-γ also induces the expression of the c-Myc gene and promotes cell proliferation [45]. Moreover, IFN-γ is a potent enhancer of lung colonization of B16 melanoma [48]. Low-dose IFN-γ treatment of B16 cells enhanced their resistance to NK cells, the first line of defense against foreign or transformed cells [48]. Moreover, intra-tumoral expression of IFN-γ was associated with a more aggressive phenotype of human melanomas [13]. Thus, IFN-γ leads to either stimulation or inhibition of cell proliferation, depending on the signaling cascade downstream of receptor binding [45].

Despite the dual effects of IFN-γ, clinical trials administering IFN-γ alone and in combination with IFN-α to treat chronic myelogenous leukemia have been conducted [46]. The clinical trials failed to demonstrate any benefit of IFN-γ administration [49, 50]. More recent attempts have tested IFN-γ administration in several cancers, including ovarian and colorectal, and bladder carcinoma [51], but trial results have been mixed or unsatisfactory [46], highlighting the challenge of targeting IFN-γ, a cytokine with widespread effects in cellular signaling processes.

As with other cytokines like TGF-β and TNF, IFN-γ clearly displays context-dependent contrasting behavior, which may be explained by its homeostatic functions [46]. While IFN-γ enhances inflammatory response, it also limits destruction of tissue in the aftermath of inflammation – this function could help cells harboring oncogenic mutations evade

destruction by immune cells and contribute to their immune privilege [46]. The role of IFN-γ in breast cancer progression, though, remains as yet unclear. Whether the interaction between IL-18 (IFN-γ inducer) and its binding protein has an effect on the expression levels of IFN-γ remains to be tested.

The feedback loop of IL-18/IFN-γ/IL-18BP, detailed above, likely plays a critical role in breast cancer progression. While the role of IL-1β in breast cancer is elucidated to an extent [6,7,11], the role of IL-18 and its interaction with IL-18BP remains to be fully characterized. Indeed, IL-18BP, via its feedback signaling [42, 44] interactions with IL-18 and IFNγ may be a more relevant therapeutic target. Thus, assessing the effects of IL-18, IL-18BP, in contrast with previously described IL-1β, on tumor cells and associated macrophages could open up new therapeutic venues for metastatic breast cancer.

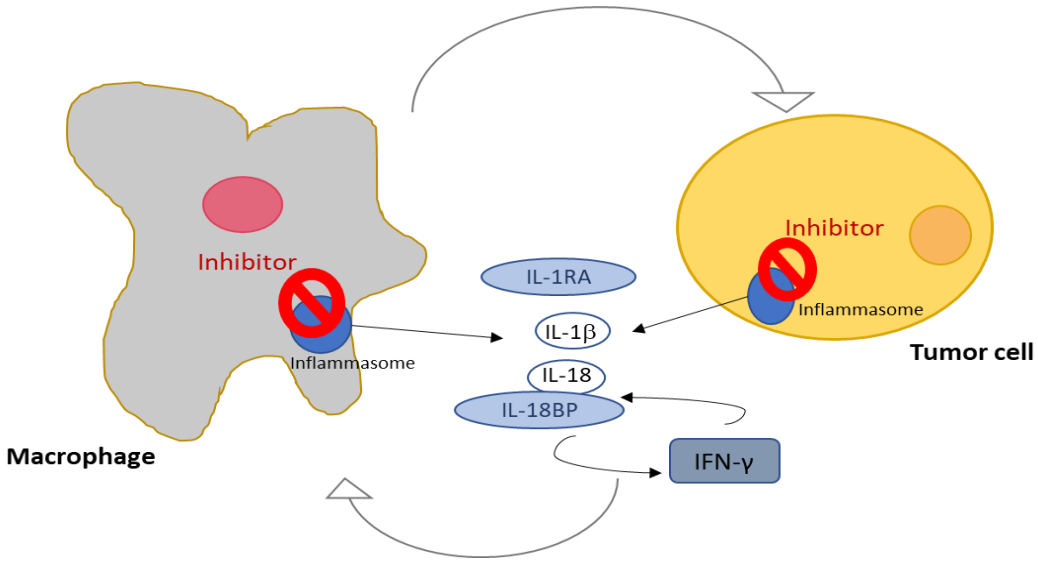

Fig 2. Schematic of the proposed model of macrophage-tumor crosstalk, and the cytokine signaling involved in the interaction.

1.6 Hypothesis and Aims

To further our understanding of NLRP3 inflammasomes and their activities in macrophages and their role in breast cancer progression, here we tested the hypothesis that *macrophage and tumor cell proliferation and secretions are regulated by the inflammasome-driven pro-inflammatory cytokines IL-1β and IL-18, and that IL-18BP critically regulates IL-18 activity, thereby altering tumor progression*.

Specifically, the following aims were tested.

Aim 1: *determine whether IL-1β and IL-18 effects are antagonistic to each other in macrophages and tumor cells.* The direct effects of IL-18 and IL-1β treatments on the growth/proliferation and IFN-γ production of J774, RAW and BMDM macrophages, as well as 4T1 tumor cells were assessed. Additionally, the differences in IL-18, IL18BP and IL-1β secretions by macrophages and 4T1 cells following inflammasome activation and inhibition were tested.

Aim 2: *determine whether imbalance of the IL-18/IL-18BP ratio significantly alters macrophage and tumor cell activities.* The secretions of IFN- γ in macrophages following treatments with varying ratios of IL-18 and IL-18BP were measured. The differences in growth/proliferation of 4T1 cells following incubation with increasing ratios of IL-18/IL-18BP were also determined.

CHAPTER 2: MATERIALS AND METHODS

2.1- Cells and culture conditions: Macrophage cells (J774A.1, hereafter J774, and RAW 264.7, hereafter RAW, ATCC, Manassas, VA) and bone marrow-derived macrophages (BMDMs) were assessed. BMDMs were sterilely cultured in DMEM supplemented with L-glutamine, Amphotericin, gentamycin (all from Thermo-Fischer Scientific) and 10% FBS [Atlanta biologic, Atlanta, GA]. The mammary mouse tumor 4T1 cells mimic aggressive human breast tumor cells were cultured in the same media. Prior to experiments, cells were seeded in either 96-well or 6-well tissue culture plates in concentrations leading to confluency within 2-3 days of the seeding. All experiments were conducted following starving period (>2hrs for macrophages, >6hrs for 4T1 cells) during which cells were incubated with culture media as described above but without FBS.

2.*2- Conditioned media:* Conditioned media (CM) for each of the cells tested were obtained by incubating cells for 48hrs in 0% FBS (4T1 cells) or ~1%FBS (J774, RAW, BMDM cells) and sterilely collecting and filtering the culture supernatants (0.2 um filters, Fischer Scientific). Filtered supernatants were aliquoted and stored at -20°C until use. Additionally, CM from macrophages cells treated with 4T1CM media were also sterilely collected and stored at -20°C.

2.3- LADMAC media BMDM: LADMAC media was used to derive BMDM was similarly collected except that media with 10% was used and collected. LADMAC media contains high concentrations of M-CSF a key cytokine that promote the differentiation pluripotent bone marrow cells in monocytes/macrophages [52]. LADMAC media (20% of 10% FBS media) was used to differentiate BMDMs from C57bl/6 and CD1 mouse bone marrow cell suspensions. During the differentiation (7-10 days, media was replaced every 2-3 days).

2.4- Inflammasome activators and inhibitors and cytokines: The classical inflammasome activator LPS (10ul of 1mg/ml stock for 1ml Sigma) and ATP (25ul/ml of a 0.2M stock Sigma) were used along with 4T1 conditioned media (4T1CM; 50% v:v). Additionally, the inhibitor MCC950 [53] was used at concentrations ranging 5-10 ug/ml. Recombinant cytokines IL-1ß, IL-18 were obtained from Sino biological Inc (China) as was IL-18BP.

2.5- Cell treatments: 4T1, J774, RAW and BMDM cells were seeded in 96 well or 6 well plates at ~60-70 % confluence with 10% FBS DMEM culture media and incubated overnight in a 5% CO_2 and >85% humidity. Following a starvation period (incubation with no (4T1) or reduced FBS media (macrophages), cells were incubated with media alone (negative control), either LPS (10ug/ml), ATP (5mM), 4T1CM, J774CM, RAWCM alone or in combination. After a 6hr incubation, supernatants were collected for dot-blots, Western blots and ELISAs and stored at -20°C. Additionally, cells were mechanically detached and cell suspension used to prepare lysates following addition of cell lysis solutions (T-PER and Pro-Prep^{TM}) or fixed in buffered formalin and stored at 4°C until immunostaining and flow-cytometry analyses.

2.6- Detection and co-localization of inflammasome proteins by Flow-cytometry and immunohistochemistry and confocal microscopy: Post-treatment, cells were fixed in formalin and stored at 4°C prior to permeabilization with saponin and immunostaining with antibodies to NLRP3 (R&D system, Minneapolis, MN); ASC1 (Santa Cruz Biotechnology, Santa Cruz CA) and Caspase 1 (Santa Cruz) as described previously [54]. The presence of NLRP3+, ASC1+ cells was determined by flow-cytometry using a Fortessa flow-cytometer (BD BioSciences, CA). Additionally, cells were mounted and visualized using confocal microscopy. 3D image stacks were collected at 0.3-µm z increments on a DeltaVision

workstation (GE) based on an inverted microscope (IX-70; Olympus) using a 60×1.4NA oil immersion lens. Images were captured at 24°C with a 12-bit charge-coupled device camera (CoolSnap HQ; Photometrics) and deconvolved using the iterative-constrained algorithm and the measured point spread function.

2.7- Detection and quantification of IL-1ß, IL-18, IL-18BP, IFN-γ in supernatants by dot-blots and ELISAs: Cell supernatants were analyzed by dot blots as described previously [54]. Samples (65µl) were mixed with CTAB buffer and loaded onto to a nitrocellulose membrane using a dot-blot apparatus (Life Technologies) and incubated for 20-30 minutes. In subsequent steps, the membranes were treated similarly to Western Blots [54]. Briefly, after blocking, incubation with primary antibodies against IL-1ß, IL-18, IL-18BP, IFNγ, a specific secondary HRP conjugated antibody was used along with a chemiluminescent substrate to detect the presence of proteins. The signal detecting the protein of interest was quantified using Image J and the Protein Array Analyzer plugin. Protein expression are presented as % of control conditions i.e., normalization of the expression of the protein of interest to the signal (pixel density) in control conditions.

ELISAs were conducted as detailed previously [54], using sandwich ELISA developed with pairs of antibodies specific for IL-1ß, IL-18 and IL-18BP. Samples were run along with increasing purified protein concentrations. Following incubation with TMB substrate and stopping the reaction, 96-well plates were read in a plate reader and the concentrations in pg/ml derived from the standard curve.

2.8- Western-blot detection and quantification of NLRP3, ASC1, Caspase 1: Cell lysates obtained following incubations with the different treatment tested were sonicated and the protein concentration determined using a BCA assay (ThermoScientific). After the addition

of loading buffer (1/2; v:v) to 20μg of protein for each sample tested and heat-denaturation, samples were was loaded onto a 12% SDS-PAGE gel. Following electrophoresis, transfer to a nitrocellulose membrane, blocking and incubation with antibody against either NLRP3, ASC1, or active Caspase 1 and subsequent incubation with matching HRP-conjugated secondary antibodies, the presence of these proteins was detected by chemiluminescence using UVP system.

2.9- Evaluation of cell proliferation/growth using MTT assays: J774, RAW and BMDM, 4T1 cells seeded in 96-well plates were incubated with CM, LPS (10μg/ml), along with increasing concentrations of IL-1ß (0 - 1 ng/mL) or IL-18 (0 - 10 ng/mL) cytokine for 24hrs. The cell growth was evaluated by measuring the increase in fluorescence of Hoechst nuclear dye (Excitation: 350, Emission: 461nm). In addition, viability MTT (i.e., tetrazolium dye) assays determining the metabolic activities of the cells following a 24hr incubation were conducted. The MTT chemical metabolized by active cells into an insoluble formazan serves as a colorimetric indicator of viable cells. After a 3.5-hour incubation, the cells were permeabilized, formazan solubilized, and the presence of formazan measured at 570 nm subtracting the background (630nm).

2.10- Evaluation of cell proliferation/growth using Hoechst nuclear vital dye: BMDM, J774, RAW and 4T1 cells seeded in 96-well plates were incubated with CM, LPS (10μg/ml), ATP (5μM) along with varying concentrations of IL-18 (0 - 10 ng/mL) supplemented with increasing concentrations of IL-18BP (0 - 10 ng/mL) for 24hrs. The cell growth was evaluated by measuring the increase in fluorescence of Hoechst nuclear dye (Excitation: 350, Emission: 461nm). Briefly following addition of sterile Hoechst

(Sigma; 1/40,000) to cell media, changes in fluorescence were measured using a plate reader (ID5 Molecular Device) and compared overtime and between treatments.

2.11- Statistical analysis: Data are presented as mean ± SEM. At least, 3 independent repeats were conducted. Difference in the parameters measured between treatments were analyzed by one- or two-way ANOVA and appropriate post-hoc tests. Parameters measured following incubation with increasing cytokine doses were tested using Pearson r correlations. Significance level was set to 0.05 *a priori*.

CHAPTER 3: RESULTS

1.1 Inflammasome proteins are expressed in J774 and BMDM macrophages, and in 4T1 tumor cells

As demonstrated previously in our lab, ASC-1 co-localized with NLRP3 and Caspase-1 in J774 macrophages and in BMDMs. In contrast, RAW cells did not express the inflammasome scaffold protein ASC-1 (Fig 3 Confocal (A,B) & Western blot (C)). Additionally, 4T1 also expressed those proteins (not shown). Interestingly, the incubation of 4T1 cells with RAW macrophage secretions was associated with a drastic downregulation of ASC-1 (Fig 4 - Flow).

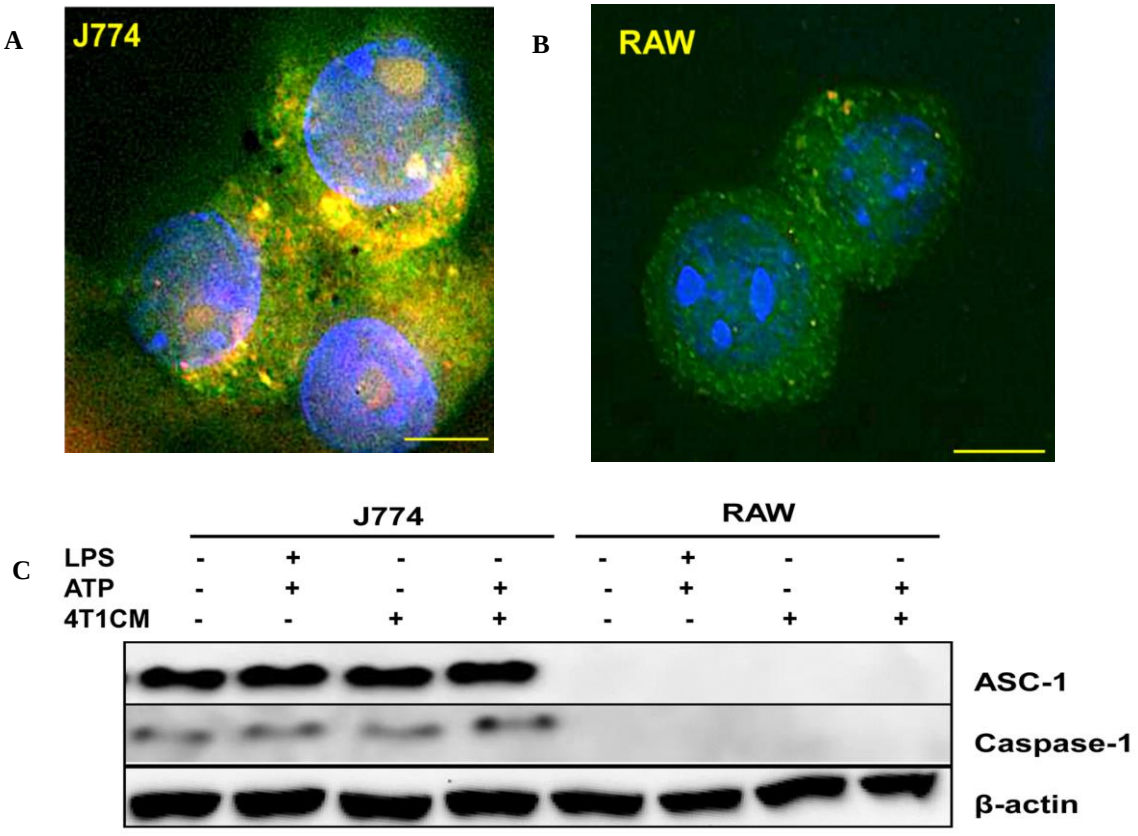

Fig 3. Confocal microscopy microphotographs of J774 (A) and RAW (B) cells depicting the NLRP3 inflammasome components. Cell nuclei (blue) highlight the cytoplasmic co-localization (yellow) of NLRP3 (green) and ASC-1 (red). The expression of active caspase 1 (p10) in J774 cells but not in RAW cells was confirmed by Western blot (C).

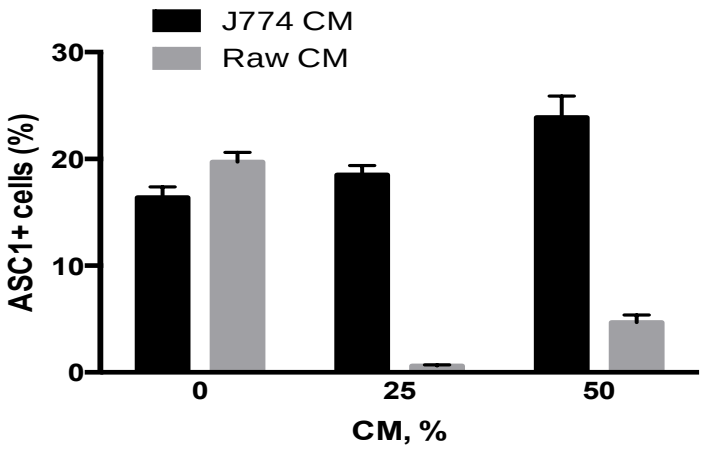

Fig 4. ASC-1 protein expression in 4T1 cells was drastically reduced following incubation with RAW CM but not with J774 CM ($p<0.0001$). Briefly, 4T1 grown to confluence were incubated with 0, 25, 50% (v:v) of RAWCM or J774CM for 24hrs. Cells were then collected, permabilized and stained for ASC-1 and the percentage of ASC-1^+ cells determined by flow-cytometry.

1.2 4T1CM along with classical inflammasome activators promoted macrophages inflammasome-related cytokine secretions

IL-1β secretions in BMDM cells, but not in J774 or RAW cells, were markedly increased by treatment with the combinations LPS-ATP, 4T1CM and 4T1CM-ATP (Fig 5A, $P<0.01$). In contrast, along with the combination LPS-ATP, 4T1CM and 4T1CM-ATP significantly increased the secretions of IL-18 by J774 macrophages but not by RAW or BMDM macrophages (Fig 5B, $p<0.0001$). Interestingly, the increase in IL-18 secretion by activated J774 cells was accompanied with increase in IL-18BP secretions (Fig 5C, $p<0.05$, Fig 5C, $p<0.001$). This was not observed in RAW cells (Fig 5C).

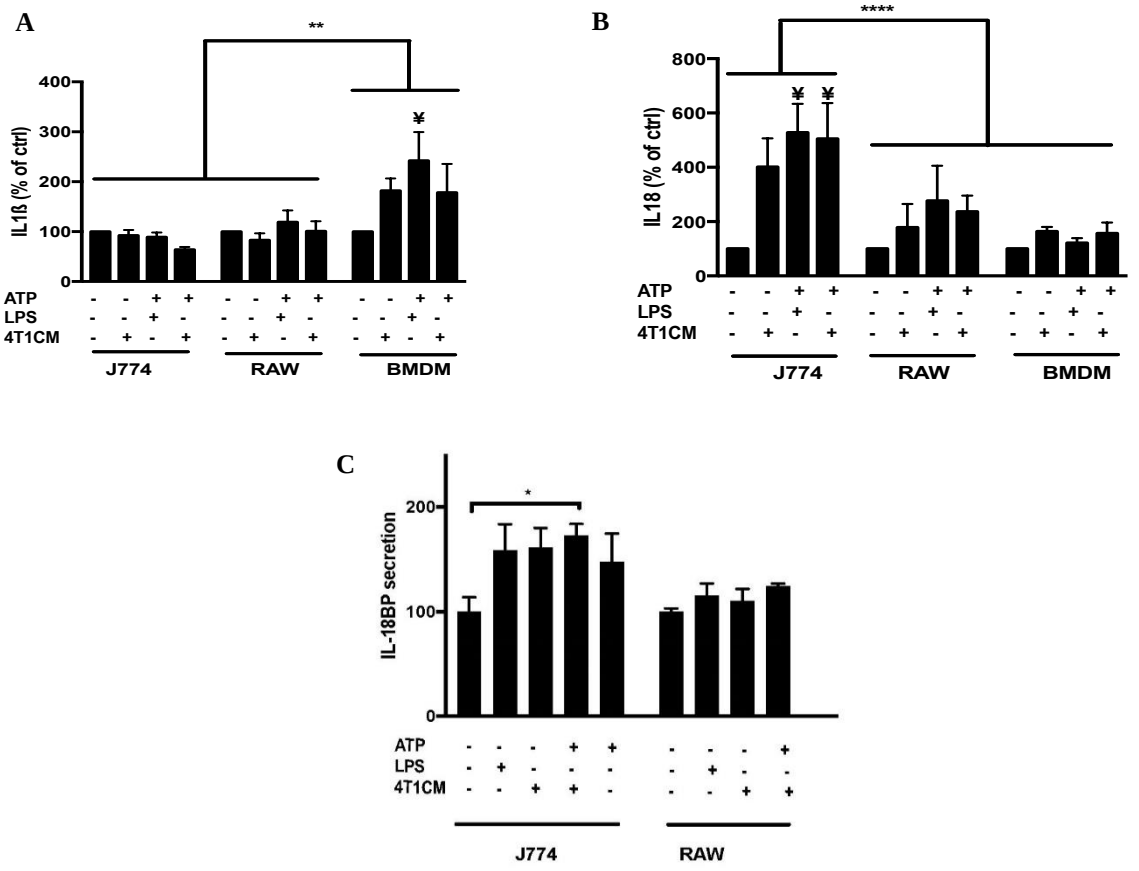

Fig 5. Secretions of IL-18 (**A**) and IL-1ß (**B**) IL-18BP (**C**) determined (by dot-blots) in media of J774, RAW and BMD macrophages following incubation with classic inflammasome activators and 4T1CM. **p<0.01 ****p<0.0001 ¥ significant compared to control.

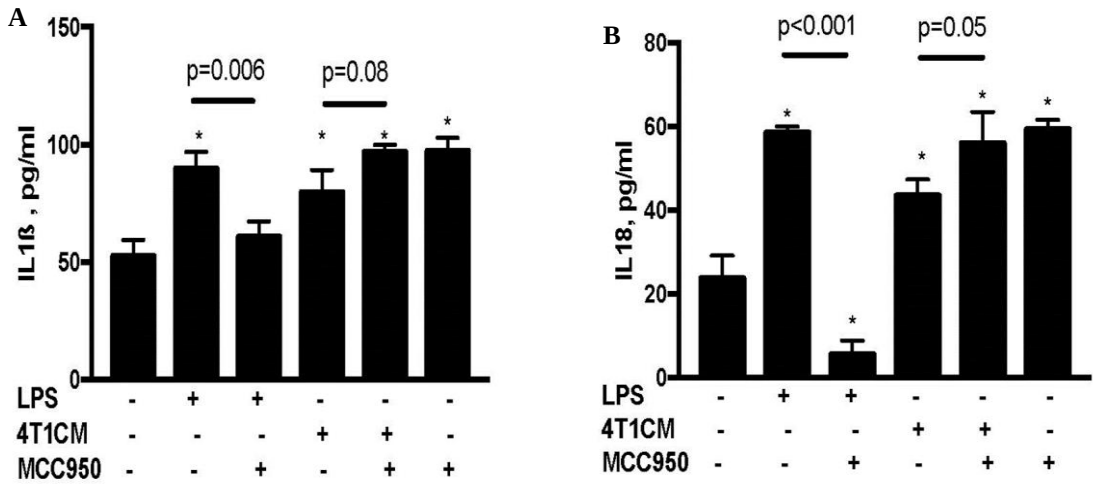

Fig 6. Secretions of IL-1β (**A**) by BMDM cells and IL-18 (**B**) by J774 macrophages determined (by ELISAs) following treatments with classic inflammasome activators without and with the specific NLRP3 inflammasome inhibitor MCC950 (5μM). *p<0.05 compared to control.

The secretions of IL-1β by BMDM (Fig 6A) and IL-18 by J774 (Fig 6B) cells following activation of inflammasomes were inhibited in the presence of the NLRP3 inflammasome inhibitor MCC950. Downstream of the IL-1ß and IL-18 signaling, IFNγ secretions were significantly decreased in the presence of LPS in the supernatant of BMDM cells (Fig 7B, $p<0.05$). Additionally, IL18BP concentrations secreted by J774 cells were increased in the presence of MCC950 alone and drastically in the presence of LPS or 4T1CM (Fig 7A, $p<0.001$).

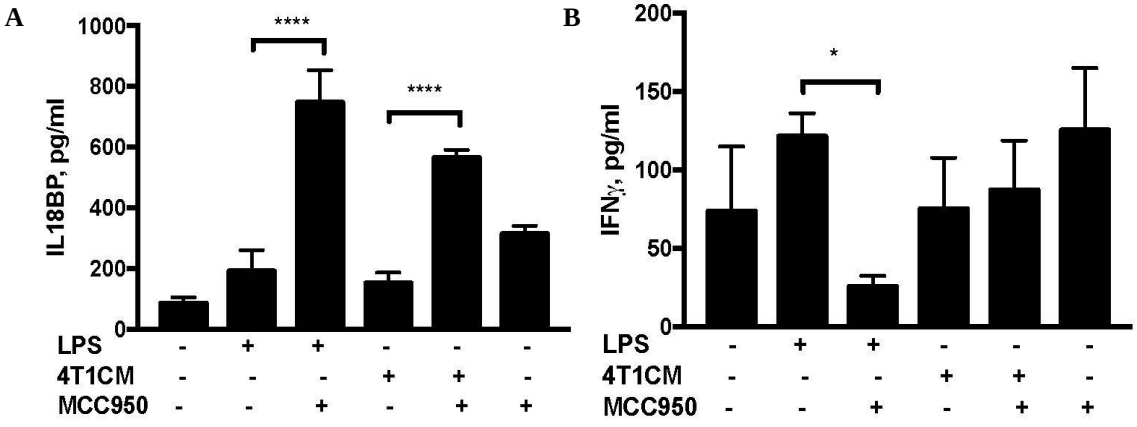

Fig 7. Secretions of IL-18BP (**A**) by J774 and IFN-γ (**B**) by BMD macrophages determined by ELISAs following incubation with classic inflammasome activators and the NLRP3 inflammasome inhibitor MCC950 (5μM). *p<0.05 compared to control.

1.3 J774 and BMDM secretions following inflammasome activation promoted 4T1 tumor cell growth

J774 and BMDM secretions promoted 4T1 tumor growth ($p<0.05$, Fig 8A,B). Notably, concurrent treatments with MCC950 abrogated 4T1 tumor growth significantly for J774CM (Fig 8A, $p<0.05$).

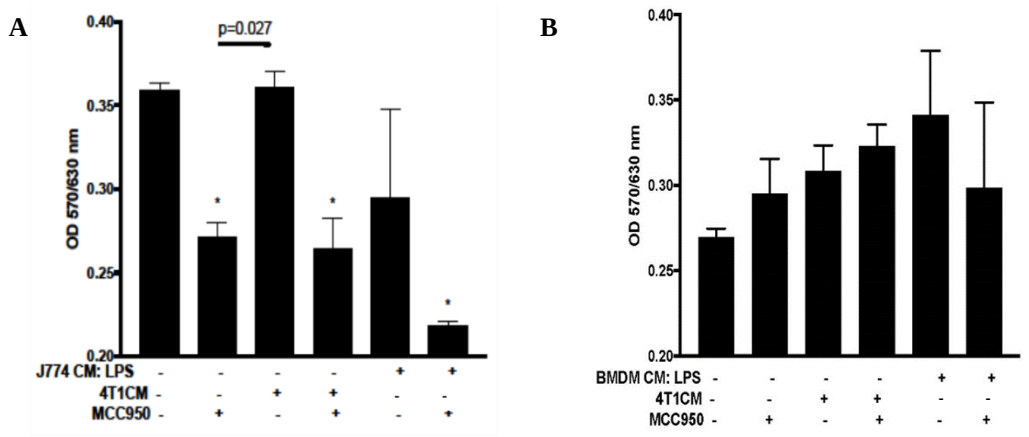

Fig 8. 4T1 cell growth as measured by MTT assays (presented as absorbance at 570/630nm) following incubation with J774-conditioned media (**A**) and BMDM-conditioned media (**B**) collected from cells previously treated with inflammasome activators and inhibitor MCC950 (5μM). *p<0.5 compared to control.

1.4 Recombinant IL-18 prevented 4T1 cell growth

Incubation of 4T1 cells with increasing IL-18 concentrations was associated with a dose-dependent decrease in 4T1 cell growth (Fig 9A, p<0.01). Similarly, increasing doses of IL-18BP also reduced 4T1 viability at higher concentrations (Fig 9B, p<0.0001). Exposure to IL-1ß of 4T1 cells reduced cell viability, but had no dose-dependent effect (Fig 9C).

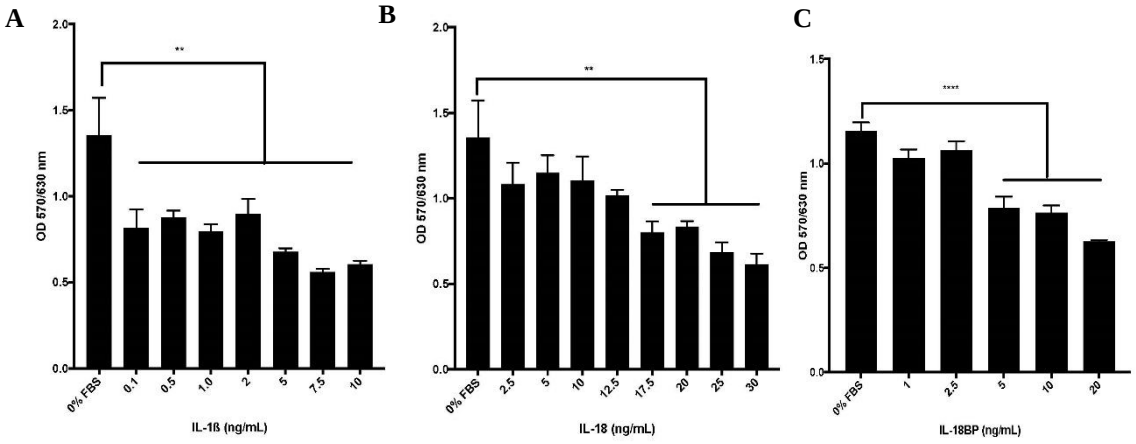

Fig 9. 4T1 cell growth as determined by MTT assays (presented as absorbance at 570/630 nm) following incubation with increasing concentrations of recombinant IL-1β (**A**), IL-18 (**B**), and IL-18BP (**C**).**p<0.01.

1.5 Increasing concentrations of recombinant IL-1ß supported BMDM growth

Inflammasome-driven IL-18 and IL-18BP had no significant dose-dependent effects on BMDM cell growth (Fig 10B,C). In contrast, exposure to increasing IL-1ß concentration led to a dose-dependent increase in BMDM cell growth (Fig 10A, P<0.01).

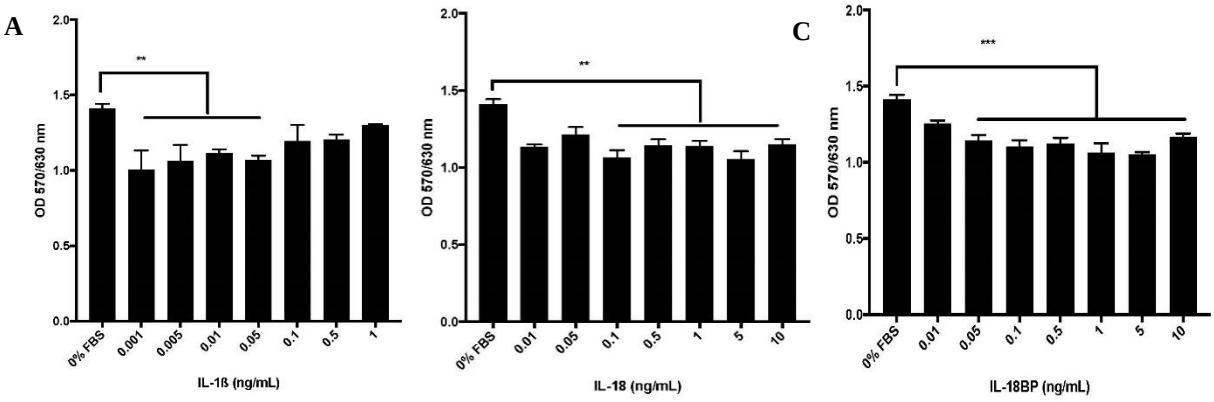

Fig 10. BMDM cell growth as determined by MTT assays (presented as absorbance at 570/630 nm) following incubation with increasing concentrations of recombinant IL-1β (**A**), IL-18 (**B**), and IL-18BP (**C**).**p<0.01.

1.6 The IL-18 inhibition of J774 cell growth was prevented by IL-18BP

Incubation with increasing concentrations of recombinant IL-18 led to a decrease in J774 cell growth (Fig 11A p<0.05). In contrast, RAW macrophage growth increased following incubation with increasing concentrations of recombinant IL-18 (Fig 11B p<0.05). Concurrent treatment with recombinant IL-18BP at 10 ng/mL and IL-18 led to an increase in the growth of J774 cells (Fig 11A). In constrast, IL-18BP combined with increased doses of IL-18 was associated with a marked IL-18 dose-dependent growth decrease of RAW cells (Fig 11A, ANOVA, P<0.001).

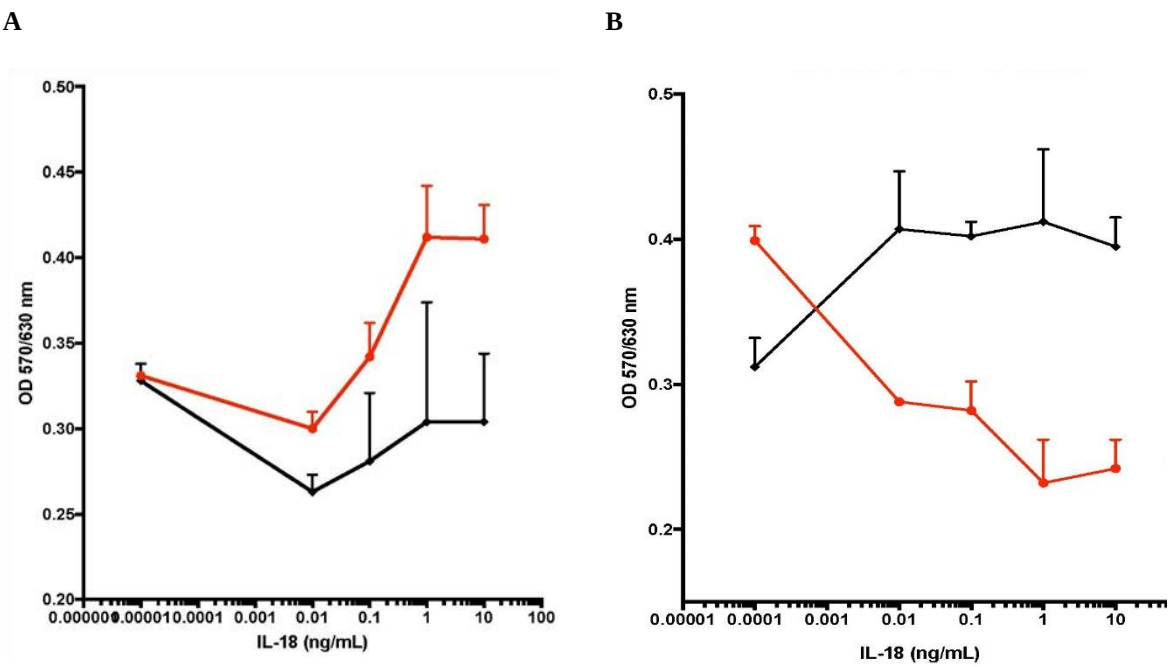

Fig 11. J774 (**A**) and RAW (**B**) macrophage growth as determined by MTT assays (presented as absorbance at 570/630 nm) following incubation with increasing concentrations of recombinant IL-18 alone (black) and with increasing concentrations of IL-18 and IL-18BP at 10 ng/mL (red). ANOVA , $p<0.01$ for RAW cells. Note: On the log scale used (X axis) to display IL-18 concentrations 0 is plotted as 0.0001.

1.7 Recombinant IL-18BP also inhibited the IL-18-driven IFN-γ secretions by J774 cells

Following incubation with increasing IL-18 concentrations, IFN-γ secretions remained unchanged in the supernatants of J774 cell cultures (Fig 12A) and significantly decreased in the supernatants of RAW cell cultures (Fig 12B). Interestingly, the concurrent incubation with IL-18BP (10 ng/mL) was associated with an IL-18 dose-dependent decrease in IFN-γ secretions by J774 cells (Fig 12A $p<0.05$).

The secretion of IFN-γ following increasing IL-18 treatments remained stable in J774 supernatant and decreased in RAW supernatants (Fig 12 AB). Interestingly, the concurrent incubation with IL-18BP (10 ng/mL) was associated with a IL-18 dose-dependent decrease in IFN-γ secretions by J774 cells (Fig 12A $p<0.05$).

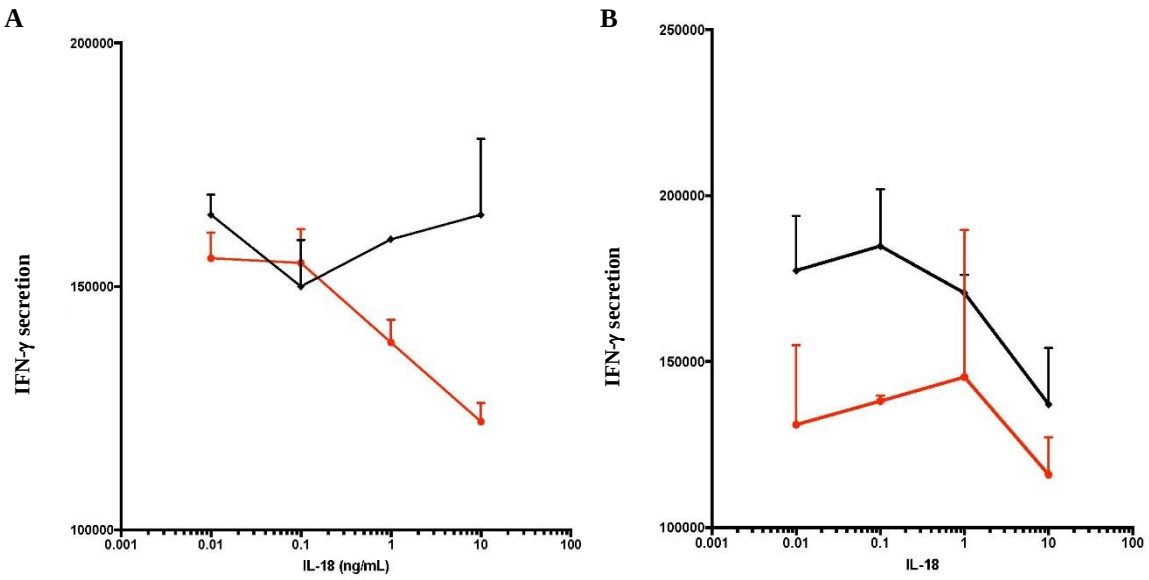

Fig 12. IFN-γ secretions by J774 (**A**) and RAW (**B**) cells as determined by dot-blot analyses (pixel intensity) when treated with increasing concentrations of recombinant IL-18 (black) alone and with recombinant IL-18 along with 10 ng/mL of IL-18BP (red). ANOVA , $p<0.01$ for J774 cells.

CHAPTER 4: DISCUSSION

The dysregulation of inflammasomes has been implicated in several pathological states [1], including inflammatory diseases like rheumatoid arthritis and systemic lupus erythematosus [2]. The activation of the inflammasome leads to the secretion of mature pro-inflammatory cytokines IL-1β and IL-18 [8], which critically regulate the tumor microenvironment [6,7,11]. The mortality of breast cancer is associated with its tendency to metastasize to crucial tissues such as bones, brain and lungs [23]. The roles of pro-inflammatory cytokines, including IL-1β and IL-18, in cancer metastasis have been elucidated [14, 28, 35, 38]. IL-1β is known to be crucial for tumor invasiveness and angiogenesis [14]; its role in the bone metastasis of breast cancer has also been recently described [55]. The functions of IL-18 in breast cancer and its metastasis are yet to be clearly characterized.

The NLRP3 inflammasome is arguably the best characterized inflammasome to date [8], and its assembly and activation is crucial in many inflammatory disease states, including cancer metastasis [17]. The NLRP3 inflammasome is expressed and assembled in both tumor cells and macrophages (Fig 3), with the latter having greater activity [8]. The crosstalk between tumor cells and macrophages through signaling is key to breast cancer metastasis [1]. Here, using *in vitro* approaches, we assessed the presence of inflammasomes in both macrophages and tumor cells and the functional effects of activating inflammasomes in these cells on the secretions of IL1ß and IL-18, IL-18BP and IFN-γ. We further determined whether IL-1ß, and especially IL-18 /IL-18BP, promoted the growth of macrophages and tumor cells.

Our results demonstrate *in vitro* that (1) the inflammasome proteins are expressed and colocalized in 4T1 tumor cells and in J774 macrophages; (2) Conditioned media from inflammasome-activated 4T1 cells promoted macrophage secretion of inflammasome-related cytokines (Fig 13), (3) Conditioned media from inflammasome-activated macrophages promoted 4T1 cell growth. Furthermore, (4) Recombinant IL-18 reduced 4T1 cell growth in a dose-dependent manner and also reduced J774 cell growth. Finally, IL-18BP abrogated IL-18-driven J774 growth inhibition.

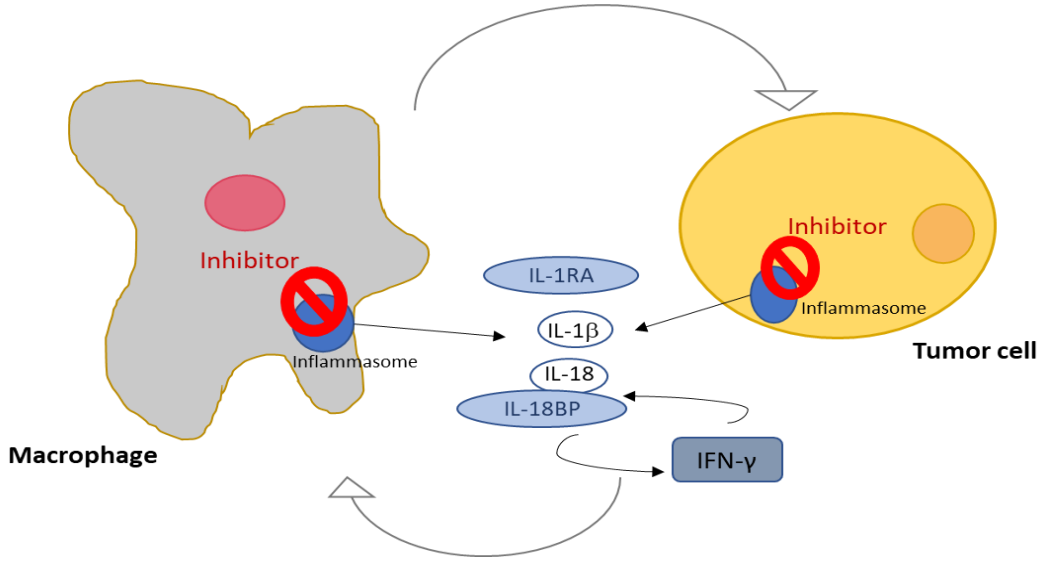

Fig 13. Schematic of the model of macrophage-tumor crosstalk, and the cytokine signaling involved in the interaction.

In vitro approaches offer distinct advantages including simplicity when teasing out cell-cell interactions and cell signaling [26]. Here, we investigated the murine 4T1 mammary epithelial cells, the murine J774 and RAW cells and murine BMDM cells. The focus on murine cells complements our efforts to assess inflammasome inhibition in the immunocompetent orthotopic 4T1 / Balb/c murine model. Indeed, RAW and 4T1 cells are syngeneic to Balb/c mice. When implanted in the mammary fat pad of Balb/c mice, the

growth and metastasis of 4T1 mammary tumor cells mimics human metastatic progression closely [56]. RAW macrophages uniquely lack expression of the ASC-1 scaffolding protein [57], as we confirmed by confocal microscopy, flow-cytometry and western blots. Additionally, *in vitro* models using cell lines are more reproducible and suitable for investigation of cell signaling [57]. However, primary cells and established cell lines behave differently in many contexts, J774 cells included [58]. Therefore, we also investigated primary macrophages derived from the bone marrow of C57bl/6 and CD1 mice (i.e., bone marrow-derived macrophages, BMDM) to further our understanding of the cross-signaling between breast tumor cells and macrophages.

Our data demonstrated via confocal microscopy, flow-cytometry and western blot that the inflammasome proteins NLRP3 and ASC-1 were expressed and co-localized in J774 macrophages and in 4T1 tumor cells. As previously suggested [57], RAW cells did not express ASC-1 protein, confirming that RAW cells have a defective inflammasome. Interestingly, the presence of inflammasome protein in mammary tumor cells and breast cells has seldomly been demonstrated. Remarkably, RAW-conditioned media drastically reduced the expression of the inflammasome scaffolding protein ASC-1 in 4T1 cells thereby modulating the participation of the 4T1 cells in the generation of a local inflammatory microenvironment.

Following inflammasome activation, J774 and BMDM macrophage secretions promoted the growth of 4T1 mammary tumor cells. Moreover, the tumor cell growth was inhibited when the conditioned media was collected from cells concurrently treated with inflammasome activators and the NLRP3 inflammasome inhibitor MCC950 [53]. Interestingly, the growth increase was also observed with CM from J774 and BMDM

treated with 4T1 CM. This suggests a possible cross stimulation between macrophages and tumor cells within the tumor microenvironment; tumor cells activate inflammasome in macrophages, and the subsequent secretions of pro-inflammatory cytokines IL-1ß and IL-18 stimulate 4T1 tumor cells growth. Of note, direct incubation with MCC950 or 4T1 CM has no effect on 4T1 tumor cell growth confirming that macrophage inflammasome activation related molecules promoted tumor growth.

As NLRP3 inflammasomes chiefly secrete IL-1ß and IL-18, we assessed the direct effects of the inflammasome-driven cytokines IL-1β and IL-18 on 4T1 cell growth. At the doses tested, both cytokines led to decreases in 4T1 cell growth. In addition, the IL-18-driven decrease was dose-dependent. Contrasting with previous works on other cancer types [38, 39], here IL-1ß and IL-18 had no antagonistic effects on 4T1 tumor cell growth. Interestingly, our data also showed that 4T1-CM increased the production of IL-1β in BMDM and IL-18 in J774, respectively. This result confirms the cross talk between tumor cell and macrophages, which through pro-inflammatory cytokine upregulation, significantly alters the tumor microenvironment and supports tumor maintenance [14]. Whether those pro-inflammatory cytokines also change the macrophage phenotype i.e. from an anti- (M1) to a pro-tumorigenic (M2) phenotype [13], remains to be determined. Furthermore, 6 hours post inflammasome activation, IL-1β and IL-18 cytokine secretions were markedly different between J774 and bone marrow-derived macrophages, suggesting either time dependence or cell type dependence of these secretions. Similar variations are often observed between established cell lines and primary cells [58], and warrant further investigations.

Further, 4T1CM also increased IL-18BP secretions in J774 cells, as did the classic inflammasome activators. This supports the presence of a IL-18/IFN-γ/IL-18BP feedback signaling loop. As suggested previously, IFN-γ upregulates the expression of IL-18BP [44]. Thus, inflammasome activation in J774, followed by IL-18 secretion, induces the production of IFN-γ, leading to an increase in expression of IL-18BP (Fig 2).

The addition of inflammasome inhibitor MCC950 led to significant decreases in LPS-activated inflammasome-driven secretion of IL-1β in BMDM cells and that of IL-18 in J774 cells, respectively, confirming the NLRP3 inflammasome involvement in IL-1ß and IL-18 secretions. Our data also indicated that IL-18BP secretions increased when J774 macrophages were concurrently treated with inflammasome activators and the inhibitor MCC950. IFN-γ secretions in BMDM, however, decreased upon concurrent treatment MCC950, supporting the feedback signaling loop. While why these two macrophages have different pro-inflammatory secretions remains to be elucidated., regardless of these different secretion patterns the MCC950 NLRP3 inflammasome inhibitor abrogated both IL-1β and IL-18 secretions. In contrast, at the dose tested, MCC950 did not significantly prevent cytokine secretion promoted by 4T1CM. Whether increased MCC950 concentrations would prevent 4T1CM induced pro-inflammatory cytokine secretion remains to be determined. Alternatively, other inflammasomes, independent of NLRP3, may be involved [59, 60].

Our investigations of the effects of increasing concentrations of IL-1β, IL-18 and IL-18BP macrophage growth indicate that IL-1β led to a dose dependent increase in growth of BMDM. In contrast, at the concentration tested, IL-18 and IL18BP independently led to an overall significant decrease in macrophage growth. These observations are in support

of the antagonistic effects of IL-18 and IL-1β on primary macrophages growth but contrast with the effects observed in 4T1 cells. Since IL-18 appears to promote the growth of tumor cells but not of macrophages, it may, among others, point to either a down regulation of IL-18 in TAMs or a concurrent up regulation of IL-18BP decreasing the IL-18/IL-18BP and thus the IL-18 signaling efficacy.

Moreover, increasing concentrations of IL-18 had no significant effect on growth of J774 macrophages, but led to an increase in growth of RAW macrophages. Since RAW cells have defective inflammasomes, the increase in RAW growth is supported by exogenous IL-18. J774 cells, on the other hand, secrete IL-18 thus extra exogenous IL-18 even in increasing concentrations had no effect. Whether more targeted alterations of the IL-18/IL-18BP will more successfully modulate the inflammasome-driven IL-18 growth inhibition remains to be determined.

Further, we demonstrated that increasing concentrations of IL-18, along with IL-18BP affected macrophage growth and IFN-γ secretions. The IL-18-driven decrease in growth was partly mitigated by the addition of IL-18BP supporting the inhibitory effects of IL-18BP - IL-18 binding on IL-18 signaling. In RAW cells, addition of IL-18BP to increasing IL-18 concentrations abrogated the growth-inducing effect of IL-18 alone highlighting the dose-dependent blocking of IL-18 signaling by IL-18BP [42]. Similarly, our data on IFN-γ secretions also corroborates this result, with IL-18 alone leading to a significant increase in IFN-γ secretions in J774 cells but not in RAW cells; while following the addition of IL-18BP, IFN-γ secretions decreased significantly in J774 cells, confirming an active IL-18-IL-18BP-IFN-γ regulatory feedback loop.

Overall, we provided evidence of inflammasome–driven IL-1ß and IL-18 cytokine production and of their functions in the interplay between tumor cells and macrophages. Our data also highlight the key IL-18/IFN-γ/IL-18BP feedback signaling loop. IL-18 induces the production of IFN-γ, and IL-18BP participates by inhibiting the activity of IL-18, in turn affecting cell growth and production of IFN-γ. Over the years, IL-18 and IFN-γ have been held as possible cancer immunotherapy options [18, 33, 42, 50, 51]. As both cytokines, especially IFN-γ have widespread roles, therapies targeting these cytokines have led to limited clinical benefits [49-51]. Targeting IL-18BP, a key regulator of IL-18 signaling, and in turn of IFN-γ, could offer a new perspective into clinical therapy for breast cancer.

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APPENDIX – SUPPLEMENTARY DATA

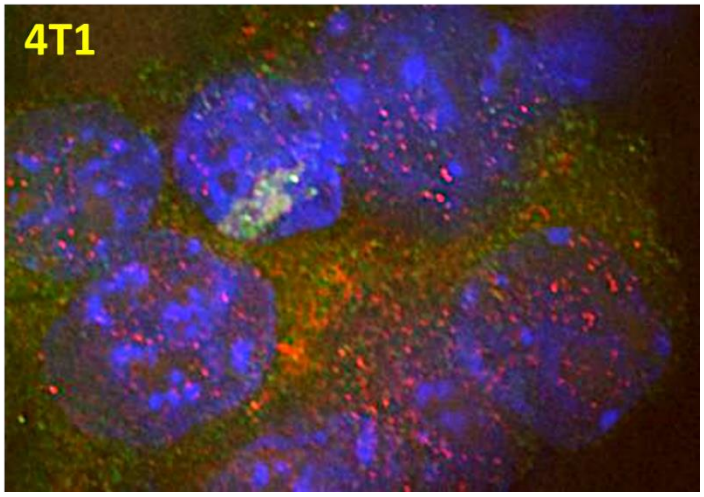

Fig S1. Confocal microscopy photographs of 4T1 tumor cells depicting the NLRP3 inflammasome components. Cell nuclei (blue) highlight the cytoplasmic co-localization (yellow) of NLRP3 (green) and ASC-1 (red).

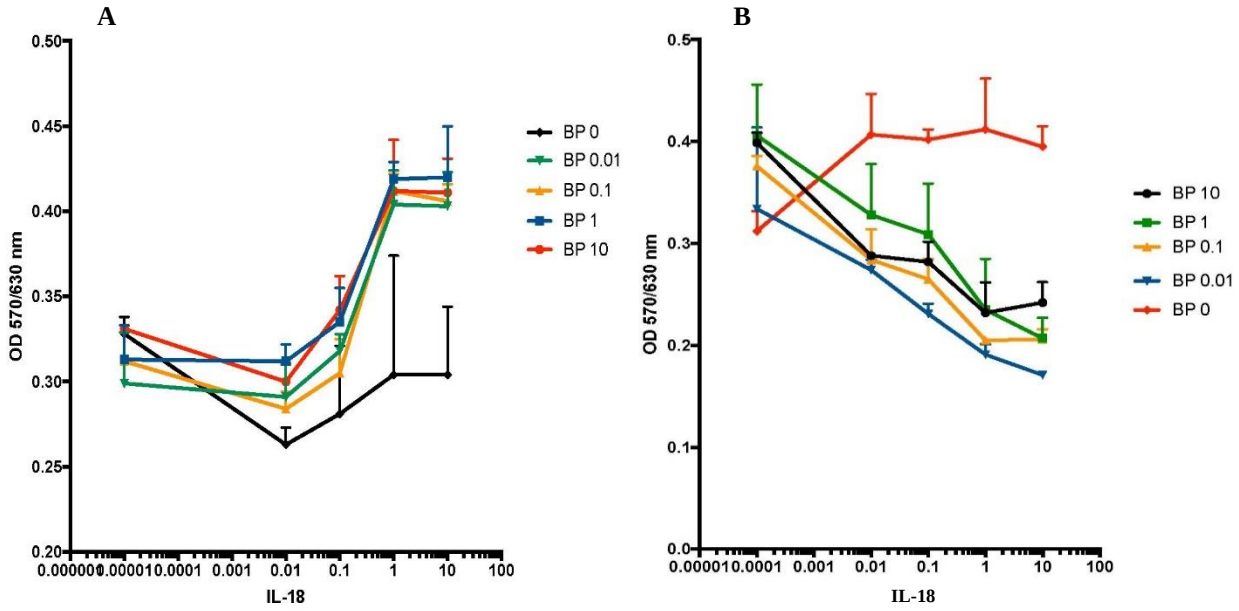

Fig S2. J774 (**A**) and RAW (**B**) macrophage growth as determined by MTT assays (presented as absorbance at 570/630 nm) following incubation with increasing concentrations of recombinant IL-18 alone (black) and with various ratios of IL-18 and IL-18BP (ng/mL). ANOVA, $p<0.01$ for RAW cells. Note: On the log scale used (X axis) to display IL-18 concentrations 0 is plotted as 0.0001.

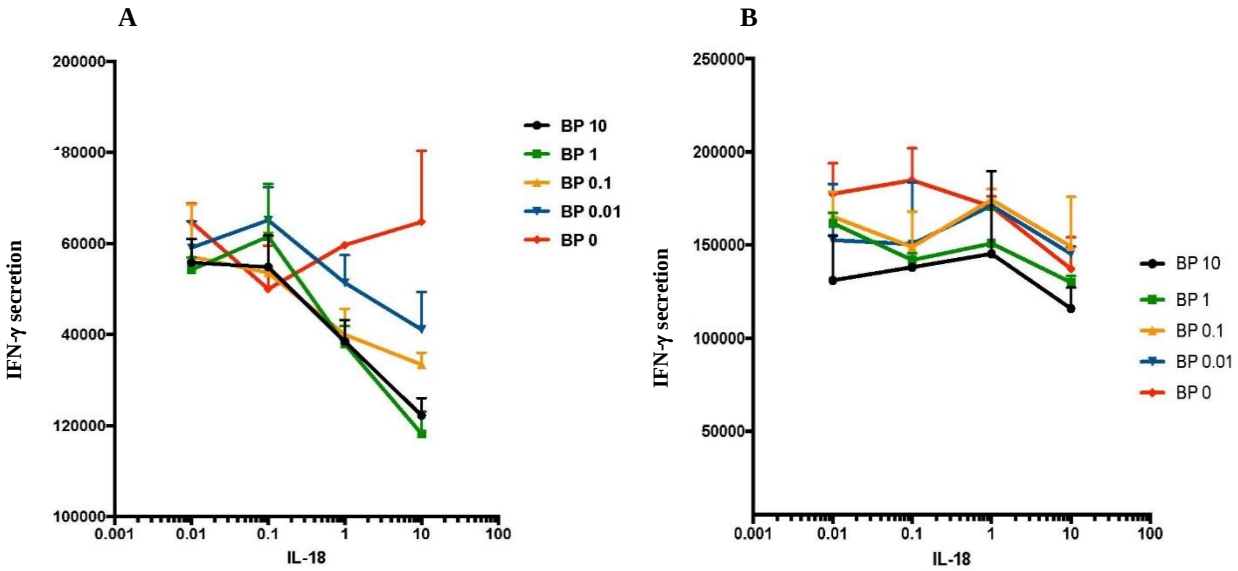

Fig S3. IFN-γ expression by J774 (**A**) and RAW (**B**) macrophage as determined by dot blots following incubation with increasing concentrations of recombinant IL-18 alone (black) and with various ratios of IL-18 and IL-18BP. ANOVA , $p<0.01$ for J774 cells.

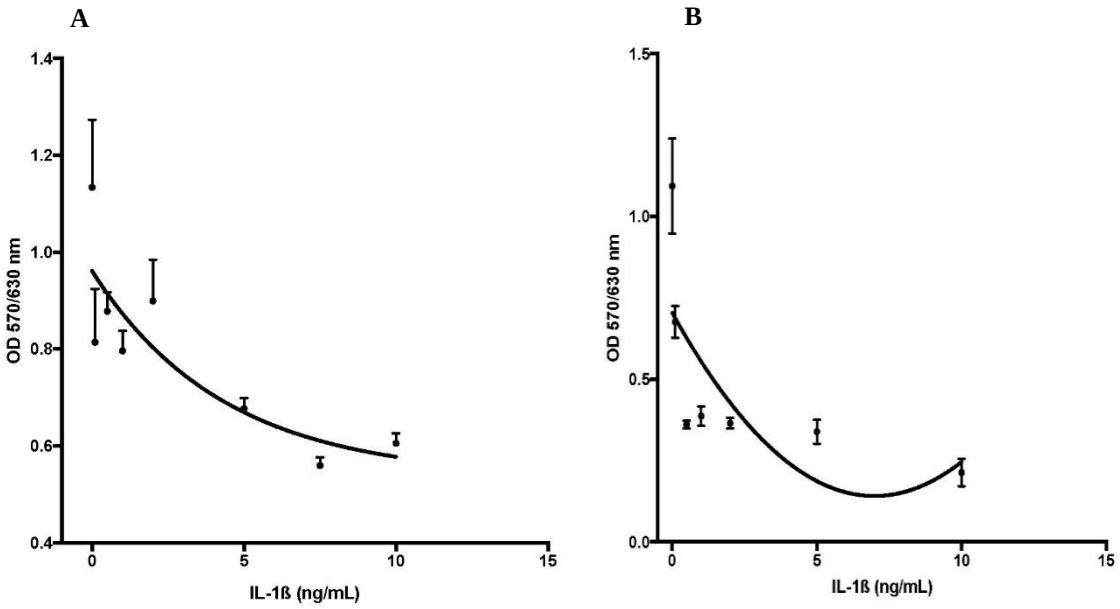

Fig S4. 4T1 tumor (**A**) and BMDM (**B**) cell growth as determined by MTT assays (presented as absorbance at 570/630 nm) following incubation with increasing concentrations of recombinant IL-1β. ANOVA, $p<0.01$.

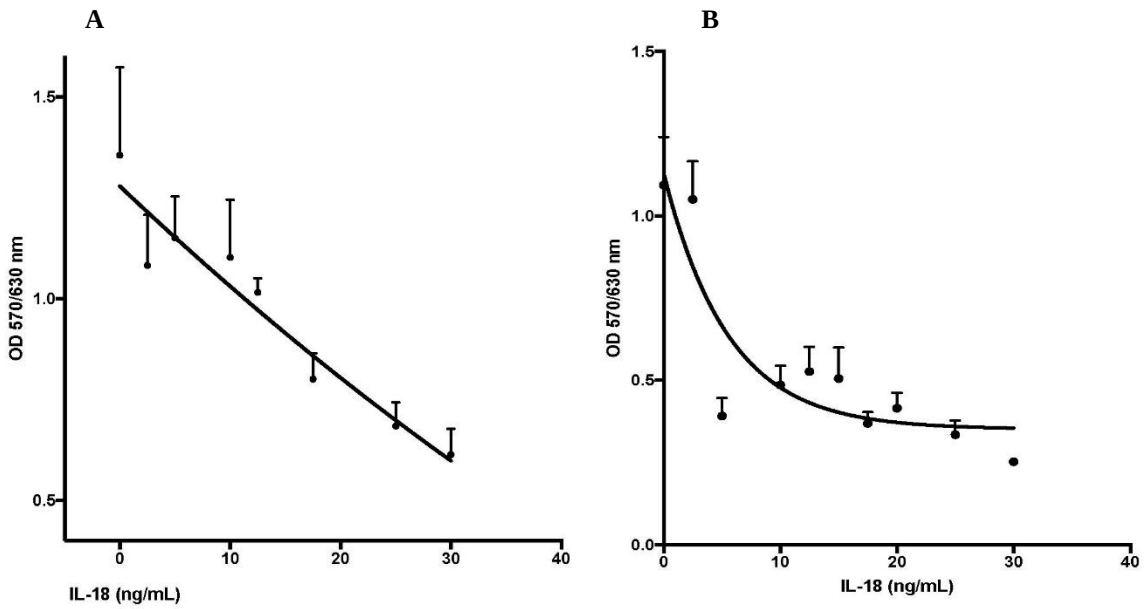

Fig S5. 4T1 tumor (**A**) and BMDM (**B**) cell growth as determined by MTT assays (presented as absorbance at 570/630 nm) following incubation with increasing concentrations of recombinant IL-18. ANOVA, $p<0.01$.

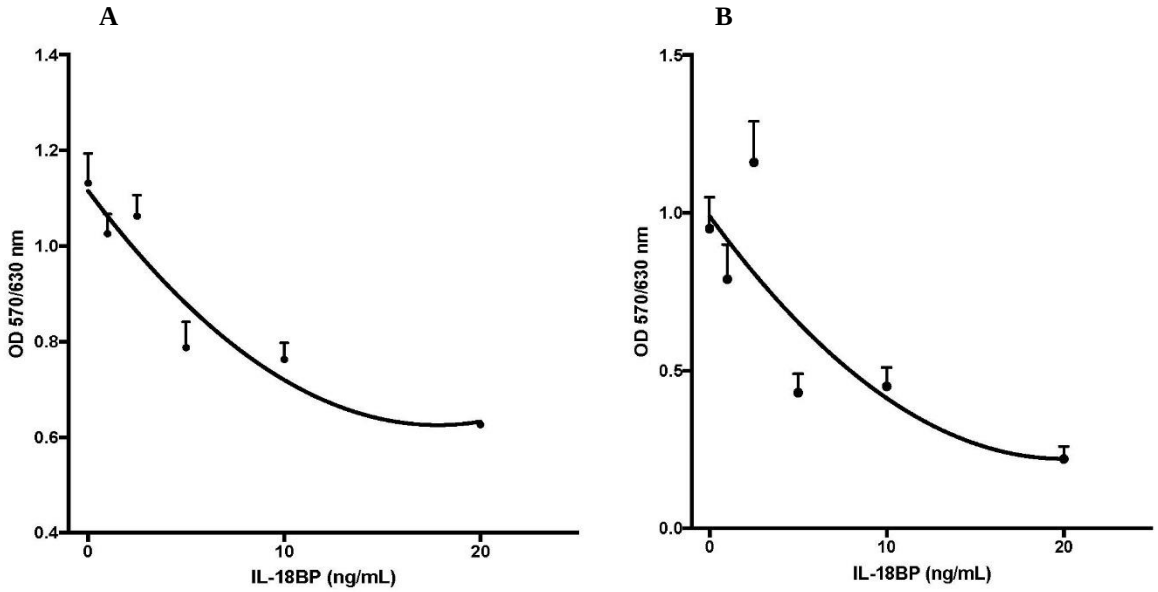

Fig S6. 4T1 tumor (**A**) and BMDM (**B**) cell growth as determined by MTT assays (presented as absorbance at 570/630 nm) following incubation with increasing concentrations of recombinant IL-18BP. ANOVA, $p<0.01$.