The Role of the NLRP3 Inflammasome in the Response to Anti-PD-1 Therapy in Metastatic Breast Cancers

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I. Abstract

The Interleukin 1 (IL-1) family of cytokines is a diverse class of secreted and membrane bound messenger proteins. The IL-1 family includes the exceptionally important proinflammatory cytokine IL-1β. IL-1β release is dependent on the nucleotide-binding domain, leucine-rich containing family, pyrin domain-containing 3 (NLRP3) inflammasome expressed in cells of the myeloid lineage. The NLRP3 inflammasome cleaves inactive pro-IL-1β into its active, secreted form (Latz, Xiao, & Stutz, 2013). In advanced metastatic breast cancer, there is a high expression of IL-1β as a result of the substantial activation of infiltrating and resident myeloid cells within the tumor microenvironment (TME) (Wu et al., 2018), (Bergenfelz et al., 2020), (Jang et al., 2020). These infiltrating myeloid cells can participate in immunosuppression by the upregulation of T cell inhibitor programmed cell death ligand (PD-L1). This immunosuppression is exasperated by the TME, and tumor cells can express PD-L1 to evade T cell toxicity. For these reasons, we investigated the role of the NLRP3 mechanism of IL-1β release in relation to breast cancer with a clinically tested cancer treatment, anti-PD-1 therapy[†]. We observed that human myeloid cells, stimulated with conditioned media from triple-negative breast cancer MDA-MB-468 cells led to NLRP3 activation and increased gene expression of PD-L1. *In vivo*, mice lacking NLRP3 and implanted with the metastatic breast cancer cell line E0771 showed a significant reduction in tumor growth ($p < 0.05$) and improved survival ($p < 0.01$). Using OLT1177®, an NLRP3 inhibitor, we showed reduced gene expression of PD-L1 ($p < 0.001$), caspase-1, the protein responsible for cleaving and activating IL-1β in the NLRP3 inflammasome ($p < 0.01$), and IL-1β ($p < 0.01$) in primary tumors. Combining NLRP3 inhibition with anti-PD-1 treatment significantly reduced tumor growth compared to monotherapies ($p < 0.05$). We show inhibition of NLRP3 accompanied by anti-PD-1 therapy yields a promising treatment for metastatic breast cancers.

[†] The data I contributed have been published, thus all figures and methods have been cited (Tengesdal et al., 2022).

II. Background:

Interleukins, described perfectly by the nomenclature, are produced by leukocytes. IL-1 cytokines are either pro- or anti-inflammatory cytokines that are produced predominantly by activated myeloid cells in an inflammatory response (Dinarello, 2018). Cytokines in this family include, but are not limited to, IL-1α, IL-1β, IL-1Ra (receptor antagonist), IL-18, and IL-38 (Krumm, Xiang, & Deng, 2014). The main character in this inflammatory response is IL-1β (Dinarello, 2018). IL-1β has been the focal point of inflammatory research since its introduction to science as "endogenous pyrogen" by Paul Beeson in the early 1940s (Dinarello, 2007). It was originally called "endogenous pyrogen" because of its ability to induce fever during an immune response. In early days of research, human neutrophil supernatants were injected into rabbits where fever could be induced in the absence of an exogenous stimuli such as infection (Dinarello, 2015). However, it was still heavily disputed in the scientific community because many proposed that fever was caused by products of infections. Until the 1960s, many still argued that fever was induced by an exogenous stimuli, ignoring the earlier studies of endogenous pyrogen. In 1967, Phyllis Bodel and Elisha Akins reported that supernatants from human blood monocytes produced a fever, a discovery that affected cytokine research. In the 1970s, Charles Dinarello started his studies to purify endogenous pyrogen. During this time scientists such as Barry Wood, Elisha Atkins, Phyllis Bodel, Ralph Kampschmidt and Patrick Murphy worked to purify endogenous pyrogen. With the purification of "endogenous pyrogen" to a single protein in 1977, the term IL-1β replaced the term "endogenous pyrogen". In 1984, Dinarello successfully isolated the IL-1β precursor cDNA (Dinarello, 2015). This began the discovery of other members of the IL-1 family of cytokines, and the mechanism of release of each. The proteins that modulate the release of these cytokines have become targets for research in pathology.

During an immune response, innate immunity is the first line of defense. The innate immune system is made up of cells that are capable of signaling and moving to the site of an infection. These cells all come from the same pool of progenitor cells, referred to as hematopoietic stem cells (HSCs) (Ballbach et al., 2017). These cells undergo hematopoiesis, or the process by which an HSC receives a signal to differentiate into a particular type of blood component. In most cases, cytokines act as the main signal, activating transcription factors which direct proliferation and differentiation programs in the nucleus. In humans, hematopoiesis takes place primarily in the bone marrow and thymus (Al-Shura, 2014). The HSCs can follow either the myeloid progression or lymphoid progression. For cells that are the "first responders" in innate immunity, the HSCs will become common myeloid progenitors. For the "secondary responders" and those that participate mainly in adaptive immunity, HSCs become common lymphoid progenitors. It is important to note that an immune response is not linear in the cell types that respond; innate and adaptive immunity over a person's life span closely work together to regulate immune responses to infection, damage, and illness such as cancer.

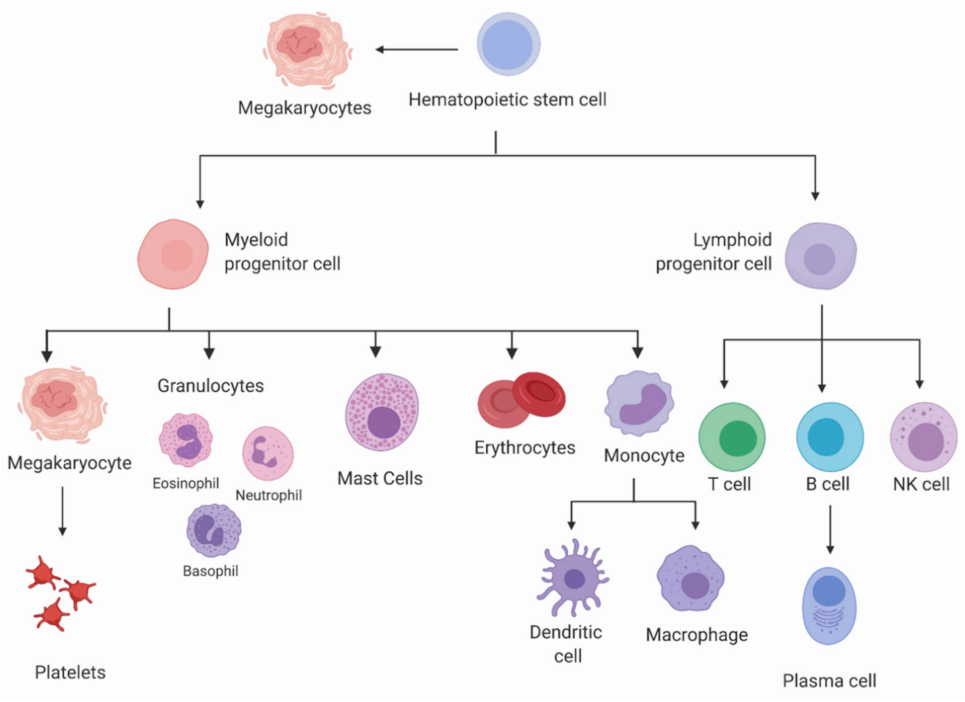

Figure 1. Hematopoiesis schematic. Illustrates comprehension of how hematopoietic cells commit to specific lineages, forming a hierarchical structure (Raza et al., 2021).

Cells of the myeloid progeny can become erythrocytes (red blood cells), mast cells, megakaryocytes, and myeloblasts (Al-Shura, 2014). Megakaryocytes continue to release thrombocytes (platelets) that help with clotting. Myeloblasts continue in differentiation to become granulocytes or monocytes. Granulocytes include basophils, eosinophils, and neutrophils. Monocytes continue to differentiate into macrophages or dendritic cells. The function of each myeloid leukocyte is crucial to the viability and survival of the organism. Mast cells play an important role in inflammatory cascades, such as an allergic reaction. Mast cells release histamine, TNFα, and prostaglandins (Fong & Crane, 2023). Granulocytes help the organism fight against pathogenic invaders and inflammatory cascades. Neutrophils are the most prolific of the granulocytes (Broudy et al., 1987). Neutrophils are released in acute inflammation during an innate immune response and release cytotoxic contents of their internal granules to kill bacterial and fungal invaders. Eosinophils work under the same mechanism to destroy larger multicellular parasites. Basophils, similar to mast cells, release histamine during an allergy response. Leukocytes in the monocyte lineage differentiate into macrophages and dendritic cells. Dendritic cells act as antigen presenting cells (APCs) to T cells. Macrophages are professional phagocytes. Macrophages also are the primary secreter of cytokines during an inflammatory response (Ward, Cherian, & Linden, 2018). Cytokines are small proteins released by cells to facilitate communication during both normal cellular activities and inflammatory responses to perceived threats. Cytokines are vital in numerous biological and physiological processes, including embryonic development, immune response to infections, aging, stem cell differentiation, vaccine effectiveness, and pain regulation (Zhang & An, 2007).

Cells of lymphoid progeny can become either a large lymphocyte or a small lymphocyte. A large lymphocyte is known as a natural killer (NK) cell. A small lymphocyte can either be a T

cell or a B cell. B cells can differentiate further into plasma cells. These plasma cells are specialized to secrete specialized antibodies that recognize specific antigens. These antibodies tag the antigen and subsequently the pathogen, damaged site, or cancer cell that is then phagocytosed by resident macrophages. The two main types of T cells that are present in the immune system are $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells (Ward, Cherian, & Linden, 2018).

Myeloid cells, primarily macrophages and dendritic cells, act as APCs to T cells during an adaptive immune response. APCs contain antigen presenting proteins known as major histocompatibility complexes (MHCs) or human leukocyte antigens (HLAs) in humans. MHCs on myeloid cells load the antigen onto its cell surface and present it to the naive T cell receptor (TCR). Naive T cells (T cells who have not been in contact with antigen) circulate through the body. They stop briefly in lymph nodes in anticipation of antigen presentation by an APC. Dendritic cells encounter and internalize antigen for digestion into peptides to be loaded onto the MHC. They then travel to lymph nodes (LNs) to encounter T cells. Macrophages phagocytose pathogen or damaged material and primarily stay at the sites of recognition. After recognition, macrophages secrete cytokines to attract T cells, a process that will be described later. Epithelial cells that have been infected internally by a virus act as an APC to be killed before infection can spread. During presentation, the TCR recognizes the fragment of the antigen on the MHC and activates an immune response. Activation of $CD4^+$ helper T cells (Th1, Th2, Th17, Tfh, and Treg cells) causes a secretion of cytokines to regulate and activate myeloid cells. Activation of $CD8^+$ cytotoxic T cells causes the secretion of cytokines and cytotoxins to kill an infected cell (Parham et al., 2021).

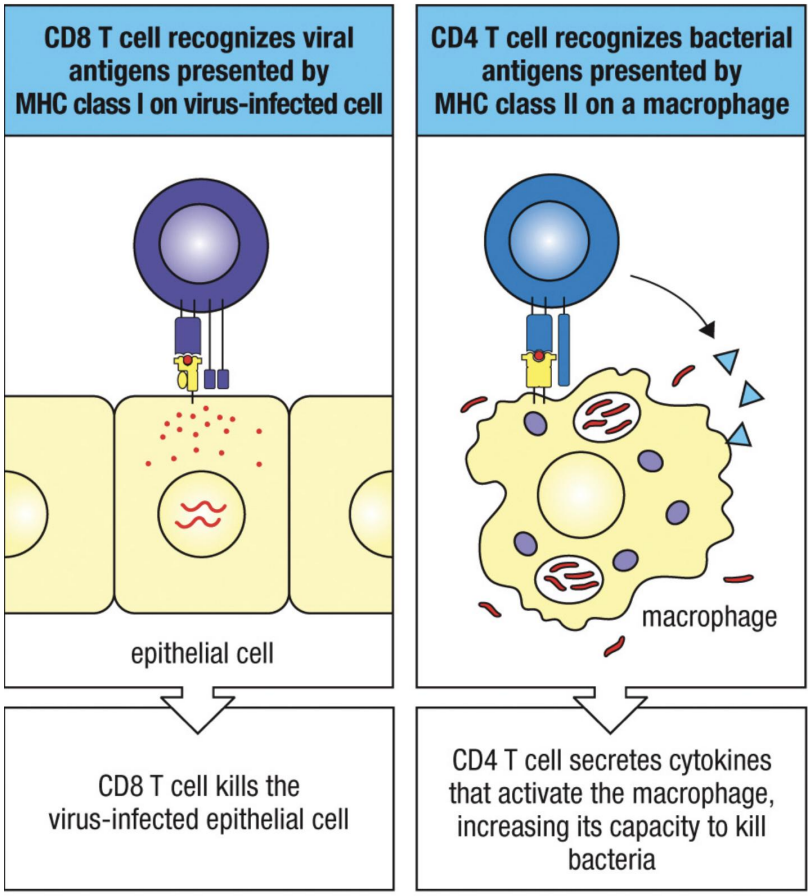

Figure 2. T Cell:MHC complex and response. The introduction of antigen to T cells via MHC presenting cells. Epithelial cells infected with internal viral pathogens use MHC class I to present to CD8+ cytotoxic T cells leading to the death of the infected cell (left). Antigen presenting cells use MHC class II to present extracellular antigen to CD4+ helper T cells to recognize and eradicate the pathogen (right) (Parham, et. al., 2021).

Receptors for “self”, “non-self”, and “altered-self” on cells allow for an immune response to occur. The receptors activated determine the signaling pathway. These pathways can lead to pro-inflammatory cytokine release, phagocytosis (the engulfing of soluble particulates by a phagocytic cell), or even the attraction of other leukocytes to the area. Cells of the innate immune response recognize conserved motifs of pathogens known as pathogen-associated molecular patterns (PAMPs), or non-pathogenic microbe-associated molecular patterns (MAMPs). These cells also recognize injury in the body through damage-associated molecular patterns (DAMPs). Receptors responsible for the recognition of these motifs are known as

pattern recognition receptors (PRRs). PRRs come in four different classifications in order to recognize a multiplicity of MAMPs and PAMPs. These categories are toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG 1-like receptors (RLRs) (Parham et al., 2021). Binding of MAMPs or PAMPs to these receptors causes a signaling cascade that leads to a release of inflammatory cytokines. TLR4 is a TLR on macrophages that recognizes lipopolysaccharide (LPS), a sugar shed by gram-negative bacteria. This binding activates the nuclear factor κB (NFκB) signaling pathway that results in the transcription of proform of IL-1β and IL-18, as well as the post translational modification of NOD-like receptor, NLRP3. This step signifies the "priming" step of the activation of the NLRP3 inflammasome. NLRP3 is a PRR in the intracellular NLR family (Tengesdal et al., 2021a). NLRP3 is the major modulatory receptor of IL-1β release. NLRP3 is activated following many things such as reactive oxidative species found in the mitochondria due to metabolic stress, or from heavy influx of ions such as calcium or potassium. It can also occur due to the milieu that exists of soluble particulates that may have been phagocytosed by a myeloid cell. This triggers the binding of NIMA-related kinase 7 (NEK7) and an adaptor apoptosis-associated speck-like protein containing a CARD (ASC). This recruits procaspase 1, which binds to ASC to be cleaved into caspase-1 (Swanson, Deng, & Ting, 2019). Tothether, this forms the NLRP3 inflammasome. The NLRP3 inflammasome is responsible for cleaving pro-IL-1β and pro-IL-18 into active IL-1β and IL-18 (Latz, Xiao, & Stutz, 2013).

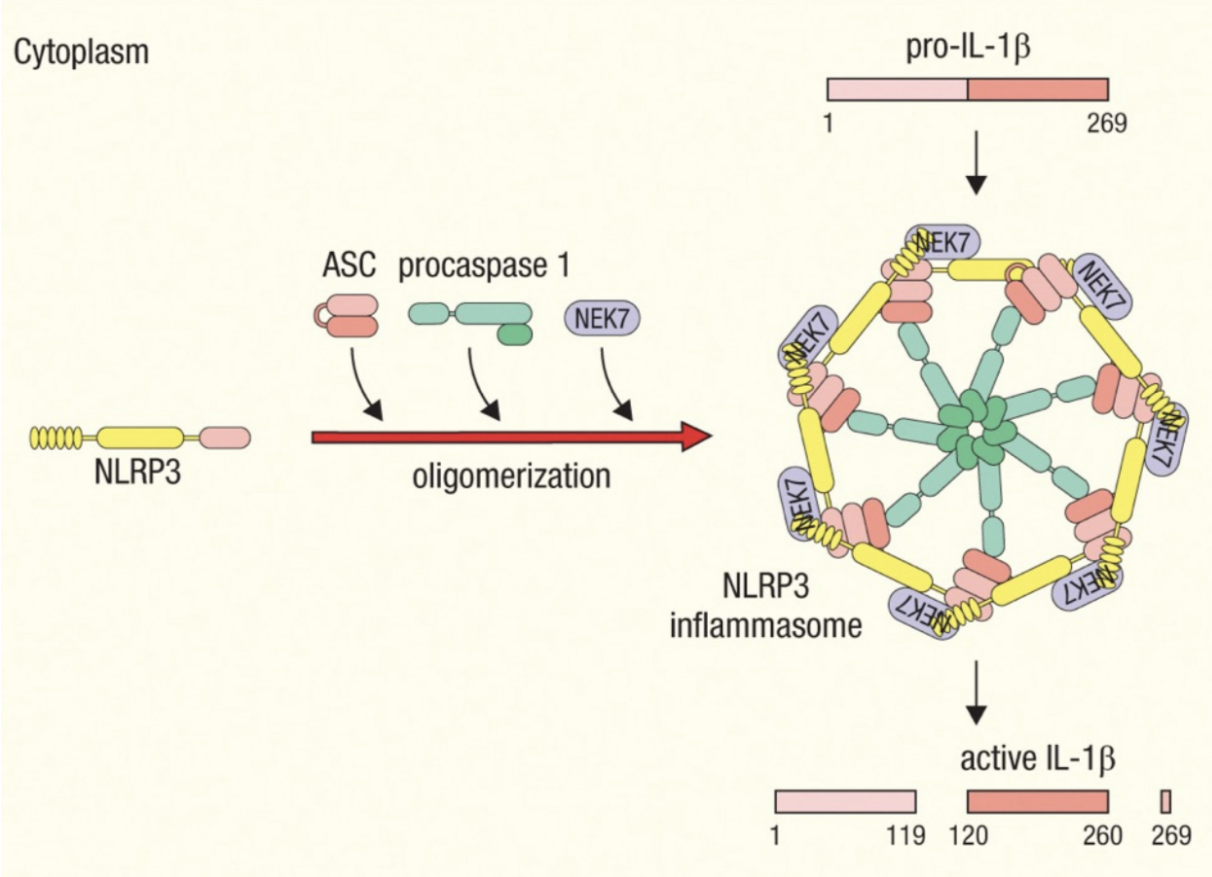

Figure 3. NLRP3 inflammasome formation. The NLRP3 subunit is recruited with ASC, NEK7, and the proform of caspase 1, procaspase 1. This inflammasome then cleaves the pro-form of IL-1β to its active form for release (Parham, et. al., 2021).

IL-1β is released in large quantities after a myeloid cell is activated. In some scenarios, NLRP3 activation results in inflammatory cell death, termed pyroptosis. Pyroptosis is the death of the myeloid cell that is secreting IL-1β. The activation of the myeloid cell not only triggers the formation of the NLRP3 inflammasome, but triggers the gasdermin D pathway. Inactive gasdermin D is cleaved into its active monomer (Parham et al., 2021). This monomer self-assembles into a pore structure that implants into the cell membrane. This allows for the sizable amount of IL-1β to be released into the extracellular space. The pores in the membrane cause the myeloid cell to die a similar death to apoptosis. This response occurs during disease states, particularly in areas of high damage or infection, such as cancer or sepsis.

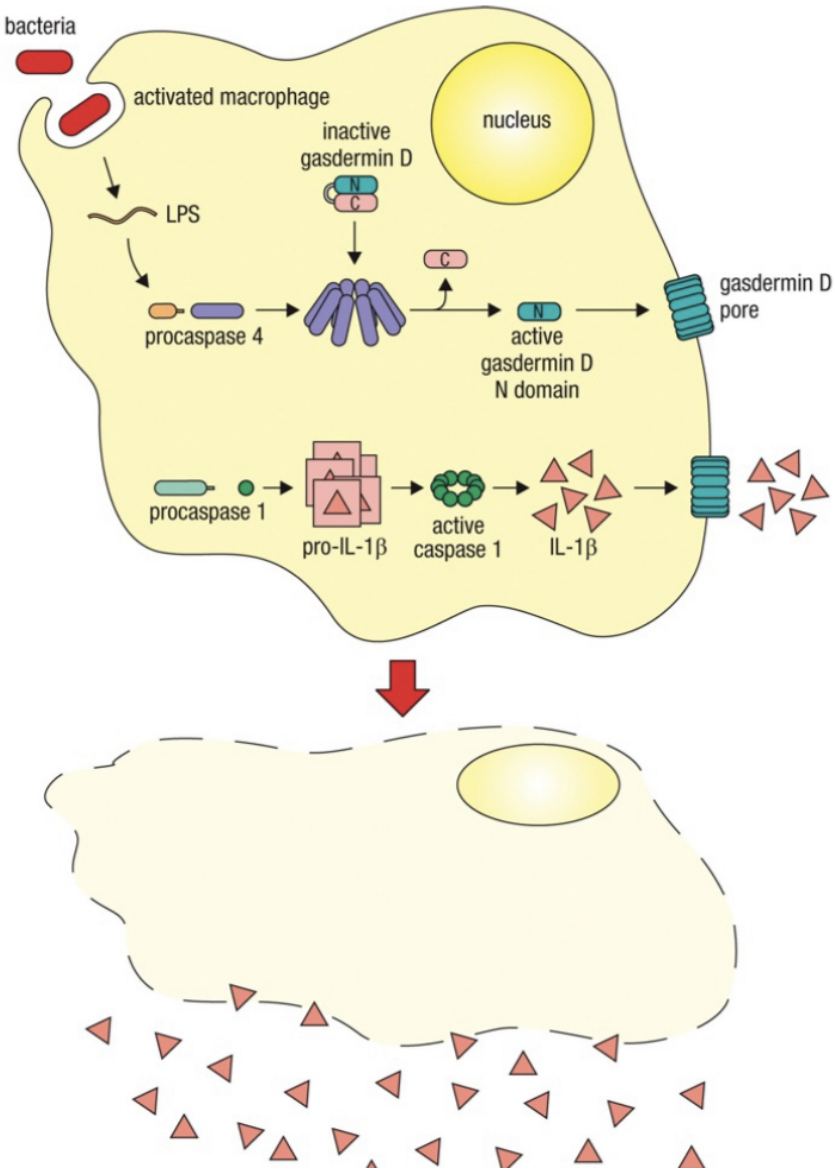

Figure 4. Macrophage pyroptosis diagram caused by the mass secretion of IL-1β. Bacterium is phagocytosed to activate NLRP3 inflammasome formation and gasdermin D pore. Multiple pores in the cell membrane causes cell death (Parham, et. al., 2021).

The dysregulation of the NLRP3 inflammasome is pathologic in many instances. Diseases involving this disruption include those involving constant NLRP3 inflammasome signaling causing chronic inflammation. The mounting evidence from animal models, reinforced by human studies, strongly suggests that the inflammasome plays a pivotal role in the onset and advancement of conditions with significant public health implications. These encompass

metabolic disorders (such as obesity, type 2 diabetes, and atherosclerosis), cardiovascular diseases (both ischemic and non-ischemic heart diseases), inflammatory conditions (including liver diseases, inflammatory bowel diseases, gut microbiome disturbances, and rheumatoid arthritis), as well as neurologic disorders (like Parkinson's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, and various other neurological conditions)(Fusco et al., 2020). This prominence of the inflammasome sets it apart from other molecular platforms in terms of its impact on these health issues. NLRP3 inhibitors are becoming a new approach to treating these inflammatory disorders. OLT1177 (dapansutrile, 3-(Methanesulfonyl)propanenitrile) is a newly synthesized β-sulfonyl nitrile compound that works as a potent inhibitor of the NLRP3 inflammasome (Marchetti et al., 2018b). OLT1177 prevents the release of active IL-1β and IL-18. OLT1177 has been used to reduce acute and chronic inflammation in patients clinically and has been proven safe to use in humans (Marchetti et al., 2018a). This drug is used in studies to pharmacologically challenge NLRP3 to test mechanisms involving the inflammasome.

Cancer biology is a prolific research avenue due to being the second cause of death in the United States after cardiovascular disease. Cancer is the name of a disease that describes thousands of types of mutations that result in and originate from uncontrolled cellular growth. Two broad categories of tumor classes exist. Benign tumors are those that are unable to metastasize. Malignant tumors are able to invade normal tissue outside of the area that the cancer originated. Further classification is determined by the tumor’s cell type, tissue, or organ of origin. All tumors arise from a single cell with a mutation in cell division regulation. Mutations in two main groups of genes are involved in cancer progression. Proto-oncogenes contribute to the normal process of cell division, including growth factors, signaling enzymes, and receptors. Tumor suppressor genes contribute to proteins that prevent proliferation. Put simply,

gain-of-function oncogenes or loss-of-function in tumor suppressor genes can result in unchecked cell proliferation resulting in a tumor.

Essential characteristics of cancer cells
Stimulating their own growth
Ignoring growth-inhibiting signals
Avoiding death by apoptosis
Developing a blood supply: angiogenesis
Seeding from site of origin to invade other tissues: metastasis
Constant replication to expand the tumor-cell population
Evasion and outrunning the immune response

Figure 5. List of essential characteristics of cancer cells used for identification of tumorous cells (Parham, et. al., 2021)

Classic hallmarks of cancer include a shift towards anaerobic metabolism, inflammation, angiogenesis, immunosuppression, proliferation and metastasis. Cancer is the professional evader of the immune system. Cancer cells are highly mutagenic, which often results in the immune system identifying and clearing tumors. However, cancer cells have been known to take advantage of the immune cell surface markers that shut off immune responses to evade leukocytes that check for abnormalities such as NK cells and cytotoxic $CD8^+$ T cells (Bi & Tian, 2017), . For this reason, cancers can evade immune surveillance and tumor growth is able to become out of control.

Though one goal of the immune system is to destroy cancerous cells, it can accidentally aid in the progression of cancer. Aspects of the tumor microenvironment (TME) can prime the

NLRP3 inflammasome to process more IL-1β (Sceneay et al., 2013). IL-1β, the pro-inflammatory cytokine, has been shown to be a pro-cancer molecule by modulating tumor-promoting mechanisms such as angiogenesis, metastasis and immunosuppression (Apte et al., 2006). Angiogenesis is marked by the formation of new blood vessels to supply the tumor with nutrients and oxygen. IL-1β induces the production of proangiogenic factor vascular endothelial growth factor (VEGF), as well as fibroblast growth factor (FGF) (Voronov et al., 2003). Chronic inflammation due to IL-1β induces immunosuppression and exhaustion of cells responsible for the clearing of tumor cells, the NK and $CD8^+$ T cells (McLane et al., 2019). A contributor to this immunosuppression by tumor cells is the upregulation of programmed cell death ligand (PD-L1) on either the tumor or tumor-infiltrating macrophage cell surface. Programmed cell death-1 (PD-1) is an immunoinhibitory receptor of the CD28 family which regularly is used to inhibit T lymphocyte proliferation, survival, and effector functions. PD-L1 is found on immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs)(Abiko et al., 2015), (Bae et al., 2007). It helps in the developmental pathway in the thymus to inhibit self-reactive T cells. Programmed cell death (PD-1) receptor is expressed on T cells, meaning $CD8^+$ T cells are consecutively turned off when they interact with PD-L1 (Qian et al., 2018). The T cell therefore is tricked and cannot kill the tumorous cell.

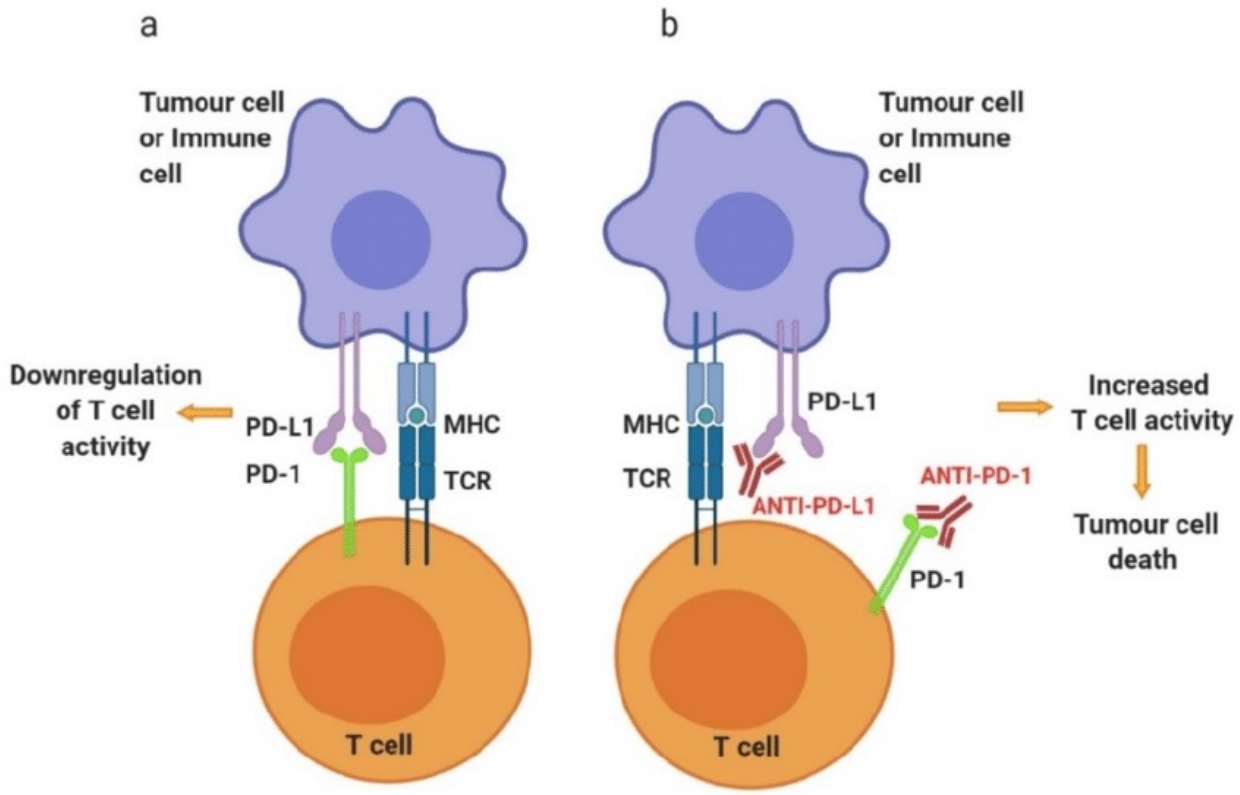

Figure 6. T cell interacting with an immune system evading tumor cell and antigen presenting cell (APC)/myeloid derived suppressor cell (MDSC). The tumor/APC/MDSC cell uses PD-L1 to bind to PD-1 on the T cell surface to turn the T cell "off" (left). When there is anti-PD-l/anti-PD-L1 antibodies present, the T cell is able to continue the immune response (May, 2023).

Metastasis is the indication of malignant tumors, or the spread of a primary tumor to a secondary tissue. Metastasis is facilitated by IL-1β in that IL-1β has been shown to regulate epithelial-mesenchymal transition (EMT) (Kalluri & Weinberg, 2009). EMT allows cancerous cells to travel by degrading cell adhesion molecules landmark to stationary epithelial cells. This allows the cancerous cell to differentiate into a mobile cell that travels to a secondary site to create metastases.

III. Introduction:

As stated previously, the proinflammatory cytokine interleukin-1β (IL-1β) plays a key role in the modulation of tumor angiogenesis, metastasis and immunosuppression. IL-1β

production was shown to be correlated with disease severity in breast tumor biopsy (Wu et al., 2018), (Aggen et al., 2021). The authors demonstrated that the IL-1β was sourced from infiltrating $CD11c^+$ myeloid cells. They also found that mice deficient in IL-1β had significantly reduced macrophages in the tumors. This was associated with limited tumor growth and an increase in infiltrating $CD8^+$ T cells. This study concludes that IL-1β and the myeloid cells that produce the cytokine play a key role in the severity and progression of breast cancer (Cao et al., 2014).

We had seen multiple previous studies in which there was an increase in tumor-NLRP3 inflammasome expression in solid tumors (Tengesdal et al., 2021a). For example, a melanoma model was described to have shown tumor-NLRP3 activation drives chronic inflammation, contributing to immunosuppression of cancer killing cells (Hamarsheh & Zeiser, 2020). Though these data suggest the promotion of tumor proliferation by NLRP3 modulation, the mechanism remains unclear. We observed activation of NLRP3 promotes chronic inflammation due to the hypersecretion of IL-1β. Chronic IL-1β exposure induces the expansion and activation of mononucleated, developmentally stunted neutrophils and monocytes. These cells are known as myeloid-derived suppressor cells (MDSCs) (Belyavsky, Petinati, Drize, 2021). MDSCs display immunosuppressive characteristics. These cells express the checkpoint inhibitor characteristic to cancer evading tumor cells, PD-L1, and secrete T cell and NK cell suppressing cytokines TGFβ or IL-10 (Bi & Tian, 2017). NLRP3 induced IL-1β modulates the secretion of cytokines and chemokines such as IL-6, G-CSF and CCL2 (Oh et al., 2013). These secreted molecules expand MDSC populations in breast cancers (Cao et al., 2014). Thus, these data suggest a possible mechanism for IL-1β mediated immunosuppression. The tumor microenvironment (TME) has been shown to induce PD-L1 upregulation by chronic inflammation in melanoma studies (Thiem et al., 2019). Recently, NLRP3 activity in melanoma was shown to upregulate PD-L1 in both tumor cells and MDSCs through tumor-intrinsic and tumor-host signaling pathways

(Theivanthiran et al., 2020). A report using 4T1 murine model of breast cancer showed a complete tumor retraction while using anti-PD-1 in combination with anti-IL-1β therapies (Kaplanov et al., 2019). Anti-PD-1 therapies are currently being assessed for efficacy in metastatic breast cancers with an overall response rate (ORR) only ranging from 5.3–18.5% (Adams et al., 2019). Therefore, we wanted to explore further into the response to anti-PD-1 treatment in breast cancers by investigating the activation of NLRP3 inflammasome along with the PD-1 inhibition.

IV. Results:

NLRP3 Promotes Breast Cancer Progression

In order to gauge the role of NLRP3 in an *in vivo* breast cancer model, we used murine derived metastatic medullary breast adenocarcinoma cell line, E0771. We orthotopically implanted onto the mammary fat pad to emulate a naturally occuring breast cancer tumor in female wild type (WT) and NLRP3-deficient mice (nlrp3−/−) mice. Figure 1A verifies significant growth reduction of the tumors in NLRP3 deficient mice compared to WT ($p < 0.05$). After verifying with the knockout mice, we pharmacologically inhibited NLRP3 by orally administering OLT1177 to WT mice. OLT1177 was administered using a drug-enriched diet (7.5 g/kg of food), or a matching standard diet was used as a negative control. Mice were implanted with E0771 cells and given the previously described diets 3 days post-implantation. Mice given the OLT1177 diet showed similar results to the NLRP-deficient mice (Fig. 1A) as there was significantly decreased tumor growth (Fig. 1B) ($p < 0.05$). Survival curve analysis for E0771 tumor-bearing mice was performed on WT and NLRP3 deficient mice. Tumor-bearing NLRP3-deficient mice survived significantly longer than tumor-bearing WT mice (Fig. 1C) (p =

0.0037).

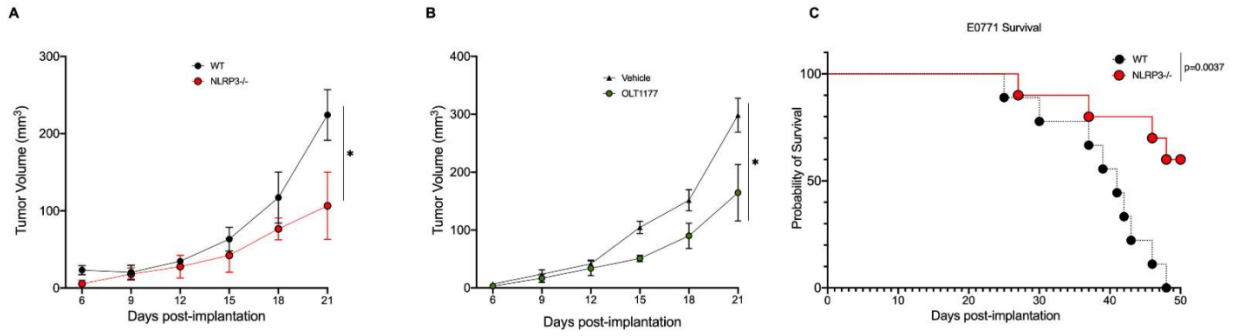

Figure 1. Genetic and pharmacologic inhibition of NLRP3 reduces breast cancer progression. (A) E0771 tumor growth in WT or nlrp3−/− mice (n = 3). **(B)** E0771 tumor growth in mice fed standard or OLT1177 diet (n = 6). **(C)** E0771 survival curve in WT or nlrp3−/− (n = 10). ** $p < 0.01$, * $p < 0.05$. (Tengesdal et al., 2022)

Breast Cancer Secretome Induces Myeloid Expression of PD-L1 and NLRP3 Components

In consideration of the NLRP3 inflammasome being contained within myeloid cells, we wanted to use our results showing NLRP3-deficient mice tumor progression was stunted to hypothesize that the infiltrating myeloid cells were responsible for pro-tumor signaling. To model this, we used the human monocyte line THP-1. To mimic a tumor micro-environment (TME), we stimulated WT and NLRP3-deficient THP-1 (THP-1defNLRP3) cells with breast cancer-conditioned media (BCM) obtained from MDA-MB-468 cells. We found that THP-1 cells treated with BCM had a significantly increased IL-1β ($p < 0.001$) secretion compared to the cells in control (Fig. 2A). The NLRP3-deficient THP-1 cells showed reduced IL-1β ($p < 0.01$) secretion compared to native THP-1 cells stimulated with BCM (Fig. 2A). This data suggest that the increase in IL-1β is dependent on NLRP3. We assessed immunosuppressive cell surface marker *Pdcd111* mRNA expression following BCM treatment to determine if it was linked with NLRP3 activation. WT THP-1 cells have a significant upregulation in expression of *Pdcd111* after stimulation with BCM (Fig. 2B) ($p < 0.05$). This increase was not observed in the

THP-1defNLRP3 cells (Fig. 2B) ($p < 0.001$). Similar to the above experiments, we wanted to assess whether pharmacological inhibition of NLRP3 would yield similar results of NLRP3 deficiency. WT THP-1 cells were stimulated with BCM as described previously and were treated with or without OLT1177 (10 μM). Again, we measured *Pdcd1l1* mRNA expression. THP-1 cells treated with OLT1177 showed no increase in Pdcd1l1 expression following stimulation with BCM (Fig. 2B) ($p < 0.01$). Next, we assessed the effect of secreted factors from 4T1 cells on IL-1 signaling in the myeloid compartment. WT mice were sacrificed and harvested for bone marrow. The adherent cells were separated and stimulated in a similar manner to the BCM but instead with 4T1-conditioned media or RPMI for 3 days. Western blot analysis was performed on NLRP3, IL-1β and PD-L1. As shown in Figure 2C–G, BCM (right) upregulated expression of PD-L1, NLRP3, pro-IL-1β (p37) and mature IL-1β (p17) when compared to the same cells in control (left) (Fig. 2F). These data suggest that the TME of breast cancer cells have tumor secreted soluble factors that act on myeloid cells. This activates the NLRP3 inflammasome which acts to upregulate immunosuppressive markers.

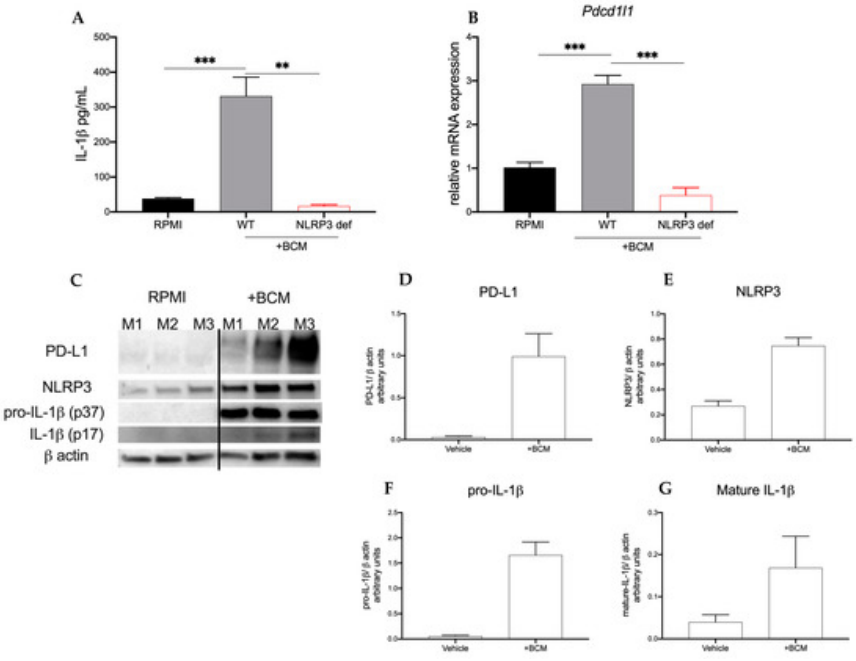

Figure 2. Breast cancer cells induce myeloid NLRP3 and PD-L1. (A,B) THP-1 or THP-1-NLRP3def cells were stimulated with MDA-MB-468-conditioned media (+BCM). **(A)** Mean ± SEM IL-1β production from THP-1 or THP-1-NLRP3def cells left unstimulated (RPMI) or treated with conditioned media after 48 h (n = 3). **(B)** Mean ± SEM of relative gene expression of Pdcd1l1 from cells described in (A,B) or stimulated with (+BCM) and treated with OLT1177 (n = 3). **(C–G)** Bone marrow adherent cells stimulated with 4T1-conditioned media (+BCM). **(C)** Representative Western blot images from (C–G); mean ± SEM of PD-L1/β-actin ratio **(D)**, NLRP3/β-actin ratio **(E)**, pro-IL-1β/β-actin ratio **(F)** and mature IL-1β/β-actin ratio **(G)** from cells described in (C–G) (n = 3). *** $p < 0.001$, ** $p < 0.01$. (Tengesdal et al., 2022)

NLRP3 Inhibition Reduces Immunosuppression in TME

We wanted to investigate how NLRP3 inhibition affected the composition of the TME. Considering the impact of BCM on protein expression in bone marrow adherent cells as described earlier, we also investigated gene expression in primary tumors. To evaluate this, we examined the expression of the *Pdcd1l1*, *Il1b*, *Casp1*, and *Nlrp3* genes in the 4T1 tumors obtained from the aforementioned mice. Notably, mice receiving the OLT1177 diet exhibited significantly lower levels of *Pdcd1l1* (Fig. 3A), *Il1b* (Fig. 3B), and *Casp1* (Fig. 3C) compared to those on the standard diet. Consistent with the known mechanism of action of OLT1177, no discernible changes were observed in Nlrp3 gene expression (Fig. 3D). We concluded that NLRP3 activation played a role in a diminished anti-tumor immunity and ultimately promoting tumor progression.

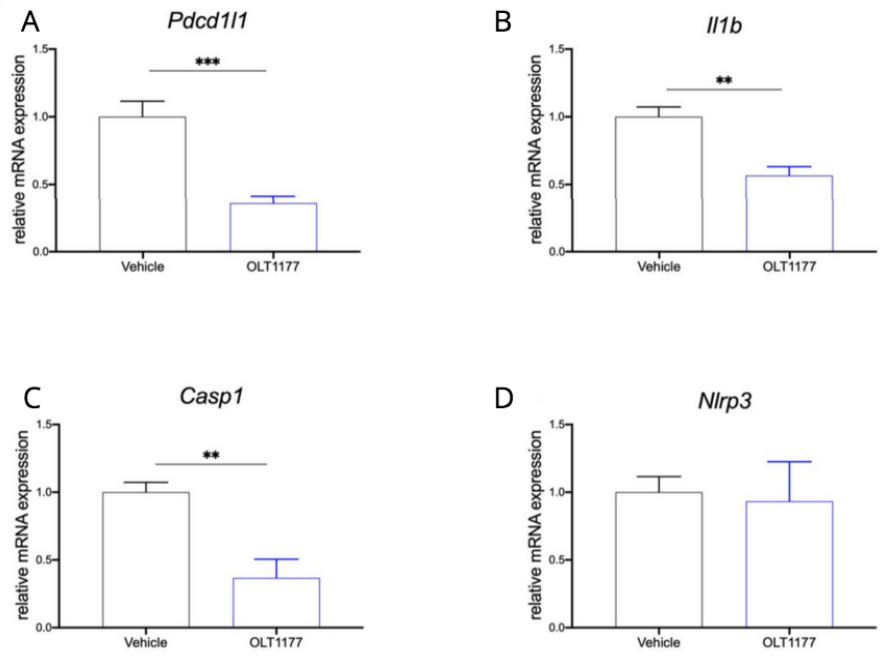

Figure 3. NLRP3 drives immunosuppressive TME. (A–D) Mean ± SEM of relative mRNA expression of *Pdcd1l1* **(A),** *Il1b* **(B),** *Casp1* **(C)** and *Nlrp3* **(D)** from primary 4T1 tumors in mice fed standard or OLT1177 diet (n = 6). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (Tengesdal et al., 2022)

NLRP3 Inhibition Enhances Anti-PD-1 Efficacy

Given the observed decrease in PD-L1 expression, we explored the impact of anti-PD-1 therapy in mice lacking NLRP3. We implanted E0771 tumors in both wild-type (WT) and NLRP3 deficient mice. Ten days after tumor implantation, we administered a single dose of anti-PD-1 (8 mg/kg) or an equivalent dose of IgG (8 mg/kg) intraperitoneally (Fig. 4A). As consistent with our previous findings, Figure 4B illustrates that NLRP deficient mice treated with control IgG exhibited reduced tumor growth compared to WT mice treated with control IgG ($p < 0.05$). Tumor growth was significantly decreased in WT mice receiving anti-PD-1 compared to WT mice treated with IgG control ($p < 0.05$). Notably, the administration of anti-PD-1 in NLRP3 deficient mice led to nearly complete suppression of tumor growth by day 21 ($p < 0.01$),

demonstrating a significant reduction compared to anti-PD-1 or NLRP3 deficient mice alone ($p < 0.05$) (Fig. 4B).

Subsequently, we explored the therapeutic potential of combining OLT1177 with anti-PD-1 treatment. In this experiment, mice were implanted with 4T1 tumors. On day 7 post-implantation, they were either maintained on a standard diet or switched to an OLT1177 diet. On day 10, we administered either IgG or anti-PD-1 (Fig. 4C). As depicted in Figure 4D, mice receiving either OLT1177 or anti-PD-1 exhibited significant reductions in tumor growth compared to the IgG control group (with reductions of 50% and 38%, respectively) ($p < 0.01$). Notably, the combination of both therapies resulted in a further reduction in tumor growth compared to either anti-PD-1 (44%) or OLT1177 (29%) alone ($p < 0.05$) (Fig. 4D).

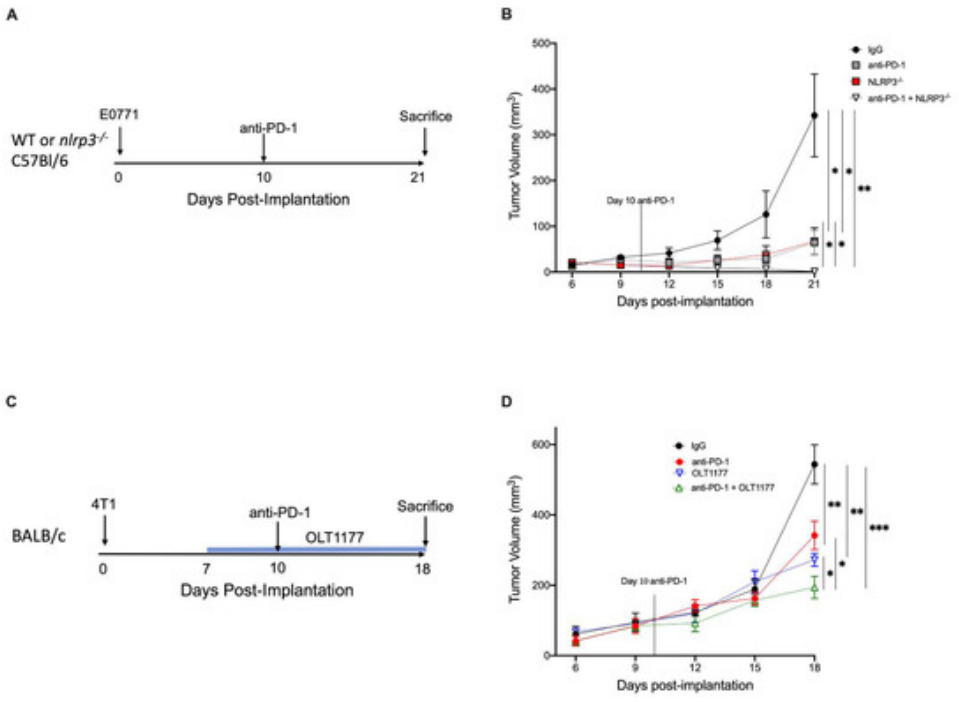

Figure 4. NLRP3 inhibition enhances anti-PD-1 efficacy. (A) Experimental design for (B). **(B)** E0771 tumor growth in WT and *nlrp3*−/− mice treated with anti-PD-1 or IgG (n = 4)**. (C)** Experimental design for (D)**. (D)** 4T1 tumor growth in mice fed standard or OLT1177 diet and treated with anti-PD-1 or IgG (n = 5). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (Tengesdal et al., 2022)

V. Discussion:

The implications of these data lay out a future for breast cancer immunotherapies that could be implemented in the near future. NLRP3 inflammasome has been studied for many years due to the downstream effects of IL-18 and IL-1β release. We found that metastatic breast cancer cells induce activation of NLRP3 inflammasome leading to immunosuppressive impacts. OLT1177 emerged as a potential therapeutic adjunct to anti-PD-1 therapy for metastatic breast cancer. Elevated levels of IL-1β were associated with disease severity in advanced-stage breast cancers, with NLRP3 being the primary contributor to breast tumor-induced IL-1β production in infiltrating myeloid cells. This finding underscored the significance of NLRP3 in driving IL-1β production and its potential as a target for therapeutic interventions. The research also addressed the limited efficacy of checkpoint inhibitors like Pembrolizumab in triple-negative breast cancer and proposed combining anti-PD-1 with NLRP3 inhibition as a promising approach. Inhibiting NLRP3-dependent PD-L1 expression in myeloid cells was shown to enhance the effectiveness of anti-PD-1 therapy. This combination therapy was supported by pre-clinical and clinical data, suggesting its potential as a valuable treatment strategy for metastatic breast cancer.

The use of the NLRP3 deficient mice was to validate that there was a relationship between proliferation of the breast cancer cell lines E0711 and 4T1 and the NLRP3 inflammasome. We then used OLT1177 to see if we could emulate this pharmacologically. We found that that size reduced significantly to signify there was a connection between the inhibition of NLRP3 and tumor proliferation. We then wanted to see if NLRP3 inflammasome containing myeloid cells could be stimulated by the tumor microenvironment (TME). This would confirm that the infiltrating myeloid cells were responsible for pro-tumor signaling. We used THP-1 monocytes (WT and NLRP3 deficient/OLT1177 inhibited) and stimulated with breast cancer conditioned media (BCM). We found that the NLRP3 inhibited monocytes did not secrete

significantly as much IL-1β as shown in WT THP-1 cells. We examined the expression of the immunosuppressive cell surface marker *Pdcd111* mRNA after exposure to BCM, aiming to ascertain its association with NLRP3 activation. WT THP-1 cells showed an increase that was not seen in the NLRP3 deficient THP-1 cells. We repeated pharmacologically with OLT1177 and the same results were found. An *ex vivo* study of WT mice was done to surveillance if there would be a similar result as seen above. Bone marrow cells were removed and cultured and analyzed or were treated with BCM. Using western blot to validate our hypothesis, we saw an upregulated expression of PD-L1, NLRP3, pro-IL-1β (p37) and mature IL-1β (p17) in the BCM treated compartment in comparison to the non-treated cells of the same origin. We wanted to investigate how NLRP3 inhibition using an OLT1177 diet would affect in vivo TME. We found using mRNA analysis that the OLT1177 diet exhibited significantly lower levels of the anti-tumor immunity genes *Pdcd1l1*, *Il1b*, *Casp1*, and *Nlrp3* genes in the 4T1 tumors. Finally, we examined the impact of anti-PD-1 therapy. We implanted tumors into WT and NLRP3 deficient mice. After ten days, we treated the mice with either anti-PD-1 or a control. Results showed reduced tumor growth in the NLRP3 deficient mice with the control treatment and in WT mice with anti-PD-1. Notably, NLRP3 deficient mice treated with anti-PD-1 almost completely stopped tumor growth by day 21. We repeated this pharmacologically with OLT1177, yielding very similar results. Our data suggest that NLRP3 inhibition and anti-PD-1 therapy can be a viable option for cancer treatment.

The potential of NLRP3 inflammasome inhibition as a therapeutic target extends beyond breast cancer, as it has been implicated in several other cancer types. NLRP3-regulated IL-1β release has been observed to exacerbate the progression of various cancers, broadening the scope of its relevance (Tengesdal et al., 2021c). In the context of human melanoma, NLRP3 activation is a notable factor. Additionally, melanoma cells have been shown to constitutively secrete IL-1β through the NLRP3 and IL-1R pathway, even in the absence of external stimuli (Tengesdal et al.,

2021a), (Dagenais et al., 2017). In clinical practice, anti-PD-1 treatments have emerged as a valuable option for managing melanomas that cannot be surgically removed or advanced cases that have invaded lymph nodes (Okamoto et al., 2010). These treatments have paved the way for innovative strategies in melanoma therapy. Lymphomas also demonstrate a connection to the NLRP3 inflammasome, specifically through its influence on IL-18, which contributes to lymphoma cell proliferation and the suppression of apoptosis (Geng, 2018). This effect is mediated by the regulation of critical genes such as C-MYC and BCL2, as well as the downregulation of TP53 and BAX. Notably, patients with relapsed or refractory lymphoma who exhibit PD-L1 overexpression have displayed a more favorable response to anti-PD-1 therapy. These observations underscore the significance of targeting the NLRP3 inflammasome in lymphoma treatment. Leukemias, including CMML, JMML, and AML, present yet another arena where NLRP3/IL-1β-dependent mechanisms come into play (Urwanisch, 2021). In particular, KRAS-mutant leukemias show myeloproliferation and cytopenias driven by RAC1 activation and the production of reactive oxygen species (ROS) (Urwanisch, 2021). This provides a potential pathway for therapeutic intervention aimed at NLRP3 modulation. Acute lymphoid leukemia (ALL), primarily affecting adolescents, exhibits an upregulation of NLRP3 and caspase-1, which results in glucocorticoid resistance through the cleavage of the glucocorticoid receptor by caspase-1. Furthermore, ALL has been associated with the upregulation of PD-L1 in the bone marrow samples of both new and relapsed patients (Urwanisch, 2021). This reinforces the potential relevance of targeting NLRP3 in leukemia therapy. In light of the findings from our study and the precedent set by the use of anti-PD-1 therapies, it is evident that melanomas, lymphomas, and leukemias should be primary candidates for further investigation (Adams et al., 2019). Furthermore, OLT1177, which was instrumental in our study, stands out as a point of interest in research endeavors exploring the possibilities of anti-PD-1/NLRP3 inhibition therapies. This emerging field of study holds significant promise for improving the treatment

outcomes of various cancers, potentially transforming the landscape of cancer therapy as a whole.

† The data I contributed have been published, thus all figures and methods have been cited.

VI. Conclusion

In summary, our study has unveiled that NLRP3 inflammasome inhibition leads to a disruption in metastatic breast cancer proliferation. We have suggested that inhibiting NLRP3 could serve as a promising therapeutic strategy to overcome resistance to anti-PD-1 treatment.

VII. Methods:

1. Mice

All animal protocols were approved by the University of Colorado School of Medicine Animal Care and Use Committee. 6-8 week old wild type female BALB/c, C57Bl/6 or nlrp3−/− (B6.129S6-Nlrp3tm1Bhk/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). (Tengesdal et al., 2022)

2. Cell Lines

Mammary carcinoma cells lines 4T1, E0771 and MDA-MB-468 were purchased from ATCC (Manassas, VA, USA), and native THP-1 and NLRP3-deficient (nlrp3-/-) THP-1 cells were purchased from InvivoGen (San Diego, CA, USA). MDA-MB-468 and 4T1 cells were cultured in DMEM (Corning, Corning, NY, USA) that was supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 0.1 mg/mL streptomycin. E0771 cells were cultured in RPMI (Corning) that was supplemented with 10% FBS, 1% HEPES, 100 units/mL penicillin and 0.1 mg/mL streptomycin. Cells were maintained in a humidified 5% CO2 atmosphere at 37 °C. (Tengesdal et al., 2022)

3. **OLT1177®**

OLT1177 was administered in an OLT1177-enriched diet at the concentration of 7.5 g of OLT1177 per kg of food. A matching diet that did not contain OLT1177 was used as a negative control (standard diet). Both diets were purchased from Research Diets Inc (New Brunswick, NJ, USA). OLT1177 used *in vitro* was provided by Olatec Therapeutics. (Tengesdal et al., 2022)

4. **Breast Cancer Conditioned Media Assays**

Supernatants from MDA-MB-468 cells (BCM) were added to native THP-1 or NLRP3-deficient THP-1 cells (1 × 105) (InvivoGen) and cultured in a 96-well plate and activated with 10 ug/mL of LPS (Sigma Aldrich, Burlington, MA, USA) for 3 hrs. BCM was then added at 1:2 ratio to fresh media. Cells were incubated for 3 days before collection of the supernatants or cells for gene analysis. Bone marrow adherent cells were obtained from WT BALB/c female mice, and 5 million cells were plated into a 12-well plate with cell culture media. The next day the non-adherent fraction was removed, and cells were then treated with 4T1-conditioned media (1:3 conditioned media to RPMI) or normal media. On day 3, cells were lysed in RIPA buffer and prepared for Western blots. (Tengesdal et al., 2022).

5. ***In Vivo* Tumor Model**

Cell lines were trypsinized, and 2 × 105 cells/mouse were suspended in Matrigel (Corning) and injected orthotopically into single mammary fat pad of BALB/c (for 4T1) or C57Bl/6 mice (for E0771). Tumor growth was assessed every 3 days using a caliper, and tumor volume was calculated as ½ (L × W × H). Mice were sacrificed after 15/18 (4T1) or 18/21 (E0771) days from tumor cell implantation. Mice were fed an OLT1177-enriched diet or a standard food diet after tumor cell implantation. Survival curves were calculated with tumor volume > 500 mm3 set as end point with 5 × 104 cells injected for E0771 and 2 × 105 for 4T1. (Tengesdal et al., 2022).

6. **Combination Therapy Model**

4T1 or E0771 cells were implanted as described above. On day 7 after implantation, mice were started on the OLT1177 diet or continued on the standard diet. At day 10, a neutralizing antibody against PD-1 (RMP1.14) (Catalog#BP0146) (8 mg/kg in 200 μL saline; BioXCell, Lebanon, NH, USA) or IgG (8 mg/kg in 200 μL saline; BioXCell) (Catalog#BP0089) was injected. Mice were sacrificed 21 days post tumor implantation. (Tengesdal et al., 2022).

7. Cytokine Measurements

Cytokines were measured by specific DuoSet ELISAs according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). A Bio-tek EL × 800 microplate reader was used to measure optical density (Agilent, Santa Clara, CA, USA). (Tengesdal et al., 2022).

8. Gene Expression

Primary tumors were collected as described above. RNA was then isolated using Trizol (Thermo Fisher Scientific, Waltham, MA, USA) and synthesized in cDNA using SuperScript III First-Strand (Thermo Fisher Scientific). Quantitative PCR (qPCR) was performed on cDNA using Power SYBR Green PCR master mix (Thermo Fisher Scientific) on a Biorad CFX96 Real time system. Cycles were set per manufacturer's suggestion. Gene expression was assessed for the following mRNAs: Pdcd1l1 (forward 5′-GCTCCAAAGGACTTGTACGTG-3′ and reverse 5′-TGATCTGAAGGGCAGCATTTC-3′), Il1b (forward 5′-CTAAACAGATGAAGTGCTCCTTCC-3′ and reverse 5′-CACATAAGCCTCGTTATCCCA-3′), Casp1 (forward 5′-AAGTCGGCAGAGATTTATCGA-3′ and reverse 5′-GATGTCAACCTCAGCTCCAG-3′), Nlrp3 (forward 5′-GAATCTCACGCACCTTTACC-3′ and reverse 5′-GCAGTTGTCTAATTCCAACACC-3′). Relative gene expression was normalized with 18s for mice and Gapdh for human: 18s (forward 5′-GTAACCCGTTGAACCCCATT-3′ and reverse 5′-CCATCCAATCGGTAGTAGCG-3′), Gapdh (forward 5′-TGTGGGCATCAATGGATTTGG-3′ and reverse 5′-ACACCATGTATTCCGGGTCAAT-3′). (Tengesdal et al., 2022).

9. Western Blotting

Bone marrow adherent cells were cultured as previously detailed. Following culture, cells were lysed in RIPA buffer (Sigma, St. Louis, MS, USA) containing protease inhibitors (Roche, Indianapolis, IN, USA). The lysates were then centrifuged at 13,000 × g for 20 minutes at 4 °C to obtain supernatants. Protein concentration in the clarified supernatants was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were separated by electrophoresis on Mini-PROTEAN TGX 4−20% gels (Bio-Rad Laboratories) and transferred to 0.2 mm nitrocellulose membranes (GE Water & Process Technologies, Feasterville-Trevose, PA, USA). After transfer, the membranes were blocked with 5% rehydrated non-fat milk in PBS-Tween 0.5% for 1 hour at room temperature. Primary antibodies against PD-L1 (1:250) (Catalog#AF1019) (R&D Systems), NLRP3 (1:1000) (Catalog#AG-20B-0014-C100) (Adipogen, San Diego, CA, USA), and IL-1β (1:250) (Catalog#AF-401-NA) (R&D Systems) were used along with peroxidase-conjugated secondary antibodies. β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) was used as a primary antibody to assess protein loading. Chemiluminescence signals were captured using the Bio-Rad Chemidoc MP Imaging System (Bio-Rad Laboratories), and images were quantified using ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA). (Tengesdal et al., 2022).

10. Statistical Analysis

Statistical significance of differences was evaluated with a two-tailed Students' t test with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ and log-rank (Mantel–Cox and Gehan–Breslow–Wilcoxon) tests for survival analysis using Prism version 7.0 software (GraphPad Software, La Jolla, CA, USA). (Tengesdal et al., 2022).

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IX. References

Abiko, K.; Matsumura, N.; Hamanishi, J.; Horikawa, N.; Murakami, R.; Yamaguchi, K.; Yoshioka, Y.; Baba, T.; Konishi, I.; Mandai, M. (2015). IFN-gamma from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer. British Journal of Cancer, 112, 1501–1509.

Adams, S.; Schmid, P.; Rugo, H.S.; Winer, E.P.; Loirat, D.; Awada, A.; Cescon, D.W.; Iwata, H.; Campone, M.; Nanda, R.; et al. (2019). Pembrolizumab monotherapy for previously treated metastatic triple-negative breast cancer: Cohort A of the phase II KEYNOTE-086 study. Annals of Oncology, 30, 397–404.

Aggen, D.H.; Ager, C.R.; Obradovic, A.Z.; Chowdhury, N.; Ghasemzadeh, A.; Mao, W.; Chaimowitz, M.G.; Lopez-Bujanda, Z.A.; Spina, C.S.; Hawley, J.E.; et al. (2021). Blocking IL1 beta promotes tumor regression and remodeling of the myeloid compartment in a renal cell carcinoma model: Multidimensional analyses. Clinical Cancer Research, 27, 608–621.

Alard, E., Butnariu, A.-B., Grillo, M., Kirkham, C., Zinovkin, D. A., Newnham, L., Macciochi, J., & Pranjol, M. Z. (2020). Advances in anti-cancer immunotherapy: Car-T cell, checkpoint inhibitors, dendritic cell vaccines, and oncolytic viruses, and emerging cellular and molecular targets. Cancers, 12(7), 1826. https://doi.org/10.3390/cancers12071826

Al-Shura, A. N. (2014). Chapter 7 - Immune System 1: Leukocytes. In A. N. Al-Shura (Ed.), Hematology in Traditional Chinese Medicine Cardiology (pp. 29-31). Academic Press. ISBN 9780128001240. https://doi.org/10.1016/B978-0-12-800124-0.00007-5

Apte, R.N.; Dotan, S.; Elkabets, M.; White, M.R.; Reich, E.; Carmi, Y.; Song, X.; Dvozkin, T.; Krelin, Y.; Voronov, E. (2006).The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions. Cancer Metastasis Reviews, 25, 387–408.

Bae, J.Y.; Lee, S.W.; Shin, Y.H.; Lee, J.H.; Jahng, J.W.; Park, K. (2007). P2X7 receptor and NLRP3 inflammasome activation in head and neck cancer. Oncotarget, 8, 48972–48982.

Ballbach, M.; Dannert, A.; Singh, A.; Siegmund, D.M.; Handgretinger, R.; Piali, L.; Rieber, N.; Hartl, D. (2017). Expression of checkpoint molecules on myeloid-derived suppressor cells. Immunology Letters, 192, 1–6.

Bergenfelz, C.; Roxa, A.; Mehmeti, M.; Leandersson, K.; Larsson, A.M. (2020). Clinical relevance of systemic monocytic-MDSCs in patients with metastatic breast cancer. Cancer Immunology and Immunotherapy, 69, 435–448

Bi, J., & Tian, Z. (2017). NK Cell Exhaustion. Frontiers in Immunology, 8, 760. https://doi.org/10.3389/fimmu.2017.00760

Broudy, V.C.; Kaushansky, K.; Harlan, J.M.; Adamson, J.W. (1987). Interleukin 1 stimulates human endothelial cells to produce granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor. Journal of Immunology, 139, 464–468.

Cao, Y.; Slaney, C.Y.; Bidwell, B.N.; Parker, B.S.; Johnstone, C.N.; Rautela, J.; Eckhardt, B.L.; Anderson, R.L. (2014). BMP4 inhibits breast cancer metastasis by blocking myeloid-derived suppressor cell activity. Cancer Research, 74, 5091–5102.

Chen, J.; Sun, H.W.; Yang, Y.Y.; Chen, H.T.; Yu, X.J.; Wu, W.C.; Xu, Y.T.; Jin, L.L.; Wu, X.J.; Xu, J.; et al. (2021). Reprogramming immunosuppressive myeloid cells by activated T cells promotes the response to anti-PD-1 therapy in colorectal cancer. Signal Transduction and Targeted Therapy, 6, 4.

Chen, S.; Crabill, G.A.; Pritchard, T.S.; McMiller, T.L.; Wei, P.; Pardoll, D.M.; Pan, F.; Topalian, S.L. (2019). Mechanisms regulating PD-L1 expression on tumor and immune cells. Journal of Immunotherapy, 7, 305.

Dagenais, M.; Dupaul-Chicoine, J.; Douglas, T.; Champagne, C.; Morizot, A.; Saleha, M. (2017). The Interleukin (IL)-1R1 pathway is a critical negative regulator of PyMT-mediated mammary tumorigenesis and pulmonary metastasis. Oncoimmunology, 6(3), e1287247. https://doi.org/10.1080/2162402X.2017.1287247

Diaz-Montero, C.M.; Salem, M.L.; Nishimura, M.I.; Garrett-Mayer, E.; Cole, D.J.; Montero, A.J. (2009). Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunology and Immunotherapy, 58, 49–59.

Dinarello C. A. (2007). Historical insights into cytokines. European Journal of Immunology, 37 Suppl 1(Suppl 1), S34–S45. https://doi.org/10.1002/eji.200737772

Dinarello, C. A. (1996). Biologic Basis for Interleukin-1 in Disease. Blood, 87(6), 2095-2147. https://doi.org/10.1182/blood.V87.6.2095.bloodjournal8762095

Dinarello, C. A. (2015). The history of fever, leukocytic pyrogen, and interleukin-1. Temperature, 2(1), 8-16. https://doi.org/10.1080/23328940.2015.1017086

Dinarello, C. A. (2018). Overview of the IL-1 family in innate inflammation and acquired immunity. Immunological Reviews, 281(1), 8–27. https://doi.org/10.1111/imr.12621

Elkabets, M.; Ribeiro, V.S.; Dinarello, C.A.; Ostrand-Rosenberg, S.; Di Santo, J.P.; Apte, R.N.; Vosshenrich, C.A. (2010). IL-1beta regulates a novel myeloid-derived suppressor cell subset that impairs NK cell development and function. European Journal of Immunology, 40, 3347–3357.

Ershaid, N.; Sharon, Y.; Doron, H.; Raz, Y.; Shani, O.; Cohen, N.; Monteran, L.; Leider-Trejo, L.; Ben-Shmuel, A.; Yassin, M.; et al. (2019). NLRP3 inflammasome in fibroblasts links tissue damage with inflammation in breast cancer progression and metastasis. Nature Communications, 10, 4375.

Feng, X.; Luo, Q.; Zhang, H.; Wang, H.; Chen, W.; Meng, G.; Chen, F. (2017). The role of NLRP3 inflammasome in 5-fluorouracil resistance of oral squamous cell carcinoma. Journal of Experimental & Clinical Cancer Research, 36, 81.

Fong, M.; Crane, J. S. (2023). Histology, Mast Cells. In StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. Retrieved May 1, 2023, from https://www.ncbi.nlm.nih.gov/books/NBK499904/

Fusco, R.; Siracusa, R.; Genovese, T.; Cuzzocrea, S.; Di Paola, R. (2020). Focus on the Role of NLRP3 Inflammasome in Diseases. International Journal of Molecular Sciences, 21, 4223.

Geng, Zhe (2018). Anti-PD-1 therapy for clinical treatment of lymphoma: a single-arm meta-analysis. Oncotarget, 9, 35343-35355.

Gonda, K.; Shibata, M.; Ohtake, T.; Matsumoto, Y.; Tachibana, K.; Abe, N.; Ohto, H.; Sakurai, K.; Takenoshita, S. (2017). Myeloid-derived suppressor cells are increased and correlated with type 2 immune responses, malnutrition, inflammation, and poor prognosis in patients with breast cancer. Oncology Letters, 14, 1766–1774.

Hamarsheh, S; Zeiser, R. (2020). NLRP3 Inflammasome Activation in Cancer: A Double-Edged Sword. Frontiers in Immunology, 11, 1444.

Jagannathan-Bogdan, M.; Zon, L. I. (2013). Hematopoiesis. Development (Cambridge, England), 140(12), 2463–2467. https://doi.org/10.1242/dev.083147

Jang, J.H.; Kim, D.H.; Lim, J.M.; Lee, J.W.; Jeong, S.J.; Kim, K.P.; Surh, Y.J. (2020). Breast cancer cell-derived soluble CD44 promotes tumor progression by triggering macrophage IL1beta production. Cancer Research, 80, 1342–1356.

Kalluri, R.; Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. The Journal of Clinical Investigation, 119(6), 1420–1428. https://doi.org/10.1172/JCI39104

Kaplanov, I.; Carmi, Y.; Kornetsky, R.; Shemesh, A.; Shurin, G.V.; Shurin, M.R.; Dinarello, C.A.; Voronov, E.; Apte, R.N. (2019) Blocking IL-1beta reverses the immunosuppression in mouse breast cancer and synergizes with anti-PD-1 for tumor abrogation. Proceedings of the National Academy of Sciences, 116, 1361–1369.

Krumm, B.; Xiang, Y.; Deng, J. (2014). Structural Biology of the IL-1 Superfamily: Key cytokines in the regulation of immune and inflammatory responses. Protein Science: A Publication of the Protein Society, 23. https://doi.org/10.1002/pro.2441

Latz, E.; Xiao, T.S.; Stutz, A. (2013). Activation and regulation of the inflammasomes. Nature Reviews Immunology, 13, 397–411.

Law, A.M.K.; Valdes-Mora, F.; Gallego-Ortega, D. (2020). Myeloid-derived suppressor cells as a therapeutic target for cancer. Cells, 9, 561.

Lim, J.H.; Um, H.J.; Park, J.W.; Lee, I.K.; Kwon, T.K. (2009). Interleukin-1beta promotes the expression of monocyte chemoattractant protein-1 in human aorta smooth muscle cells via multiple signaling pathways. Experimental Molecular Medicine, 41, 757–764.

Marchetti, C.; Swartzwelter, B.; Gamboni, F.; Neff, C. P.; Richter, K.; Azam, T.; Dinarello, C. A. (2018a). OLT1177, a β-sulfonyl nitrile compound, safe in humans, inhibits the NLRP3 inflammasome and reverses the metabolic cost of inflammation. Proceedings of the National Academy of Sciences, 115(7), E1530-E1539. https://doi.org/10.1073/pnas.1716095115

Marchetti, C.; Swartzwelter, B.; Koenders, M.I.; Azam, T.; Tengesdal, I.W.; Powers, N.; de Graaf, D.M.; Dinarello, C.A.; Joosten, L.A.B. (2018b). NLRP3 inflammasome inhibitor

OLT1177 suppresses joint inflammation in murine models of acute arthritis. Arthritis Research & Therapy, 20, 169.

McLane, L. M.; Abdel-Hakeem, M. S.; Wherry, E. J. (2019). CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer. Annual Review of Immunology, 37, 457–495. https://doi.org/10.1146/annurev-immunol-041015-055318

Meyer, C.; Cagnon, L.; Costa-Nunes, C.M.; Baumgaertner, P.; Montandon, N.; Leyvraz, L.; Michielin, O.; Romano, E.; Speiser, D.E. (2014). Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab. Cancer Immunology and Immunotherapy, 63, 247–257.

Nanda, R.; Chow, L.Q.; Dees, E.C.; Berger, R.; Gupta, S.; Geva, R.; Pusztai, L.; Pathiraja, K.; Aktan, G.; Cheng, J.D.; et al. (2016). Pembrolizumab in patients with advanced triple-negative breast cancer: Phase Ib KEYNOTE-012 study. Journal of Clinical Oncology, 34, 2460–2467.

Okamoto, M.; Liu, W.; Luo, Y.; Tanaka, A.; Cai, X.; Norris, D.A.; Dinarello, C.A.; Fujita, M. (2010). Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta. Journal of Biological Chemistry, 285, 6477–6488.

Oh, K.; Lee, O.Y.; Shon, S.Y.; Nam, O.; Ryu, P.M.; Seo, M.W.; Lee, D.S. (2013). A mutual activation loop between breast cancer cells and myeloid-derived suppressor cells facilitates spontaneous metastasis through IL-6 trans-signaling in a murine model. Breast Cancer Research, 15, R79

Parham, P. (2021). The immune system (5th ed.). W.W. Norton & Company.

Qian, J.W.; Wang, C.; Wang, B.; Yang, J.; Wang, Y.D.; Luo, F.F.; Xu, J.Y.; Zhao, C.J.; Liu, R.H.; Chu, Y.W. (2018). The IFN-gamma/PD-L1 axis between T cells and the tumor

microenvironment: Hints for glioma anti-PD-1/PD-L1 therapy. Journal of Neuroinflammation, 15, 290.

Raza, Y.; Salman, H.; Luberto, C. (2021). Sphingolipids in Hematopoiesis: Exploring Their Role in Lineage Commitment. Cells, 10(10), 2507. https://doi.org/10.3390/cells10102507

Sceneay, J.; Parker, B.S.; Smyth, M.J.; Moller, A. (2013). Hypoxia-driven immunosuppression contributes to the pre-metastatic niche. *Oncoimmunology*, 2, e22355.

Silva, M., Martins, D., & Mendes, F. (2022). The Role of Immune Checkpoint Blockade in Acute Myeloid Leukemia. Onco, 2(3), 164–180. https://doi.org/10.3390/onco2030011

Swanson, K.V.; Deng, M.; Ting, J.P. (2019). The NLRP3 inflammasome: Molecular activation and regulation to therapeutics. Nature Reviews Immunology, 19, 477–489.

† Tengesdal, I. W.; Li, S.; Powers, N. E.; May, M.; Neff, C. P.; Joosten, L. A. B.; Marchetti, C.; Dinarello, C. A. (2022). Activation of Host-NLRP3 Inflammasome in Myeloid Cells Dictates Response to Anti-PD-1 Therapy in Metastatic Breast Cancers. Pharmaceuticals, 15(5), 574. https://doi.org/10.3390/ph15050574

Tengesdal, I. W.; Menon, D. R.; Osborne, D. G.; Neff, C. P.; Powers, N. E.; Gamboni, F.; Mauro, A. G.; D'Alessandro, A.; Stefanoni, D.; Henen, M. A.; Mills, T. S.; De Graaf, D. M.; Azam, T.; Vogeli, B.; Palmer, B. E.; Pietras, E. M.; DeGregori, J.; Tan, A. C.; Joosten, L. A. B.; Fujita, M.; Marchetti, C. (2021a). Targeting tumor-derived NLRP3 reduces melanoma progression by limiting MDSCs expansion. Proceedings of the National Academy of Sciences of the United States of America, 118(10), e2000915118. https://doi.org/10.1073/pnas.2000915118

Tengesdal, I. W.; Dinarello, A.; Powers, N. E.; Burchill, M. A.; Joosten, L. A. B.; Marchetti, C.; Dinarello, C. A. (2021b). Tumor NLRP3-Derived IL-1β Drives the IL-6/STAT3 Axis

Resulting in Sustained MDSC-Mediated Immunosuppression. Frontiers in Immunology, 12, Article 661323. (https://doi.org/10.3389/fimmu.2021.66132)

Tengesdal, I. W.; Dinarello, C. A.; Marchetti, C., (2021c). Nlrp3 and Cancer: Pathogenesis and Therapeutic Opportunities. Available at SSRN:http://dx.doi.org/10.2139/ssrn.4501298

Theivanthiran, B.; Evans, K.S.; DeVito, N.C.; Plebanek, M.; Sturdivant, M.; Wachsmuth, L.P.; Salama, A.K.; Kang, Y.; Hsu, D.; Balko, J.M.; et al. (2020). A tumor-intrinsic PD-L1/NLRP3 inflammasome signaling pathway drives resistance to anti-PD-1 immunotherapy. Journal of Clinical Investigation, 130, 2570–2586.

Thiem, A.; Hesbacher, S.; Kneitz, H.; di Primio, T.; Heppt, M.V.; Hermanns, H.M.; Goebeler, M.; Meierjohann, S.; Houben, R.; Schrama, D. (2019). IFN-gamma-induced PD-L1 expression in melanoma depends on p53 expression. Experimental and Clinical Cancer Research, 38, 397.

Urwanisch, Laura (2021). The NLRP3 Inflammasome and Its Role in the Pathogenicity of Leukemia. International Journal of Molecular Sciences, 22, 1271.

Voronov, E.; Shouval, D. S.; Krelin, Y.; Apte, R. N. (2003). IL-1 is required for tumor invasiveness and angiogenesis. Proceedings of the National Academy of Sciences, 100(5), 2645-2650. https://doi.org/10.1073/pnas.0437939100

Ward, J. M.; Cherian, S.; Linden, M. A. (2018). Hematopoietic and Lymphoid Tissues. In P. M. Treuting, S. M. Dintzis, & K. S. Montine (Eds.), Comparative Anatomy and Histology (Second Edition) (pp. 365-401). Academic Press. ISBN 9780128029008. [https://doi.org/10.1016/B978-0-12-802900-8.00019-1](https://doi.org://10.1016/B978-0-12-802900-8.00019-1)

Weber, R.; Fleming, V.; Hu, X.; Nagibin, V.; Groth, C.; Altevogt, P.; Utikal, J.; Umansky, V. (2018). Myeloid-derived suppressor cells hinder the anti-cancer activity of immune checkpoint inhibitors. Frontiers in Immunology, 9, 1310.

Wen, J.; Ye, F.; Huang, X.; Li, S.; Yang, L.; Xiao, X.; Xie, X. (2015). Prognostic significance of preoperative circulating monocyte count in patients with breast cancer: Based on a large cohort study. Medicine, 94, e2266.

Winer, E.P.; Lipatov, O.; Im, S.A.; Goncalves, A.; Munoz-Couselo, E.; Lee, K.S.; Schmid, P.; Tamura, K.; Testa, L.; Witzel, I. (2021). Pembrolizumab versus investigator-choice chemotherapy for metastatic triple-negative breast cancer (KEYNOTE-119): A randomized, open-label, phase 3 trial. The Lancet Oncology, 22, 499–511.

Wu, T.C.; Xu, K.L.; Martinek, J.; Young, R.R.; Banchereau, R.; George, J.; Turner, J.; Kim, K.I.; Zurawski, S.; Wang, X.; et al. (2018). IL1 receptor antagonist controls transcriptional signature of inflammation in patients with metastatic breast cancer. Cancer Research, 78, 5243–5258.

Wunderlich, M.; Manning, N.; Sexton, C.; O'Brien, E.; Byerly, L.; Stillwell, C.; Perentesis, JP.; Mulloy, JC.; Mizukawa, B.. (2021). PD-1 Inhibition Enhances Blinatumomab Response in a UCB/PDX Model of Relapsed Pediatric B-Cell Acute Lymphoblastic Leukemia. Frontiers in Oncology, 11, 642466.

Zhang, J. M., & An, J. (2007). Cytokines, inflammation, and pain. International Anesthesiology Clinics, 45(2), 27–37. https://doi.org/10.1097/AIA.0b013e318034194e

Zheng, Q.; Yao, D.; Cai, Y.; Zhou, T. (2020). NLRP3 augmented resistance to gemcitabine in triple-negative breast cancer cells via EMT/IL-1beta/Wnt/beta-catenin signaling pathway. Bioscience Reports, 40.

Zheng, Y.; Fang, Y.C.; Li, J. (2019). PD-L1 expression levels on tumor cells affect their immunosuppressive activity. Oncology Letters, 18, 5399–5407.

Zheng, Y.; Fang, Y.C.; Li, J. (2020). PD-L1 promotes tumor growth and progression by activating WIP and beta-catenin signaling pathways and predicts poor prognosis in lung cancer. Cell Death & Disease, 11, 506.

Zitvogel, L. & Kroemer, G. (2012). Targeting PD-1/PD-L1 Interactions for Cancer Immunotherapy. Oncoimmunology, 1, 1223-1225.