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Cooperative interplay between macrophages and cancer cells for inflammasome activation in breast tumor microenvironment

유방종양미세환경에서 대식세포와 암 세포의 상호작용에 의한 인플라마좀의 활성화

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서울대학교 대학원

약학과 의약생명과학 전공

장 정 훈

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Cooperative interplay between macrophages and cancer cells for inflammasome activation in breast tumor microenvironment

by

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ABSTRACT

Cooperative interplay between macrophages and cancer cells for inflammasome activation in breast tumor microenvironment

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Similar to heterogeneous tumor mass made up of multiple subtypes of cancer cells, stromal cells are also highly variable and heterogeneous. Heterogeneity of tumor is considered to be a major cellular feature of cancer due to the presence of not only different populations of cancer cells, but also distinct composition of stromal cells and their expression signatures within the tumor

microenvironment. Stromal cells in the tumor microenvironment include lymphatic endothelial cells, T and B lymphocytes, tumor-associated macrophages, cancer-associated fibroblast, adipocytes, etc. These cells interact with cancer cells as well as each other through complex and dynamic network of cytokines, chemokines, growth factors, and hormones. Crosstalk among the cells in the tumor microenvironment exerts fate-determining roles in many types of cancer.

The presence of inflammatory cells and pro-inflammatory mediators in the tumor microenvironment has been considered as another hallmark of cancer. The inflammatory tumor microenvironment has both tumor progressive and tumor suppressive aspects, and such interchangeable roles of the inflammatory tumor microenvironment mainly depend on the stages of cancer development and types of cancer.

Inflammasomes are multi-protein complexes, which play essential roles in maintaining body's homeostasis as the first line of host defense against infections and damaged tissues by releasing interleukin (IL)-1β and IL-18 in innate immunity. IL-1β, one of the most potent pro-inflammatory cytokines, is involved in establishment of inflammatory conditions by mediating a

systemic pro-inflammatory cascade. Overexpression/secretion of IL-1β, as a consequence of aberrant activation of inflammasomes mainly in tumor-associated macrophages, has been observed in many types of human malignancies, such as liver, lung, gastric, and colorectal cancer. However, the roles of IL-1β in the breast tumor microenvironment, especially in the context of interplay between cancer cells and tumor-associated macrophages, are still unclear.

In the present study, increased serum levels of IL-1β, IL-6, IL-18, and TNF-α were observed in breast cancer patients. Secretion of IL-1β was increased by the co-culture of human monocyte-like cells and triple negative breast cancer (TNBC) cells. Additionally, the conditioned-media from TNBC cells potently induced IL-1β secretion by macrophages. Consistent with these observations, macrophage depletion reduced the serum levels of IL-1β, and alleviated breast cancer progression in a murine orthotopic breast tumor model. Profiling the secretome of human breast cancer cells revealed that the CD44 antigen was the most differentially released protein in basal conditions of TNBC cells. Antibody neutralization of CD44 abrogated IL-1β production in macrophages, and inhibited the growth and metastasis of primary tumors.

These results suggest that IL-1β-mediated oncogenic signaling is stimulated, at least in part, by soluble CD44 (sCD44) derived from breast cancer cell membrane, and targeting sCD44 antigen may hence provide an alternative therapeutic strategy for breast cancer treatment by modulating the inflammatory tumor microenvironment.

Key words

Inflammatory tumor microenvironment; Triple negative breast cancer; Macrophages; Inflammasomes; Interleukin-1β; Soluble CD44

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List of Abbreviations

AOM	Azoxymethane
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine 5'-triphosphate
BMDM	Bone marrow derived macrophages
BMDN	Bone marrow derived neutrophils
CAC	Colitis-associated colorectal cancer
CM	Conditioned-medium
COX-2	Cyclooxygenase type 2
CRC	Colorectal cancer
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DMBA	7,12-Dimethylbenz(a)anthracene
DSS	Dextran sulfate sodium
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MMP	Matrix metalloproteinase
NLR	NOD-like receptor
PAMPs	Pathogen-associated molecular patterns

PGE2	Prostaglandin-E2
ROS	Reactive oxygen species
SFN	Sulforaphane
STAT3	Signal transducer and activator of transcription 3
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor
TPA	12-O-Tetradecanoylphobol-13-acetate
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1

Chapter I

Roles of inflammasomes in tumor microenvironment

1. Introduction

The complexity of tumor microenvironment is reflected by not only heterogeneity of cancer cells, but variable composition of their surrounding stromal cells (1). The profile of infiltrated/resident immune cells and inflammatory mediators in the proximity of cancer cells defines the inflammatory tumor microenvironment (2). The inflammatory microenvironment is now recognized as an important participant of all stages of tumor development, from an early stage of carcinogenesis to tumor progression and metastatic spread to distant organs (1-4).

The inflammasomes are multi-protein complexes, composed of an NOD-like receptor (NLR), apoptosis-associated speck-like protein containing a CARD domain (ASC), and pro-caspase-1, which are formed upon encounter of pathogen- or damage-associated stimuli (5) (**Fig. 1**). Activation of the inflammasomes is responsible for the cleavage of pro-interleukin (IL)-1β and pro-IL-18 by caspase-1 into their active form. This triggers both pro-inflammatory and host-protective responses by promoting the infiltration of inflammatory and immunocompetent cells into inflamed site in innate immunity (5) (**Fig. 1**). Due to the critical roles of host immune defense against

inflammatory damage, the inflammasomes are well-recognized as important protein complexes indispensable for maintaining homeostasis of the body.

Although the inflammasomes execute beneficial roles in innate immune defense, aberrant activation of inflammasomes and concurrent overexpression/overproduction of their components have also been observed in many different types of human malignancies (6). In tumor microenvironment, the inflammasomes are expressed in various types of cells, such as T and B lymphocytes, macrophages, and dendritic cells (DCs) as well as cancer cells. The effects of inflammasomes on cancer development appear to vary, depending on cell types and surrounding context in the inflammatory environment (Table 1) (6,7).

2. Fundamental roles of inflammasomes in innate immunity

The innate immune system is engaged by recognition of infection, injuries, and altered cellular homeostasis to initiate clearance of pathogens and to repair damaged tissues. One of the key complexes for the operation of innate immune responses is inflammasomes. The inflammasomes are multi-protein complexes activated and oligomerized upon sensing pathogen-

associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Activation of inflammasomes triggers an alarm to alert adjacent cells and tissues by releasing pro-inflammatory cytokines. This ends up with amplification of the inflammatory cascade. Inflammasome activation triggers maturation of two representative pro-inflammatory cytokines, IL-1β and IL-18, to engage innate immune defense. Release of active forms of IL-1β and IL-18 promotes recruitment of effector cell populations required for the immune responses and tissue repair.

Although acute inflammation is a physiologic response to infections or tissue damage, it must be resolved properly. Unresolved inflammation can lead to a chronic inflammatory state, which is implicated in the pathogenesis of a wide variety of human disorders including metabolic syndromes and cancer. Notably, inflammatory cells involved in the early phase of inflammation can undergo a functional repolarization to get involved in the onset and establishment of resolution. It has now become evident that coordinated resolution programs start shortly after inflammatory responses begin (8). The fine-tuning of inflammation would create a favorable environment which allows an effective transition from the pro-inflammatory

phase to the onset of pro-resolving response (9). In this context, it is noticeable that some pro-inflammatory molecules involved in the acute phase of inflammation can simultaneously initiate a program for active resolution. For instance, the prototypic pro-inflammatory cytokine IL-1β has been shown to trigger an anti-inflammatory cascade, resulting in the production of IL-10 (9).

Considering differential effects of IL-1β on acute inflammation and its resolution, it can be speculated that the inflammasomes that secrete a mature form of this cytokine may mediate both pro-inflammatory and pro-resolving responses. While activation of the inflammasomes initially mediates pro-inflammatory response, it culminates in the resolution of inflammation and thereby contributes to homeostatic processes (7). This may account for a dynamic macrophage polarity gradient from pro-inflammatory to anti-inflammatory phenotypes (10).

Inflammasomes tightly regulate the expression, maturation, and secretion of IL-1β as a master regulator. IL-1β, which is mainly generated at the injury or infection sites, induces inflammation-associated gene expression, such as cyclooxygenase type 2 (COX-2) and inducible nitric oxide synthase (iNOS) to produce prostaglandin E_2 (PGE_2) and nitric oxide (NO),

respectively (5). Moreover, IL-1β increases the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) in mesenchymal cells and vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells. This results in the recruitment of inflammatory cells and immunocompetent cells from circulation to damaged or infection sites in cooperation with other chemokines (5). Another pro-inflammatory cytokine controlled by the inflammasomes is IL-18 which is important for induction of IL-17 expression in Th17 cells, and involved in polarization of T cells toward Th1 or Th2 cells in combination with other cytokines (5). Unlike many other cytokines, IL-1β and IL-18 are not secreted through the classical endoplasmic reticulum (ER)-Golgi pathway, but recent studies have revealed that the Gasdermin D is associated with IL-1β secretion (11-13).

Due to its potential roles in innate immunity, inappropriate regulation of inflammasomes has a strong association with a variety of inflammatory and auto-immune disorders, including neurodegenerative diseases (multiple sclerosis, Alzheimer's disease, and Parkinson's disease) and metabolic disorders (atherosclerosis, type 2 diabetes, and obesity) as well as various human malignancies (14-17).

3. Involvement of inflammasomes in cancer

3-1. Colorectal Cancer

Colorectal cancer (CRC) is the third most common malignancies worldwide with the highest frequencies in western countries. CRC is frequently associated with inflammatory bowel disease (IBD) which is an inflammatory disorder in the intestinal tract. Crohn's disease and ulcerative colitis are two major types of IBD, both characterized by overactivated immune response in the intestinal tract. Patients with IBD are at high risk for colitis-associated colorectal cancer (CAC) (18-20). Numerous studies have been conducted to define the roles of inflammasome components in CAC, but results are controversial.

Accumulating evidence points to critical roles of the inflammasomes and pro-inflammatory cytokines, IL-1β and IL-18, in dextran sulfate sodium (DSS)-induced colitis and azoxymethane (AOM) plus DSS-induced CAC. In this mouse model, AOM is used as a powerful carcinogenic chemical compound causing DNA damages in intestinal epithelial cells. Repeated administration of DSS in drinking water induces damages to the intestinal epithelial barrier, resulting in the induction of inflammation and development

of CRC. In an AOM plus DSS-induced CAC mouse model, mice deficient for inflammasome components are highly susceptible to development of CAC as evidenced by increased morbidity, histopathology, and colonic polyp formation (21-25). In CAC microenvironment induced by AOM and DSS, the inflammasomes in hematopoietic-derived cells exerted more potent effects on regulation of intestinal tumorigenesis than inflammasomes in other types of cells in intestine, such as epithelial cells, tumor-derived cells or stromal cells (22,24). Allen *et al*. reported protective effects of *Pycard*, *Nlrp3*, and *Casp1* on acute and recurring colitis induced by DSS, and found that Pycard and caspase-1 were essential for survival in AOM plus DSS-induced CAC(22). In addition, significantly increased colonic lesions and an increased number of colonic macroscopic polyps were observed in WT mice receiving bone marrow from *Nlrp3*$^{-/-}$ mice when compared with those in mice receiving WT bone marrow (22). These clinical data using chimeric animals by adoptive bone marrow transplantation suggest that NLRP3 in hematopoietic-derived cells plays a tumor suppressive role in CAC microenvironment (22). In case of NLRP6, its expression in colon epithelial cells was relatively higher than that in lamina propria, granulocytes, and monocyte lineage cells, NLRP6 in

colon epithelial and stromal cells showed no tumor suppressive effect on the development of CAC (24). However, *Nlrp6*-deficient hematopoietic-derived cells were more susceptible to inflammation-induced tumorigenesis, resulting from impairment of resolution of inflammation and repairing damaged epithelium. These finding suggest predominant tumor protective effects of NLRP6 in hematopoietic-derived cells against colitis-induced tumorigenesis (24).

NLRC4 in bone marrow failed to exert an inhibitory effect on CAC development, but NLRC4 in epithelial and stromal cells played protective roles in CAC development (23,25). In line with the tumor suppressive function of NLRC4, the expression of NLRC4 was found to be involved in p53-dependent cancer cell death(26). Therefore, NLRC4 in bone marrow and cancer cells is likely to be important for limiting tumorigenesis (23,25).

On the other hand, oncogenic roles of IL-1β, a final product of inflammasome activation, in the CAC microenvironment have been reported. The expression and secretion of IL-1β, a potent pro-inflammatory cytokine, are critical for the development of CAC (27,28). In the CAC pathogenesis, the infiltrated neutrophils are considered mainly responsible for producing IL-1β

(27-29). IL-1β derived from infiltrated neutrophils induced secretion of IL-6 from lamina propria, resident dendritic cells, and macrophages in the CAC milieus (27). In addition, IL-1β was required for secretion of IL-17A from intestine-resident myeloid cells in an autocrine or a paracrine manner (28). Secretion of IL-6 and IL-17A by intestinal myeloid cells (predominantly dendritic cells and macrophages) enhanced proliferation and decreased apoptosis of pre-neoplastic intestinal epithelial cells via the signal transducer and activator of transcription (STAT) 3 and the nuclear factor-κB (NF-κB)-dependent signaling pathways in the CAC microenvironment (28,30,31). Overproduction of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-17A, is closely associated with the development of CAC (27,28,30-33).

Several studies have been conducted to determine the effects of NLRP3 inflammasome deficiency on experimental colitis, but the results are discordant, especially in the context of the role of IL-1β on intestinal inflammation and carcinogenesis (34).

IL-1β and some other members of the IL-1 family cytokines (e.g., IL-18 and IL-36γ) display a dual role in regulating inflammatory bowel diseases,

reinforcing the concept that pro-inflammatory cytokines may contribute to both pro-inflammatory responses and resolution of inflammation (9).

3-2. Breast cancer

Patients with breast cancer often exhibit up-regulated expression of pro-inflammatory cytokines including IL-1β, and show a gradually increased pattern of serum IL-1β depending on a stages of cancer (35,36). This increased expression of IL-1β is more likely to be associated with progression and metastasis of breast cancer. Recent studies have demonstrated that the inflammasomes and its effector molecule, IL-1β, play critical roles in breast cancer progression and metastasis to distant organs, such as lung and bone (37,38). In an orthotopic breast cancer mouse model, $NLRP3^{-/-}$ and $CASP1^{-/-}$ inflammasome components deficient mice showed a significantly reduced growth of primary tumor and the incidence of lung metastasis (37,38). Similarly, blockade of IL-1R signaling by use of an IL-1R antagonist and IL-1R deficient mice lowered growth of primary tumor and metastasis to lung and bone in a breast cancer mouse model (37,38).

Inflammation in the tumor microenvironment is one of the well-known hallmarks of cancer, and breast cancer is no exception to this feature (39). Although breast cancer shares inflammatory characteristics with other cancers, this malignancy is rarely associated with inflammation-induced cancer (extrinsic pathway), but rather related to cancer-associated inflammation (intrinsic pathway). Breast cancer is originated from genetic alterations within malignant or pre-malignant cells. Therefore, development of inflammatory conditions and activation of inflammasomes largely rely on interaction among the components of breast tumor microenvironment, which determines the fate of breast cancer. Accumulating evidence suggests that DCs and myeloid cells are mainly responsible for releasing IL-1β in the breast tumor microenvironment (35,40-45). According to Wu *et al*., interaction between breast cancer cells and $CD11c^+$ DCs induced caspase-1-dependent IL-1β secretion from $CD11c^+$ DCs which was stimulated by breast cancer cell membrane-derived TGFB1 (35). In addition, ATP derived from dying tumor cells formed by chemotherapy induced NLRP3 inflammasome activation and IL-1β secretion from DCs through P2X7 purinergic receptor in breast tumor microenvironment (40). IL-1β release by DCs induced differentiation of IL-

13-producing $CD4^+$ T cells and IFN-γ-producing $CD8^+$ T cells in breast cancer bearing humanized mice (35,40). $CD4^+$ T effector cells that express high levels of IL-13 promoted development of mammary carcinomas and their metastasis to the lung by enhancing pro-tumorigenic potential of tumor-associated macrophages (46,47). IFN-γ derived from $CD8^+$ T cells, when primed by IL-1β, reduced the chemotherapeutic efficacy (40). In breast cancer treatment, patients with estrogen receptor α-positive breast cancer showed positive response to Tamoxifen, but IL-1β reduced its chemotherapeutic efficacy by reducing estrogen receptor α receptor expression (48).

In addition to DCs, other types of myeloid and lymphoid cells have also potential to release IL-1β in breast tumor microenvironment (41-45). According to my study described in the following chapter, macrophages were also found to be one of the major cellular sources for IL-1β secretion. Notably, depletion of macrophages alleviated breast cancer progression in a syngeneic orthotopic breast cancer mouse model. The secretion of IL-1β from macrophages was triggered by breast cancer cell membrane-derived soluble CD44 (sCD44), and inhibition of sCD44 function reduced secretion of IL-1β from macrophages. Consistently, breast cancer cell released soluble factors

induced activation of NLRP3 inflammasome in myeloid cells. Further, obesity-induced NLRC4 inflammasome activation in $F4/80^{+}/CD11b^{+}$ macrophages facilitated tumor growth and angiogenesis through up-regulation of adipocyte-mediated *Vegfa* expression in breast tumor microenvironment (41,45). Moreover, Wellenstein *et al*. suggested WNT ligands-induced IL-1β secretion (49). Deletion mutation of *TP53* in breast cancer cells induced secretion of WNT ligands. This, in turn, promoted IL-1β secretion from tumor-associated macrophage, leading to systemic inflammation for breast cancer metastasis (49).

Although tumor progressive roles of inflammasomes and IL-1β have been reported, IL-1β also shows a tumor suppressive function in breast tumor microenvironment (50,51). At the metastatic site, the existence of metastasis-initiating cancer cells causes metastasis, recurrence, and therapeutic resistance of cancer cells. According to Castano *et al*., sustained IL-1β expression and IL-1R signaling in metastasis-initiating cells prevented colonization and secondary tumor formation in metastatic microenvironment (50). This adverse effect of IL-1β is consistent with a recent study showing that IL-1R blockade,

in combination with paclitaxel, slightly reduced primary breast tumor growth, but potently induced pulmonary metastasis (51).

3-3. Gastric cancer

Gram-negative bacterium *Helicobacter pylori* (*H. pylori*) infection is the most prevalent chronic bacterial infection, affecting ~50% of the world's population, and around 80% of patients with gastric cancer are *H. pylori* infection positive (52,53). Chronic inflammation by this bacterium is considered one of the main causes of gastric cancer (52). *H. pylori*-induced inflammasome activation has been extensively associated with gastric carcinogenesis. The inflammatory response toward *H. pylori* is characterized by recruitment of immune cells, mainly dendritic cells, macrophages, neutrophils, and B and T lymphocytes to the infection site (52). *H. pylori* induces NLRP3 inflammasome activation and IL-1β secretion from innate immune cells, such as bone marrow-derived dendritic cells (BMDCs), bone marrow-derived macrophages (BMDMs), and bone marrow-derived neutrophils (52,54,55).

In patients with gastric cancer, IL-1β is notably correlated with clinical and pathological features of gastric cancer, and levels of IL-1β were significantly increased as the tumor size enlarged (52). Elevated levels of inflammasome-regulated cytokines were associated with polymorphisms in the IL-1 and IL-18 genes, predisposing to the development of gastric cancer (56). Stomach-specific expression of IL-1β in a transgenic mouse model resulted in spontaneous inflammation, dysplasia, and gastric cancer (56). The gastric carcinogenesis correlated with early recruitment of myeloid-derived suppressor cells to the stomach which was attributable to overexpression of IL-1β (56). IL-1β secretion induced by *H. pylori* was shown to enhance gastric carcinogenesis (57-59), and IL-18 facilitated immune escape of gastric cancer cells by suppressing CD70, and increasing metastatic potential through upregulation of CD44, VEGF, AP-1/c-fos, MMP2, and MMP9 (57,60). IL-1β and IL-18 are largely involved in gastric carcinogenesis through inhibition of gastric acid secretion, induction of epigenetic changes, promotion of angiogenesis, mobilization of bone marrow cells, and induction of adhesion factor expression (52-56,60).

3-4. Skin cancer

The most important environmental risk factor for skin cancer is ultraviolet (UV) radiation which is able to induce photocarcinogenesis by causing DNA damage, immunosuppression, and inflammation (61-63). The involvement of inflammation in melanoma, the most aggressive form of skin cancer, is characterized by upregulation of several pro-inflammatory cytokines including IL-6, IL-8, CCL5, and IL-1β, all of which can be regulated by IL-1β (5,63,64). UV-radiation is a powerful inducer of IL-1β in skin cancer (63,65), and melanoma-derived IL-1β has been shown to promote tumor growth, angiogenesis, invasion, and metastasis (2,62,66-73).

The roles of inflammasomes and IL-1β in skin cancer may depend on the type of cells. Several studies using a two-stages mouse skin carcinogenesis model revealed that the inflammasomes and IL-1 have dual functions in the development of inflammation-induced skin cancer. According to the study by Drexler *et al*., *Il-1r1*$^{-/-}$ and *Caspase-1*$^{-/-}$ mice treated with 7,12-dimethylbenz(a)anthracene (DMBA) as an initiator and 12-o-tetradecanoylphobol-13-acetate (TPA) as a promoter showed a reduced number of skin tumors and a tumor incidence compared to WT mice,

suggesting the tumor supportive role of IL-1β-IL-1 receptor axis in two-stage skin carcinogenesis. On the other hand, *ASC*$^{-/-}$ mice showed no significant functional difference in tumor burden and incidence compared to WT mice in response to DMBA and TPA treatment. Interestingly, ASC, showed completely different behavior in either keratinocytes or myeloid cells of ASC tissue-specific conditional knockout mice. *ASC*-deficient myeloid cells developed fewer tumors than WT mice, but the tumor number and the incidence rapidly exceeded those in *ASC*-deficient keratinocytes, although the onset of tumor formation was delayed for a few weeks when compared to WT control (74). The results from ASC tissue-specific conditional knockout mice imply cell-type dependent dual functions of inflammasome components in a skin carcinogenesis model. Consistent with these pleiotropic results, ASC expressed in primary melanoma exerted an inhibitory effect on tumorigenesis by suppressing IKKα/β phosphorylation and NF-κB transcriptional activity. On the other hand, relatively up-regulated expression of ASC in metastatic melanoma increased NF-κB activity and inflammasome-mediated IL-1β secretion (75). In mice, keratinocytes do not express NLRP3, and thus myeloid cells have major responsibility for NLRP3 inflammasome-mediated

development of skin cancer (43,76). In addition, knock down of NLRP1 in melanoma cells attenuated their tumor-promoting properties through regulation of inflammasomes and the apoptotic pathway in both *in vitro* and *in vivo* (77).

3-5. Other malignancies

In addition to the cancers discussed above, inflammasome activation and IL-1β secretion play also essential roles in other types of cancer. Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver. Hepatitis C virus (HCV) infection leads to development of chronic liver disorders in ~70% of infected patients, some of which may develop HCC (78). Kupffer cells (resident macrophages in liver) and HCC cells are known as major sources of IL-1β secretion in HCV-mediated progression of HCC (79-81). HCV also induced assembly of the NLRP3 inflammasome complex in human HCC cells (81), and HCV-induced NLRP3 inflammasome activation in kupffer cells caused high serum levels of IL-1β in HCV-infected patients (79,80). In HCC microenvironment, secretion of IL-1β from macrophages was increased by necrotic debris of HCC cells formed

under hypoxic conditions. The IL-1β secretion enhanced epithelial-mesenchymal transition and metastasis of HCC cells through stabilization of HIF-1α (82). Unlike tumor progressive effects of IL-1β in HCC microenvironment, the expression of NLRP3 inflammasome components showed negative correlation with pathological grades and clinical stages in the HCC patient tissues (83). Compared with peritumoral non-cancerous liver tissues, NLRP3 inflammasome components were down-regulated in hepatic parenchymal cells in human HCC, which is associated with more advanced clinical stages. This negative correlation between the expression of NLRP3 inflammasome components and clinical stages of HCC may arise from lacking of fundamental roles of inflammasomes. During development of HCC, massive loss of hepatocytes is accompanied by chronic hepatic inflammation. Dying hepatocytes release danger signals, and the accumulation of danger signals causes liver damage, which further increases the risk of liver cancer (83). NLRP3 inflammasome acts essential roles in sensing danger signals. Therefore, loss of NLRP3 inflammasome components during development of HCC leads to failure of sensing danger signals, which results in promoting liver cancer development (83-85).

Secretion of IL-1β and IL-18 increased in peripheral blood leukocytes from patients with non-small-cell lung cancer and small-cell lung cancer compared to healthy donor, but impairment of IL-1β secretion was also observed in alveolar macrophages from lung cancer patients (86). Depletion of tumor-associated macrophages decreased secretion of IL-1α and IL-1β, resulting in reduced lung tumorigenesis in a murine pulmonary cancer model (87). IL-1β derived from neutrophils impaired the therapeutic efficacy of NF-κB inhibitors for lung cancer treatment (88).

4. Cancer preventive/therapeutic strategies targeting inflammasomes

Due to the oncogenic potential of inflammasomes and IL-1β involving in the whole range of tumorigenesis steps, regulation of inflammasomes can be a practical strategy for prevention or treatment of inflammation-associated cancers. For several decades, natural and synthetic compounds have been investigated with regards to their capability to regulate inflammasomes (Table 2).

Curcumin [1,7-*bis*(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] isolated from the rhizomers of *Curcuma longa* is a lipid soluble

polyphenol. Curcumin has diverse pharmacological effects, such as anti-oxidant, anti-inflammatory (89), anti-microbial (90,91), and anti-cancer (92-94) effects. Curcumin markedly alleviated DSS-induced colitis in mouse, and suppressed LPS or LPS plus DSS-induced NLRP3 inflammasome activation and concurrently, IL-1β secretion in BMDM and PM by preventing potassium efflux, intracellular generation of reactive oxygen species (ROS), and cathepsin B release (35,95). Similarly, curcumin significantly reduced the expression of NLRP3, caspase-1 cleavage, and IL-1β secretion through inhibition of TLR4/MyD88/NF-κB/P_2X_7R signaling in PMA-primed THP-1 cells (96).

Resveratrol (3,5,4-trihydroxystilbene) is present in the peel of grapes, blueberries, peanut, and red wine (97). Resveratrol has anti-oxidant, anti-inflammatory, anti-cancer, and anti-diabetic effects (98). Resveratrol showed specific inhibition of NLRP3 inflammasome by disrupting assembly and function of ASC and NLRP3 in the mitochondria and ER, respectively in response to a TLR1/2 agonist and an inflammasome activator, such as nigericin, ATP, MSU, and silica in BMDMs (99). Interestingly, resveratrol did not exert inhibitory effects on flagellin- or dsDNA-induced NLRC4 and AIM2

inflammasome activation (99). Administration of resveratrol alleviated high-fat diet-induced hepatic inflammation with reduced serum levels of pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) in the mouse liver (100). This improvement was accompanied by alteration in NLRP3 inflammasomes (100). In addition, *cis*-resveratrol inhibited IL-1β secretion from THP-1 cells induced by LPS plus ATP through suppression of P_2X_7R (purinergic receptor), glucose-regulated protein 78 (ER stress marker), and ROS production (101).

Sulforaphane (SFN) is a natural compound derived from cruciferous vegetables, such as broccoli, cabbage, Brussel sprouts, mustard, and radish. It has been known that SFN has chemopreventive and therapeutic properties against lung, breast, colorectal, pancreatic, and prostate cancer (102). In addition, SFN has been known as an inducer of Nrf2-mediated antioxidant gene expression and an inhibitor of the NF-κB signaling pathway (103). SFN has been considered for its potential use in the management of Alzheimer's disease (AD) treatment due to its capability to penetrate the blood-brain barrier. According to An *et al*., SFN inhibited caspase-1-dependent inflammasome activation induced by $A\beta_{1-42}$ through inhibition of STAT-1 phosphorylation and activation of the Nrf2/heme oxygenase (HO)-1 signaling cascade (104).

In another study, SFN significantly reduced MSU crystal-induced IL-1β secretion in a murine peritonitis model and inhibited activation of NLRP1b, NLRP3, NAIP5/NLRC4, and AIM2 inflammasomes in BMDMs (105). The inhibitory effects of inflammasomes were not associated with ROS regulation (105). Moreover, inhibition of NLRP3 and NLRC4 inflammasomes by SFN was not reversed in *Nrf2*$^{-/-}$ BMDMs (105).

As discussed earlier, failure of inflammation resolution is directly linked to tumor development in many types of cancers, such as CRC, gastric cancer, and HCC. Therefore, timely alleviation of inflammation through inflammasome regulation can be suggested as a promising strategy for chemoprevention using natural compounds. The majority of previous studies on inhibition of inflammasome activation/assembly by phytochemicals have been conducted in cell culture or in short-term animal inflammatory models, further investigations will be necessary to assess their chemopreventive/cancer therapeutic effects through regulation of inflammasomes.

Biopharmaceutical drugs (e.g., anakinra and canakinumab) targeting IL-1β are now used to treat several inflammatory or auto-immune diseases, such

as rheumatoid arthritis (Table 3) (106,107). IL-1β significantly contributes to the establishment of systemic inflammation in tumor microenvironment and tumor progression (108). Based on these findings, several trials have been attempted using an IL-1R antagonist. According to Holen *et al*., an IL-1R1 antagonist (anakinra) significantly reduced the growth of primary tumor and its bone metastasis in a mouse model (38). Although anakinra did not induce apoptosis of tumor cells, cancer cell proliferation and angiogenesis were inhibited by anakinra (38). In agreement with these results, Wu *et al*. reported inhibitory effects of an IL-1R1 antagonist on breast cancer progression (109). Notably, anakinra treatment prevented breast cancer progression with substantial decrease in the proportion of IL-13 producing tumor-infiltrating $CD4^+$ T cells and increase of IFN-γ producing $CD4^+$ T cells in a humanized mouse model (109). In other studies, administration of anakinra suppressed tumor growth and lymph node metastasis in the LNM35 xenograft mice (110). In addition, rhIL-1R antagonist plus one of the standard chemotherapy regimens, gemcitabine, significantly reduced tumor burden when compared to the gemcitabine treatment alone in a pancreatic ductal adenocarcinoma orthotopic xenograft mouse model (111). In the Palucka pilot clinical trial with

eleven HER2-negative metastatic breast cancer patients, 100 mg/daily of anakinra was subcutaneously administered for a two-week run-in treatment period. This was followed by continuous daily anakinra administration along with one of the standard chemotherapy regimens for HER2-negative breast cancer patients for a median duration of 4 months (109). In this pre-clinical study, 2 of 11 patients had considerably reduced tumor size, 4 had stable disease, 2 stopped anakinra administration due to injection site reactions and the other 3 had progressive disease. Some patients showed reduced pain and increased quality of life on anakinra plus chemotherapy (109). Anakinra also reduced chemotherapy-associated 'sickness syndromes'.

Canakinumab was approved for use as an anti-IL-1β neutralizing mAb by US FDA in 2009. In a randomized, double-blind, placebo-controlled trial with canakinumab with 10,061 atherosclerosis patients, canakinumab showed a significantly reduced incidence and a mortality of lung cancer compared to the placebo group (112).

5. Conclusion and perspective

In this review, fate-determining roles of inflammasomes and their downstream effector cytokines, IL-1β and IL-18 in tumor microenvironment were discussed. The inflammasomes affect the whole range of tumorigenesis steps, and the consequence of aberrantly activated inflammasomes on cancer development and progression varies depending on the composition and profile of tumor microenvironment. The pleiotropic roles of inflammasomes in tumor microenvironment may depend on types of inflammasomes, types of cancers, types of stromal cells in tumor microenvironment, and stages of cancers.

In inflammation-induced cancer, deficient or impaired activation of inflammasomes and expression/processing of their components may fail to mediate inflammation and induce inflammation resolution in an early stage of chemically induced carcinogenesis [22]. Failure of systemic inflammatory responses, such as inflammation initiation, amplification, and resolution, leads to severe tissue damages, accumulation of PAMPs or DAPMs, and delayed tissue repair (83). The roles of inflammasomes, especially in hematopoietic lineage in stimulating tumor progression have been demonstrated in CRC and breast cancer (22,24,35,40). However, during metastasis, high levels of IL-1β released by activated inflammasomes inhibited colonization of metastasis-

initiating cells (50). Therefore, the inflammasomes should be timely regulated due to their differential roles depending on the stage of cancer progression.

There have been many efforts to repress the aberrant activation of inflammasomes. Although some natural compounds have been shown to inhibit overactivation of inflammasomes or overexpression of inflammasome components, most studies aimed to test their ability to ameliorate inflammatory disorders, not to prevent/treat cancer. Therefore, it will be worthwhile determining whether chemopreventive or chemotherapeutic effects of natural compounds are mediated through regulation of inflammasomes. In order to target cancer, antibody neutralization of IL-1β and use of an IL-1R antagonist showed significant anti-cancer effects in pre-clinical studies using animal models and its safety was confirmed. However, these regimens are still not authorized to use for the treatment of cancer patients. Further studies will be necessary to achieve better clinical results qualified for use in cancer patients.

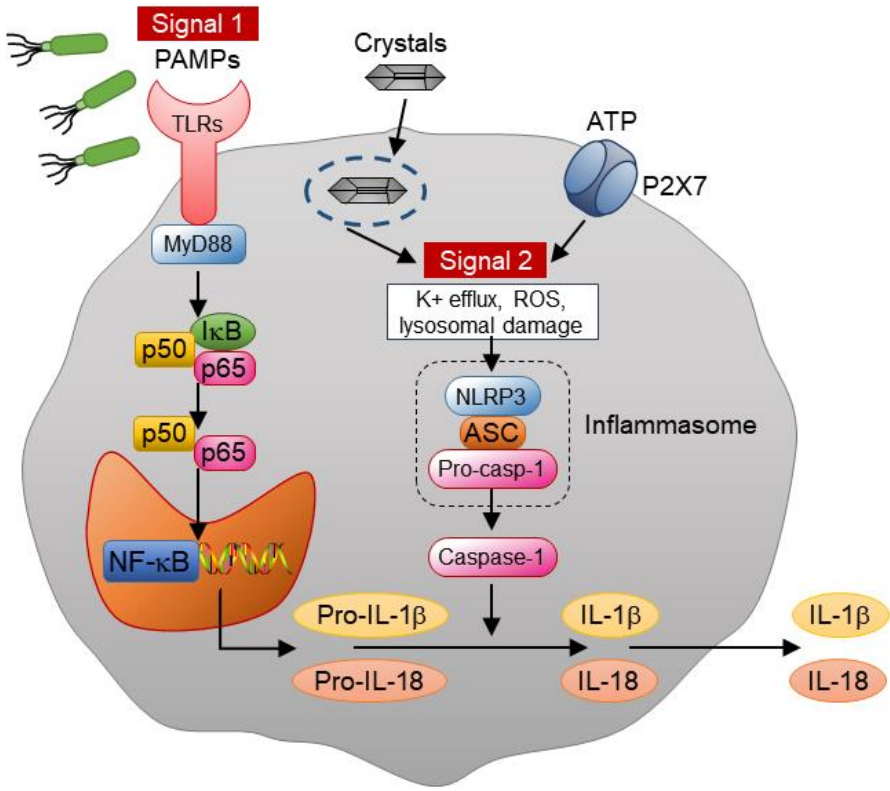

Figure 1. Inflammasome activation pathway.

Table 1. Effects of cell type-dependent expression of inflammasome components on cancer progression.

Cancer	Inflammasome components	Types of cell	Outcome	Ref.
Colorectal cancer	*Nlrp3*$^{-/-}$	Hematopoietic-derived cells	Inhibition of tumor progression	(22)
	Nlrp6$^{-/-}$	Hematopoietic-derived cells	Inhibition of tumor progression	(24)
	Nlrc4$^{-/-}$	Hematopoietic-derived cells	No effect on CAC	(23,25)
	Nlrc4$^{-/-}$	Epithelial & stromal cells	Increased tumor burden	(23,25)
	IL-1β	Neutrophils	Induction of IL-6 release from lamina propria, DCs, macrophages	(27)
Breast cancer	IL-1β	CD11c^{+}DCs	Induction of breast cancer progression	(35)
	NLRP3 activation IL-1β	DCs	Induction of IL-13-producing CD4^{+}T cell and IFN-γ-producing CD8^{+}T cell differentiation	(40)
	Obesity-induced NLRC4 activation	F4/80^{+}/CD11b^{+} macrophages	Induction of tumor growth and angiogenesis by increased expression of *Vegfa*	(41,45)

	IL-1β	Metastasis-initiating cells	Prevention of colonization and secondary tumor formation in metastatic microenvironment	(50)
Gastric cancer	IL-1β	Stomach specific expression	Induction of stomach inflammation and tumorigenesis	(2)
Skin cancer	IL-1β	Melanoma	Promotion of tumor growth, angiogenesis, invasion, and metastasis	(2,62,66-72).
	Asc$^{-/-}$	Myeloid cells	Decreased tumor burden	(74)
	Asc$^{-/-}$	Keratinocytes	Increased tumor burden and incidence	(74)
Hepatocellular carcinoma	IL-1β	Macrophages	Induction of EMT and metastasis	(82)
Lung cancer	IL-1β	Macrophages	Induction of lung tumorigenesis	(87)
	IL-1β	Neutrophils	Impairment of NF-κB inhibitor efficacy for lung cancer treatment	(88)

Table 2. Regulation of inflammasomes by natural compounds.

Natural compound	Mechanism	Ref.
Curcumin	Reduced expression of NLRP3, inhibition of caspase-1 cleavage, and secretion of IL-1β	(96)
Curcumin	Inhibition IL-1β secretion in BMDM by preventing K+ efflux, ROS generation, and cathepsin B release	(35,95)
Resveratrol	Inhibition of NLRP3 inflammasome via preventing assembly of ASC and NLRP3 on the mitochondria and ER	(99)
cis-resveratrol	Inhibition of IL-1β secretion by suppression of P2X7R and ROS production	(101)
Sulforaphane	Inhibition of Aβ1-42-induced caspase-1 dependent inflammasome activation via inhibition of STAT-1 phosphorylation and activation of the Nrf2/HO-1 signaling cascade	(104)
Sulforaphane	Reduced MSU crystal-induced IL-1β secretion in peritonitis model, and inhibited NLRP1b, NLRP3, NAIP5/NLRC4 and AIM2 inflammasomes in BMDMs	(105)

Table 3. Pharmacological drugs targeting IL-1.

Pharmaceutical drugs (Commercial)	Mechanism of action	FDA approval	Administration
Anakinra (Kineret)	IL-1R1 antagonist	US FDA approved in 2001	SC
Canakinumab (Ilaris)	Anti-IL-1β neutralizing monoclonal antibody	US FDA approved in 2009	IV, SC
Rilonacept (Arcalyst)	IL-1 trap (soluble receptor)	US FDA approved in 2009	SC

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PURPOSE OF THE STUDY

Inflammatory context is the most common feature of tumor microenvironment. Although each of cancers shows different prognosis by inflammatory condition in its microenvironment, it has become clear that the inflammation exerts fate-determining roles in tumor microenvironment. The inflammatory context in tumor microenvironment is able to be categorized into two different mechanisms, intrinsic (cancer-associated inflammation) and extrinsic (inflammation-induced carcinogenesis) pathway. Many human malignancies are linked to inflammation-induced carcinogenesis, such as colitis-induced colorectal cancer, hepatitis B/C virus-mediated hepatocellular carcinoma, and *Helicobacter pylori*-induced gastric cancer, and failure of inflammation resolution is major cause of cancer. Inflammatory condition in breast tumor microenvironment is established through cancer-associated inflammation (intrinsic pathway). Compared to inflammation-induced carcinogenesis, it has not been well-defined the underlying mechanism for cancer-associated inflammation in breast tumor microenvironment.

Inflammatory response is initiated by sensing pathogen-associated molecular patterns (PAMPs) and danger (damage)-associated molecular patterns (DAMPs) which signals are detected by inflammasomes in immune cells. Inflammasomes, multi-protein complexes, engage systemic inflammation by releasing IL-1β. IL-1β is potent pro-inflammatory cytokine, aberrant expression of IL-1β has been reported in many types of human malignancies. Hence, lots of studies have been done to regulate inflammatory tumor microenvironment by modulation of inflammasomes and IL-1β, but the mechanisms of inflammasome activation and IL-1β induction have not been elucidated in breast tumor microenvironment. In this study, therefore, I attempted to elucidate the roles of IL-1β on breast cancer progression and underlying mechanism how IL-1β is released in breast tumor microenvironment.

Chapter II

Inflammasome activation by soluble CD44 released by breast cancer cells: Implications for breast tumor progression

1. Abstract

Interleukin (IL)-1β is a central regulator of systemic inflammatory response in breast cancer, but the precise regulatory mechanisms that dictate the overproduction of IL-1β are largely unsolved. Here we show that IL-1β secretion is increased by the co-culture of human monocyte-like cells and triple negative breast cancer (TNBC) cells. In addition, macrophages robustly produced IL-1β when exposed to the conditioned-media of TNBC cells. Consistent with these observations, macrophage depletion decreased serum IL-1β, and reduced breast cancer progression in an orthotopic breast cancer mouse model. Profiling the secretome of human breast cancer cells revealed that the CD44 antigen was the most differentially released protein in basal conditions of TNBC cells. Antibody-mediated neutralization of CD44 abrogated IL-1β production in macrophages, and inhibited the growth of primary tumors. These results suggest IL-1β-mediated oncogenic signaling is triggered by breast cancer cell membrane-derived soluble CD44 (sCD44) antigen, and targeting sCD44 antigen may provide an alternative therapeutic strategy for breast cancer treatment by modulating inflammatory tumor microenvironment.

Keywords

Inflammatory tumor microenvironment, Triple negative breast cancer, Macrophages, Inflammasomes, IL-1β, Soluble CD44

2. Introduction

Tumor microenvironment is considered to exert a decisive effect on tumor progression (1). In addition to the cancer cells at a primary tumor site, there are various subsets of resident and infiltrated inflammatory cells including innate and adaptive immune cells, myeloid cells and lymphoid cells in the tumor microenvironment (2,3). These cells interplay with one another through complex and dynamic network of chemokines, cytokines, and growth factors (4-6). Tumor-associated inflammatory signaling and their microenvironmental crosstalk play pivotal roles in different stages of tumor development (7-10).

Inflammasomes are multi-protein complexes that promote inflammation through secretion of interleukin (IL)-1β in response to microbial infection and endogenous damage-associated molecular patterns (DAMPs), such as uric acid, ATP, high mobility group box 1, and the heat shock proteins 70 and 90 (11). IL-1β, one of the pro-inflammatory cytokines, engages innate immune responses through infiltration of inflammatory cells (e.g., neutrophils, macrophages, and monocytes) into infection sites (11). Due to its essential roles in innate immune responses, inflammasomes are indispensable for host

defense against external infections and tissue damages. In addition to the inflammatory conditions, elevated levels of IL-1β have been reported in various types of cancer patients (7,12). In breast cancer, it has been demonstrated that aberrant expression of IL-1β and inflammasomes is closely associated with progressive and metastatic potential of breast cancer, resulting in poor prognosis (13-16). Nonetheless, the mechanism by which IL-1β is released in breast tumor microenvironment remains elusive.

The connection between inflammation and cancer can be categorized into two pathways. One is inflammation-induced carcinogenesis, also known as the extrinsic pathway, and the other is cancer-associated inflammation or the intrinsic pathway (17,18). Many human malignancies are related to inflammation-induced carcinogenesis. Examples are colitis-induced colorectal cancer (19), hepatitis B/C virus-mediated liver cancer (20), *Helicobacter pylori*-induced gastric cancer (21), liver-fluke-associated cholangiocarcinoma (22,23), and asbestos-associated mesothelioma (24). Compared to inflammation-induced carcinogenesis, the mechanism underlying cancer-associated inflammation in breast tumor microenvironment is not fully elucidated.

Here we report a novel mechanism responsible for IL-1β production in the breast tumor microenvironment. Notably, triple negative breast cancer (TNBC) cell-derived soluble CD44 (sCD44) antigen promotes IL-1β secretion from macrophages. This finding suggests an importance of intercellular communication in tumor microenvironment between breast cancer cells and macrophages via sCD44 antigen-IL-1β signaling axis as a novel immunotherapeutic target for better clinical outcomes of TNBC patients.

3. Materials and Methods

Reagents

DMEM, RPMI 1640, DMEM/F-12 medium, and FBS were purchased from Gibco BRL (Grand Island, NY, USA). Recombinant human (rh) IL-1β and recombinant mouse (rm) IL-1β were purchased from R&D Systems (201-LB and 401-ML, Minneapolis, MN, USA). Clophosome and control liposomes were obtained from FormuMax Scientific Inc. (F70101C-NC, Sunnyvale, CA, USA). Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), ATP, apyrase, and dithiothereitol (DTT) were products of Sigma-Aldrich (St. Louis, MO, USA). D-Luciferin was purchased

from Gold Biotechnology (LUCK, St. Louis, MO, USA). Brefeldin A was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Primary antibody for IL-1β was purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain (ASC), β-actin, E-cadherin, and N-cadherin were obtained from Santa Cruz Biotechnology (CA, USA). Antibodies for CD44 and mouse IgG2a isotype, and Cytokeratin were purchased from Novus Biologicals (Centennial, CO, USA). Antibody against amyloid-β was obtained from Merk (Kenilworth, NY, USA), and Ki-67 was purchased from Abcam (Cambridge, UK). .

Cells and cell culture

MDA-MB-231 and MCF-7 cells were obtained from Korean Cell Line Bank (KCLB) in 2013, and THP-1 cells were obtained from KCLB in 2018. MCF-10A and MDA-MB-468 cells were kindly provided by Prof. Marc Diederich (College of pharmacy, Seoul National University) and Prof. Dong-Young Noh (College of medicine, Seoul National University), respectively. The genetic identity of the cell lines was confirmed by short tandem repeat

profiling, and all cell lines were routinely tested for *Mycoplasma* contamination by PCR test method (iNtRON Biotechnology; Burlington, MA, USA). All cell lines were maintained and used at the ≤ 25 passage number. Human breast cancer MDA-MB-231 and MDA-MB-468 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Human breast cancer MCF-7 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS,100 U/ml penicillin and 100 μg/ml streptomycin. Immortalized human benign breast epithelial MCF-10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 100 ng/ml cholera toxin, 20 ng/ml human epidermal growth factor, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 2 mM L-glutamin, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human monocyte-like THP-1 cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamin, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. For inducing differentiation of monocyte-like THP-1 cells to macrophages, the cells were seeded and incubated in 60 mm dishes at a density of 5×10^5/ml in medium containing 100 nM PMA for 48 h, followed by incubation in medium without

PMA for another 24 h. All cell lines were maintained in an incubator at 37ºC in a humidified atmosphere of 5% CO_2.

Human serum samples

Human serum samples from patients diagnosed with stage III breast cancer and healthy donors were obtained from Korea Institute of Radiological and Medical Sciences (KIRAMS) Radiation Biobank. Human serum samples were analyzed to measure levels of IL-1β, IL-6, IL-18, TNF-α, and CD44 by ELISA. This study was performed in accordance with the Declaration of Helsinki of the World Medical Association, and the study was approved by Institutional Review Board (IRB) of Seoul National University (No. E1812/003-001). Informed written consent for this study was waived by IRB approval.

Mouse orthotopic allograft (transplant) breast cancer model

All animal experiments were conducted on protocols approved by the Institutional Animal Care and Use Committee at Seoul National University (SNU-180123-1-1). Five-week-old female mice with BALB/c genetic

background were purchased from Orient Bio Inc. (Seongnam, Gyeonggi-do, Korea). The mice were acclimated for 1 week before use, and maintained throughout the study in a controlled environment: 22 ± 2ºC, 50 ± 5% relative humidity, and a 12 h light/dark cycle.

For the orthotopic transplant breast cancer mouse model, 4T1-Luc cells or sh-IL1R1a 4T1 cells suspended in PBS were transplanted into 4th mouse mammary fat pad. Mice for each experiment were randomly assigned to groups. The primary tumor size was measured every 3-4 days using a caliper. The tumor volume was calculated as follows: (width^2 × length)/2. For bioluminescence measurement, D-Luciferin (150 mg/kg) was administered intraperitoneally. Mice were then anesthetized with isoflurane (2-3% isoflurane in oxygen flow rate of 1 liter/min). Bioluminescence images were obtained using the IVIS Spectrum *in vivo* imaging system (PerkinElmer; Waltham, MA, USA) and the Living Image software. After 4-5 week of orthotopic transplantation, mice were euthanized by CO_2 inhalation. Their blood samples were collected through cardiac puncture, and then, primary tumors and lung tissues were excised for measurement of tumor weight and assessment of lung metastasis.

ELISA

Sera from human and mouse, and supernatants from cell culture were analyzed using IL-1β, IL-6, IL-18, TNF-α, and CD44 ELISA kits according to the manufacturer's instruction. Human IL-1β (ELH-IL1b), IL-6 (ELH-IL6), TNF-α (ELH-TNFa), and CD44 (ELH-CD44), and mouse IL-1β (ELM-IL1b) ELISA kits were purchased from RayBiotech (Norcross, GA, USA). Human IL-18 (ab215539) ELISA kit was purchased from Abcam (Cambridge, UK), and the mouse CD44 (LS-F8020) ELISA kit was obtained from LS Bio (Seattle, WA, USA).

Tissue immunofluorescence staining

The dissected mouse primary tumor tissues were prepared for immunofluorescence analysis of the expression of IL-1β, N-cadherin, E-cadherin, Cytokeratin, Ki-67, and CD44. Four-μm sections of 10% formalin-fixed, paraffin embedded tissues were placed on glass slides and deparaffinized 3 times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated by using microwave and boiled

twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. The slides were stained with corresponding primary antibodies in 5% bovine serum albumin (BSA) at 4ºC for overnight, and then washed and stained with secondary antibodies at room temperature for 1 h. Nuclei were stained with 1 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) for 5 min.

Flow cytometry

For preparation of macrophage and dendritic cells from mouse primary tumor, isolated mouse primary tumors were teased into small pieces by using a gentleMACS dissociator (Miltenyi Biotec; Germany), then digested with RPMI1640 medium containing digestion enzymes as manufacturer's instructions for 40 min. Red blood cells (RBCs) were lysed with 1 ml of RBC lysis buffer (iNtRON Biotechnology) for 1 min, and cells were separated using LSM (MP Biomedicals; France) gradient centrifugation. For macrophage isolation from spleen, spleen was smashed and ground onto the 100 μm nylon cell strainer (Corning Inc.; NY, USA), and the cells were resuspended in DMEM medium containing 10% FBS. For lysis of RBCs, the cells were incubated with 1 ml of RBS lysis buffer for 1 min, and then the cells were

separated using LSM gradient centrifugation. For Fc blocking, the cells were incubated with an anti-CD16/32 antibody in staining buffer containing 1% BSA and 0.1% sodium azide in PBS for 15 min. Specific antibodies for macrophage membrane (F4/80, CD11b, Ly6G, and Ly6C) and dendritic cell membrane (F4/80, CD11c, and MHCII) were added to samples and incubated for 1 h. All antibodies for FACS analysis were purchased from BioLegend Inc. Cells were analyzed by a BD FACSCalibur, FACS Aria III or LSRFortessa (BD Biosciences; Franklin Lakes, NJ, USA). FlowJo software (Ashland, OR, USA) was used to analyze the data.

Co-culture experiment

Human monocyte-like THP-1 cells (6×10^5/ml) were seeded and differentiated in the bottom layer of 6 well-culture plate, and a day after, breast cancer cells (5×10^5/ml) were seeded onto 0.4 μm porous insert layer (Corning Inc.; NY, USA). On day 4, both differentiated THP-1 cells in the bottom layer and breast cancer cells in the insert layer were combined for co-culture.

Western blot analysis

Cells were lysed in lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl, pH 7.4, and protease inhibitors). The protein concentration was determined by using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific; Waltham, MA, USA). Culture supernatant between 3-50 kDa was concentrated by Amicon ultracentrifugal filter units (Millipore; Burlington, MA, USA). Protein samples were resolved by SDS-PAGE, and transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk for 1 h at room temperature prior to incubation with appropriate primary antibodies. Subsequently membranes were washed and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. Following 3 times washes, transferred proteins were detected with an enhanced chemiluminescence detection kit (Abclone; Seoul, South Korea).

Isolation and differentiation of bone marrow-derived macrophages (BMDM) and peritoneal macrophages (PM)

To obtain BMDM, bone marrow cells from femurs and tibias of 6-8-week-old BALB/c mouse were flushed out by cold PBS containing 2% heat inactivated FBS. RBCs were lysed in RBC lysis buffer. After lysis RBCs,

remaining cells were plated on sterile petri dishes and incubated for 3 days in DMEM medium containing 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml M-CSF (BioLegend Inc.) at 37ºC in a humidified atmosphere of 5% CO_2. After 3 days, unattached cells were removed by changing medium containing 20 ng/ml M-CSF. To obtain PM, peritoneal cells were harvested from peritoneal cavity of 6-8-week-old BALB/c mouse by cold PBS containing 2% heat inactivated FBS. RBCs were lysed in RBC lysis buffer. After lysis of RBCs, remaining cells were incubated in DMEM medium containing 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin for 3 h at 37ºC in a humidified atmosphere of 5% CO_2. After 3 h of incubation, unattached cells were removed by changing medium.

ASC oligomerization assay

For ASC oligomer cross-linking, cells were dissolved in lysis buffer (1% NP-40, 150 mM KCl and 20 mM HEPES-KOH, pH 7.7) supplemented with protease inhibitors (0.1 mM PMSF, 1 μg/ml leupeptin, 11.5 μg/ml aprotinin, and 1 mM sodium orthovanadate), followed by shearing 25 times through 20-gauge needle. The lysates were then centrifuged at 5,000 × *g* for 10 min at 4ºC,

and the pellets were washed twice with PBS, and resuspended in 500 μl PBS. The resuspended pellets were cross-linked with disuccinimidyl suberate (2 mM) for 30 min and centrifuged at 5,000 × *g* for 10 min at 4ºC. The cross-linked pellets were lysed in 20 μl 1× SDS sample buffer, and boiled for 5 min at 95ºC. The samples were separated by 10% non-reducing SDS-PAGE.

ASC speck formation assay

Human monocyte-like THP-1 cells were seeded and differentiated at a density of 1.2×10^5/ml in a 4 chamber slide. Differentiated THP-1 cells were treated with conditioned-medium (CM) from breast cancer cells (MDA-MB-231, MDA-MB-468, and MCF-7) and immortalized human benign breast epithelial cells (MCF-10A) CM for 12 h. Chamber slides were fixed with 4% paraformaldehyde for 10 min at room temperature, and then permeabilized with 0.5% Triton X-100 for 5 min at room temperature. Chamber slides were incubated with ASC primary antibody in PBST for overnight at 4ºC, and then washed and incubated with secondary antibody for 1 h at room temperature. Nuclei were stained with 1μg/ml of DAPI for 5 minutes. ASC specks were counted under the microscope (Nikon; Tokyo, Japan).

Real-time PCR

Total RNA was isolated from cells with Trizol® Reagent (Thermo Fisher Scientific; Waltham, MA, USA), and 1 μg of RNA was reverse transcribed using the Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega; Madison, WI, USA). Real-time quantitative PCR was performed on a 7500 Real-Time PCR instrument (Thermo Fisher Scientific) using the RealHelix Premier Quantitative PCR Kit (NanoHelix Co. Ltd; Daejeon, South Korea). Dissociation curve analysis was performed to verify the identity of PCR products. Target gene expression was normalized to *L32* or *Actb*. Data were analyzed using the comparative cycle threshold (Ct) (ΔΔCt) method.

Breast cancer cell secretome analysis

The protein concentration of breast cancer cell secretome was measured using the BCA assay, and 100 μg of proteins were mixed with 8 M Urea at a 1:3 ratio. The final concentration of urea was set as 6 M, and the mixture was sonicated for 30 min in 4ºC. Then, DTT was treated for reduction of proteins to a final concentration of 10 mM and incubated for 45 min at 37ºC. Then

iodoacetamide (Sigma-Aldrich) was treated for alkylation to the final concentration of 30 mM and incubated in dark at room temperature. Trypsin was added to the samples (1:50, trypsin:sample) and incubated for overnight at 37ºC.

Digested peptides were dried and re-suspended in 0.1% formic acid in water and analyzed using the Q Exactive Mass Spectrometer coupled with the Waters ACQUITY UPLC system. For the proteome profiling analysis, the gradient was as follows (T min / % of solvent B): 0/5, 5/10, 100/40, 102/80, 112/80, 114/5, 120/5. The peptides were eluted through a trap column, ionized through an EASY-spray column (50 × 75 μm ID) packed with 2 μm C18 particles at an electric potential of 1.8kV. Full MS data were acquired in a scan range of 400-2,000 T at a resolution of 70,000 at m/z 200, with an automated gain control target value of 1.0 × 106 and a maximum ion injection of 100 ms. The maximal ion injection time for MS/MS was set to 50 ms at a resolution of 17,500. Dynamic exclusion time was set to 30 s.

The MS2 spectra were searched with the MaxQuant (v. 1.5.1.2) against the Uniprot human database (containing 20,417 proteins). Carbamidomethylation of cysteine as a fixed modification and oxidation of

methionine as variable modifications was used for each search. A false discovery rate cut-off of 1% was applied at the peptide spectrum match and protein levels. An initial precursor mass deviation up to 4.5 ppm and a fragment mass deviation up to 20 ppm were allowed. Protein identification required at least one peptide using the 'razor plus unique peptides' setting in MaxQuant. Proteins were quantified using the XIC-based label-free quantification algorithm in MaxQuant.

Neutralization assay

CM of MDA-MB-231 and MDA-MB-468 was extracted for 24 h. The CM was incubated with neutralizing antibodies (6 ug of anti-CD44 mAb and anti-amyloid-β mAb) for 6 h in rotator at room temperature. To remove antibody-bound proteins, CM was cut-off with 100 kDa of ultracentrifugal filter unit. Under 100 kDa fraction of CM was treated to PMA-differentiated THP-1 cells for 12 h.

Wound healing assay

MDA-MB-231 and MCF-7 cells were seeded at the density of 3×10^5/ml into Ibidi Culture-Inserts (2×0.22 cm^2; Regensburg, Germany) in 60 mm dishes. After 24 h incubation, Ibidi Culture-Inserts were gently removed. Cells were treated with rhIL-1β for another 48 h. Wound width was measured under the microscope (Nikon).

Cell cycle analysis

Cells were trypsinized, collected, and fixed with 70% EtOH for 24 h. Then, the cells were washed with ice-cold PBS, and incubated in propidium iodide (PI) staining solution [1 mg/ml PI (Invitrogen), 0.2 mg/ml RNase A (Promega) and 0.1% v/v Triton X-100 in PBS] for 30 min at room temperature. PI stained cells were analyzed on a BD FACSCalibur. FlowJo V10 software was used to analyze cell cycle distribution.

Statistical analysis

All data are given as mean ± SD or mean ± SEM, and are based on experiments performed at least 3 times. All statistical analysis and graphical displays were done with Prism software (GraphPad; La Jolla, CA, USA).

Differences between 2 experimental conditions were analyzed using student's *t*-test. Differences among more than 3 conditions were analyzed using ANOVA test. A value of $P < 0.05$ was considered statistically significant

4. Results

Correlation between IL-1β and breast cancer development

As an initial approach to explore the inflammatory microenvironment in breast cancer, pro-inflammatory cytokines were analyzed in serum samples from healthy donors and patients diagnosed with stage III breast cancer (Table 1 and 2). Serum levels of representative pro-inflammatory cytokines (IL-1β, IL-6, IL-18, and TNF-α) were significantly elevated in breast cancer patients compared to healthy controls (Fig. 1A-D). The most striking difference was observed for IL-1β with its mean serum concentration 40-fold higher in breast cancer patients than that in healthy individuals (Fig. 1E). While the increased serum levels of IL-1β were moderately correlated with elevated serum levels of IL-6 and IL-18, there was no such correlation between IL-1β and TNF-α in breast cancer patients (Fig. 1F-H). Next, we analyzed the serum IL-1β in a 4T1-BALB/c syngeneic orthotopic breast cancer mouse model (Fig. 2A).

Consistent with finding in breast cancer patients, the serum concentration of IL-1β was significantly elevated in 4T1 tumor bearing mice (191 ± 17.49 pg/ml) compared to non-tumor bearing mice (29.8 ± 20.33 pg/ml). Moreover, expression of IL-1β was markedly up-regulated in primary tumor tissues of mice sacrificed at 5 weeks after inoculation of 4T1 cells compared with primary tumor tissues of mice sacrificed at 3 weeks after inoculation of 4T1 cells (Fig. 2B and C). These results provide evidence that elevated levels of IL-1β are likely to be associated with breast cancer development and progression.

Tumor progressive effects of IL-1β

To further investigate potential involvement of IL-1β in breast cancer progression, we first performed a wound healing assay with two representative human breast cancer cell lines, MDA-MB-231 and MCF-7 cells. As shown in Fig. 3A, rhIL-1β stimulated the proliferation of both MDA-MB-231 and MCF-7 cells as evidenced by increased Ki-67 expression and the proportion of cells in the S phase of the cell cycle (Fig. 3B-E). Next, direct effects of IL-1β on breast cancer progression were assessed by intratumoral injection of

rmIL-1β into primary tumor in a 4T1-BALB/c syngeneic orthotopic breast cancer mouse model. Administration of rmIL-1β significantly promoted the growth of primary tumor and pulmonary metastasis of breast cancer cells (Fig. 4A-G).

IL-1β exerts its pro-inflammatory functions by binding to its receptor, IL-1R1. In order to assess the importance of IL-1β-IL-1R1 signaling on breast cancer cell growth and metastasis, *Il1r1a* knockdown murine mammary carcinoma 4T1 cells (Fig. 5A) were orthotopically transplanted into the 4th mammary fat pad of BALB/c mice. Although there was no significant difference between two groups in serum levels of IL-1β (Fig. 5B), silencing of its receptor in 4T1 cells significantly retarded the growth (Fig. 5C), and reduced the size of primary tumors as compared to those in the 4T1 sh-control cell inoculated group (Fig. 5D and E). Additionally, knockdown of *Il1r1a* in 4T1 cells alleviated pulmonary metastasis of 4T1 cells and formation of lung metastatic foci (Fig. 5F-H). During metastasis, epithelial tumor cells lose their adhesion and polarity, and eventually acquire capability of cell motility to become mesenchymal stem cells through epithelial-mesenchymal transition (EMT) (25). Epithelial and mesenchymal cells express high levels of E-

cadherin and N-cadherin, respectively. Decreased expression of N-cadherin with concomitant increase in that of E-cadherin was observed in primary tumor tissues of the 4T1 sh-*Il1r1a* cell inoculated group (Fig. 5I). Collectively, these results suggest that the IL-1β-IL-1R1 signaling axis in the breast tumor microenvironment has critical roles in breast cancer progression.

Cellular source of secreted IL-1β in breast tumor microenvironment

Cells of the myeloid lineage, such as macrophages and dendritic cells, are largely recognized as major sources for releasing IL-1β in the context of immune responses (11). However, cells outside of the myeloid compartment, including epithelial cells, are also able to release IL-1β. For instance, IL-1β secretion was observed in keratinocytes upon UV irradiation (26). In order to identify a cellular source of secreted IL-1β in the breast tumor microenvironment, we first determined whether breast cancer cells themselves could release IL-1β. Both human breast cancer (MDA-MB-231, MDA-MB-468, and MCF-7) and immortalized human benign breast epithelial (MCF-10A) cell lines barely expressed basal *IL1B* mRNA expression (Fig. 6A). Human monocyte-like THP-1 cells, well-known for releasing IL-1β upon LPS

plus ATP stimulation, were used as a positive control. Furthermore, neither human breast cancer cells nor immortalized human benign breast epithelial cells released IL-1β into culture supernatant (Fig. 6B). We further examined whether crosstalk between breast cancer cells and myeloid cells, such as macrophages and dendritic cells, could induce IL-1β release in the breast tumor microenvironment. Herein, we focused on the interaction between breast cancer cells and macrophages due to 10 times more predominant population of macrophages than dendritic cells in primary tumors formed in the 4T1-BALB/c syngeneic orthotopic breast cancer mouse model (Fig. 7). Therefore, PMA-primed THP-1 cells differentiated into macrophages were co-cultured with human breast cancer cells to mimic mutual interaction in the breast tumor microenvironment using a transwell co-culture system. Secretion of IL-1β was dramatically increased by co-culturing THP-1 cells and TNBC cells (MDA-MB-231 and MDA-MB-468) (Fig. 8A and B), and this secretion of IL-1β was time-dependently enhanced (Fig. 8C-F). We next examined intracellular expression of pro- and cleaved-IL-1β in both TNBC cells and THP-1 cells to identify cellular origin of IL-1β released in the co-culture system. Protein expression of both pro- and cleaved-IL-1β was not evident in

MDA-MB-231 and MDA-MB-468 cells, but elevated in THP-1 cells (Fig. 9A-F), indicating that IL-1β secretion in co-culture supernatant was derived from THP-1 cells, not from the breast cancer cells. To ensure that macrophages represent a major cellular source of secreted IL-1β which stimulates cancer progression in the breast tumor microenvironment, they were depleted by i.p. injection of clodronate liposomes in a 4T1-BALB/c syngeneic orthotopic breast cancer mouse model (Fig. 10A and B). Injection of clodronate liposomes for 2 weeks starting from 2 weeks, after inoculation of 4T1 cells (Fig. 10A), significantly reduced the growth and the size of mouse primary tumors (Fig. 10D-F), as well as the serum levels of IL-1β (Fig. 10C). However, there were no significant differences in the incidence of lung metastasis and the formation of lung metastatic foci upon macrophage depletion (Fig. 10G-I). In general, it takes over 1 month for breast cancer cells to metastasize to lung tissue in a 4T1-BALB/c syngeneic orthotopic breast cancer mouse model, but the *in vivo* experiment could not continue further due to a lethal condition caused by immunosuppression following macrophage depletion. Therefore, to elucidate the effects of macrophage depletion on lung metastasis in a 4T1-BALB/c syngeneic orthotopic breast cancer mouse model, we depleted

macrophages for 2 weeks starting from 3 weeks after inoculation of 4T1 cells (Fig. 11A and B). Although there were no significant differences in the growth and the size of mouse primary tumors (Fig. 11D-F), depletion of macrophages significantly alleviated lung metastasis of 4T1 cells (Fig. 11G-I). Consistent with the previous result, serum levels of IL-1β were significantly reduced as a consequence of macrophage depletion in the 4T1-BALB/c syngeneic orthotopic breast cancer mouse model (Fig. 11C). These data indicate that macrophage depletion made after propagation of orthotopic breast tumor is not sufficient to suppress the breast cancer growth, whereas the subsequent metastasis can be attenuated. Taken all together, the above findings strongly suggest that by interplaying with breast cancer cells, macrophages become competent to secrete IL-1β in the breast tumor microenvironment.

Breast cancer cell-induced inflammasome activation in macrophages

It is well defined that inflammasome activation mediates IL-1β production in immune cells. To determine whether inflammasome activation in macrophages could be triggered by breast cancer cell-derived soluble factors, we treated PMA-primed THP-1 cells with CM from human breast

cancer cells or immortalized human benign breast epithelial cells. In agreement with results from the co-culture experiment (Fig. 8A and B), CM of MDA-MB-231 or MDA-MB-468 cell culture substantially enhanced the IL-1β production in THP-1 cells (Fig. 12A and B). Likewise, secretion of IL-1β was also induced by CM from murine mammary carcinoma 4T1 cells treated to both murine BMDM and PM (Fig. 12C). Oligomerization and speck formation of ASC are read-outs for inflammasome activation (27,28). Both events were markedly increased following treatment with CM derived from MDA-MB-231 or MDA-MB-468 cells (Fig. 12D and E). Moreover, THP-1 cells treated with CM from both TNBCs exhibited the enhanced expression of the M1-type macrophage markers (Fig. 13A and B). These results indicate that secreted molecule(s) from breast cancer cells, especially triple negative MDA-MB-231 and MDA-MB-468 cells, induce inflammasome activation, leading to IL-1β production in macrophages.

Human breast cancer cell secretome analysis

To identify the breast cancer cell-derived soluble factor(s) responsible for inflammasome activation and IL-1β secretion in macrophages, we

analyzed secretome of human breast cancer cell lines by LC-MS/MS using a label-free quantification approach (Fig. 14A). Total 1,560 human proteins were identified from CM of MDA-MB-231, MDA-MB-468, and MCF-7 human breast cancer cells (Fig. 14B). Compared to other released protein fractions, the commonly released protein fraction (A) was strongly associated with the NF-κB signaling pathway, which is responsible for IL-1β expression (Fig. 14C). To narrow down the candidate proteins, MDA-MB-231 CM was fractionated by molecular weight (MW) (<50, 50-100, >100 kDa) using ultracentrifugal filter units. Among 3 fractions collected based on MW, the 50-100 kDa fraction of MDA-MB-231 CM induced the highest secretion of IL-1β from THP-1 cells (Fig. 15A). Additionally, blocking protein transport from endoplasmic reticulum (ER) to Golgi apparatus by Brefeldin A in MDA-MB-231 cells did not influence the secretion of IL-1β from THP-1 cells (Fig. 15B), suggesting that the candidate proteins were not released through the ER-Golgi-mediated secretion mechanism.

Exosomes are cell-derived vesicles that are not released via the conventional ER-Golgi-mediated secretion pathway, and cancer cell-derived exosomes have a function in cell-cell communication using their contents (e.g.

proteins, lipids, RNA species, and DNA), (29-31). However, exosome-removed MDA-MB-231 CM by ultracentrifugation exerted no significant effects on IL-1β secretion in THP-1 cells (Fig. 15C). Moreover, to exclude involvement of a well-known inflammasome activator, ATP (11), we utilized Apyrase to hydrolyze ATP in MDA-MB-231 CM. The hydrolysis of ATP in the MDA-MB-231-derived CM failed to alter the production of IL-1β in THP-1 cells (Fig. 15D). Considering the MW information of protein candidates and the IL-1β induction patterns of breast cancer cells, 7 protein candidates were narrow-downed from the commonly released protein fraction 'A' of human breast cancer cell secretome analysis (Table 3).

Involvement of soluble CD44 derived from breast cancer cells in IL-1β secretion by macrophages

Among 7 protein candidates identified through the breast cancer cell secretome analysis, CD44 antigen was found to be the most differentially released protein in triple negative MDA-MB-231 and MDA-MB-468 cells, compared to MCF-7 cells (Table 3). CD44 is a single span transmembrane glycoprotein with multiple oncogenic functions, including tumor growth,

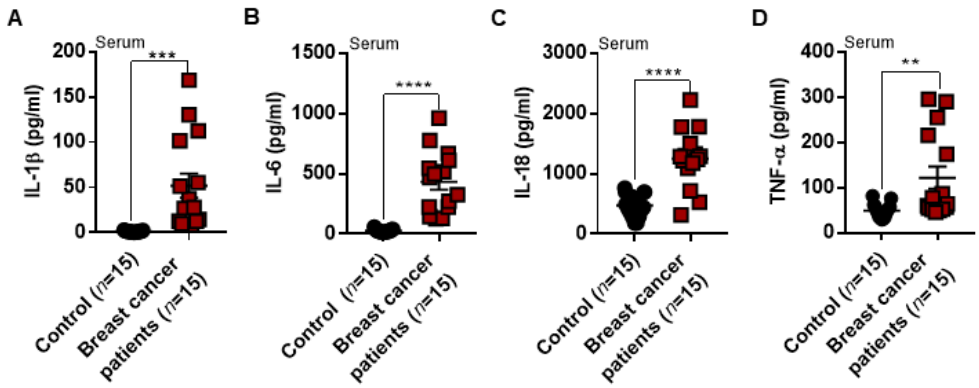
metastasis, and chemoresistance in many cancers (32-34). Elevated levels of a soluble form of CD44 (sCD44) have been reported in the serum of patients with colon, renal, and gastric cancer (35,36). In breast cancer patients, significantly elevated serum concentrations of sCD44 were observed compared to healthy controls (Fig. 16A). Such increased serum levels of sCD44 correlated with increased serum levels of IL-1β in breast cancer patients (Fig. 16B). Moreover, a low survival rate was observed in TNBC patients who had a high expression of CD44 (Fig. 16C). In line with this notion, TNBC cells released much larger amount of sCD44 in culture medium without stimulation compared to non-TNBC MCF-7 cells and immortalized human benign breast epithelial MCF-10A cells (Fig. 16D). To confirm that breast cancer cell-derived sCD44 was involved in IL-1β secretion from macrophages, sCD44-neutralized CM from MDA-MB-231 and MDA-MB-468 cells was treated to THP-1 cells. Neutralization of sCD44 by anti-CD44 mAb in the CM of MDA-MB-231 and MDA-MB-468 cells remarkably reduced IL-1β secretion (Fig. 17A and B). However, antibody neutralization of another potential candidate protein, amyloid-β (Table 3) in the CM of the both human breast cancer cell lines failed to inhibit secretion of IL-1β by THP-1 cells (Fig.

17C and D). To determine the effects of CD44 on breast cancer progression in *in vivo*, CD44 neutralizing mAb and isotype antibody as a control were injected subcutaneously into nearby mouse primary tumor tissue, starting from 1 week after 4T1 cell inoculation in a 4T1-BALB/c orthotopic breast cancer mouse model (Fig. 18A). Although the neutralizing mAb against CD44 targeted both sCD44 and membrane-bound CD44, neutralization of CD44 markedly prevented the primary tumor growth (Fig. 18B-E), and alleviated lung metastasis of 4T1 cells (Fig. 18F and G). Notably, serum levels of IL-1β were also decreased by CD44 neutralizing mAb treatment (Fig. 18H). Based on these findings, sCD44 released from breast cancer cells is most likely to be a *bona fide* stimulator of IL-1β secretion by macrophages.

Stimulation of sCD44 secretion from breast cancer cells by IL-1β

To determine whether IL-1β can influence the sCD44 expression, rhIL-1β was treated to human breast cancer cells (MDA-MB-231, MDA-MB-468, and MCF-7) and immortalized human benign breast epithelial cells (MCF-10A). rhIL-1β treatment induced sCD44 secretion from TNBC cells (MDA-MB-231 and MDA-MB-468) (Fig. 19A). It has been known that sCD44 is

produced by ectodomain cleavage of membrane-bound CD44 (34). Proteolytic cleavage of membrane-bound CD44 is mediated by various membrane-associated matrix metalloproteinases (MMPs), such as ADAM10, ADAM17, and MT1-MMP (37). However, rhIL-1β did not exert any significant effect to the mRNA expression of *ADAM10*, *ADAM17*, and *MMP14* (Fig. 19B-D), but increased expression of CD44 in human breast cancer MDA-MB-231 and MDA-MB-468 cells (Fig. 19E-H).



E

Cytokine (pg/ml)	Normal control		Breast cancer patients	
	Mean	Median	Mean	Median
IL-1β	1.27	1.18	51.81	27.7
IL-6	27.73	25.65	432.63	469.29
IL-18	425.5	442.9	1251	1282.95
TNF-α	49.16	49.56	121.98	64.14

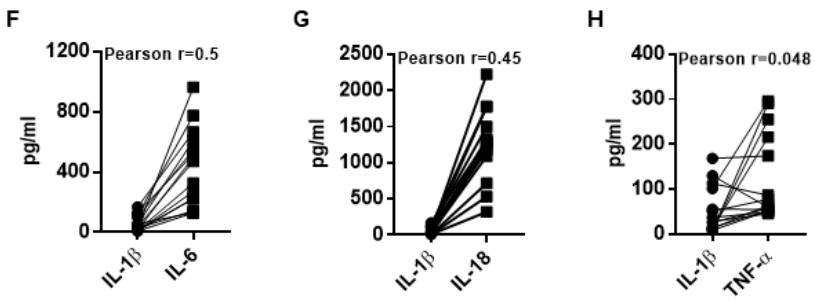


Figure 1. **Expression of pro-inflammatory cytokines in breast cancer patients. A-D,** Serum levels of IL-1β **(A)**, IL-6 (**B**), IL-18 **(C)**, and TNF-α **(D)** in healthy donors (*n*=15) and breast cancer patients (*n*=15) were measured by ELISA. **E,** Mean and median values of pro-inflammatory cytokines in breast cancer patients. **F-H,** The correlation between serum levels of IL-1β and IL-6

(**F**), IL-1β and IL-18 **(G)**, and IL-1β and TNF-α **(H)** in breast cancer patients (*n*=15) was determined by Pearson's correlation analysis. Data represent mean ± S.E.M. Statistical analysis: Student's *t*-test.

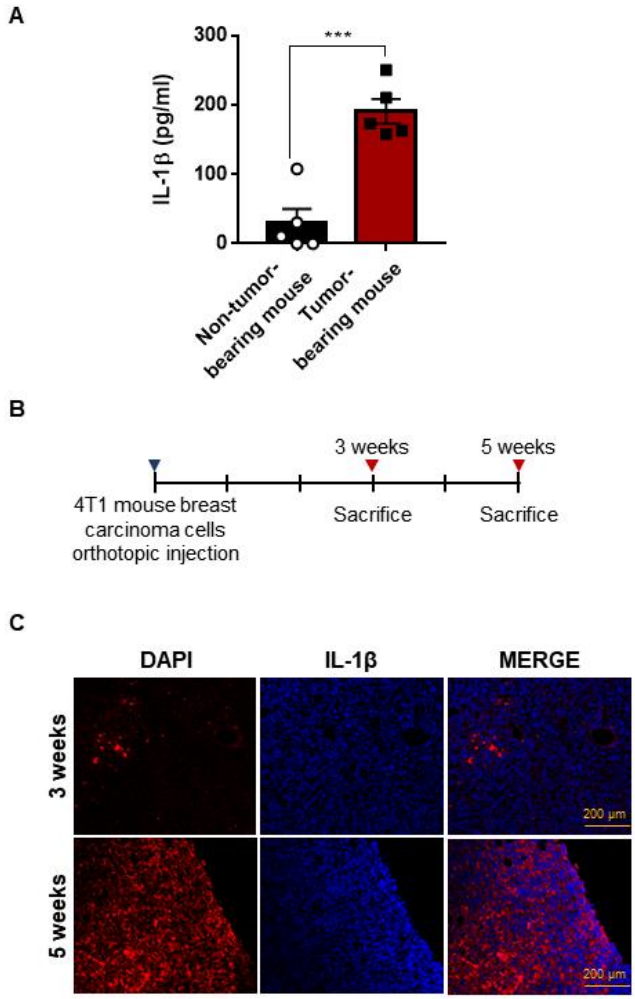


Figure 2. **Expression of IL-1β in a 4T1-BALB/c orthotopic breast cancer mouse model. A,** Serum levels of IL-1β in non-tumor bearing mice (*n*=5) and 4T1-BALB/c syngeneic orthotopic mice (*n*=5) were measured by ELISA. **B** and **C,** IL-1β expression in mouse primary tumor tissues. Schematic representation of the experiment (**B**). Immunofluorescence staining of IL-1β

(red) and DAPI (blue) in mouse primary tumor tissues obtained at 3 and 5 weeks after inoculation of 4T1 cells to the 4^{th} mammary fat pad of BALB/c mouse (**C**). Data represent mean ± S.E.M. Statistical analysis: Student's *t*-test.

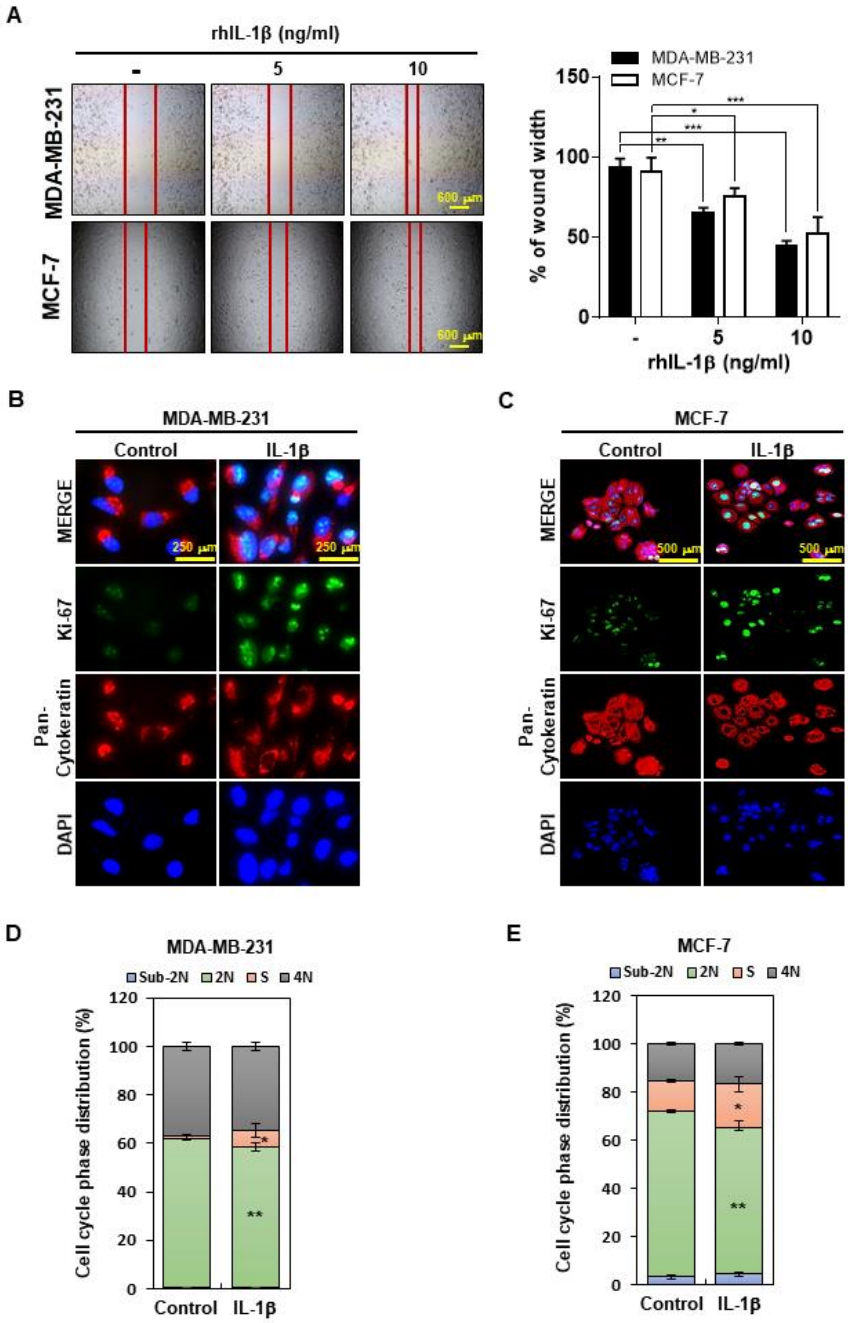

A
rhIL-1β (ng/ml)
-
5
10
MDA-MB-231
MCF-7
600 μm
MDA-MB-231
MCF-7
% of wound width
150
100
50
0
*
**
***
***
-
5
10
rhIL-1β (ng/ml)
B
MDA-MB-231
Control
IL-1β
MERGE
Ki-67
Pan-Cytokeratin
DAPI
250 μm
C
MCF-7
Control
IL-1β
MERGE
Ki-67
Pan-Cytokeratin
DAPI
500 μm
D
MDA-MB-231
Sub-2N
2N
S
4N
Cell cycle phase distribution (%)
120
100
80
60
40
20
0
*
**
Control
IL-1β
E
MCF-7
Sub-2N
2N
S
4N
Cell cycle phase distribution (%)
120
100
80
60
40
20
0
*
**
Control
IL-1β

Figure 3. **IL-1β-induced proliferation of human breast cancer cells. A,** Wound healing assay of MDA-MB-231 and MCF-7 cells in the absence or presence of rhIL-1β. Cells were treated with indicated concentrations of rhIL-1β for 48 h. The representative images of cells and quantification of wound width were displayed. **B** and **C**, Immunofluorescence staining of Ki-67 (green), Pan-cytokeratin (red), and DAPI (blue) in MDA-MB-231 (**B**) and MCF-7 cells (**C**). rhIL-1β (10 ng/ml) was treated to human breast cancer cells for 24 h. **D** and **E**, Cell cycle was analyzed by flow cytometry in MDA-MB-231 (**D**) and MCF-7 cells (**E**). rhIL-1β (10 ng/ml) was treated to human breast cancer cells for 9 h. Data are presented as mean ± S.D., One-way ANOVA (A), Two-tailed Student's *t*-test (D, E). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

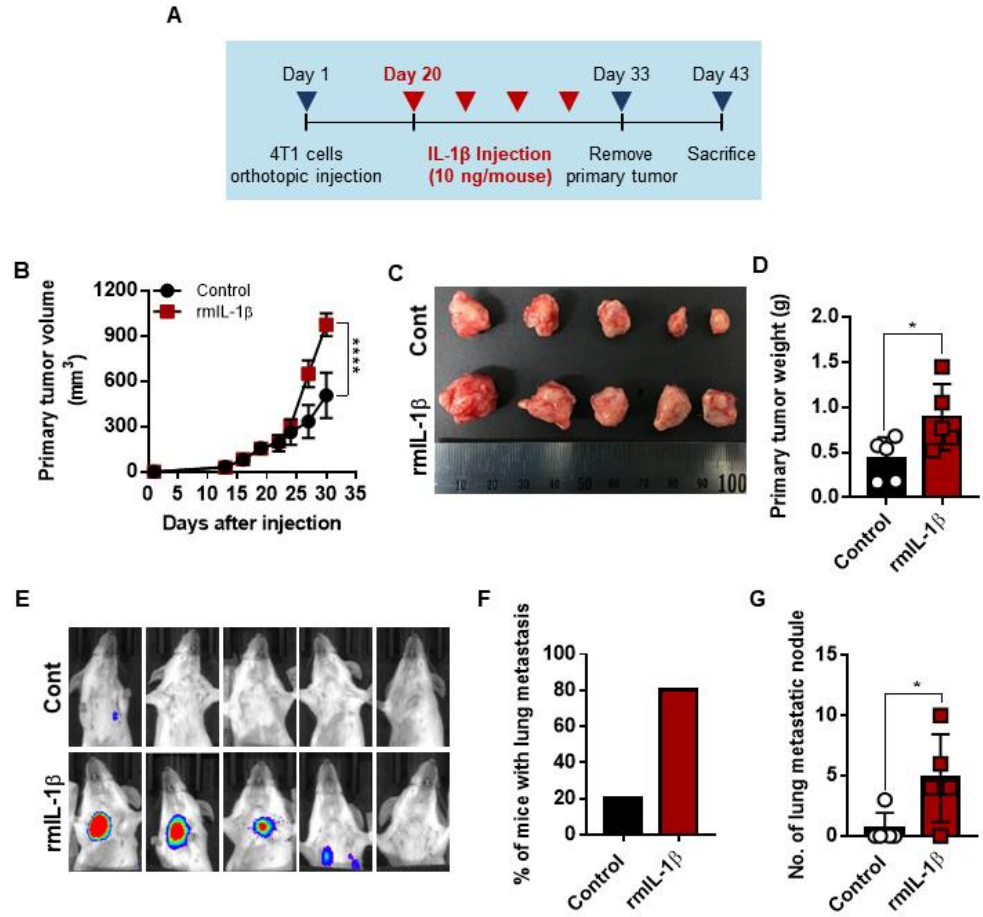


Figure 4. **Effects of IL-1β on breast cancer progression in a 4T1-BALB/c orthotopic breast cancer mouse model. A-G,** 4T1-BALB/c syngeneic orthotopic mice were treated with intratumoral injection of rmIL-1β (10 ng/mouse) or saline bi-weekly for 2 weeks starting from 3 weeks after inoculation of 4T1-Luc cells (*n*=5 for each group). The rmIL-1β

administration scheme (**A**). Growth of primary tumors (**B**). Photograph (**C**) and quantification of primary tumors (**D**). IVIS images for visualizing lung metastasis (**E**). Incidence of lung metastasis (**F**) and quantification of lung metastatic nodule (**G**). Data represent mean ± S.E.M. Statistical analysis: Two-way ANOVA (B), Student's *t*-test (D, G). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

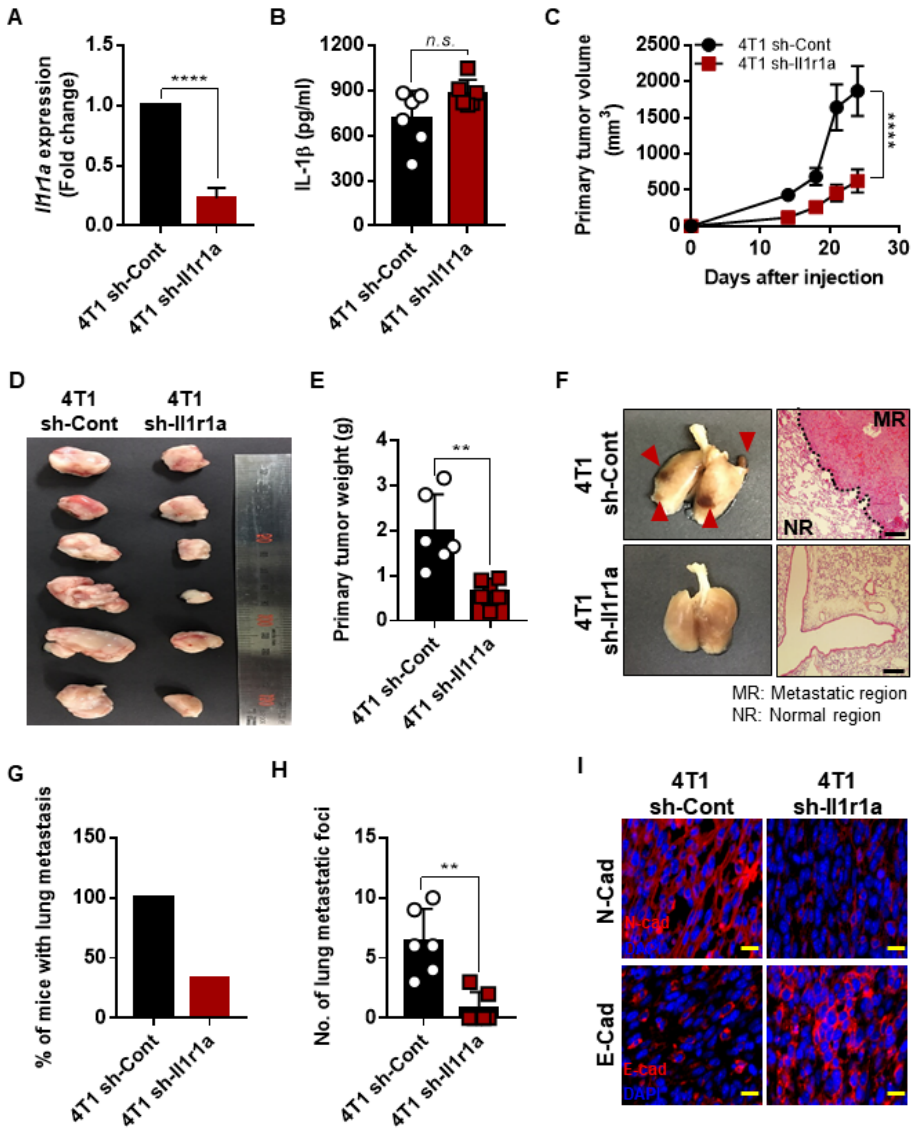


Figure 5. Potential role of IL-1β and IL-1R1 signaling on breast cancer progression in a 4T1-BALB/c orthotopic breast cancer mouse model. A-I, 4T1 cells with/without knockdown of *Il1r1a* were orthotopically inoculated,

and mice were euthanized at the end of the 5th week (n=6 for each group). RT-qPCR for measurement of basal mRNA expression of *Il1r1a* in 4T1 cells with/without *Il1r1a* knockdown (**A**). Serum levels of IL-1β in mice were measured by ELISA (**B**). Growth of primary tumors (**C**). Photograph (**D**) and quantification of primary tumors (**E**). Representative photographs of lung metastatic foci and hematoxylin & eosin (H&E) staining (**F**), incidence of lung metastasis (**G**), and quantification of lung metastatic foci (**H**). Red arrowheads indicate lung metastatic foci. Scale bar, 500 μm. Immunofluorescence staining of N-cadherin and E-cadherin in primary tumors (**I**). Scale bar, 100 μm. Data represent mean ± S.D. (A) and mean ± S.E.M. (B, C, E, H). Statistical analysis: Student's *t*-test (A, B, E, H) and Two-way ANOVA (C). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

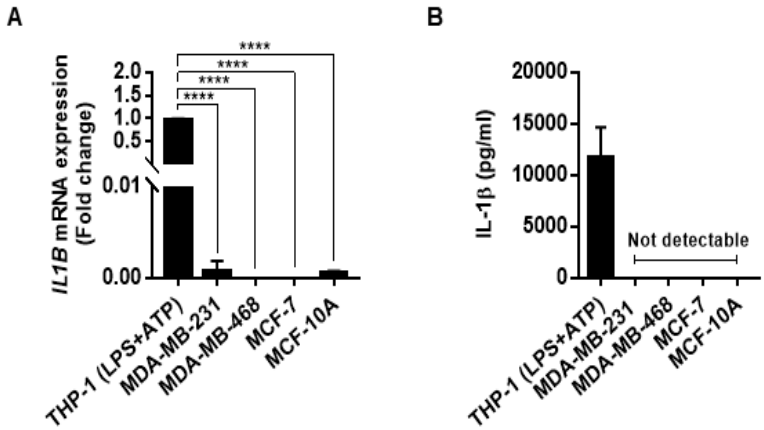


Figure 6. Expression of IL-1β in human breast cancer cells. A, RT-qPCR for measurement of basal mRNA expression of *IL1B* in human breast cancer cells (MDA-MB-231, MDA-MB-468, and MCF-7) and normal human breast epithelial cells (MCF-10A). PMA-differentiated THP-1 cells were primed with LPS (100 μg/ml) for 12 h followed by exposure to ATP (5 mM) for 30 min. These cells were used as positive control for expression of *IL1B*. **B,** IL-1β levels in CM of human breast cancer cells and normal human breast epithelial cells were measured by ELISA. Data represent mean ± S.D. Statistical analysis: ANOVA. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

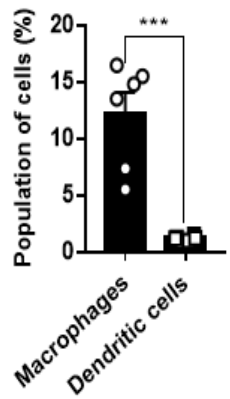


Figure 7. Population of myeloid cells in primary tumors of 4T1-BALB/c orthotopic breast cancer mouse model. Proportion of F4/80^{+}CD11b^{+}Ly6G^{-} Ly6C^{-} macrophages and F4/80^{-}CD11c^{+}MHCII^{high} dendritic cells in primary tumors of 4T1-BALB/c syngeneic orthotopic mice (*n*=6). Data represent mean ± S.E.M. Statistical analysis: Student's *t*-test.

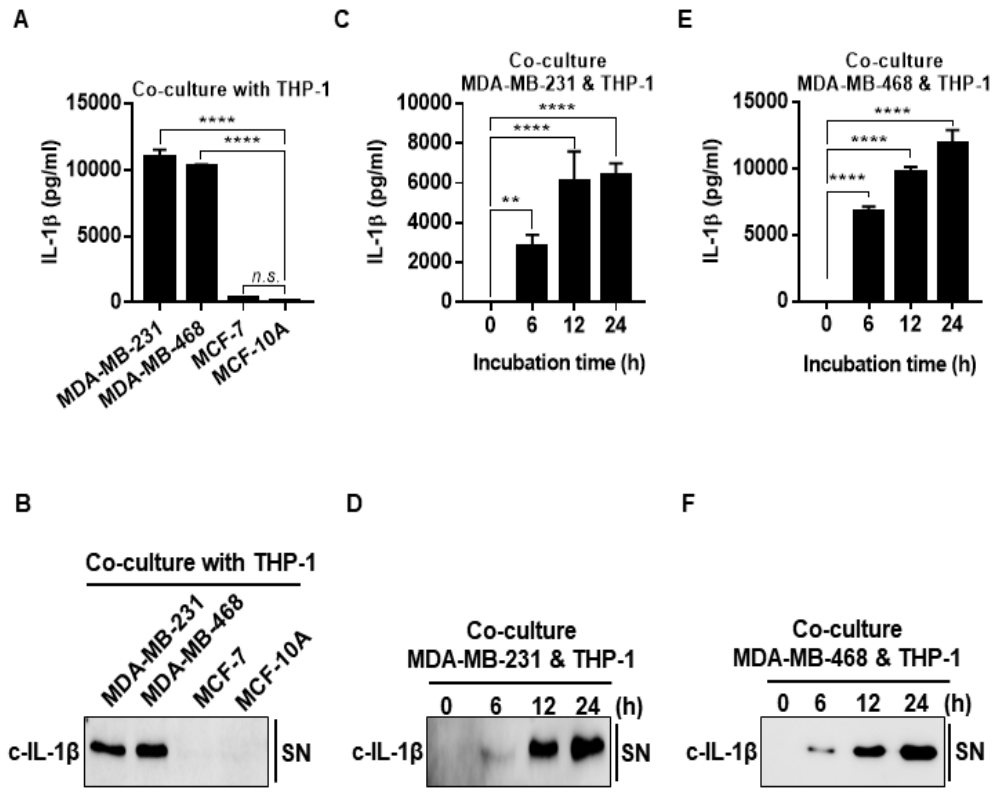


Figure 8. IL-1β secretion from co-culture between PMA-primed THP-1 cells and human breast cancer cells. A-F, Co-culture of PMA-primed THP-1 cells with human breast cancer cells or normal human breast epithelial cells using the trans-well co-culture system. IL-1β levels in co-culture supernatant were measured by ELISA (**A**) and immunoblot analysis (**B**). Time-dependent secretion of IL-1β in the supernatant from co-culture of MDA-MB-231 cells and THP-1 cells (**C**, **D**), and MDA-MB-468 cells and THP-1 cells (**E**, **F**) was

determined by ELISA and immunoblot analysis, respectively. Data represent mean ± S.D. Statistical analysis: ANOVA. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

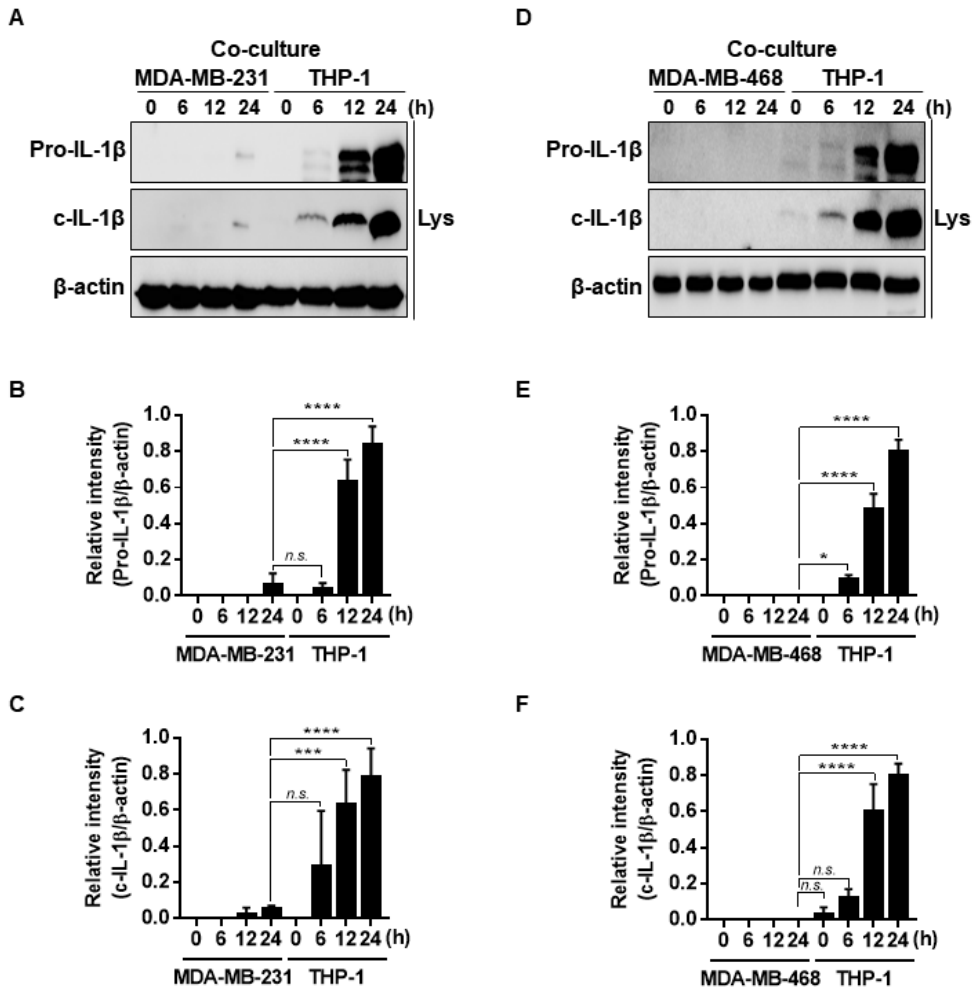


Figure 9. Origin of released IL-1β from co-culture system. Time-dependent co-culture of PMA-primed THP-1 cells with MDA-MB-231 or MDA-MB-468 cells using the trans-well co-culture system. Intracellular expression levels of pro- and cleaved-IL-1β proteins were determined by immunoblot analysis (**A**, **D**) and the bands intensities were quantified (**B**, **C**, **E**, **F**). Data

represent mean ± S.D. Statistical analysis: ANOVA. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

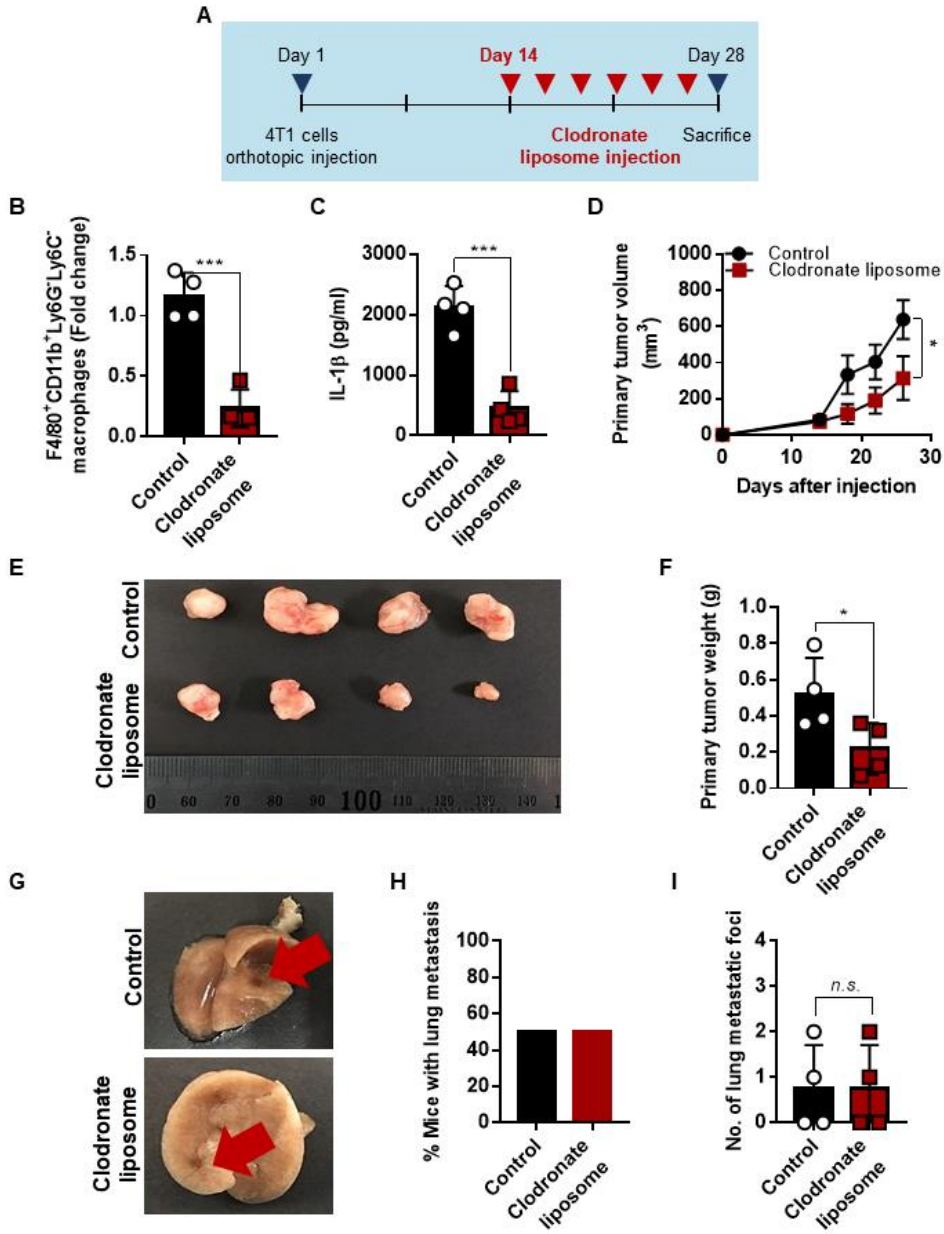


Figure 10. Effects of early phase macrophage depletion on breast cancer progression in a 4T1-BALB/c orthotopic breast cancer mouse model. A-I,

Schematic representation of clodronate liposome injection (n=4 for each group) (**A**). Flow cytometric analysis of $F4/80^{+}CD11b^{+}Ly6G^{-}Ly6C^{-}$ macrophages from spleen of 4T1-BALB/c syngeneic orthotopic mice (**B**). Serum levels of IL-1β were measured by ELISA (**C**). Growth of primary tumors (**D**). Photograph of primary tumors (**E**). Quantification of primary tumor weights (**F**). Photographs (**G**), incidence (**H**), and quantification of lung metastatic foci (**I**). Red arrowheads indicate lung metastatic foci. Data represent mean ± S.E.M. Statistical analysis: Student's *t*-test (B, C, F, I), Two-way ANOVA (D). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

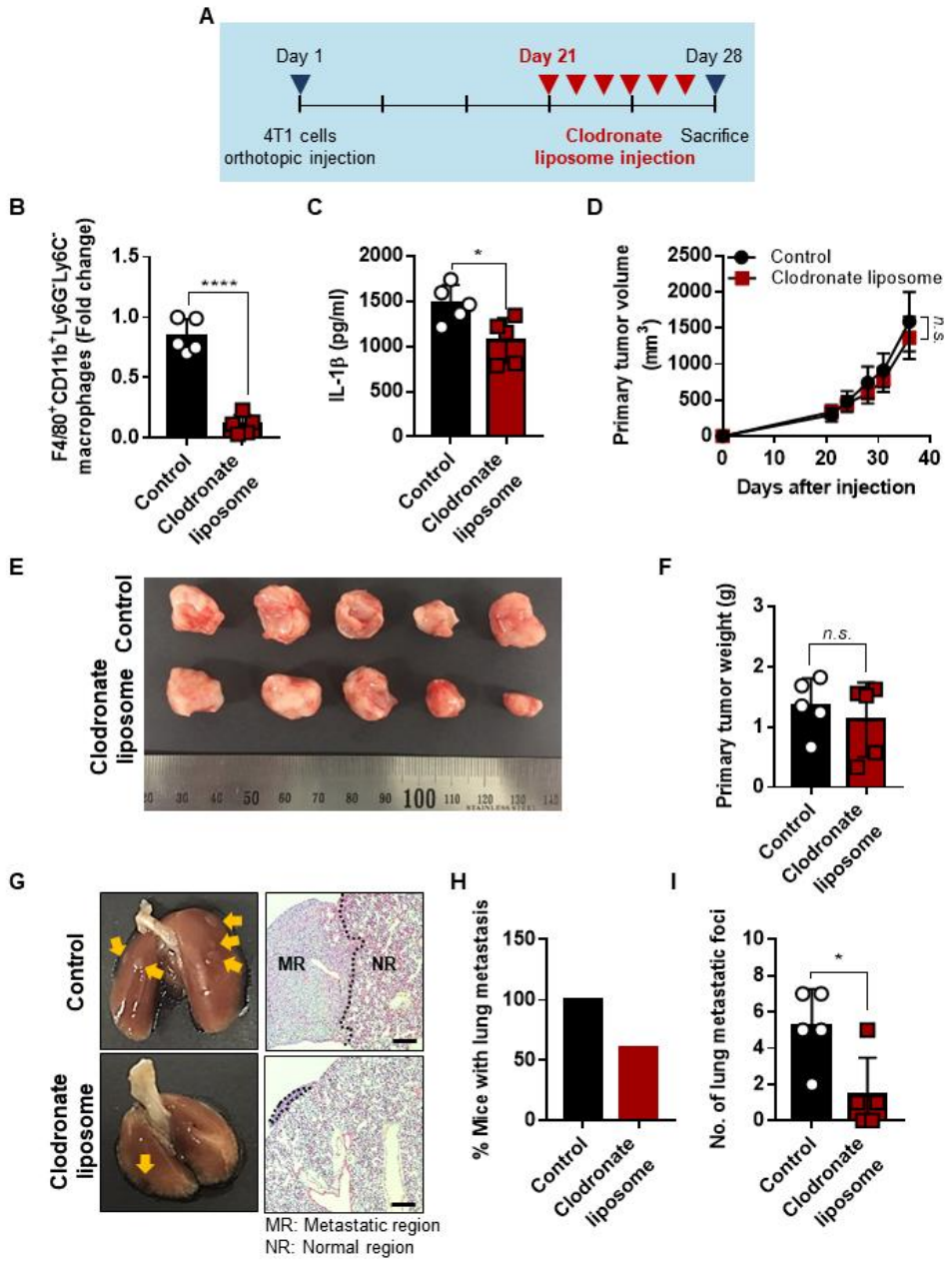


Figure 11. Effects of late phase macrophage depletion on breast cancer progression in a 4T1-BALB/c orthotopic breast cancer mouse model. A-I,

Schematic representation of clodronate liposome injection (n=5 for each group) (**A**). Flow cytometric analysis of $F4/80^{+}CD11b^{+}Ly6G^{-}Ly6C^{-}$ macrophages from spleen of 4T1-BALB/c syngeneic orthotopic mice (**B**). Serum levels of IL-1β were measured by ELISA (**C**). Growth of primary tumors (**D**). Photograph of primary tumors (**E**). Quantification of primary tumor weights (**F**). Representative photographs of H&E staining (**G**), incidence (**H**), and quantification of lung metastatic foci (**I**). Yellow arrowheads indicate lung metastatic foci. Data represent mean ± S.E.M. Statistical analysis: Student's *t*-test (B, C, F, I), Two-way ANOVA (D). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

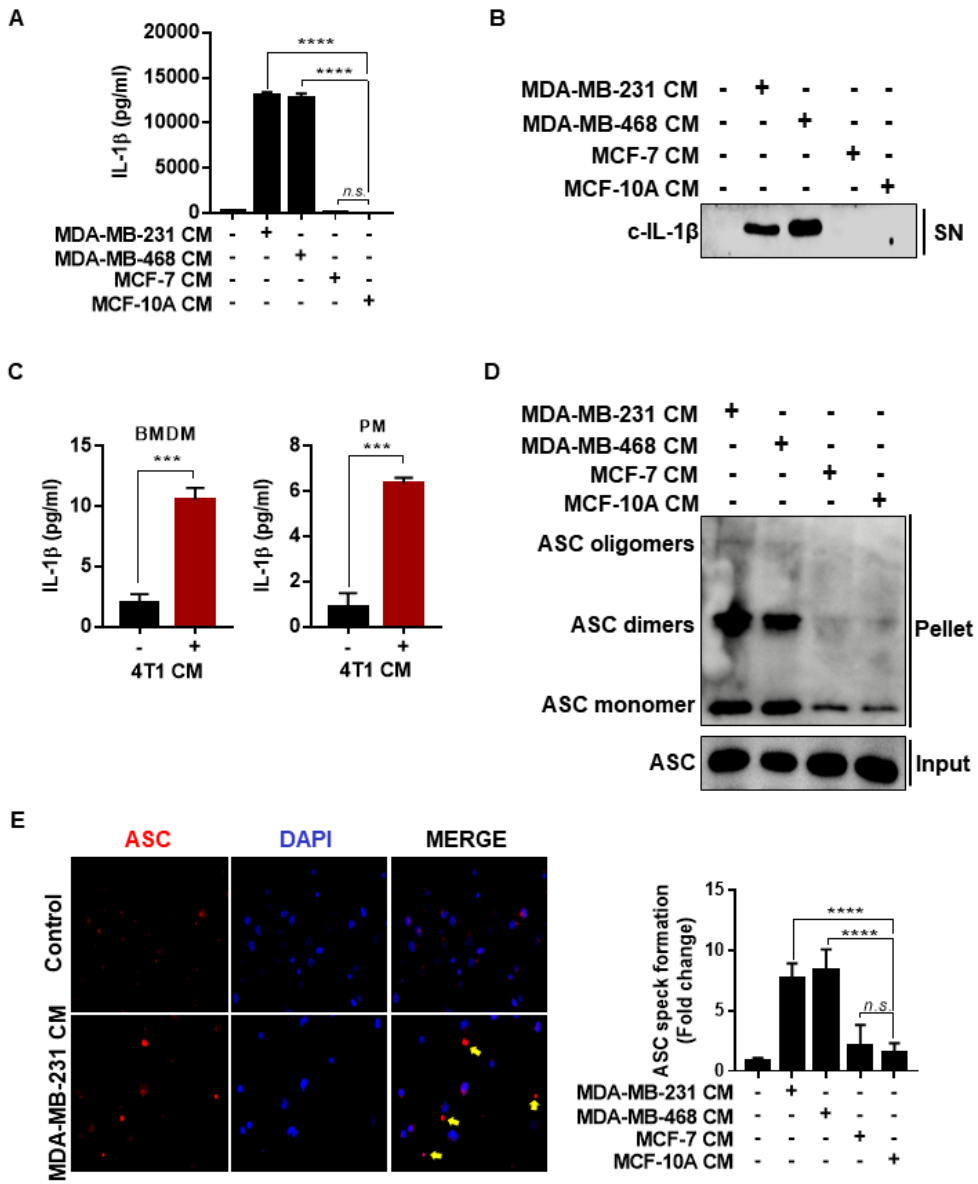


Figure 12. Inflammasome activation by breast cancer cell-derived soluble factors. A and **B,** IL-1β levels in culture supernatant of PMA-primed THP-1 cells treated with CM of human breast cancer cell culture or normal human

breast epithelial cell culture for 24 h were measured by ELISA (**A**) and immunoblot analysis (**B**). **C**, IL-1β levels in culture supernatant of BMDM and PM treated with 4T1 CM for 24 h were measured by ELISA. **D,** Immunoblot image of ASC oligomerization after cross-linking of pellets of THP-1 cells treated with human breast cancer cell CM for 24 h. **E,** ASC speck formation (yellow arrows) in PMA-primed THP-1 cells treated with human breast cancer cell CM for 12 h was determined by immunofluorescence staining. Data represent mean ± S.D. Statistical analysis: ANOVA (A, E) and Student's *t*-test (C). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

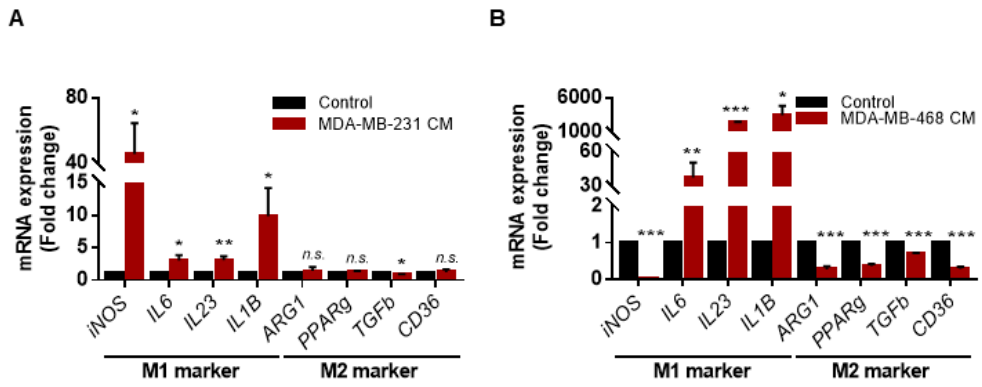


Figure 13. Effect of breast cancer cell CM on macrophage polarization. A and **B**, RT-qPCR for measurement of M1-type macrophage markers (*iNOS*, *IL6*, *IL23,* and *IL1B*) and M2-type macrophage markers (*ARG1*, *PPARG*, *TGFB1,* and *CD36*) in monocyte-like THP-1 cells upon treatment with CM from MDA-MB-231 (**F**) and MDA-MB-468 (**G**) cells. Data represent mean ± S.D. Statistical analysis: ANOVA. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

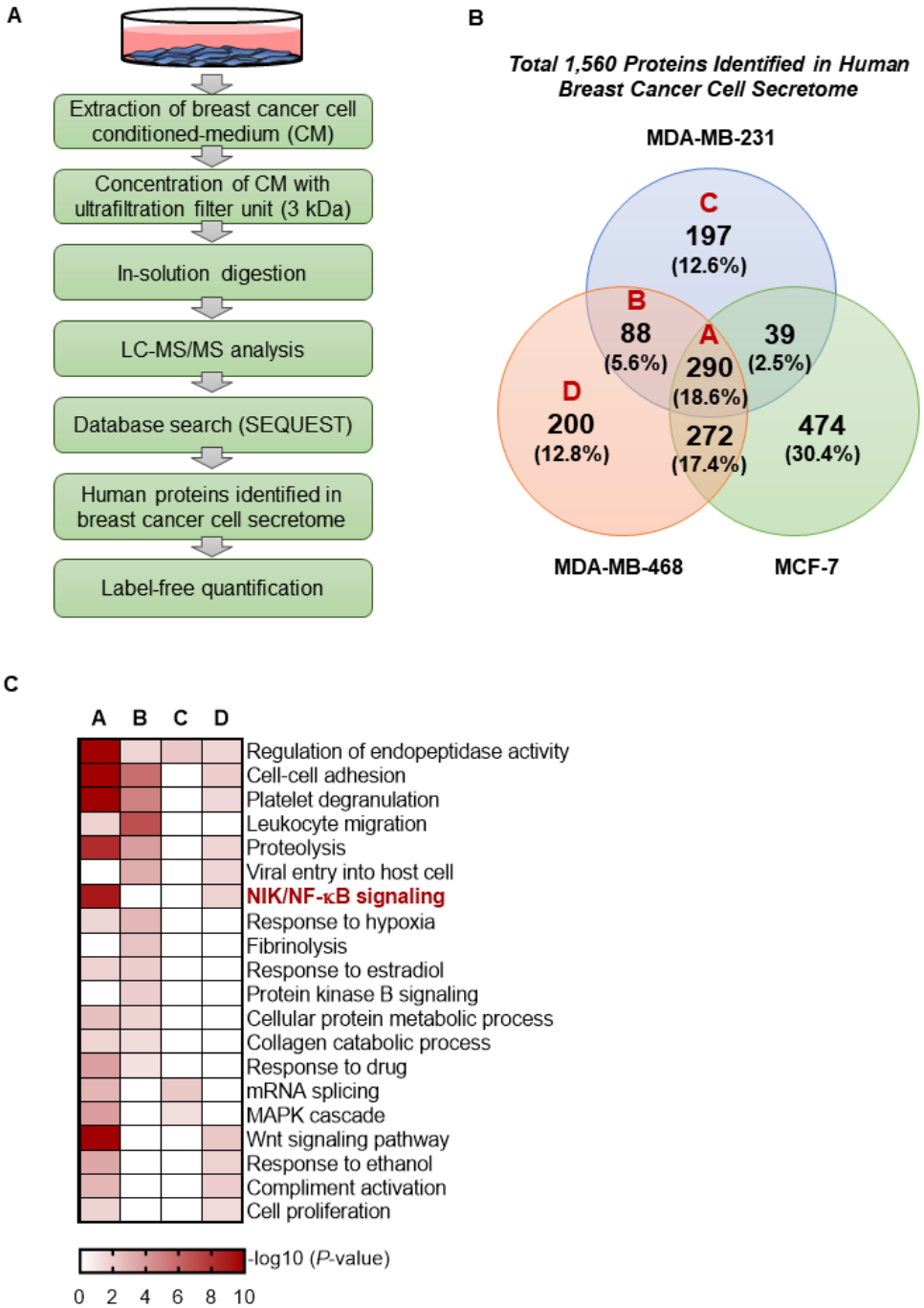

A
Extraction of breast cancer cell conditioned-medium (CM)
Concentration of CM with ultrafiltration filter unit (3 kDa)
In-solution digestion
LC-MS/MS analysis
Database search (SEQUEST)
Human proteins identified in breast cancer cell secretome
Label-free quantification
B
Total 1,560 Proteins Identified in Human Breast Cancer Cell Secretome
MDA-MB-231
C
197
(12.6%)
B
88
(5.6%)
A
290
(18.6%)
39
(2.5%)
D
200
(12.8%)
272
(17.4%)
474
(30.4%)
MDA-MB-468
MCF-7
C
A B C D
Regulation of endopeptidase activity
Cell-cell adhesion
Platelet degranulation
Leukocyte migration
Proteolysis
Viral entry into host cell
NIK/NF-κB signaling
Response to hypoxia
Fibrinolysis
Response to estradiol
Protein kinase B signaling
Cellular protein metabolic process
Collagen catabolic process
Response to drug
mRNA splicing
MAPK cascade
Wnt signaling pathway
Response to ethanol
Compliment activation
Cell proliferation
-log10 (P-value)
0 2 4 6 8 10

Figure 14. Secretome analysis of human breast cancer cell lines. A, Experimental workflow of secretome analysis of human breast cancer cell lines. **B,** Venn diagrams and the number of identified proteins from secretome analysis of human breast cancer cell lines (MDA-MB-231, MDA-MB-468, and MCF-7). **C,** Relationship of released proteins from human breast cancer cells various signaling pathways. A, B, C, and D are depicted in Fig. 14B. A: Commonly released proteins from MDA-MB-231, MDA-MB-468, and MCF-7. B: Commonly released proteins from MDA-MB-231 and MDA-MB-468. C: Released proteins from MDA-MB-231. D: Released proteins from MDA-MB-468.

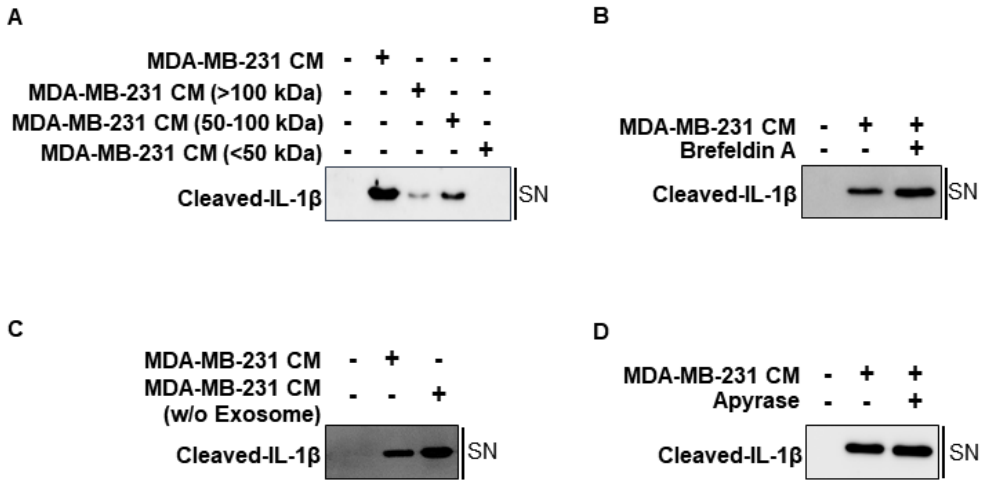


Figure 15. Characteristics of breast cancer cell-derived soluble factors for IL-1β release. A, Cleaved-IL-1β levels in the supernatant of PMA-primed THP-1 cells treated for 24 h with fractionated MDA-MB-231 CM based on MW were determined by immunoblot analysis. MDA-MB-231 CM was fractioned by MW (<50, 50-100, >100 kDa) using ultracentrifugal filter units. **B,** Cleaved-IL-1β levels in culture supernatant of PMA-primed THP-1 cells treated with MDA-MB-231 CM for 24 h were determined by immunoblot analysis. MDA-MB-231 CM was extracted with/without Brefeldin A for 6 h. **C,** Cleaved-IL-1β levels in culture supernatant of PMA-primed THP-1 cells treated with MDA-MB-231 CM or exosome removed-MDA-MB-231 CM for

24 h were determined by immunoblot analysis. Exosomes were removed by ultracentrifuge (90Ti rotor, 150,000 × *g* for 2 h) from MDA-MB-231 CM. **D,** Cleaved-IL-1β levels in culture supernatant of PMA-primed THP-1 cells treated with MDA-MB-231 CM for 24 h were determined by immunoblot analysis. MDA-MB-231 CM was extracted with/without Apyrase for 12 h.

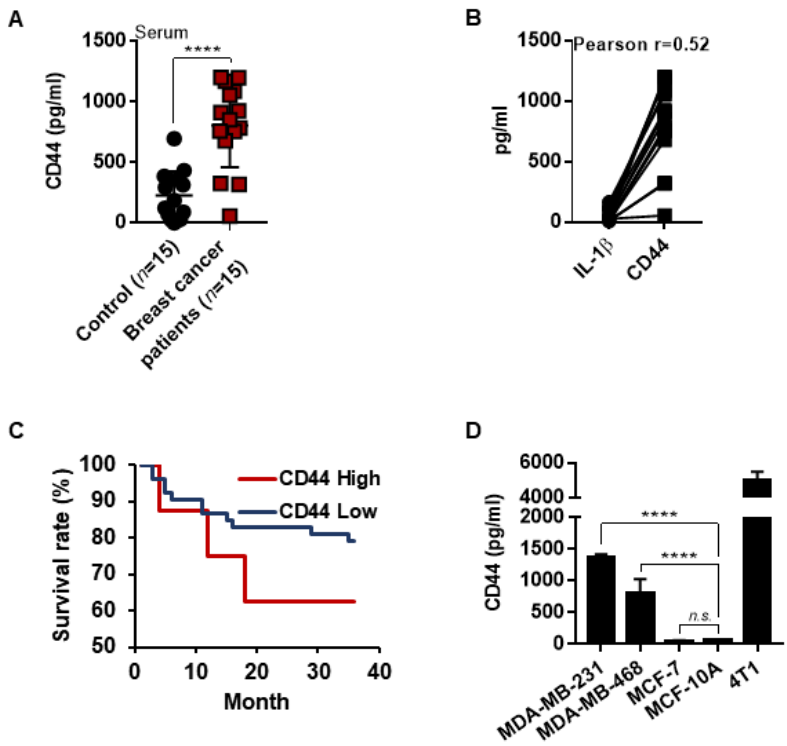


Figure 16. CD44 expression in breast cancer patients. A, Serum levels of CD44 in healthy donors (*n*=15) and breast cancer patients (*n*=15) were determined by ELISA. **B,** The correlation between serum levels of IL-1β and sCD44 in breast cancer patients was determined by Pearson's correlation analysis. **C,** Survival rate of TNBC patients depending on CD44 expression. **D,** sCD44 levels in CM of human breast cancer cells and normal human breast epithelial were measured by ELISA. Data represent mean ± S.E.M. (A) and

mean ± S.D. (D) Statistical analysis: Student's *t*-test (A) and ANOVA (D). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

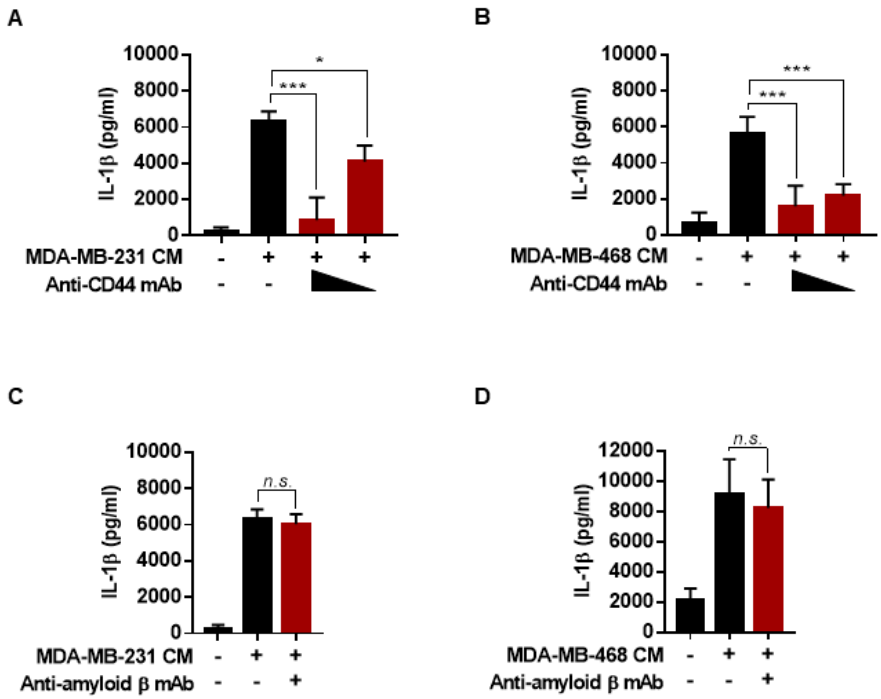


Figure 17. Effects of CD44 neutralization from breast cancer cell CM for IL-1β secretion. A and **B,** IL-1β levels in culture supernatant of PMA-primed THP-1 cells treated with sCD44-neutralized MDA-MB-231 CM (**A**) or MDA-MB-468 CM (**B**) for 12 h were measured by ELISA. MDA-MB-231 CM and MDA-MB-468 CM were treated with 3 and 6 μg of CD44 neutralizing antibody for 6 h. **C** and **D,** IL-1β levels in culture supernatant of PMA-primed THP-1 cells were treated with amyloid β-neutralized MDA-MB-231 CM (**C**) or MDA-MB-468 CM (**D**) for 12 h, and the IL-1β levels in the culture

supernatant were measured by ELISA. MDA-MB-231 CM and MDA-MB-468 CM were treated with 6 μg of amyloid β neutralizing antibody for 6 h. Data represent mean ± S.D. Statistical analysis: ANOVA. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

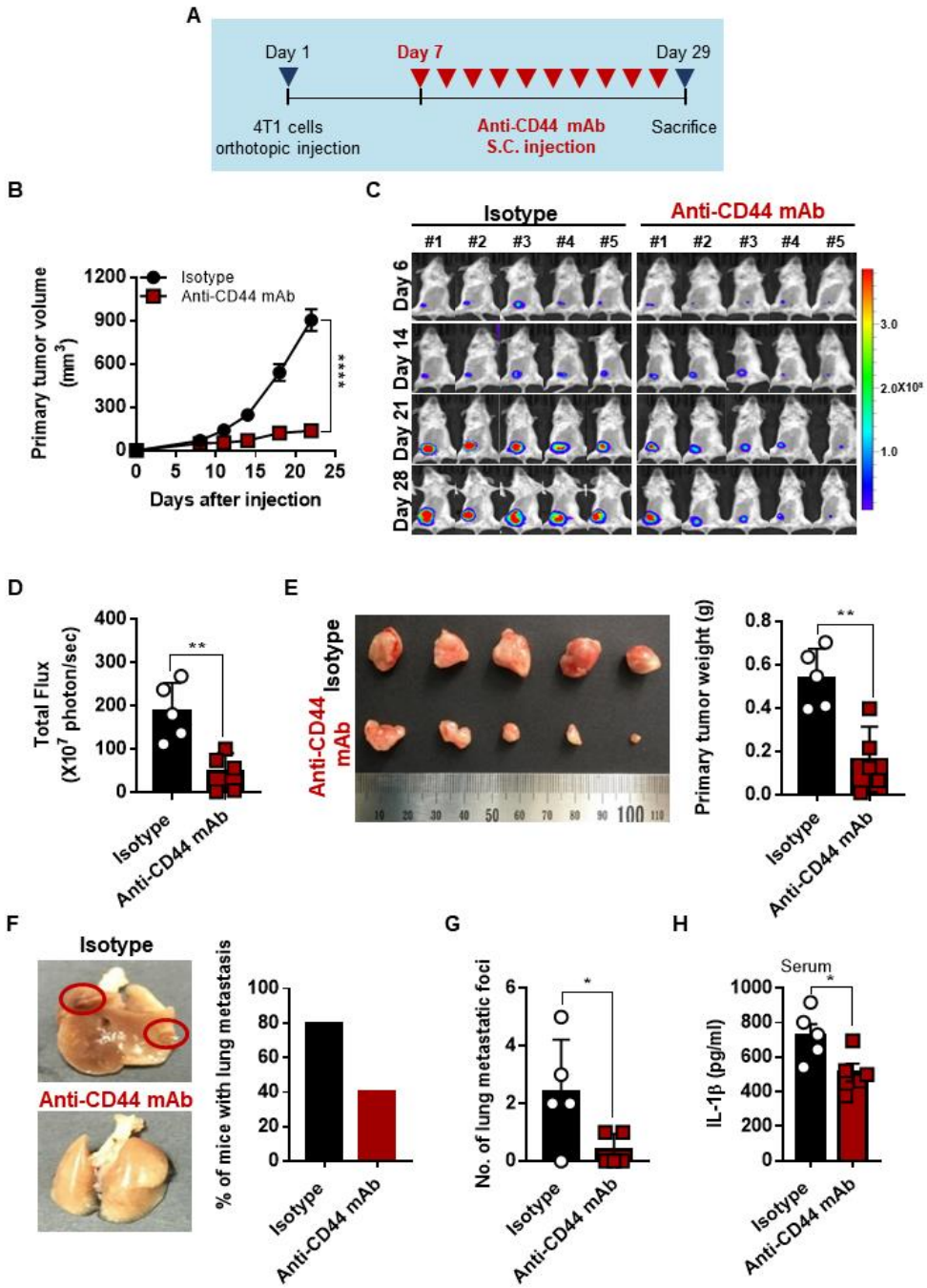

A
Day 1
Day 7
Day 29
4T1 cells
orthotopic injection
Anti-CD44 mAb
S.C. injection
Sacrifice
B
Primary tumor volume (mm3)
Isotype
Anti-CD44 mAb
Days after injection
****
C
Isotype
Anti-CD44 mAb
#1 #2 #3 #4 #5
Day 6
Day 14
Day 21
Day 28
D
Total Flux (X10^7 photon/sec)
**
E
Isotype
Anti-CD44 mAb
Primary tumor weight (g)
F
Isotype
Anti-CD44 mAb
% of mice with lung metastasis
G
No. of lung metastatic foci
H
Serum
IL-1β (pg/ml)

Figure 18. Effects of CD44 neutralization on breast cancer progression in a 4T1-BALB/c orthotopic breast cancer mouse model. A-H, CD44 was neutralized by anti-CD44 neutralizing mAb in a 4T1-BALB/c orthotopic breast cancer mouse model. Anti-CD44 mAb was s.c. injected into nearby mouse primary tumor starting from 1 week after 4T1-luc cell inoculation (**A**). Primary tumor growth (**B**). IVIS images for visualizing primary tumor (**C**). Quantification of bioluminescence of primary tumor (**D**). Photograph and quantification of primary tumors (**E**). Representative photographs of lung metastatic foci and incidence of lung metastasis (**F**), and quantification of lung metastatic foci (**G**). Serum levels of IL-1β were measured by ELISA (**H**). Data are presented as mean ± S.E.M. (B, D, E, G, H), Two-tailed Student's *t*-test (D, E, G, H), One-way ANOVA (B). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

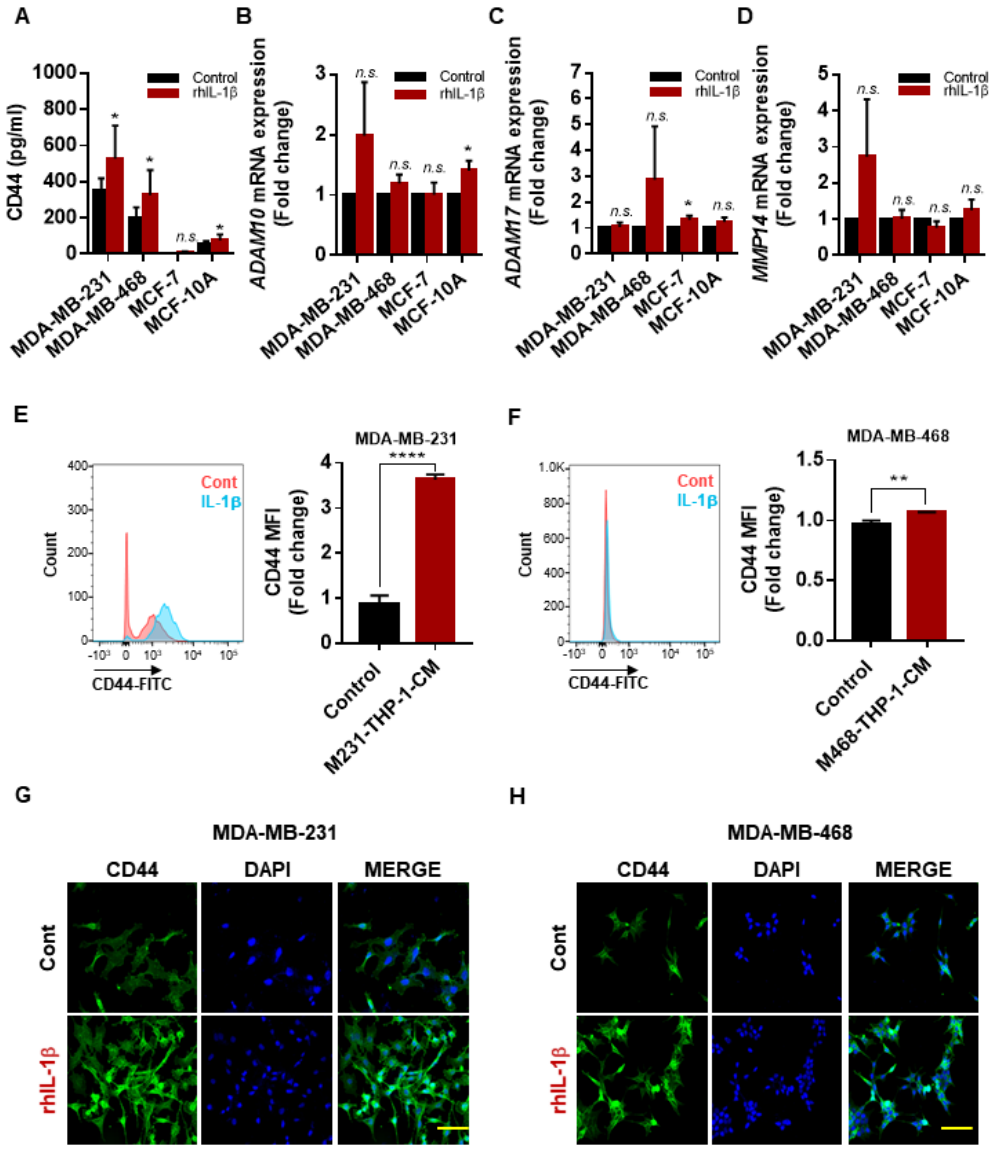


Figure 19. IL-1β-induced sCD44 secretion from human breast cancer cells.

A, sCD44 levels in culture supernatant were determined by ELISA. rhIL-1β (100 ng/ml) was treated to human breast cancer cells and human normal breast

epithelial cells for 24 h. **B-D,** RT-qPCR for measurement of mRNA expression of *ADAM10*, *ADAM17*, *MMP14* in human breast cancer cells and human normal breast epithelial cells. rhIL-1β (100 ng/ml) was treated to human breast cancer cells and human normal breast epithelial cells for 24 h. **E and F,** CD44 expression in MDA-MB-231 (**E**) and MDA-MB-468 (**F**) was examined by flow cytometry after 24 h of rhIL-1β (10 ng/ml) treatment. The expression of CD44 was quantified based on mean fluorescence intensity (MFI). **G and H,** Immunofluorescence staining of CD44 (green) and DAPI (blue) in MDA-MB-231 (**G**) and MDA-MB-468 cells (**H**). rhIL-1β (100 ng/ml) was treated to human breast cancer cells for 6 h. Scale bar, 100 μm. Data are presented as mean ± S.D., Two-tailed Student's *t*-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

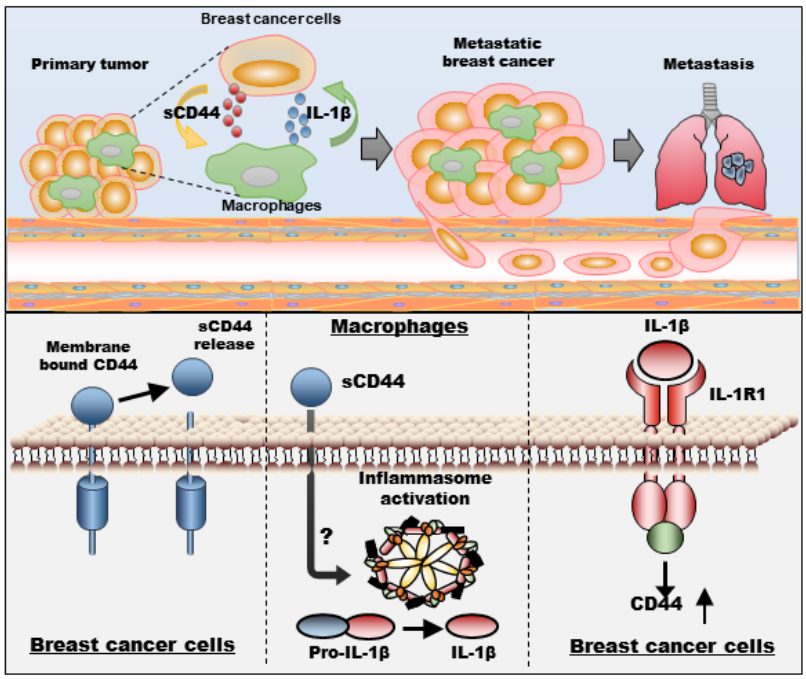


Figure 20. Proposed model of CD44-mediated IL-1β secretion from macrophages for breast cancer progression.

Table 1. Clinical characteristics of breast cancer patients.

Characteristics	No. of patients
Age	
Median 40 years	
Range 32 - 45 years	
Tumor size (T)	
T1	2
T2	10
T3	3
T4	-
Nodal status (N)	
N0	-
N1	1
N2	7
N3	7
Metastasis (M)	
M0	15
M1	-

Table 2. Stage of breast cancer patients.

	No.	Sex	Age	Cancer stage
Breast cancer patients	1	F	35	IIIA
	2	F	44	IIIC
	3	F	39	IIIC
	4	F	38	IIIA
	5	F	44	IIIA
	6	F	42	IIIA
	7	F	43	IIIA
	8	F	37	IIIC
	9	F	37	IIIC
	10	F	45	IIIA
	11	F	34	IIIC
	12	F	40	IIIA
	13	F	42	IIIC
	14	F	45	IIIC
	15	F	32	IIIA

Table 3. Selected protein candidates from breast cancer cell secretome analysis.

No.	Accession No.	Protein name	Gene name	Fold change		MW (kDa)
				MDA-MB-231 / MCF-7	MDA-MB-468 / MCF-7	
1	**P16070**	**CD44**	***CD44***	**16.1**	**7.7**	**81.5**
2	P05067	Amyloid-beta A4 Protein	*APP*	6.0	3.0	86.9
3	O00391	Sulfhydryl oxidase 1	*QSOX1*	5.5	1.0	82.5
4	Q06481	Amyloid-like protein 2	APLP2	5.5	1.5	86.9
5	Q14118	Dystroglycan	*DAG1*	3.4	2.6	97
6	P02787	Serotransferrin	*TF*	2.4	1.7	77.0
7	Q9H2G4	Testis-specific Y-encoded-like protein 2	*TSPYL2*	2.1	2.3	79.4

5. Discussion

IL-1β is a potent pro-inflammatory cytokine released as a consequence of inflammasome activation in response to infection and injury, and plays fundamental roles in innate immunity (38). Upon activation of inflammasomes, IL-1β triggers infiltration of inflammatory and immune cells into infected and damaged regions by inducing expression of adhesion molecules (e.g., intercellular adhesion molecule-1 and vascular cell adhesion molecules-1) in mesenchymal and endothelial cells (39), and release of inflammatory mediators (e.g., chemokines, and other cytokines) (40-42). Although IL-1β is involved in host defense mechanism, aberrant inflammasome activation and consequently sustained production of IL-1β are closely related to pathogenesis of autoimmune and inflammatory disorders (40). In addition to inflammatory diseases, many cancers have inflammatory characteristics in their tumor microenvironment. Therefore, attention has been focused on regulation of cancer progression by modulating inflammatory microenvironment. Breast tumor microenvironment is closely linked to cancer-associated inflammation, also called the intrinsic pathway (43). The present study demonstrated tumor progressive potential of IL-1β in a 4T1-

BALB/c orthotopic breast cancer mouse model. Depletion of macrophages reduced serum levels of IL-1β, and alleviated growth of primary tumor and lung metastasis. Although IL-1β showed tumor supportive effects in the present study, the roles of IL-1β in tumor microenvironment can be varied depending on cell and organ types for its expression. Consistent with our present study, Wu et al. have reported that dendritic cell-derived IL-1β in primary breast tumor microenvironment is associated with advanced disease stage (15). In another study, however, sustained IL-1β-mediated systemic inflammatory responses prevent metastasis-initiating cancer cells from differentiating and colonizing at metastatic site (44).

In addition to cancer cells, various types of surrounding stromal cells comprise tumor microenvironment. These include myeloid cells, lymphoid cells, cancer-associated fibroblasts, and sometimes adipocytes (3). Although IL-1β is mainly secreted by innate immune cells, non-professional immune cells are also able to release IL-1β (45). For instance, substantial amount of IL-1β was found to be secreted from human primary keratinocytes irradiated with UV (45). In the present study, however, we excluded the possibility of human breast cancer cells to release IL-1β because of substantially low

expression of *IL1B* mRNA and undetectable amount of secreted IL-1β in the culture medium of several human breast cancer cell lines. Innate immune cells, such as macrophages and dendritic cells, are well known for their capability of releasing IL-1β in response to pathogen-associated molecular patterns or DAMPs. Both macrophages and dendritic cells also comprise a large portion of inflammatory tumor microenvironment except tumor cells. Wu and colleagues have reported that dendritic cells represent one of primary sources of IL-1β secretion in the breast tumor microenvironment (15). In our 4T1-BALB/c orthotopic breast cancer mouse model, a much greater proportion of infiltrated and resident macrophages was observed in mouse primary tumor tissues than that of dendritic cells. Further, macrophage depletion significantly reduced serum levels of IL-1β as well as tumor growth and metastasis.

Through human breast cancer cell secretome analysis and a neutralizing experiment, we were able to identify sCD44, released by breast cancer cells, as a potential inducer of IL-1β secretion by macrophages. CD44 is a non-kinase transmembrane glycoprotein that is expressed in connective tissues and bone marrow as well as cancer cells. CD44 has been considered as one of the cancer-stemness markers, and contributes to cancer cell proliferation,

differentiation, migration, angiogenesis, and chemoresistance (33,34,46). In contrast to the well-known roles of cell-surface CD44 in cancer, the role of sCD44 in cancer development and progression has been poorly understood. High serum levels of sCD44 have been observed in several human malignancies, including colon cancer, breast cancer, gastric cancer, and liver cancer (36,47-49). In our present study, high concentrations of sCD44 were also detected in the serum of breast cancer patients compared to the healthy control. Notably, MDA-MB-231 and MDA-MB-468 TNBC cells released much larger amounts of sCD44 than non-TNBC cells. The existence of TNBC cells in the breast tumor microenvironment represents poor prognosis and aggressive behavior, which are characterized by a larger tumor size, a higher tumor grade, and enhanced dissemination to distant organs (50,51). Therefore, increased secretion of sCD44 might contribute to an aggressive phenotype of TNBC in breast cancer patients.

Although sCD44 is a key molecule responsible for breast cancer cell-driven IL-1β secretion from macrophages in the breast tumor microenvironment, we do not exclude the possibility of involvement of other factors in this process. Indeed, a recent study has revealed that breast cancer

cell-derived TGF-β induces IL-1β secretion from dendritic cells (15). In addition, Wellenstein *et al*. reported WNT ligands-induced IL-1β secretion (52). Deletion mutation of *TP53* induced secretion of WNT ligands from breast cancer cells, and released WNT ligands stimulated IL-1β secretion from tumor-associated macrophages, thereby driving systemic inflammation for breast cancer metastasis (52). Therefore, in addition to sCD44, inflammatory tumor microenvironment can be established by other soluble mediators (e.g., TGF-β and the WNT ligand) derived from other cancer cells.

Given the importance of IL-1β and inflammasomes in tumor progression, several clinical and pre-clinical trials targeting IL-1 have been attempted to treat breast cancer as well as other cancers malignancies. Of the approved IL-1 blocking regimens, administration of anakinra (IL-1 receptor antagonist) provided a significantly increased survival benefit for patients with metastatic colorectal cancer and pancreatic cancer (53,54). In targeting metastatic breast cancer using anakinra with one of the chemotherapeutics (nab-paclitaxel, eribulin, or capecitabine), 2 of 11 patients showed a considerably reduced tumor size and *IL1B* gene expression in peripheral blood leukocytes (15). In addition, canakinumab, approved as an anti-IL-1β

neutralizing mAb, significantly reduced the incidence of lung cancer and showed 77% reduction in mortality of lung cancer in patients with atherosclerosis (55). Although IL-1β targeting cancer therapy showed good therapeutic outcome in some cancers, there is still an increased risk of infection resulting from blockade of IL-1β because of its fundamental roles in innate immunity. Therefore, precise timing and dosage of IL-1β blocking agents should be determined before applied to cancer patients. In this context, regulation of cancer cell-induced IL-1β production might be a better option.

In conclusion, the present work demonstrates that the breast cancer cell-derived soluble mediator, sCD44 induces IL-1β secretion by macrophages in the breast tumor microenvironment, which promotes breast cancer progression and metastasis (Fig. 20). Accordingly, targeting the sCD44-IL-1β axis would be considered as an alternative promising strategy for the immunotherapy of breast cancer progression.

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CONCLUSION

Tumorigenesis is not only attributable to genetic alterations within malignant or pre-malignant cells, but also involvement of tumor microenvironment. In particular, inflammatory tumor microenvironment is one of the hallmarks of cancer, a large proportion of tumor microenvironment is composed of inflammatory cells and mediators. Interaction of the cells in tumor microenvironment is recognized as an important regulator of all stages of tumor development.

One of the effective pro-inflammatory cytokines to induce systemic inflammation in tumor microenvironment is IL-1β which is produced upon the inflammasome activation. Due to the fundamental roles of IL-1β, the IL-1β is an indispensable cytokine to maintain body homeostasis in innate immune system, and uncontrolled expression of IL-1β is closely associated with various immune related diseases as well as cancer. However, the effects of IL-1β on breast cancer progression are still controversial in breast tumor microenvironment. Therefore, in the present study, I attempted to elucidate the

roles of IL-1β and regulatory mechanism that dictates overproduction of IL-1β in breast tumor microenvironment.

Inflammatory context was observed in stage III breast cancer patients with high levels of pro-inflammatory cytokines including IL-1β. In syngeneic orthotopic breast cancer mouse model, IL-1β was contributed to breast cancer progression, and IL-1β-IL-1R1 signaling axis played tumor progressive roles in breast tumor microenvironment. Various types of cells are able to release IL-1β. In breast tumor microenvironment, breast cancer cells were excluded from the cellular source for secretion of IL-1β due to the undetectable levels of IL-1β into the extracellular. The secretion of IL-1β, however, was potently induced by the interaction of breast cancer cells with human monocytic THP-1 cells through soluble mediators, and TNBC cells released soluble factors activated the inflammasomes and released IL-1β from macrophages. In syngeneic orthotopic breast cancer mouse model, macrophage depletion significantly decreased serum IL-1β and breast cancer progression. In human breast cancer cell secretome analysis, 1,560 proteins were analyzed, and CD44 was the most differentially released proteins by TNBC cells than non-TNBC

cells. Indeed, significantly increased serum CD44 was observed in breast cancer patients. The sCD44 was involved in IL-1β secretion from macrophages, inhibition of CD44 biological activity significantly reduced progression and metastasis of breast cancer in orthotopic breast cancer mouse model.

Taken together, IL-1β-engaged oncogenic signaling triggered by breast cancer cell membrane-derived soluble CD44 (sCD44) antigen induces breast cancer progression, and targeting sCD44 antigen may provide an alternative therapeutic strategy for breast cancer treatment by modulating inflammatory tumor microenvironment. The vicious circle between breast cancer cells and macrophages is operated by these two soluble mediators, sCD44 and IL-1β.

국 문 초 록

종양미세환경은 암세포, 내피세포, 섬유아세포, 골수유래세포, 암-연관 섬유아세포, 면역세포 등 다양한 세포들로 이루어져 있고, 이러한 종양미세환경 내에서의 이질성(heterogeneity)은 종양이 갖는 특징 중 하나이다. 그중 높은 비율의 염증성 세포와 염증 매개 물질들의 존재에 따른 염증성 종양미세환경은 많은 종류의 암이 가지는 또 다른 특징 중 하나이며, 이들이 분비하는 사이토카인(cytokine), 키모카인(chemokine), 성장인자(growth factor) 등을 통한 상호작용은 종양의 증식 및 발달, 전이에 중요한 역할을 수행한다고 알려져 있다.

인플라마좀(inflammasome)은 거대 단백질 복합체로서 외부로부터의 병원균의 감염에 의한 pathogen-associated molecular patterns (PAMPs), 혹은 손상된 조직이나 세포들이 분비하는 damage-associated molecular patterns (DAMPs)과 같은 비 미생물 성분의 자극에 의해 IL-1β를 분비함으로써 염증반응을 촉진시킨다. IL-1β는 강력한 전 염증성 사이토카인 중 하나로 감염 부위로 다양한 염증성 세포들을 불러들여 선천면역반응을 증폭시킴으로써, 생체 항상성 유지와 방어기전으로 필수적인 역할을 수행한다. 면역반응에 있어서 인플라마좀과 IL-1β가 가진 이러한 필수적인 역할로 인해, 비 특이적인 발현의 인플라마좀과 IL-1β는 암을 비롯한 다양한 면역관련 질환과 밀접한 관련이 있다고 보고되었다. 하지만 이러한 비 특이적인 발현의 인플라마좀과 IL-1β가 유방 종양의 발달 및 증식, 전이에 미치는 영향 및 그 역할에 관해서는 아직 논란이 있으며, 유방 종양 미세환경에서 비 특이적인 인플라마좀과 IL-1β의 발현 기작에 관해서는 아직 연구가

미비한 실정이다. 따라서, 본 논문에서는 IL-1β가 유방암의 증식 및 전이에 미치는 영향과 유방 종양 미세환경에서 IL-1β의 분비 기작에 관해 연구를 진행하였다.

먼저 유방암과 염증성 미세환경사이의 관련성을 확인하기 위하여, 유방암 환자와 정상 대조군의 혈청 내 염증성 사이토카인을 확인한 결과, 전 염증성 사이토카인인 IL-1β, IL-6, IL-18, TNF-α 모두 유방암 환자의 혈청 내에서 정상 대조군에 비해서 월등히 높은 수치를 보였고, 특히, 그 중에서 IL-1β의 경우에는 정상 대조군 보다 약 40 배 이상의 증가된 평균 수치를 보였다. 이와 유사하게, 4T1-BALB/c 동소위 유방암 마우스 모델에서 4T1 세포를 이식하지 않은 정상 마우스와 비교하였을 때, 유방암이 발생한 마우스의 혈청 내 IL-1β가 정상 마우스에 비해 증가되는 것을 확인하였다. 또한, IL-1 수용체가 결핍된 4T1 세포를 이용한 4T1-BALB/c 동소위 유방암 마우스 모델에서 감소된 원발성 유방 종양의 증식과 폐 전이가 확인되었다. 위 결과들을 미뤄보았을 때, 유방암의 발달 및 진행, 전이는 IL-1β-IL-1 수용체를 통한 신호전달 체계와 밀접한 관련이 있다는 것을 시사한다. 유방 종양 미세환경에서 증가된 IL-1β의 출처를 확인하기 위해 실시한 동시 배양 실험과 유방암세포주 배양액 처리 실험을 통해서 삼중 음성 유방암 세포(triple negative breast cancer)가 분비한 수용성 물질에 의해 대식세포의 인플라마좀 활성화를 통해 IL-1β의 분비가 증가된 것을 확인하였다. 또한, 4T1-BALB/c 동소위 유방암 마우스 모델에서 대식세포를 결핍시켰을 때, 마우스 혈청 내 IL-1β 농도가 감소하였고, 원발성 유방 종양의 크기와 폐 전이가 모두 감소하였다. 이는 유방 종양

미세환경에서 증가된 IL-1β는 유방암 세포가 분비하는 수용성 물질에 의해 대식세포에서 IL-1β의 분비가 촉진된 것임을 시사한다.

유방암 세포주가 분비하는 수용성 물질 중 대식세포에서 IL-1β의 분비를 증가시키는 물질을 동정하기 위하여 인간 유방암 세포주의 secretome 을 분석한 결과, CD44 가 비 삼중음성유방암 세포주와 비교하였을 때 삼중 음성 유방암 세포주에서 가장 많이 분비되는 것을 확인하였고, 또한 유방암 환자의 혈청 내 CD44 의 농도가 정상 대조군에 비해 월등히 높은 수준을 보였다. 대식세포에서 IL-1β의 분비는 CD44 를 중성화 시킨 삼중 음성 유방암 세포주의 세포 배양액을 대식세포에 처리하였을 때 유의미하게 감소하였고, 4T1-BALB/c 동소위 유방암 마우스 모델에서 CD44 를 중성화 시켰을 때 원발성 유방 종양의 증식과 폐 전이, 그리고 혈청 내 IL-1β의 농도가 감소한 것을 확인하였다. 위 결과들을 종합했을 때, 유방 종양 미세환경에서 유방암 세포주의 세포막으로부터 분비된 CD44 는 대식세포에서 인플라마좀의 활성화를 일으키고, 그로 인해 증가된 IL-1β의 분비로인하여 유방암의 발달 및 진행, 그리고 전이가 촉진된다는 것을 알 수 있다. 따라서 CD44 의 표적 치료는 CD44 가 지닌 유방 종양의 악성화에 미치는 영향뿐만 아니라, IL-1β에의해 유도된 염증성 종양미세환경의 조절을 통한 유방 종양의 증식 및 전이 억제 효과까지 기대할 수 있다.

Key words

Inflammatory tumor microenvironment; Triple negative breast cancer; Macrophages; Inflammasomes; Interleukin-1β; Soluble CD44

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AWARDS

Young Investigator Award (Oral presentation) Dec. 2019

The Korean Society of Cancer Prevention

(Breast cancer cell-derived soluble CD44-induced IL-1β secretion from macrophage: Implications for breast tumor progression

Best Presentation Award (Oral presentation) Jul. 2019

The Korean Society of Cancer Prevention

(Cooperative crosstalk between macrophages and cancer cells for inflammasome activation in breast tumor microenvironment)

Young Investigator Award (Oral presentation) Jul. 2018

The Korean Society of Cancer Prevention

(Crosstalk between breast cancer cells and macrophages through interaction mediators in tumor microenvironment induces inflammasome activation

Young Scientist Award (Oral presentation) Aug. 2017

The 12th International Conference & 5th Asian Congress on Environmental Mutagens

(Interaction between breast cancer cells and macrophages induces inflammasome activation: Implications for poor prognosis)

Young Scientist Award Jul. 2014

The Korean Society of Cancer Prevention

(AME is a mammalian target of rapamycin kinase inhibitor on EGF-induced neoplastic cell transformation)

ORAL & POSTER PRESENTATION

2019 The Korean Society of Cancer Prevention Meeting in South Korea (Cooperative crosstalk between macrophages and cancer cells for inflammasome activation in breast tumor microenvironment)	Oral	Jul. 2019
2018 AACR Annual meeting at Chicago in U.S.	Poster	Apr. 2018

(Crosstalk between breast cancer cells and macrophages in tumor microenvironment induces inflammasome activation)		
2018 The Korean Society of Cancer Prevention Meeting in South Korea (Crosstalk between breast cancer cells and macrophages through interaction mediators in tumor microenvironment induces inflammasome activation	Oral	Jul. 2018
24th IUBMB Congress & FAOBMB Congress at Seoul in South Korea (Inflammasome activation through crosstalk between breast cancer cells and macrophages in tumor microenvironment)	Poster	Jun. 2018
The 12th International Conference & 5th Asian Congress on Environmental Mutagens at Incheon in South Korea (Interaction between breast cancer cells and macrophages induces inflammasome activation: Implications for poor prognosis)	Oral	Aug. 2017
The 13th Asia Pacific Federation of Pharmacologist Meeting at Bangkok in Thailand (Aschantin, an ATP-competitive and specific inhibitor of mTOR kinase)	Poster	Feb. 2016
2014 The Korean Society of Cancer Prevention Meeting in South Korea	Poster	Jul. 2014

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