Proceedings of Anticancer Research, 2022, Volume 6, Issue 6
http://ojs.bbwpublisher.com/index.php/PAR
ISSN Online: 2208-3553
ISSN Print: 2208-3545

Macrophage CCL22 Secretion Under Hypoxic Conditions Promotes the Metastasis of Triple-Negative Breast Cancer

Wenming Zhao, Bingjuan Zhou, Jirui Sun, Haizhi Qiao, Jinmei Li, Qiushuang Ma, Xu Wang, Jinku Zhang*

Department of Pathology, Baoding First Central Hospital, Baoding 071000, Hebei Province, China

***Corresponding author:** Jinku Zhang, zjkblk@sina.com

Abstract: *Objective:* To explore the mechanism by which macrophages secrete CCL22 to promote the metastasis of triple-negative breast cancer under hypoxic conditions. *Methods:* 20 ng/mL mass concentration of phorbol 12-myristate 13-acetate (PMA) cell culture medium, 4′,6-diamidino-2-phenylindole (DAPI), dimethyl sulfoxide (DMSO), trypsin digestion solution, CCL18 Kit , Interleukin (IL)-10 Kit, CCL17 Kit, CCL22 Kit, TRIzol™ Reagent Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Kit, triple-negative breast cancer cells MDA-MB-231 and BT-549, as well as other reagents were used to culture triple-negative breast cancer (TNBC) cells MDA-MB-231 and BT-549 as well as human mononuclear cells THP-1, analyze and observe the metastasis of triple-negative breast cancer cells to the lungs, the secretion of CCL22, the migration of triple-negative breast cancer, and the situation of CCR4. *Results:* Compared with normal tumor-associated macrophage (TAM), hypoxic TAM further promoted the migration of tumor cells. The number of tumor metastases in the lungs, induced by hypoxic TAM, was significantly higher than that of normoxic TAM. Hypoxia can significantly stimulate the expression of CCL22. CCL22 can significantly promote the migration of MDA-MB-231 and BT-549 cells. The expressions of CCR3, CCR4, and CCR5 in tumor tissues were significantly increased compared with normal tissues, in which CCR4 showed the most significant increase. *Conclusion:* TAM cultured under hypoxia significantly enhanced the migration ability of TNBC cells and promoted the metastasis of cancer cells to the lungs *in vivo*. The hypoxic condition induced TAM to secrete CCL22; the expression of CCL22 receptor, CCR4, in breast cancer tissues was significantly higher than that in normal tissues.

Keywords: Hypoxia; Macrophages; Triple-negative breast cancer; CCL22

Online publication: November 16, 2022

1. Introduction

Breast cancer is one of the malignant tumors with the highest incidence in women worldwide. There are more than 1.5 million new cases of breast cancer every year across the globe. It threatens the safety of female patients [1]. Although the mortality rate of patients has significantly dropped with the use of various comprehensive treatments, approximately 23.67% of women still die from tumor metastasis or recurrence, in which invasion and metastasis are the main causes of death [2]. The pathogenesis of breast cancer has not been fully elucidated, and its corresponding molecular mechanism also lacks strong evidence. The internal environment of a tumor is known as the tumor microenvironment (TME). A large number of studies have confirmed that TME plays a crucial role in the occurrence and development of breast cancer [3]. Macrophages are an important part of the body's innate immune response, and they are a heterogeneous cell population with high plasticity. Macrophages can be recruited around tumors under the action of

chemokines and cytokines [4]. Stimulated by cytokines in the TME, it polarizes into different types of tumor-associated macrophages (TAMs) [5]. Generally speaking, macrophages can be polarized into M1 or M2 macrophages. M2 macrophages are activated by Th2 cytokines, such as interleukin (IL)-4, IL-10, and IL-13. These alternatively activated macrophages are able to produce IL-10, CCL17, CCL22, CCL18, and IL-1 receptor antagonist (Ra)/IL-1R inducers, promote angiogenesis, local tissue reconstruction and repair, as well as promote the occurrence, development, and metastasis of cancer cells. At the same time, M2 macrophages can also inhibit inflammatory responses by regulating M1 macrophage-mediated functions and adaptive immunity, thereby indirectly promoting tumor growth [6].

2. Materials and methods

2.1. Reagents

20 ng/mL mass concentration of phorbol 12-myristate 13-acetate (PMA) cell culture medium, 4′,6-diamidino-2-phenylindole (DAPI), dimethyl sulfoxide (DMSO), trypsin digestion solution, CCL18 Kit, IL-10 Kit, CCL17 Kit, CCL22 Kit, TRIzol^{TM} Reagent Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Kit, and triple negative breast cancer (TNBC) cells MDA-MB-231 and BT-549.

2.2. Methods

Cell culture: TNBC cells MDA-MB-231 and BT-549 and human mononuclear cells THP-1 were cultured, and the basal medium was ψ = 10% fetal bovine serum; they were divided into two groups, the normoxia group (37°C, ψ = 5% carbon dioxide (CO_2), and saturated humidity) and the hypoxia group (37°C, $V(N_2):V(CO_2):V(O_2)$ = 94:5:1 gas mixture was continuously pumped into the incubator to maintain saturated humidity). 1×10^6 THP-1 was inoculated into a 6-well plate, and PMA was added to each well to make the final mass concentration of 20 ng/mL. After culturing for 72 h, non-adherent cells were removed; the adherent cells were M0 macrophages. Transwell method was used to detect tumor cell migration, followed by RT-PCR detection. Computed tomography (CT) was used to determine tumor metastasis, while enzyme-linked immunosorbent assay (ELISA) was used to detect the concentration of cytokines.

2.3. Observation indicators

Lung metastasis, CCL22 secretion, migration of TNBC cells, and CCR4 of TNBC cells were observed and analyzed.

2.4. Statistical analysis

SPSS 18.0 was used for statistical analysis of the experimental data. All experiments were independently repeated at least three times. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and t-test was performed for data comparison and analysis. $P < 0.05$ indicated statistical significance.

3. Results

3.1. Macrophages promote triple-negative breast cancer cell migration under hypoxic conditions

Experiments were carried out according to the aforementioned methods, and after 48 h, the images were observed under the microscope as shown in **Figure 1**.

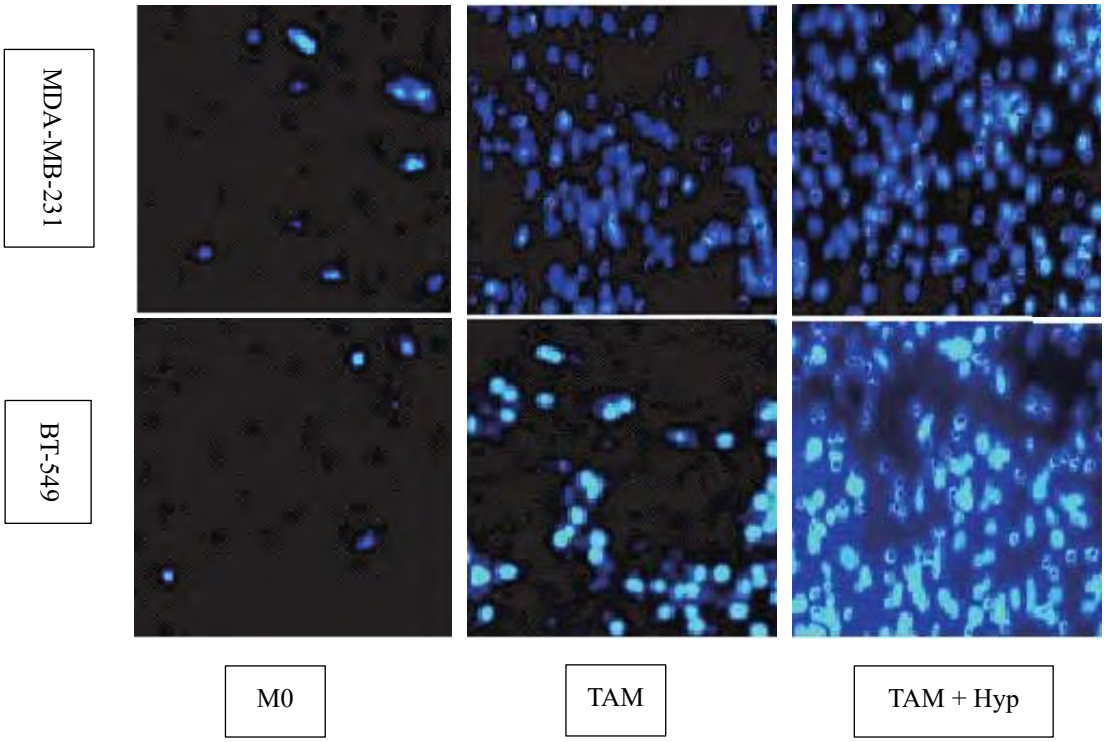

Figure 1. Transwell assay of triple-negative breast cancer cells treated with three conditional medias (CMs)

3.2. Macrophages promote lung metastasis of triple-negative breast cancer cells

Hypoxia-treated TAMs, which were cultured *in vitro* under hypoxic conditions, significantly induced lung metastasis of MDA-MB-231 cells. The number of metastases in the lung induced by hypoxia-treated TAMs were significantly higher than those of normoxia-cultured TAMs.

3.3. Hypoxia induces macrophage secretion of CCL22

CCL22 may be an important factor in the stimulation of TNBC metastasis by hypoxic TAM. In TAM, the expression of CCL18 was the highest, followed by CCL22 and IL-10 expressions, which were relatively low. Hypoxia significantly stimulated an increase in CCL22 expression, without any significant effects on the expressions of the other three cytokines.

3.4. CCL22 promotes lung migration of triple-negative breast cancer cells

10 ng/mL of CCL22 can significantly promote the migration of MDA-MB-231 and BT-549 cells (**Figure 2**).

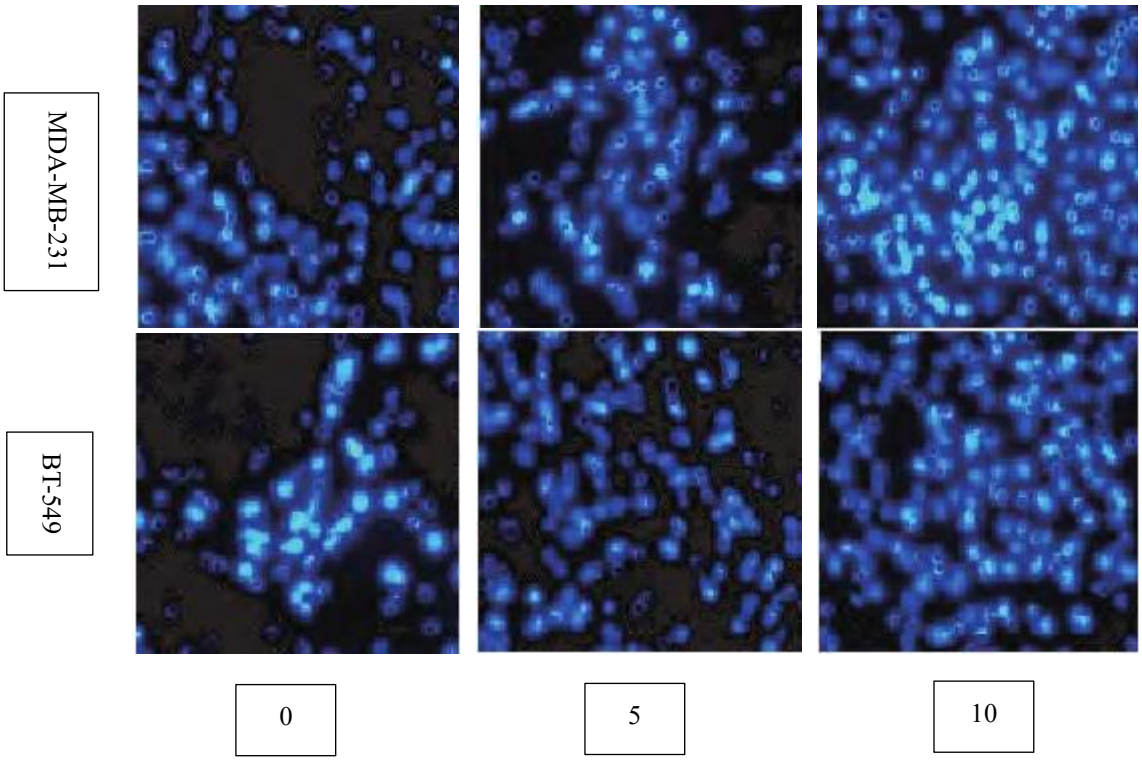

Figure 2. Transwell assay of triple-negative breast cancer cells induced by different concentrations of CCL22

3.5. CCR4 mediates macrophages to promote triple-negative breast cancer cell migration under hypoxic conditions

The combination of CCL22 and CCR4 plays an important role in promoting breast cancer metastasis. The expressions of CCR3, CCR4, and CCR5 were significantly elevated in tumor tissues compared with normal tissues, in which the elevation of CCR4 was the most significant, as shown in Table 1.

Table 1. Expression ratio of CCR family genes in breast cancer tissue and normal tissue

Gene	Normal tissue expression value (N)	Tumor tissue expression value (T)	T/N
CCR1	280	330	1.18
CCR2	99	110	1.11
CCR3	1.8	3.4	1.89
CCR4	14	50	3.33
CCR5	150	280	1.87

4. Discussion

Tumor-associated macrophages have been found to be associated with increased tumor progression and metastasis. They are the emerging targets for therapeutic intervention. Intratumoral macrophages can be derived from macrophages present in the tissue or from tumor-induced myeloid cells that migrate to the tumor and differentiate into macrophages. In early tumorigenesis, resident macrophages may be part of the first response, in which they bind to other innate immune cells to initiate an inflammatory response that can coordinate adaptive immune responses and, in some cases, promote progression. In established tumors, resident macrophages or bone marrow-derived macrophages (BMDMs) tend to polarize to an M2-type or activated phenotype rather than an inflamed M1 state. This M2 wound-healing phenotype promotes tumor progression and metastasis by secreting growth and angiogenic factors, remodeling the extracellular matrix,

and suppressing immune responses. In contrast, M1-activated macrophages inhibit tumor development and tumor growth through direct effects (*e.g*., secretion of reactive oxygen species [ROS]) or promotion of Th1 responses [7,8]. Macrophages have two functional phenotypes: classical activated macrophages, referred to as M1 macrophages, and alternative activated macrophages, referred to as M2 macrophages. In the pro-inflammatory circle, M1 macrophages are the main macrophages. The inducers of such macrophages include lipopolysaccharide (LPS), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), *etc*., which have the ability to present antigens, secrete a large amount of pro-inflammatory cytokines, such as IL-12, IL-23, and TNF-α, as well as other chemokines, produce nitric oxide (NO), and express inducible nitric oxide synthase, thus showing an inflammatory phenotype. Inducing factors of M2 macrophages include IL-4, IL-13, M-CSF, glucocorticoids, IL-10, *etc*. They participate in the body's balance process, such as the formation of new blood vessels, and the body's tissue remodeling and wound healing process. They highly express the anti-inflammatory cytokine IL-10 and characteristic factors, upregulate scavenger receptors and CD163, produce ornithine and polyamine, highly express Arg-1, and possess anti-inflammatory properties.

In most cases, there are two main strategies to achieve therapeutic goals by targeting TAMs. The first strategy is to reduce the density of TAMs in tumor tissue, while the second is to induce TAMs to switch from the M2 phenotype to the M1 phenotype. In breast cancer, high levels of IL-10, a marker for M2-type macrophages, have been detected *in vivo*. In addition, genetic mapping data have revealed that TAMs in breast cancer have M2-like properties. This M2-like polarization has even been observed in some cases of brain metastases from breast cancer [9], and the source of TAMs is largely dependent on the tumor type. In breast cancer models, newly recruited monocytes differentiate into TAMs, whereas in brain cancer models, TAMs originate from blood-derived monocytes and resident macrophages [10]. When TAMs are produced from monocytes recruited in the circulation, tumor cells need to secrete factors that prompt monocytes to migrate to the tumor site. In general, the phenotype of TAMs in the TME is highly plastic. In a study, human breast cancer cells were found to predispose TAMs to an anti-inflammatory phenotype by secreting M-CSF [11], whereas in an *in vivo* model of BALB/c4T1 breast cancer, tumor microenvironmental conditions promoted the differentiation of monocyte precursors into different subsets of TAMs [12-17]. These results suggest that the phenotype of TAMs differs depending on the tumor type and the activation process of TAMs is highly complex and dependent on the microenvironment in which they are located. The proportion of macrophages in tumor tissues can reach up to 50%, and the infiltration of a large number of tumor-associated macrophages is closely related to poor prognosis. Tumor-targeted therapy for macrophages has achieved excellent results in clinical trials, but its efficacy in clinical treatment is limited. At present, a variety of tumor-targeted therapy methods for tumor-associated macrophages are under investigations. The more important ones include inhibiting the recruitment of macrophages, activating the anti-tumor activity of macrophages, killing and clearing macrophages cells, as well as reversing the phenotype of macrophages.

Many cytokines promote the recruitment of tumor-associated macrophages. The CCL2/CCR2 axis plays a very important role in the recruitment of macrophages. Therefore, targeting this pathway could potentiate effective tumor therapy. In animal models, CCL2 blockers (carlumab, CNTO88) have been shown to inhibit the growth of various tumors. In addition, the chemokine CSF-1 has significant effects on the growth, differentiation, and recruitment of macrophages. Both CSF-1 (GW2580) and CSF-1R inhibitors can significantly reduce the recruitment and infiltration of M2-type cells, thereby achieving the purpose of developing specific targeted tumor therapy. Experiments have found that the activation of proto-oncogenes can increase the expression of CCL-2 and CSF-1 in mice with papillary thyroid carcinoma, leading to the accumulation of TAMs in the tumor area, thereby promoting tumor progression. Targeting the expression of chemokine receptors 2 (CCR-2) cells can reduce the number of TAMs and inhibit tumor progression.

This study showed that hypoxically cultured TAMs significantly enhanced the migration ability of TNBC cells and promoted the metastasis of cancer cells to the lung *in vivo*. TAMs were induced by hypoxic conditions to secrete CCL22. The expression of CCL22 receptor, CCR4, in breast cancer tissues was found to be significantly higher than that in normal tissues.

Funding

This work was supported by grants from The Medical Science Research Project of Hebei Province (20220282) and the Key Laboratory of Molecular Pathology and Early Diagnosis of Tumor in Hebei Province.

Disclosure statement

The authors declare no conflict of interest.

References

[1] Xu S, Zhang Z, Zhang X, et al., 2022, Study on the Correlation Between the Prognosis of Neoadjuvant Chemotherapy for Triple-Negative Breast Cancer and the Expression of Ki-67 and TOPK. Advances in Modern Biomedicine, 22(08): 1514–1518.

[2] Zhang M, Liu W, Wang L, et al., 2022, The Relationship Between BRCA Gene Mutation and the Prognosis of Triple-Negative Breast Cancer Targeted Therapy. Journal of Clinical Military Medicine, 50(04): 397–399.

[3] Yao S, Zhang J, Liu Y, et al., 2022, Expression and Clinical Significance of CD44v6 Before and After Neoadjuvant Chemotherapy in Triple-Negative Breast Cancer. China Coal Industry Medical Journal, 25(02): 162–166.

[4] Chen F, Liu F, Wu N, et al., 2022, Inhibitory Effect and Mechanism of Novel Recombinant Myricetin on Triple-Negative Breast Cancer. Journal of Bengbu Medical College, 47(04): 426–432.

[5] Jamiyan T, Kuroda H, Yamaguchi R, et al., 2022, CD68 and CD163 Positive Tumor-Associated Macrophages in Triple-Negative Breast Cancer. Journal of Clinical and Experimental Pathology, 38(03): 277.

[6] Lei H, 2022, MCM7 Gene Expression and Clinical Significance in Triple-Negative Breast Cancer Patients Before and After Neoadjuvant Chemotherapy. Chinese Journal of Gerontology, 42(06): 1337–1340.

[7] Li Z, He Y, Tang J, 2022, Expression of HIF-1α and MALAT-1 in Triple-Negative Breast Cancer and Their Correlation Analysis. Henan Journal of Surgery, 28(02): 58– 60.

[8] Zhong L, Wang J, Zheng H, 2022, Clinicopathological Significance of CD4, CD8, Foxp3 and PD-L1 Expression in Stromal Tumor-Infiltrating Lymphocytes of Triple-Negative Breast Cancer. Journal of Clinical and Experimental Pathology, 38(03): 278–283 + 288.

[9] Pei D, Li B, Dai X, et al., 2021, Expression of EGFR and VEGFR in Triple-Negative Breast Cancer and Their Correlation with Pathological Features. Jiangxi Medicine, 56(11): 1942–1945 + 1958.

[10] Zou Q, Zhou Y, Zhen Y, 2021, Expression and Prognosis of AR and ki67 in Triple-Negative Breast Cancer. Medical Diet and Health, 19(18): 160–161.

[11] Wang R, 2021, Lnc01094/miR-877-5p/Del-1 Axis Promotes the Occurrence of Malignant Phenotype in Triple-Negative Breast Cancer, thesis, China Medical University.

[12] Li L, Zhang Z, Pan B, et al., 2021, Based on PI3K/Akt/mTOR Signaling Pathway to Investigate the Effects of Shugan Jianpi Jiedu Recipe-Containing Serum on the Proliferation, Migration and Invasion of Triple-Negative Breast Cancer MDA-MB-231 Cells. Chinese Journal of Experimental Formulas, 27(15): 22–28.

[13] Zhang P, Zhong X, Zheng H, et al., 2021, Expression and Significance of Spindle Checkpoint Protein BUB1B in Triple-Negative Breast Cancer. Chinese Journal of Pathology, 50(06): 645–649.

[14] Tian X, 2021, Relationship Between PELP1/MNAR Expression and PIK3CA Gene Mutation in Molecular Subtypes of Breast Cancer, Datong Fifth People's Hospital, Shanxi.

[15] Mi X, 2021, Screening of EMT-Related RNAs in Exosomes of Triple-Negative Breast Cancer Stem Cells, Jilin Provincial People's Hospital, Jilin.

[16] Huang X, 2021, The Relationship and Significance of PD-L1 Expression and Epithelial-Mesenchymal Transition in Triple-Negative Breast Cancer, thesis, Kunming Medical University.

[17] Mi M, Wei X, Jiang H, et al., 2021, Research Progress on the Mechanism of LncRNA in the Occurrence and Development of Breast Cancer. Laboratory Medicine and Clinic, 18(06): 844–847.