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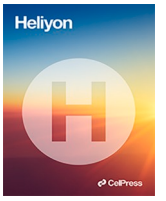

Chronic treatment with TNF-α, alone and in combination with Takinib, SB203580 and metformin induce cell death in breast cancer

Maryam Abdolvand [a], Milad Shahini Shams Abadi [b], Amin Soltani [a], Fatemeh Banisharif [b], Mahdi Ghatrehsamani [b,*]

[a] Department of Microbiology and Immunology, Shahrekord University of Medical Sciences, Shahrekord, Iran
[b] Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

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ABSTRACT

Breast cancer (BC) is the most common malignancy, and the largest cause of cancer death among women. The interactions between tumor cells and tumor micro environmental factors have a major impact on tumor progression. One of the critical pro-inflammatory cytokines present in breast cancer tumor microenvironment is TNF-α. The aim of this study was to evaluate the long-term effect of TNF-α (1 week) along with p38 or TAK1 inhibitors as well as metformin on induction of cellular death, cancer stem cell and expression of metastatic marker CXCR4. MCF-7 and MDA-MB-231 cells were treated with TNF-α for one week and then were treated with combination of Takinib, SB203580 or Metformin; after all treatments were done, cell proliferation, cellular death, surface expression of CXCR4, CD44 and CD24 were determined. The results showed that treatment with TNF-α alone or in combination with Takinib, SB203580 and metformin elevated induction of cellular death in both cell lines compared to the control group. TNF-α also increased CXCR4 expression in MCF-7 cells, but it reduced its expression in the MDA-MB-231 cells. Also, breast cancer stem cells (BCSCs) population decreased in MDA-MB-231 cells treated with TNF-α alone or in combination with SB203580 and metformin. Although, in MCF-7 cells only combination of TNF-α and Takinib reduced BCSCs population in a time dependent manner. Altogether, we showed that TNF-α alone or in combination with other treatments can affect the progression of breast cancer.

1. Introduction

Breast cancer is one of the main leading cause of death by cancer among women [1]. In 2020, breast cancer (BC) with over 2.3 million new diagnoses, is the most commonly diagnosed malignancy among women worldwide, accounting for nearly 24.5 % of all new cancer cases [2]. BC includes several subtypes with different genetic, molecular and clinical differences that lead to different proliferation and metastatic potential [3]. Treatment and patient treatments outcomes depend on the underlying BC subtype [4]. Despite the current treatment for breast cancer such as radiotherapy, surgery and chemotherapy, the rate of resistance to treatment and recurrence of the disease is still high [5]. Cancer is caused by a broad variety of genetic and epigenetic alterations that confer unique

* Corresponding author.
E-mail address: Mahdi.samani.2020@gmail.com (M. Ghatrehsamani).

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characteristics of cancer cells that enable them to display autonomous proliferation, invasiveness, cell-dead resistance, replicative immortality, immunity system escape, and metastatic potential [6]. In addition, the interactions between tumor cells with stromal components of the tumor microenvironment (TME) have a major impact on tumor progression and tumor fate [7]. Various mediators like inflammatory cytokines,which are common in TME, can govern tumor cell metastasis or cancer progression [8]. One of the critical pro-inflammatory cytokines present in the TME of breast cancer patients is TNF-α [9,10]. TNF-α is a double-edged sword for tumors, it causes tumor cells necrosis and apoptosis and strong immune responces, on the other hand it promotes tumor cells proliferation, survival, metastasis and invasion [11–14]. Nevertheless, the mechanism by which TNF-α promotes breast cancer stem cells (BCSCs) has not been fully elucidated. TNF-α also can activate TAK-1 [15]. TAK-1 is a serine/threonine kinase of mitogen-activated protein (MAP) kinase signaling cascade family [16]. TAK1 is an important mediator in inflammation/immune responses and cancer progression, by activating a list of downstream pathways, like P38 and AMP-activated kinase (AMPK) [17,18]. Interestingly AMPK activation inhibited CXCR4 upregulation, the chemotactic factor which induce tumor metastasis. Beside TAK1, metformin as an activator of AMPK seems to reduce tumor metastasis via CXCR4 inhibition. Thus, the aim of this study is to evaluate the chronic effect of TNF-α along with p38 and TAK1 inhibitors as well as metformin in inducing cell death, cancer stem cell count and expression of metastatic marker CXCR4.

2. Methods

2.1. Agents and antibodies

Soluble TNF-α was acquired from eBioscience. TAK1inhibitor (Takinib) was bought from BioVision. 3 [4,5dimethylthiazol2yl]-2,5diphenyl tetrazolium bromide (MTT), p38 inhibitor (SB203580) and Metformin were purchased from Sigma-Aldrich. Takinib and SB203580 were dissolved in Dimethyl sulfoxide (DMSO). FITC Annexin V and PI apoptosis detection kit (556,547, BD Biosciences, USA) was purchased from BD Biosciences. FITC-conjugated anti-CD24 antibody (Clone M1/69), PE -conjugated anti-CD44 antibody, (Clone IM7) and PE-Cyanine5-conjugated anti-CD184 (CXCR4) antibody (Clone 12G5) were obtained from eBioscience.

2.2. Cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from Pasteur Institute of Iran. These cell lines were maintained in RPMI 1640 (Gibco BRL) media which supplemented with 10 % fetal bovine serum (Gibco BRL, Gaithersburg, MD, U.S.A) and 100 mg/ml streptomycin and 100 mg/ml penicillin and were cultured in incubator at 37 °C with humidified atmosphere containing 5 % CO2.

2.3. Measurement of cellular viability

The cells were treated with TNF-α for one week. The cell culture medium were replaced in 3 days and TNF-α was added to each culture again. Then the cells were exposed to Takinib or SB203580 or Metformin for 24 or 48h separately. Cell viability was Assay via MTT method. The results reflect 3 independent experiments on average.

2.4. Quantification of apoptosis by annexin V labelling

Apoptosis and necrosis quantity (as a cell death) have been measured using Annexin V-FITC/PI detection kit (BD bioscience) based on the manufacturer's instructions. Briefly, the cells were suspended in the staining buffer and then stained with Annexin V-FITC/PI for the investigation of cellular death. Then, the cells were washed and investigated through a flow cytometer. Flowjo 10 software was used for analyzing data results.

2.5. Flow cytometry

To analyze the percentage of BCSC ($CD44^+$ $CD24^-$cells), FITC-conjugated anti-CD24, PE-Conjugated anti-CD44 and corresponding isotype control have been added to cell suspensions and incubated at 4 °C in darkness for 30 min. To evaluate the CXCR4 positive cells PE-Cy5-conjugated anti-CXCR4 and corresponding isotype control have been added to cell suspensions and incubated at 4 °C in darkness for 30 min. Afterward, the cells were washed and investigated through a flow cytometer. Flowjo 10 software was used for analyzing data results.

2.6. Statistical analysis

The displayed values are given as mean ± SD. The statistical analysis was done by one-way analysis of variance (ANOVA) with Tukey test using GraphPad Prism 8. In entire cases, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$ were considered to be statistically significant. Each trial was repeated three times.

3. Results

3.1. TNF-α induce cellular death in MDA-mb-231 and MCF-7 cell lines

The preceding study showed that TNF-α, SB203580 and Metformin at a concentration of 5 ng/ml, 10 μM and 10 mM, respectively; have optimal effects on breast cancer lines (21–23). The concentration of Takinib that used in previous research that had an optimal effect on MCF-7 cell line was 10 μM (24) but this dose was lethal for MDA-MB-231 cells; so we evaluated the IC50 of Takinib for MDA-MB-231 cells using MTT method. The appropriate concentration of Takinib for MDA-MB-231 cell line was as follows: for 24 h treatment, 7 μM and for 48 h treatment 4 μM.

The viability of cells that were treated with TNF-α alone or in combination with Takinib, SB203580 or Metformin were measured with the MTT test. As shown in Fig. 1, cell viability was not significantly changed after 24 h of treatment with a combination of TNF-α

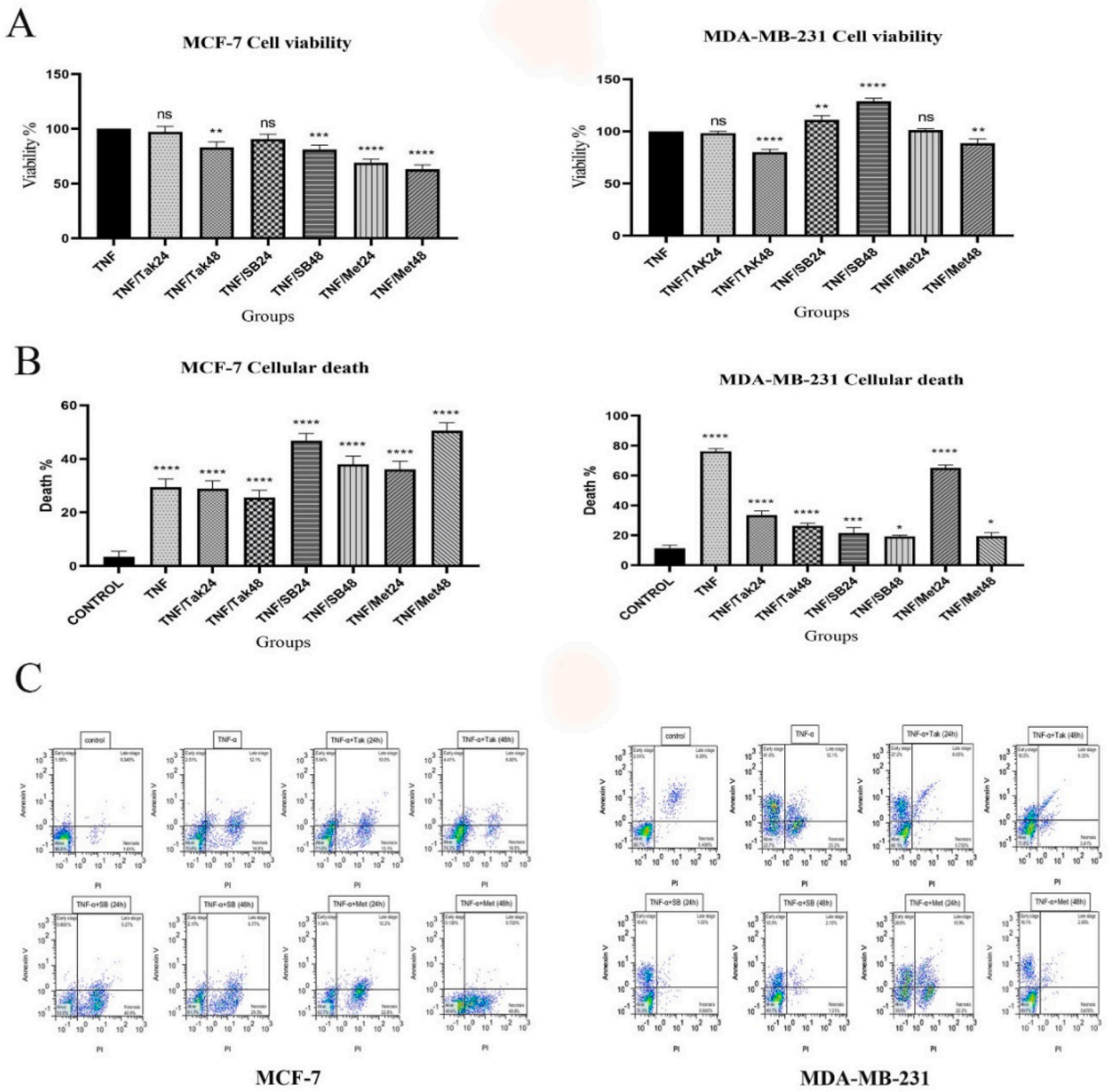

Fig. 1. Comparison of the cell viability **(A)** and cell death, apoptosis + necrosis, **(B)** in MDA-MB-231 and MCF-7 cells. Cell survival after treating with TNF-α for one week or its combination with SB203580 or Metformin or Takinib for 24 and 48 h. Example of the cell death quadrant in MCF-7 and MDA-MB-231 cells **(C)**. The data represent n = 3 (Mean ± SD). P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

and Takinib but decreased significantly after 48 h in MCF-7 and MDA-MB-231 cells ($P < 0.01$ and $P < 0.0001$, respectively) (Fig. 1A). Also, As shown in Fig. 1 cell viability was not significantly changed after 24 h of treatment with a combination of TNF-α and Metformin, but decreased significantly after 48 h in MDA-MB-231 cell line ($P < 0.01$) and it decreased in a time-depend manner in MCF-7 cells ($P < 0.0001$) (Fig. 1A). Although, combination treatment of TNF-α and SB203580 in MDA-MB-231 cell increased cell viability in a time-depend manner ($P < 0.01$ and $P < 0.0001$, respectively) but in MCF-7 cells this combination decreased cell viability after 48 h of treatment ($P < 0.001$) (Fig. 1A).

To study the effects of TNF-α on cellular death, the cells were treated with 5 ng/ml of cytokine for one week and then were exposed to Takinib, SB203580 or Metformin. TNF-α increased cellular death in both cell lines ($P < 0.0001$) (Fig. 1B). As shown in Fig. 1 cell death was significantly increased after in contrast to control group (Fig. 1B). Although, treatment with combination of TNF-α and Metformin increased cellular death higher than other combinatorial treatment.

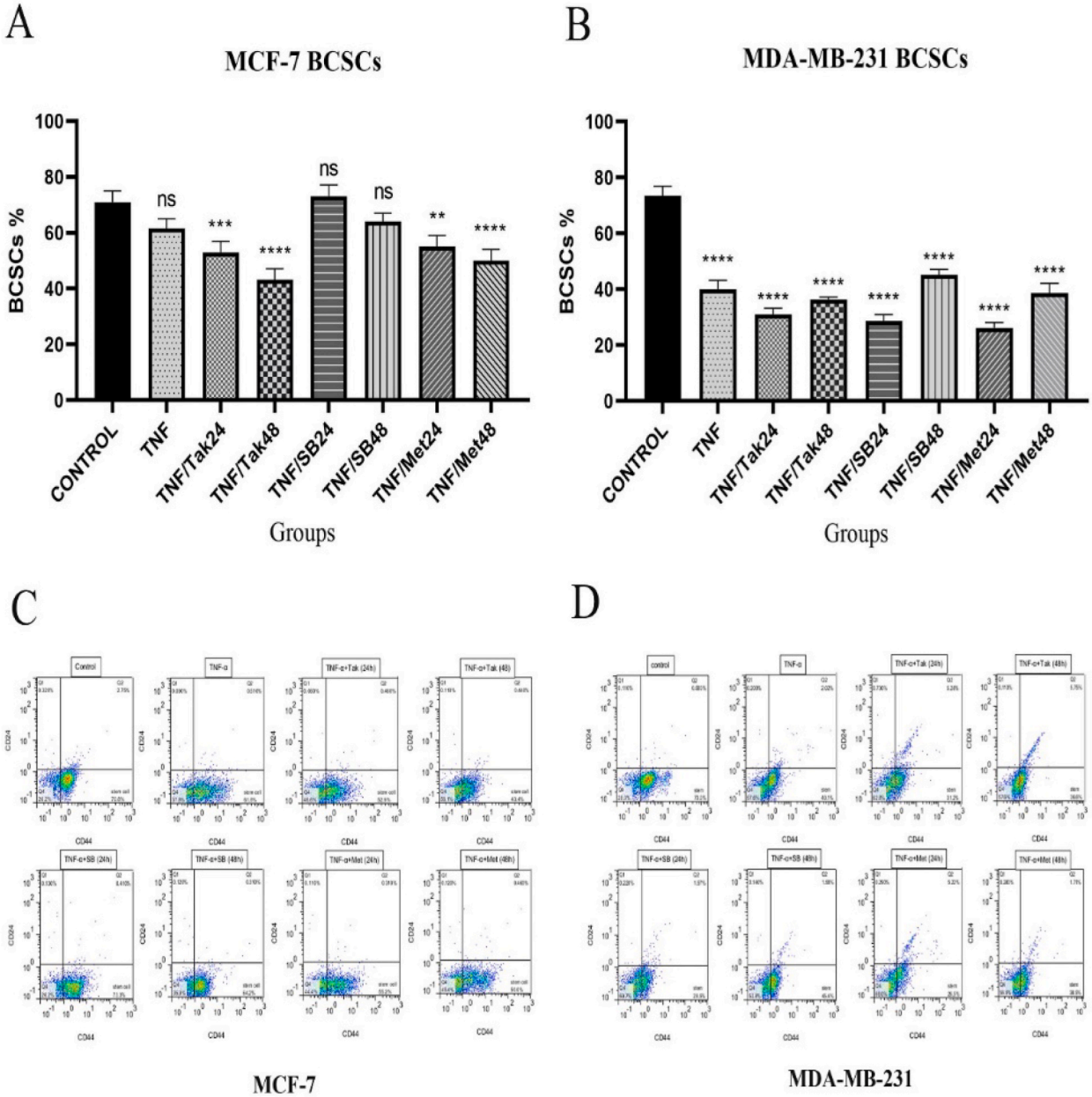

Fig. 2. Comparison of BCSCs ($CD44^+$ $CD24^-$ markers) population in MCF-7 cells **(A)** and in MDA-MB-231 cells **(B).** BCSCs population after treating cells with TNF-α for one week or its combination with SB203580 or Metformin or Takinib for 24 and 48 h. Example of the BCSCs population in MCF-7 and MDA-MB-231 cells **(C) & (D)**. The data represent n = 3 (Mean ± SD). $P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3.2. TNF-α effect on BCSCs

As shown in Fig. 2 TNF-α single treatment didn't changed BCSCs population in MCF-7 cells significantly (Fig. 2A), but it decreased BCSCs in the MDA-MB-231 cells ($P < 0.0001$) (Fig. 2B). Although, combination of TNF-α + Metformin induced a significant decrease in BCSC population in MCF-7 (Fig. 2A). Also, all single or combinatorial treatment of TNF-α induced a significant decrease in BCSC population in MDA-MB-231 cell line (Fig. 2B).

3.3. TNF-α effect on CXCR4 expression

TNF-α elevated CXCR4 expression on MCF-7 cells ($P < 0.0001$) (Fig. 3A) and decreased CXCR4 expression on MDA-MB-231 cells (P

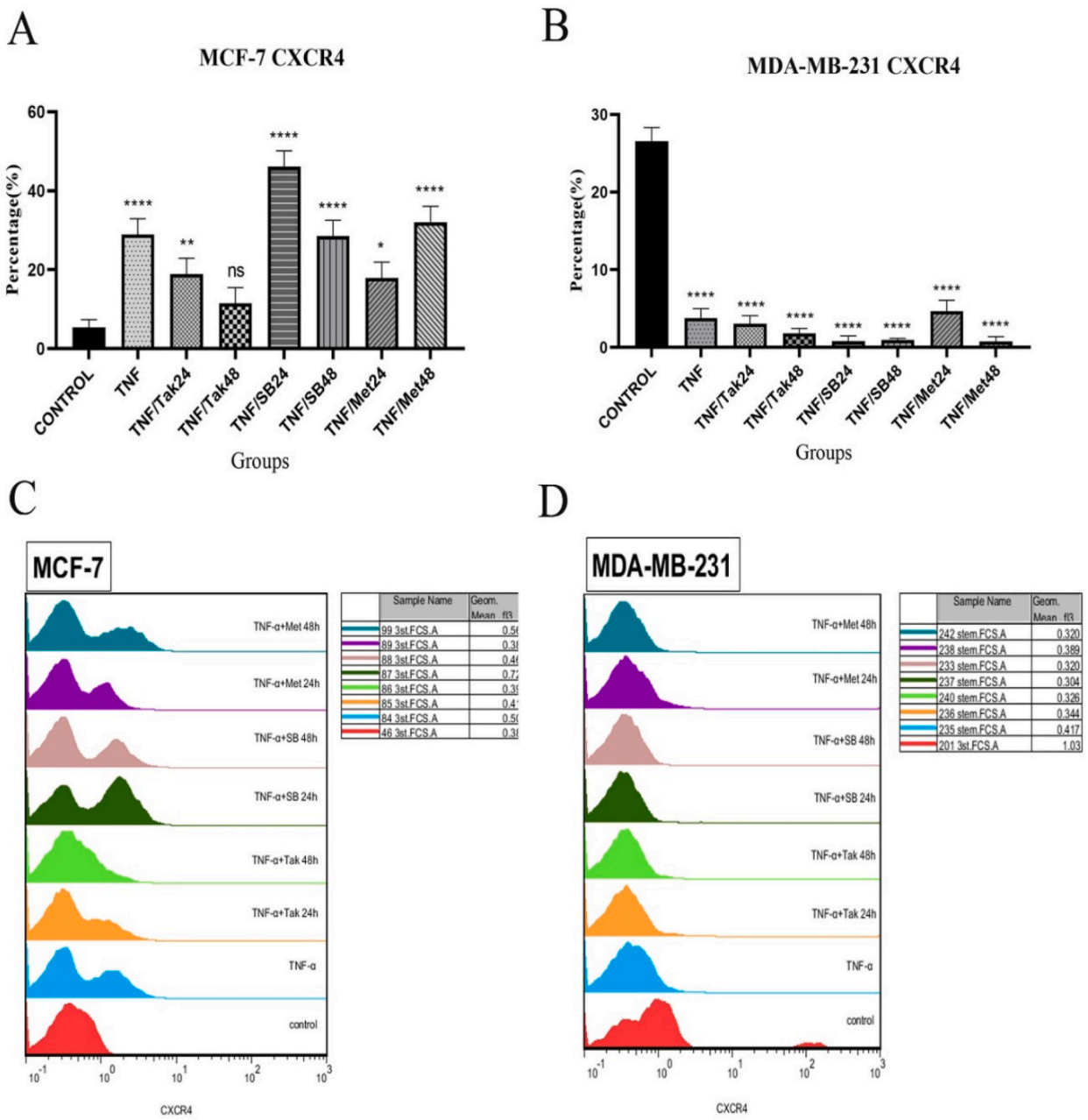

Fig. 3. CXCR4+ population after treating cells with TNF-α for one week or its combination with SB203580 or Metformin or Takinib for 24 and 48 h compared to control (untreated) cells. Percentage of CXCR4+ population in MCF-7 cells **(A)** and in MDA-MB-231 cells **(B).** Example of the CXCR4+ population in MCF-7 **(C)** and MDA-MB-231 cells **(D)**. The data represent n = 3 (Mean ± SD). $P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

< 0.0001) (Fig. 3B). While, combination of TNF-α + Takinib 48h treatment reduced CXCR4 expression on MCF-7 cells and make it's elevation insignificant, but all other treatment increased CXCR4 expression on MCF-7 cells significantly, (Fig. 3-A). On the other hand, treatment with single TNF-α or its combination with SB203580 or Metformin or Takinib reduced CXCR4 expression on MDA-MB-231 cells (Fig. 3B).

4. Discussion

Various kind of cytokines and other factors produced by tumor cells, stroma cells and tumor infiltrate immune cells; provide positive and negative feedback on tumor growth, progression, differentiation and metastasis [19]. One of the famous tumor environment factor is TNF-α, the pro-inflammatory cytokine which has been reported elevated even in the blood of breast cancer patients [20,21]. TNFα, performs its function by activating NF-κB and some other transcription factors, which regulates survival, proliferation or apoptotic [21]. Previous study showed that, TNF-α single treatment for one week, elevated cell death compared to the control group. As well, Nicholas and colleagues observed that TNF-α reduces survival, cell cycle arrest and induces apoptosis in the MCF-7 cancer cells in a time-dependent manner [22]. Pileczki et al. Showed that TNF-α gene deletion was associated with inhibition of cell proliferation and cellular death in TNBC cells [23]. Our results show that one week exposure of the breast cancer cells with TNF-α induced cellular death and decrease BCSCs population and CXCR4 expression in MDA-MB-231 cells but increase CXCR4 positive cells in MCF-7 cells. Thus, based on the expression of CXCR4, it seems presence of TNF-α in the breast tumor environment reduce metastasis of TNBC (MDA-MB-231) and increase metastasis of MCF-7 cells. Although, combination of TNF- α and Takinib for 48 h reduce the BCSCs population and CXCR4 expression in MCF-7 cells. Thus, TAK-1 inhibitor, Takinib, is much efficient in MCF-7 cells rather than TNBC cells. Thus, induction or treatment with TNF-α in combination with Takinib can be a potential treatment for breast cancer. TNF-α signaling can activate Transforming growth factor-β-activated kinase-1 (TAK-1) [15]. TAK-1 is a serine/threonine kinase of MAP kinase family that can phosphorylate and activate regulate various cellular processes, like differentiation, proliferation, metastasis and apoptosis [24].

AMPK was originally identified as a cell energy sensor and plays a crucial role in homeostasis of cellular energy. Recent research has shown that AMPK does its functions with phosphorylation and regulation of several signaling molecules downstream in normal tissues. Decreased level of AMPK or downregulation of its activity engaged in promoting of breast tumorigenesis, hence the activation of AMPK found to have benefit effects on tumor prognosis [25]. Metformin (1,1-dimethylbiguanide hydrochloride), a famous AMPK activator is a common diabetic patients' treatment. It has been shown that the risk of breast cancer has been decreased in diabetic patients who received metformin than those treated with other antidiabetic drugs [26,27]. We observed that metformin has an additive effect on TNF-α role in elevation of cellular death or reduction of BCSCs in both cell line but this combination therapy induces CXCR4 expression on MCF-7 cells. Takinib role in inhibition of CXCR4 expression may increase potential of this combination therapy in breast cancer and reduce the possible side effect like metastasis.

Inhibition of p38 is a strategy for improvement of immune response and reversion of immune senescence [28]. Nevertheless, SB203580 a specific p38 inhibitor may potentiate immune response but our finding shows that p38 inhibitor can induce breast tumor progression. Although, inhibition of P38 via SB203580 may not be a good candidate for direct tumor cells therapy but it may increase immune responses to tumor cells which is needed further research to demonstrate its beneficial or adversary effects. In addition, the combination of TNF-α + SB203580 increase cellular death in MCF-7 cells but it also highly increased CXCR4+ population in MCF-7 cells.

CXCR4 is the most commonly expressed chemokine receptor on cancer cells; that has been uncovered, in over 23 types of human tumors such as kidney, lung, brain, breast and prostate cancers [29]. CXCR4 was the first chemokine receptor diverted through breast cancer cells to proliferate and metastasize to distant organs [30]. In line with the results of Hamaguchi et al. Who stated that TNF-α, by positively regulating CXCR4, causes metastasis and invasion of breast cancer cells [31]; our study have shown that TNF-α induce CXCR4 expression on MCF-7 cells. Though, as mentioned above, Takinib can antagonize its involvement in CXCR4 expression after 48h. But in the MDA-MB-231 cells, combined treatments of TNF-α (TNF-α + Takinib, TNF-α + SB203580 and TNF-α + metformin) in the MDA-MB-231 subtype, similar to TNF-α single treatment, caused a decrease in CXCR4. Indeed, the combination of TNF-α + Takinib can be a candidate treatment that reduces resistant BCSCs population and resuces CXCR4 expression cells in both cell lines.

Metformin has been reported to inhibit the EMT process, which leading to induce CSCs, and limiting CSCs self-renewal properties [32]. TAK-1 is NF-κB enhancer, and previous studies shown that inhibiting NF-κB may reduce EMT-related events, It is therefore confirm that inhibition of TAK-1 leads to the reduction of the EMT process [33,34]. In breast cancer BSCSs are the reasons for resistance to therapy or disease relapsing is the presence of BCSCs inside the cancerous tissue increase the capacity of self-renewal and differentiation of tumor cells, and also may be responsible for initiation of the tumor. These cells are known to be highly resistant to chemotherapy and radiotherapy [35]. In our study, one week treatment of MCF-7 cells with TNF-α did not change the amount of cancer stem cells, but decreased the BCSCs in MDA-MB-231 cell. This findings were in contrast to previous studies that have shown that TNF-α causes BCSC phenotype in breast cancer and increases the self-renewal ability and invasion of BCSCs [36,37]. However, on of important difference between these studies was the duration of treatment, in previous studies the time of TNF-α exposure was different from our study, which could indicate a difference in the results. Although, many study survey the tumor environmental factor after a while, but we should consider that tumor is a chronic disease situation and it cells encountered to various kind of tumor environmental factor chronically.

Thus, long term environment factors effect can reveal more logical and real data than short term treatment study. In our study, one week exposure of breast cancer cells with TNF-α showed the benefactor effect on elevation of cellular death, reduction of BCSCs population and CXCR4 expression (except MCF-7 cells). Takinib as a TAK-1 inhibitor showed the additive effect in combination with

TNF-α. It also suppresses expression of CXCR4 on MCF-7 cells induced by TNF-α.

5. Conclusion

Altogether, TAK-1 inhibitor (Takinib) is a potential product that can prevent breast cancer progression especially when TNF-α present in tumor micro environment. In addition, in both cell line Takinib in combination with TNF-α decreased CSCs population.

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Data availability statement

We like sharing all research data which may help other researchers evaluate our findings.

CRediT authorship contribution statement

Maryam abdolvand: Data curation, Methodology. **Milad Shahini Shams Abadi:** Formal analysis, Validation. **Amin soltani:** Formal analysis, Software, Supervision, Validation. **Fatemeh banisharif:** Investigation, Validation, Writing – review & editing. **Mahdi Ghatrehsamani:** Conceptualization, Funding acquisition, Project administration, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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