DIF-1 exhibits anticancer activity in breast cancer via inhibition of CXCLs/CXCR2 axis-mediated communication between cancer-associated fibroblasts and cancer cells

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Abstract

The tumor microenvironment (TME), largely composed of tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs), plays a key role in cancer progression. A small molecule, differentiation-inducing factor-1 (DIF-1) secreted by *Dictyostelium discoideum,* is known to exhibit anticancer activity; however, its effect on the TME remains unknown. In this study, we investigated the effect of DIF-1 on the TME using mouse triple-negative breast cancer 4T1-GFP cells, mouse macrophage RAW 264.7 cells, and mouse primary dermal fibroblasts (DFBs). Polarization of 4T1 cell-conditioned medium-induced macrophage into TAMs was not affected by DIF-1. In contrast, DIF-1 decreased 4T1 cell co-culturing-induced C-X-C motif chemokine ligand 1 (CXCL1), CXCL5, and CXCL7 expression in DFBs and suppressed DFB differentiation into CAF-like cells. Additionally, DIF-1 inhibited C-X-C motif chemokine receptor 2 (CXCR2) expression in 4T1 cells. Immunohistochemical analyses of tumor tissue samples excised from breast cancer-bearing mice showed that DIF-1 did not affect the number of CD206-positive TAMs; however, it decreased the number of α-smooth muscle actin-positive CAFs and CXCR2 expression. These results indicated that the anticancer effect of DIF-1 was partially attributed to the inhibition of CXCLs/CXCR2 axis-mediated communication between breast cancer cells and CAFs.

Keywords: Cancer-associated fibroblast, C-X-C type chemokine ligand, C-X-C type chemokine receptor 2, differentiation-inducing factor-1, Tumor-associated macrophage, Tumor microenvironment

Abbreviations: AMPK, AMP-activated protein kinase; CAFs, cancer-associated fibroblasts; CXCL, C-X-C motif chemokine ligand; CXCR2, C-X-C motif chemokine receptor 2; DIF-1, differentiation-inducing factor-1; DFBs, mouse primary dermal fibroblasts; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HER2, human epidermal growth factor receptor 2; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate;

STAT1, signal transducer and activator of transcription 1; STAT3, signal transducer and activator of transcription 3; TAMs, tumor-associated macrophages; TME, tumor microenvironment.

Introduction

In 2020, 685,000 deaths were reported worldwide among 2.3 million women diagnosed with breast cancer [1]. Triple-negative breast cancer (TNBC), characterized by the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression, accounts for 15% of all breast cancer cases [2]. As TNBC is not responsive to hormone therapy and anti-HER2 treatment, the need for novel therapeutic strategies for TNBC is imperative [3-5].

Emerging evidence suggests that tumors not only include neoplastic cells but also the tumor microenvironment (TME), including the significantly altered stroma [6]. The TME is primarily composed of tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs), and the interaction of these cells with cancer cells exacerbates the pathological state by promoting tumor growth, migration, invasion, and metastasis [6, 7]. The TME is a critical element in tumor development and progression [7, 8]. As the tumor stroma occupies the majority of the tumor tissue in breast cancer, there is a growing interest in the breast cancer microenvironment for use as a potential therapeutic target [6].

Chemokines and their receptors that mediate communication between cancer cells and the TME promote metastasis in various cancers [9-11]. C-X-C motif chemokine ligands 1, 2, 3, 5, 6, 7, and 8 (CXCL1–CXCL8) bind to C-X-C motif chemokine receptor 2 (CXCR2), which is expressed in both cancer and stromal cells in the TME. In recent years, CXCR2 has attracted attention as a potential target for breast cancer treatment since it is closely associated with breast cancer progression via downstream signal transduction [12-14]. Specifically, CXCL1 and CXCL5 are secreted by TAMs, which enhance cancer cell malignancy via increased expression of SNAI1, an epithelial-mesenchymal transition-related transcription factor, and induce a positive feed-forward loop in cancer cells by increased CXCR2 expression via nuclear translocation of signal transducer and activator of transcription 3 (STAT3) [15]. CAF-mediated upregulation of SNAI1 expression in cancer cells induces cancer progression via upregulation of the expression of CXCL1 and CXCL2, which are chemokines that attract immunosuppressive cells to facilitate cancer immune evasion [11, 16].

Differentiation-inducing factor-1 (DIF-1) is a small molecule secreted by *Dictyostelium discoideum*, which is isolated and purified to yield a compound that induces differentiation into stalk cells. This molecule suppresses breast cancer cell proliferation, migration, infiltration, and colonization in lung metastasis models [17, 18]. Our earlier studies established the mechanism underlying DIF-1-mediated activation of AMP-activated protein kinase (AMPK), which leads to suppression of cell proliferation via downregulation of STAT3 expression [17] and cell motility via decreased SNAI1 expression [18]. As described above, STAT3 and SNAI1 that are regulated by DIF-1 are important factors in the regulation of CXCLs/CXCR2 axis-mediated communication between cancer cells and the TME, indicating that DIF-1 may exhibit inhibitory effects on the TME. However, this theory has not been experimentally investigated to date.

In this study, we investigated the effect of DIF-1 on the TME based on three perspectives. First, we explored the effect of DIF-1 on macrophage polarization into TAMs. Second, we examined the effect of DIF-1 on the communication between cancer cells and CAFs via the CXCLs/CXCR2 axis. Third, we assessed the effect of intragastric administration of DIF-1 on the TME based on experiments using tumor-bearing animals.

Results

2.1. DIF-1 showed no effect on macrophage polarization into TAMs

In breast cancer, the stromal tissue occupies the majority of the TME, constituting the bulk of the tumor tissue and significantly influencing the degree of cancer malignancy. Hence, stromal tissue regulation may act as a potential strategy for treating breast cancer [19]. STAT3 activation is observed in both tumor cells and macrophages, and it is responsible for cancer cell proliferation and macrophage differentiation into TAMs [20, 21] which promotes tumor growth and metastasis [22]. As our previous study showed that DIF-1 suppressed STAT3 expression and tumor growth in breast cancer [17], it is

hypothesized that macrophage differentiation into TAMs may be regulated by DIF-1. Therefore, we conducted *in vitro* experiments to examine the effect of DIF-1 on macrophage polarization into TAMs.

First, we investigated the effect of DIF-1 on TAMs using mouse macrophage RAW 264.7 cells and mouse TNBC 4T1-GFP cells. As shown in Fig. 1A, we used three culture protocols; RAW 264.7 cells were incubated with: (i) Dulbecco's modified eagle medium (DMEM) and 10% fetal bovine serum (FBS), (ii) conditioned medium of 4T1-GFP cells (4T1-CM), and (iii) 4T1-CM and 1 μM of DIF-1. After 7 days, RAW 264.7 cells cultured in the normal medium were observed to be round (Fig. 1B-i), whereas RAW 264.7 cells cultured in 4T1-CM revealed a spindle-shaped cell morphology (Figs. 1B-ii and 1B-iii). Two phenotypes of macrophages were identified: the M1 phenotype and M2 (TAMs) phenotype. The spindle-shaped cell morphology is a known characteristic trait of the M2 phenotype [23, 24], indicating that 4T1-CM induced the differentiation of macrophages into TAMs. In flow cytometric analysis, CD206 was used as a marker for the M2 phenotype, and CD80 was used as a marker for the M1 phenotype. As shown in Fig. 1C, the number of CD206-positive cells in the normal medium was negligible (Fig. 1C-i). In contrast, co-culturing with 4T1-CM increased the number of CD206-positive cells (Fig. 1C-ii). However, treatment with DIF-1 did not alter the number of CD206-positive cells (Fig. 1C-iii). Macrophage polarization into the M1 phenotype was not altered upon co-culturing with 4T1-CM (Fig. 1D-ii) or treatment with DIF-1 (Fig. 1D-iii). These results indicated that DIF-1 did not alter macrophage polarization.

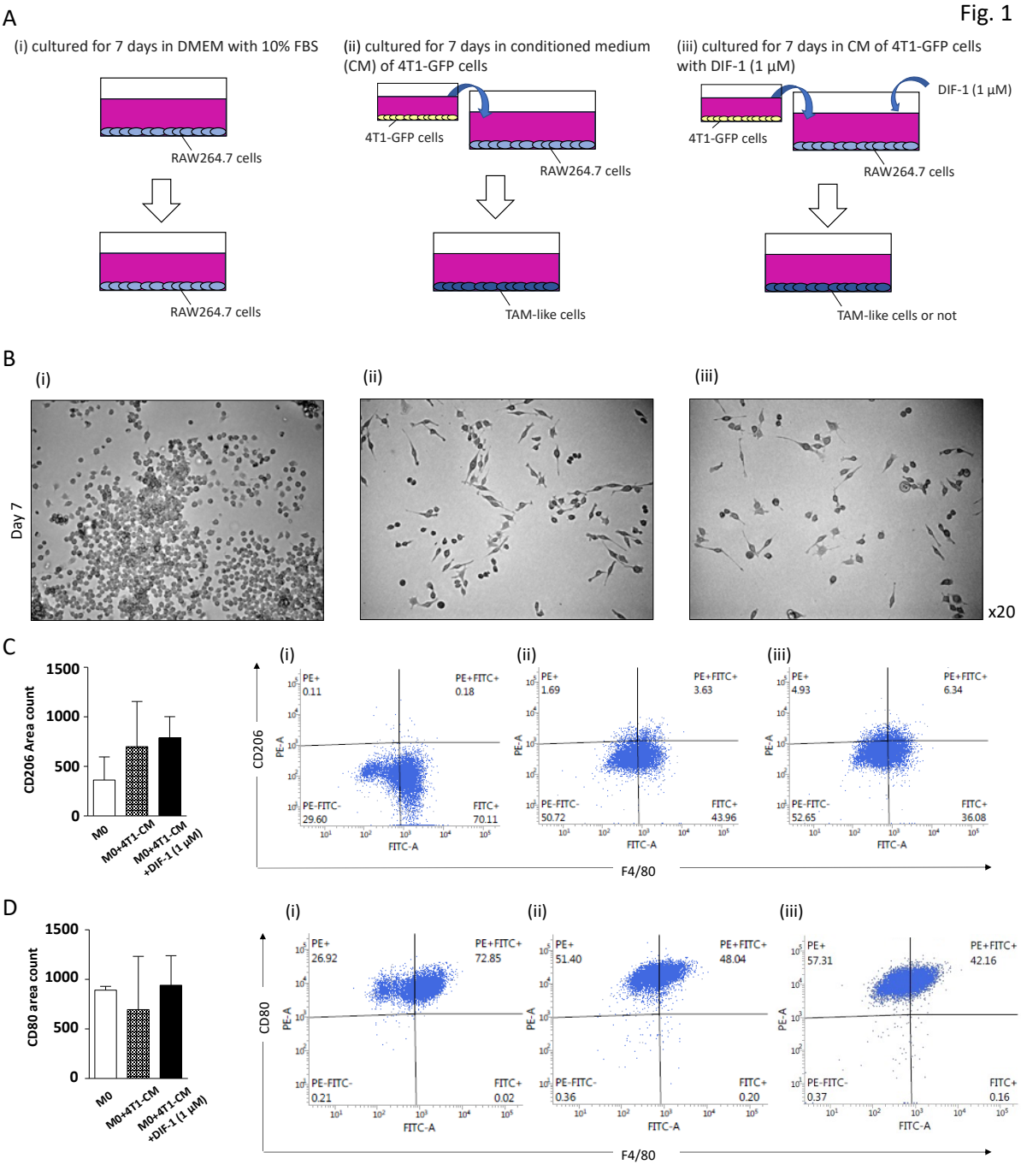


Figure 1. Effect of DIF-1 on macrophage polarization into TAMs

(A) Experimental protocols. (i) RAW 264.7 cells cultured in DMEM with 10% FBS. (ii) RAW 264.7 cells cultured in 4T1-CM. (iii) RAW 264.7 cells cultured in 4T1-CM with DIF-1 (1 μM). (B) Shape of RAW 264.7 cells after culturing for 7 days. (i–iii) Same as A(i)–A(iii). (C) Flow cytometric

analysis for CD206. (i–iii) Same as A(i)–A(iii). (C) Flow cytometric analysis for CD80. (i–iii) Same as A(i)–A(iii). The results are presented as mean ± SD of three independent experiments.

2.2. DIF-1 decreased CXCL1, CXCL5 and CXCL7 expression in CAF-like cells

As CAFs are the most prominent stromal component and secrete a variety of soluble factors, such as chemokines or growth factors, which enhance tumor growth and invasion, CAFs in close proximity to cancer cells are promising targets for cancer treatment [25, 26]. CXCR2 and CXC ligands are mediators of communication between cancer cells and CAFs [9, 10, 21]. CAFs typically produce CXCL1, CXCL2, and CXCL5, which promote breast cancer cell migration [25, 27, 28]. To confirm whether DIF-1 influences the CXCLs/CXCR2 axis-mediated interaction between CAFs and breast cancer cells, three culture protocols using mouse dermal fibroblasts (DFBs) and 4T1-GFP cells were used; DFBs were cultured: (i) without 4T1-GFP cells, (ii) with 4T1-GFP cells, and (iii) with DIF-1-treated 4T1-GFP cells for 24 h (Fig. 2A). Co-culturing with 4T1-GFP cells significantly increased the mRNA expression of CXCL1, CXCL2, CXCL5, and CXCL7 in DFBs (Fig. 2B). The increase in the expression of these cytokines increases in CAFs is well known [29, 30]. Therefore, in the present study, we identified DFBs as differentiated into CAFs by co-culturing with 4T1 cells. (Fig. 2B). However, consistent with the previous report [30], no significant difference in the expression of α-SMA, a conventional marker of CAF, was observed (Fig. S1). Hence, we named these cells as CAF-like cells instead of CAF. Treatment with DIF-1 significantly decreased CXCL1, CXCL5, and CXCL7 expression (Fig. 2B). Co-culturing with DIF-1-treated 4T1-GFP cells did not significantly alter CXCR2 mRNA expression or CXCR2 protein expression (Fig. 2C). Regarding the other CXCL/CXCR, the previous studies reported that CXCL12/CXCR4 signaling converted normal mammary fibroblasts into CAFs [17], and CXCL12 secreted by CAFs promoted the invasion of breast cancer cells [16]. Therefore, we examined the expressions of CXCR4 and CXCL12 in CAF-like cells. DIF-1 significantly decreased the expression of CXCL12 in CAF-like cells, while it did not affect CXCR4 expression (Fig. S2). These results suggested that DIF-1 decreased CXCL secretion in CAF-like cells.

Fig. 2

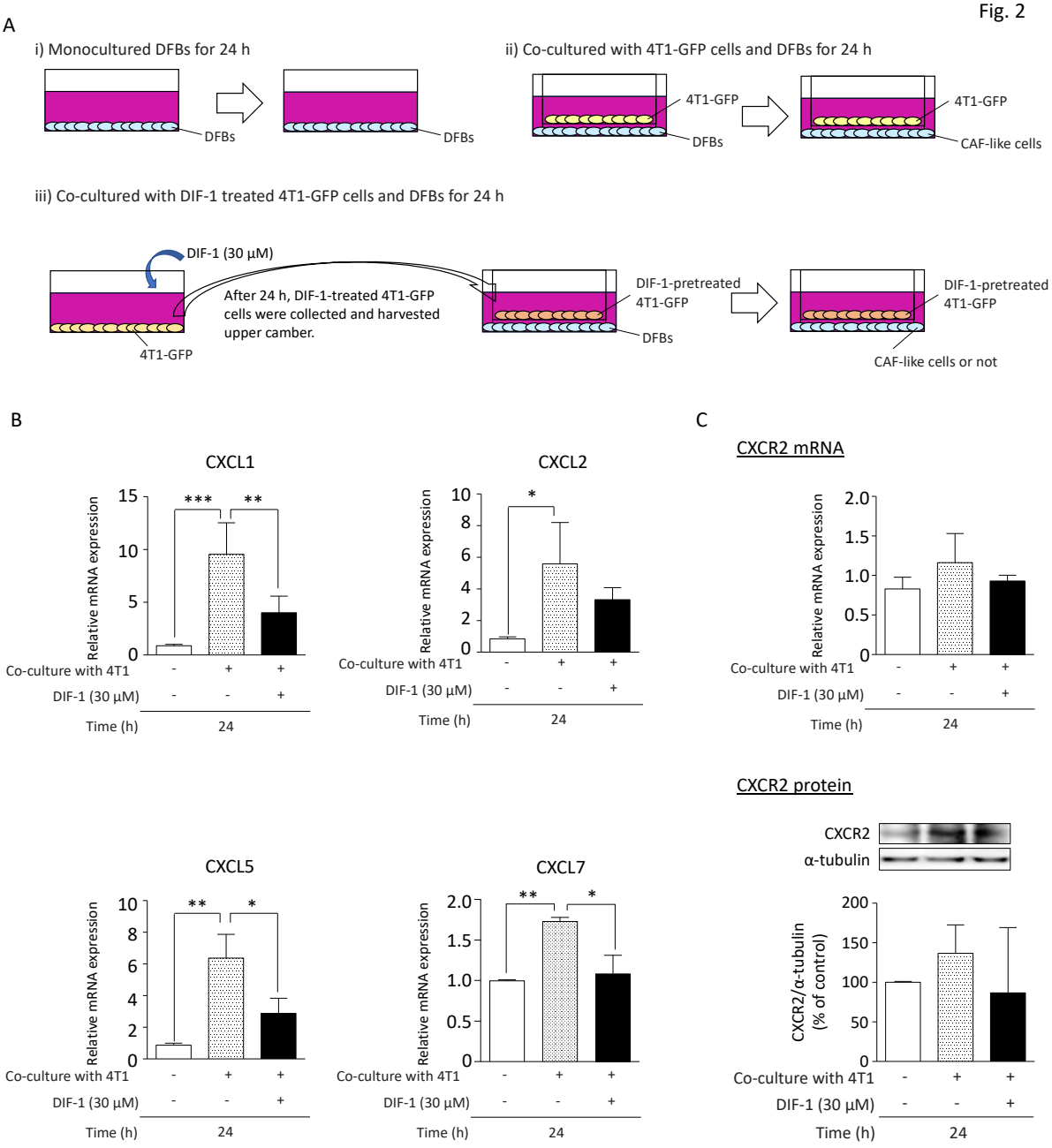


Figure 2. Effect of DIF-1 in CAF-like cells.

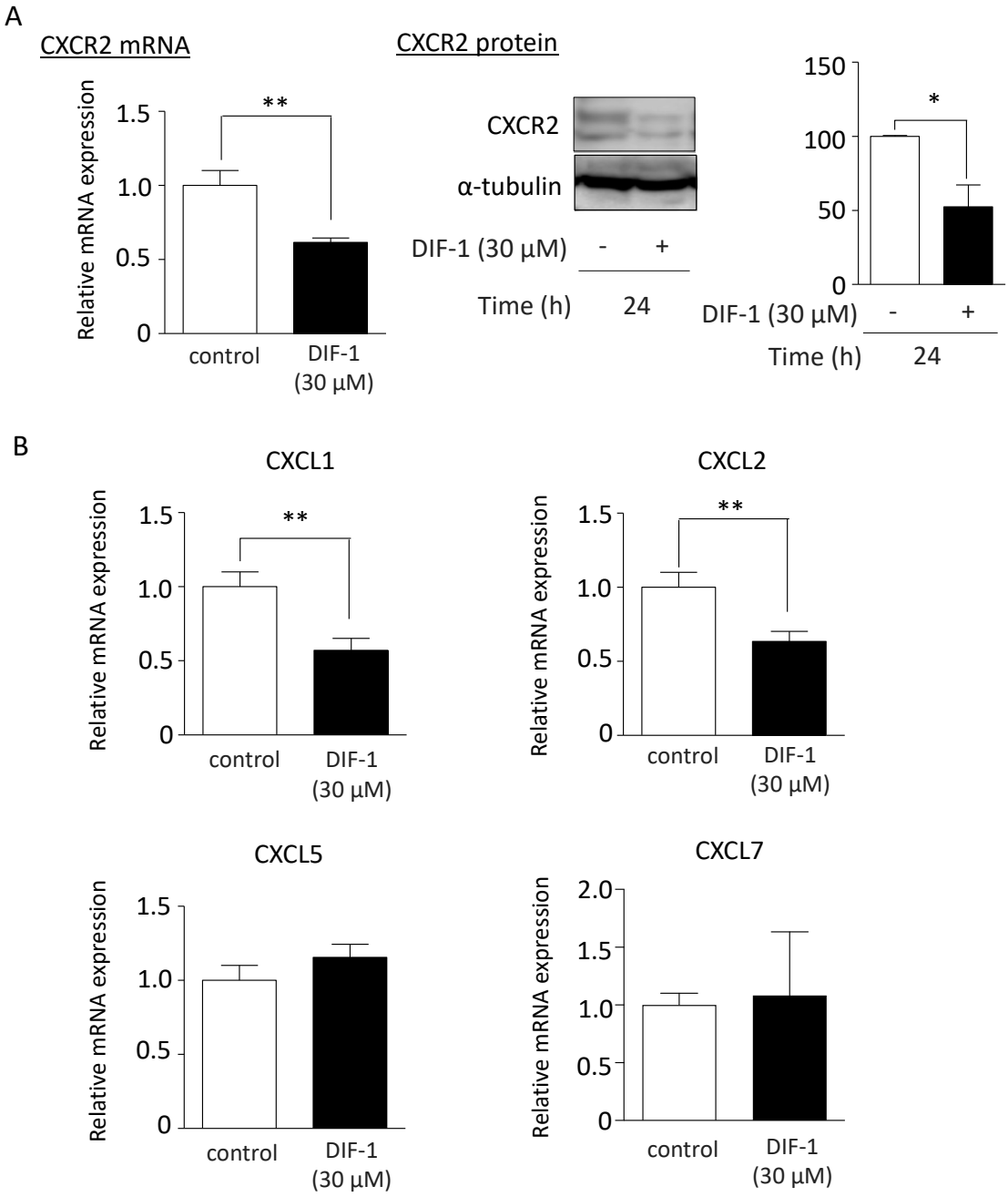
(A) Experimental protocols. (i) Mono-cultured DFBs. (ii) DFBs cultured with 4T1-GFP cells. (iii) DFBs cultured with DIF-1-pretreated 4T1-GFP cells. (B) Effect of DIF-1 on CXCLs in DFBs. (C)

Effect of DIF-1 on CXCR2 mRNA and protein expression in DFBs. The results are presented as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

2.3. DIF-1 decreased CXCR2 and CXCL expression in 4T1-GFP cells

In cancer cells, CXCLs that bind to CXCR2 exacerbate cancer malignancy and cause therapeutic resistance [25, 27, 28, 31]. Therefore, we examined CXCR2 expression in breast cancer cells with and without DIF-1 treatment (30 μM). CXCR2 was expressed in 4T1 cells cultured without DIF-1 (Fig. 3A), which is consistent with a previous study [9]. Treatment with DIF-1 significantly decreased CXCR2 mRNA and protein levels (Fig. 3A). Cancer cells secrete CXCLs to evade host immunity via migration of myeloid-derived suppressor cells [11, 32]. The expression of CXCL1 and CXCL2 in 4T1-GFP cells was significantly decreased by DIF-1 based on the examination of CXCL1, CXCL2, CXCL5 and CXCL7 expression (Fig. 3B). However, DIF-1 did not significantly affect the expression of CXCL12 and CXCR4 in 4T1 cells (Fig. S2). These results suggested that DIF-1 inhibited the communication between CXCLs and CXCR2 in 4T1-GFP cells.

Fig. 3



1

2 ***Figure 3. Effect of DIF-1 on the CXCLs/CXCR2 axis in breast cancer cells.***

3 (A) Effect of DIF-1 on CXCR2 mRNA and protein expression. (B) The effect of DIF-1 on CXCL1,

4 CXCL2, CXCL5, and CXCL7 mRNA expression in 4T1-GFP cells. **P* < 0.05 and ***P* < 0.01.

2.4. Intragastric administration of DIF-1 decreased CAFs in TNBC xenografts

We previously reported that DIF-1 exhibited anti-tumor effects in breast cancer cell lines [17, 18]. Intragastric administration of DIF-1 inhibited primary tumor growth and metastatic lung colony formation in 4T1 xenograft and spontaneous metastasis models, respectively [18]. In this study, a TNBC xenograft model was developed by injecting 4T1-GFP cells into the mammary fat pads of BALB/c mice to differentiate the stromal and parenchymal portions of the tumor. The mice were divided into two groups: control and DIF-1-treated groups. The control and DIF-1-treated groups were intragastrically administered soybean oil and DIF-1 (150 mg/kg body weight) suspended in soybean oil, respectively, every 12 h for two weeks [17, 18]. Tumors from the injected cells of the euthanized mice were excised on day 14. Our previous study demonstrated that DIF-1 administration suppressed tumor growth in the same *in-vivo* experiment as the present study [18]. To examine the TME, including macrophages, TAMs, and CAFs, the excised breast tumors were sectioned, and immunofluorescent staining was performed using CD68, CD206, and α-SMA as markers for macrophages, TAMs, and CAFs, respectively. Treatment with DIF-1 slightly altered CD68 and CD206 expression (Figs. 4A and 4B), consistent with the in vitro results (Fig. 1). In contrast, α-SMA expression was significantly decreased in the DIF-1-treated group compared to that in the control group (Fig. 4C). Furthermore, CXCR2 mRNA and protein expression were significantly attenuated in the DIF-1-treated group (Fig. 4D). These in vivo results showed the effect of DIF-1 on various TME components and their interaction with cancer cells via CXCR2, and were found to be consistent with the results obtained from the in vitro experiments.

Fig. 4

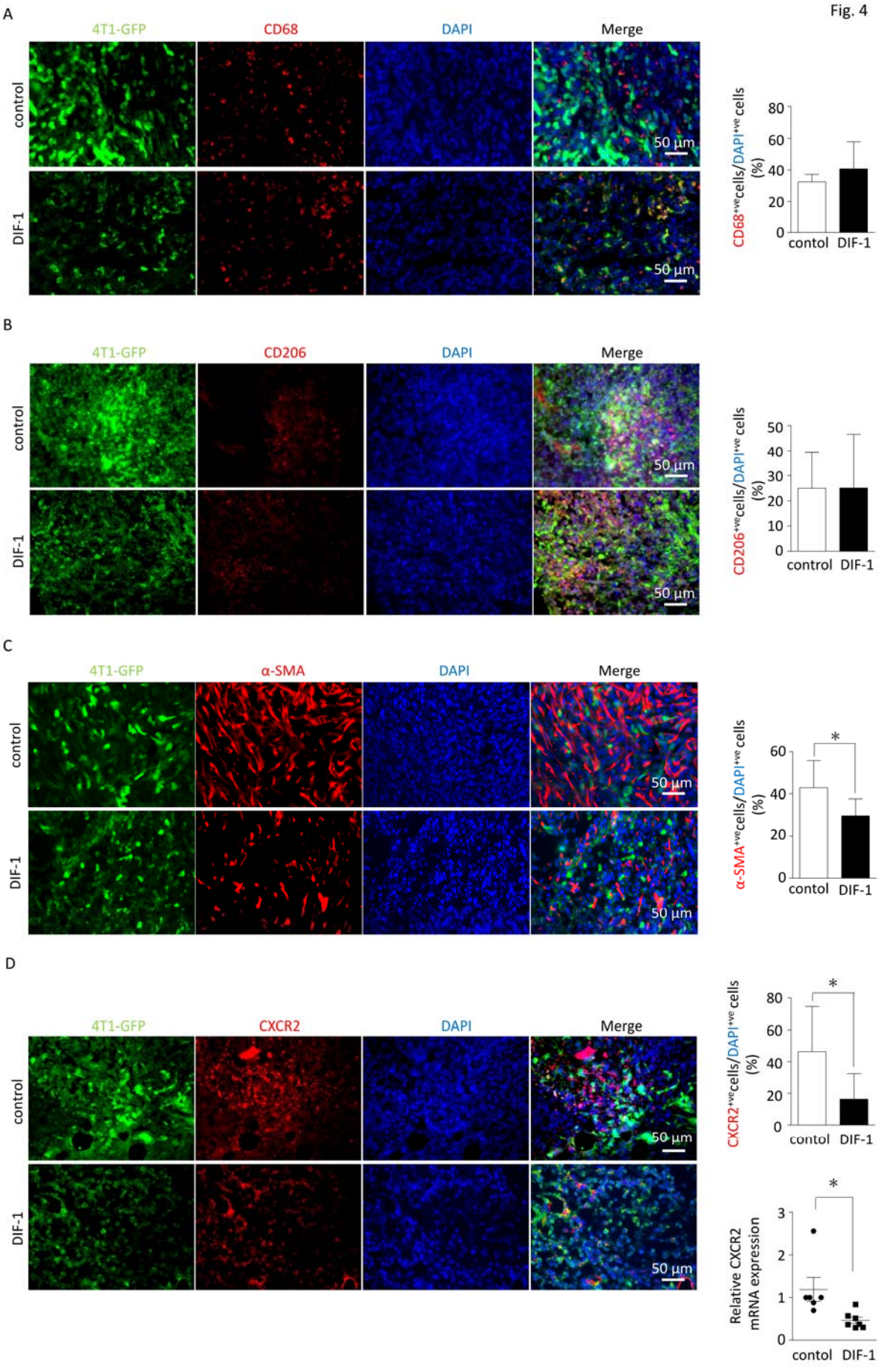

A
4T1-GFP
CD68
DAPI
Merge
control
DIF-1
50 μm
CD68+ve cells/DAPI+ve cells (%)
contol
DIF-1
B
CD206
CD206+ve cells/DAPI+ve cells (%)
C
α-SMA
α-SMA+ve cells/DAPI+ve cells (%)
D
CXCR2
CXCR2+ve cells/DAPI+ve cells (%)
Relative CXCR2 mRNA expression

Figure 4. Effect of DIF-1 on the expression of CD68/CD206 and α-SMA as markers for TAMs and CAFs, respectively.

Effect of DIF-1 on TME-related proteins. (A) Immunostaining of CD68 (red) and statistical analysis to determine the ratio of CD68-positive cells to DAPI-positive cells. (B) Immunostaining of CD206 (red) and statistical analysis to determine the ratio of CD206-positive cells to DAPI-positive cells. (C) Immunostaining of α-SMA (red) and statistical analysis to determine the ratio of α-SMA-positive cells to DAPI-positive cells. (D) Immunostaining of CXCR2 (red) and statistical analysis to determine the ratio of CXCR2-positive cells to DAPI-positive cells (top) and CXCR2 mRNA expression. Scale bar: 50 μm. Three sections were randomly selected from the tumor tissue samples. The results are presented as mean ± SD of three independent experiments. $*P < 0.05$.

3. Discussion

In this study, we reported the inhibitory effects of DIF-1 on the TME in breast cancer, which has not been reported to date, to the best of our knowledge. DIF-1 inhibited CAF differentiation and the interaction between breast cancer cells and CAFs via the CXCLs/CXCR2 axis.

Earlier studies showed that DIF-1 exhibited anticancer activity via suppression of cancer cell proliferation and motility [17, 33-38]. Our previous studies demonstrated that DIF-1 suppressed both tumor growth and metastasis via the AMPK-mammalian target of the rapamycin complex 1 (mTORC1) system in breast cancer [17, 18]. Our previous study suggested that DIF-1 binds to MDH2 and inhibits its activity [39]. As MDH2 inhibition activates AMPK [40], we hypothesized that the pharmacological target of DIF-1 may be MDH2. However, further studies are required to confirm this hypothesis. We demonstrated that DIF-1 activated AMPK, which in turn phosphorylated regulatory-associated protein of mTOR (Raptor), an essential component of mTORC1, leading to the suppression of mTORC1 activation. This inhibition of mTORC1 led to decreased $p70^{S6K}$ activity, thereby inhibiting STAT3 mRNA translation, resulting in cancer growth suppression. Suppression of mTORC1 activity also

inhibited the expression of C-C motif chemokine ligand 2 (CCL2), a protein that mediates macrophage recruitment to tissues surrounding cancer cells [41]. Furthermore, STAT3 activation induced the differentiation of mobilized M1 phenotype macrophages into TAMs [21, 42]. Based on the effect of DIF-1 on mTOR and STAT3 reported in our earlier study [18], the effect of DIF-1 on TAMs in the TME seemed worth investigating. Therefore, previously reported effects of DIF-1 (suppression of mTORC1 activity and STAT3 expression) led us to hypothesize that DIF-1 may suppress macrophage recruitment and conversion to TAMs. Although DIF-1 inhibited mTORC1 activation [18] and CCL2 mRNA expression in 4T1-GFP cells (Fig. S3), the intragastric administration of DIF-1 did not increase the number of CD68-positive macrophages in the xenografted tumors (Fig. 4A). CCL2 plays an important role in regulating TAM attractants in solid tumors, but CCL2 inhibition suppressed TAM accumulation by only 50% and did not lead to complete TAM depletion in the mouse models [43-45]. This indicated that other chemokines are involved in monocyte recruitment [46]. C-C chemokine receptor type 2 (CCR2) ligands such as CCL5, CCL7, and CCL12, and cytokines such as vascular endothelial growth factor and colony-stimulating factor-1 are involved in macrophage infiltration and monocyte recruitment in a complex and compensatory manner [47]. The failure of DIF-1 to prevent macrophage infiltration may be attributed to the presence of factors other than CCL2. Moreover, DIF-1 decreased STAT3 expression in both xenografted breast tumors and 4T1 cells [18], but DIF-1 did not affect the macrophage polarization into CD206-positive TAMs in the in vivo and in vitro experiments conducted in this study (Figs. 4B and 1C). Macrophage polarization is regulated by complicated factors; STAT3 and STAT1 activations induce differentiation into M2 (TAMs) [21, 42] and M1 phenotypes, respectively [23]. Thus, STAT1 and STAT3 have opposing actions on macrophage polarization. Since DIF-1 inhibit phosphorylation and expression of both STAT3 [18] and STAT1 (Fig. S4), the effects of DIF-1 on macrophage polarization into the M1 or M2 phenotype (TAMs) might be counteracted.

Our previous *in-vitro* studies have shown that 20–30 μM of DIF-1 significantly inhibited cell proliferation and motility of cancer cells [17, 33-38]. In addition, intragastric administration of DIF-1 (150 mg/kg body weight) increased its serum concentration to 30 μM [33]. Therefore, all experiments

were consistently conducted at 30 μM of DIF-1. However, in the experiments on macrophages, RAW264.7 cells needed to be cultured for 7 days to induce TAMs, and 30 μM of DIF-1 did not allow the macrophages to survive. After trying several concentrations of DIF-1, 1 μM emerged as the maximum concentration that would not kill macrophages for 7 days. As a limitation, this study does not clarify whether treatment with 1 μM DIF-1 is sufficient to affect macrophage differentiation in *in-vitro* experiments. However, DIF-1 did not affect TAM differentiation in an *in-vivo* model in which blood concentrations of DIF-1 increased to 30 μM. Thus, despite the different concentrations of DIF-1, the *in-vitro* and *in-vivo* results were consistent; thus, we conclude that DIF-1 does not affect TAM differentiation in this study.

Among the complex constituents of the TME, CAFs are the most prominent stromal component [25, 26]. Normal fibroblasts secrete various chemokines and utilize some of these chemokines when co-cultured with cancer cells [30, 48]. Chemokines efficiently induce the transformation of normal fibroblasts to CAFs, thereby promoting cancer growth and metastasis [25, 26]. Therefore, it is important to suppress chemokine secretion to inhibit CAF differentiation. Transformation of DFBs to CAF-like cells upon co-culturing with 4T1-GFP cells was confirmed (Fig. 2B) based on the increased levels of CXCL1, CXCL2 and CXCL5, which are normally secreted by CAFs [25, 27, 28]. However, co-culturing with DIF-1-treated 4T1-GFP cells seemed to suppress CAF differentiation and activation in vitro (Fig. 2). DIF-1 also decreased the number of α-SMA-positive CAFs in the xenografted tumors (Fig. 4). Interaction between cancer cells and fibroblasts promotes CAF differentiation [26], the release of various inflammatory modulators, crosstalk and positive feedback involving Janus kinase-STAT signaling [29], and physical changes in the extracellular matrix [49]. Our previous studies showed that DIF-1-mediated decrease in mTORC1 activity suppressed $p70^{S6K}$-mediated STAT3 expression [17, 18] and that DIF-1 suppressed matrix metalloproteinase-2 expression in melanoma cells [35]. Based on these mechanisms underlying DIF-1 activity, it was speculated that DIF-1 inhibited CAF differentiation. In contrast, the DIF-1-induced reduction in STAT3 expression suppressed cyclin D1 expression,

resulting in the inhibition of cell proliferation [17]. Therefore, DIF-1-mediated inhibition of CAF proliferation can be omitted.

CXCR2 is a promising drug target to inhibit cancer progression. Downregulation of CXCR2 inhibited cell migration and invasion in breast cancer [9] and non-small cell lung cancer [50]. Inhibition of CXCR2 suppressed the cell cycle in ovarian cancer via cyclin D1 suppression, apoptosis, and angiogenesis [51]. Decreased CXCR2 expression inhibited the recruitment of bone marrow-derived mast cells into the TME, decreased tumor growth and metastasis, and improved the survival rate of mice bearing gastric cancer [52]. DIF-1 did not decrease CXCR2 expression in CAFs (Fig. 2C). However, the results in Fig. 3B show that DIF-1 decreased the expression of CXCR2 in cancer cells. Therefore, it can be inferred that the lower CXCR2 expression in tumor tissues in the DIF-1 group is due to its effect on cancer cells (Fig. 4D). However, the reduction of the number of CAFs themselves in the xenografted tumor tissue in the DIF-1 group might have contributed to the markedly decreased CXCR2 expression (Fig. 4C). As CXCR2 dysregulation decreases the number of colonies, cancer stem cell markers, and EMT markers in colorectal cancer [53], the effect of DIF-1 on cancer stem cell and EMT markers must be investigated. Furthermore, tissues in the tumor microenvironment reportedly affect each other. In breast cancer, CAFs secrete monocyte chemotactic protein-1, CXCL12, and chitinase 3-like 1 and induce monocyte migration and polarization of M2 phenotype [17, 18]. Further studies are needed.

CAFs directly influence tumor progression and therapeutic resistance [54]. To date, there are no clinically available drugs that target CAFs; however, their therapeutic use has been validated in multiple trials. The small molecule dipeptidyl peptidase inhibitor PT-100, which inhibits CAFs by targeting fibroblast activation protein (FAP), in combination with chemotherapy, significantly suppressed tumor growth in a mouse xenograft model of colorectal cancer [55]. Similarly, AMD3100, a CXCR4 inhibitor, synergized the anti-tumor effect of anti-programmed cell death ligand 1 immunotherapy in an in vivo pancreatic ductal adenocarcinoma (PDAC) model [56]. Anti-FAP vaccination inhibited tumor growth in melanoma, breast cancer, and mouse lymphoma models [57]. In another study, Pasireotide (a

SOM230 analog), an inhibitor of the mTOR/4E-BP1 protein synthesis pathway, in combination with gemcitabine chemotherapy, reduced tumor growth and chemoresistance in PDAC xenografts in nude mice [58]. Drugs targeting CAFs could be an effective strategy for cancer treatment. Anticancer drugs developed using DIF-1 as the lead compound have the potential to be novel CAF-targeted therapeutic drugs. Furthermore, DIF-1 does not kill cancer cells but inhibits their growth without the representative side effects of conventional chemotherapy, such as weight loss and myelosuppression [17, 18, 33, 35]. Owing to its low toxicity, DIF-1 can be used efficiently in combination with conventional chemotherapeutic agents. Therefore, DIF-1 is a potential lead compound for the development of CAF-targeted anti-tumor drugs.

4. Materials and methods

4. 1. Chemicals and antibodies

DIF-1 was synthesized according to the procedure described previously [59]. Monoclonal anti-CXCR2 antibodies (cat no: 20634-1-AP) were purchased from Proteintech (Rosemont, IL, USA). Monoclonal anti-α-tubulin antibodies (#CP06) were purchased from Calbiochem (Darmstadt, Germany). Monoclonal antibodies against total STAT1 (t-STAT1) (#9172S) and phosphorylated STAT1 (p-STAT1) (#5375S) were purchased from Cell Signaling Technology (Danvers, MA, USA).

4. 2. Cell culture

Shigehiro Ohdo (Department of Pharmaceutics, Graduate School of Pharmaceutical Sciences, Kyushu University) supplied the 4T1 cells, which were cultured at 37 °C in an atmosphere of 95% air and 5% CO_2 in DMEM (Sigma-Aldrich, MA, USA) supplemented with 10% FBS, 100 U/mL penicillin G, and 0.1 μg/mL streptomycin. The 4T1 cells were transfected with the green fluorescent protein (GFP) using polyethylenimine (PEI) MAX transfection and puromycin 1 μg/mL. Passages 17–30 cells from their arrivals were used in the experiments.

DFBs were isolated from the dorsal dermis of 8-week-old female BALB/c mice purchased from CLEA, Japan Inc. (Kyudo, Tosu, Japan), according to the procedure described previously [60]. Harvested DFBs were cultured at 37 °C in an atmosphere of 90% N_2, 5% O_2, and 5% CO_2 in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 100 U/mL penicillin G, and 0.1 μg/mL streptomycin. DFBs from passages 2 and 3 were used in the experiments.

4. 3. Macrophage polarization into TAMs

Mouse macrophage RAW 264.7 cells were incubated with 4T1-CM for 7 days. The 4T1-GFP cells were cultured in DMEM with FBS 10% for 24 h, and the supernatant was collected and used as the conditioned medium. The medium was replaced every 48 h during the incubation of RAW 264.7 cells with 4T1-CM.

4. 4. Flow cytometry

The harvested cells were collected using TrypLE™ Express Enzyme (cat no: 12605036, Thermo Fisher Scientific, Waltham, MA, USA). The collected cells were washed with stain buffer (FBS) (cat no: 554653; Bioscience, Franklin Lakes, NJ, USA). The cells were rinsed and then incubated with purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™) (cat no: 553141; Bioscience) at room temperature for 10 min. The cells were stained with Alexa Fluor® 488 anti-mouse F4/80 antibody (cat no: 123120; BioLegend, San Diego, CA, USA), PE anti-mouse CD80 antibody (cat no: 104708; BioLegend), and PE anti-mouse CD206 (MMR) antibody (cat no: 141706, BioLegend) for 30 min on ice in the dark. The cells were washed with stain buffer and analyzed using a BD FACSVerse™ Flow Cytometer (Bioscience).

4. 5. Western blotting

Protein samples (10 μg/lane) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis with a 12% gel and then transferred onto a polyvinylidene difluoride membrane using the semi-dry transfer method (1 h at 12 V). Western blotting was performed as described previously

[34]. Protein bands were quantified using optical densitometry and analyzed using ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA). Data are presented as percentages of the protein levels in the control animals.

4. 6. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the FastGene® RNA Basic Kit (NIPPON Genetics, Tokyo, Japan). The purity and quantity of the RNA samples were determined using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Delaware, USA). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA).

qRT-PCR was performed using TaqMan™ Gene Expression Assays (Applied Biosystems, Foster City, USA) on a LightCycler® Nano System (Roche Molecular Biochemicals, Mannheim, Germany) using the primers for *CCL2* (assay ID: Mm00441242_m1) encoding CCL2 and *GAPDH* (assay ID: Mm99999915_g1) encoding GAPDH (loading control). qRT-PCR was performed using GeneAce SYBR® qPCR Mix α (Nippon Gene Co.) on a LightCycler® Nano System (Roche Molecular Biochemicals). The primer sequences (5'-3') used were as follows: CXCL1, forward ACTGCACCCAAACCGAAGTC, reverse TGGGGACACCTTTTAGCATCTT; CXCL2, forward CCAACCACCAGGCTACAGG, reverse GCGTCACACTCAAGCTCTG; CXCL5, forward GTTCCATCTCGCCATTCATGC, reverse GCGGCTATGACTGAGGAAGG; CXCL7, forward ACCTCCAGATCTTGCTGCTG, reverse TCCTGGCCTGTACACATTCA; CXCL12, forward GCTCTGCATCAGTGACGGTA, reverse TTCTTCAGCCGTGCAACAAT; CXCR2, forward ATGCCCTCTATTCTGCCAGAT, reverse GTGCTCCGGTTGTATAAGATGAC; CXCR4, forward TCAGTGGCTGACCTCCTCTT, reverse TTCCTTGGCCTCTGACTGTT; and GAPDH, forward ACCACAGTCCATGCCATCAC, reverse TCCACCACCCTGTTGCTGTA. The mRNA levels of various genes were quantified using GAPDH (loading control) for normalization.

4. 7. In vivo experiments

In vivo experiments using mouse tumor growth models and dosing methods were performed as described previously [18]. Briefly, mice were housed in a temperature-controlled environment with a 12 h light-dark cycle and had *ad libitum* access to feed and water. 4T1-GFP cells were trypsinized and resuspended in 50% Matrigel in phosphate-buffered saline at a concentration of 1.0×10^6 cells/mL. The suspension (0.1 mL) was injected into the left #4 mammary fat pad of 10-week-old BALB/c female mice (Kyudo, Tosu, Japan) anesthetized with 1.0%–2.0% isoflurane.

Mice were randomly segregated into two groups. Mice in the DIF-1 treatment group received DIF-1 resuspended in soybean oil using gastric gavage, while those in the control group received only soybean oil. DIF-1 was administered every 12 h (150 mg/kg in the morning and evening: 10 mL/kg per day) 5 days a week.

4. 8. Tissue preparation, histology, and immunohistochemical analysis

Primary tumors excised from the animals after euthanasia were fixed in 4% paraformaldehyde at 4 °C overnight and washed with phosphate-buffered saline (PB S). Samples were placed in 30% sucrose solution for 24 h and then embedded in the Tissue-Tek OCT embedding medium. Sections of thickness 8 μm were cut using a cryostat (Thermo Fisher Scientific, Pittsburgh, PA, USA). Sections were then subjected to immunohistochemical staining.

Immunostaining was performed as described previously [17]. Tissue sections were permeabilized using 0.5% TritonX-100. Following antigen retrieval, slides were blocked using 5% goat serum (Vector S-1000) for 1 h at room temperature and incubated with anti-CD68 (cat no: 97778, CST, Danvers, MA, USA), anti-CD206 (cat no: 60143-1-lg, Proteintech, Rosemont, IL, USA), anti-α-SMA (cat no: 60839, CST, Danvers, MA, USA), and anti-CXCR2 (Proteintech) antibodies overnight at 4 °C. The slides were washed using PBS and then incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (A27034, Thermo Fisher Scientific) for 30 min, and then mounted using 4′,6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Laboratories, Burlingame, CA, USA).

4. 9. Histomorphometric analysis

Histomorphometric measurements were performed using the Keyence software (Keyence, Osaka, Japan). Quantification of the immunopositive areas was performed using photographic analysis of tissue sections containing primary tumors at 40× magnification. A minimum of three tissue sections from each animal were analyzed using histomorphometry. The number of CD68-positive, CD206-positive, α-SMA-positive, and CXCR2-positive pixels was divided by the number of DAPI-positive pixels.

4. 10. Statistical analysis

All experiments were independently repeated three or more times (biological replicates). Results are expressed as mean ± standard deviation (SD). *P*-values were calculated using the Student's paired t-test. Statistically significant differences comparing three or more groups were analyzed using one-way or two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test (GraphPad Prism version 7.0, GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant if $P < 0.05$. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

4. 11. Ethics of animal experimentation

All animal experiments were performed in compliance with the ARRIVE guidelines. The Committee on Ethics of Animal Experiments of Kyushu University approved the study protocol. Animal handling and experimental procedures were performed in compliance with Kyushu University's guidelines for animal experiments and law no. 105 and notification no. 6 of the Japanese government. All surgical procedures were performed under isoflurane inhalational anesthesia, and every effort was made to minimize the suffering of the experimental animals.

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Data availability

All data generated during this study are included in this published article.

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Author contributions

Conceptualization, F.S-T., M.A.; methodology, F.S-T., M.A.; investigation, F.S-T., M.A.; writing-original draft preparation, F.S-T., M.A.; writing-review and editing, F.S-T., M.A., K.M., T.I., K.T., and T.S. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

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Supplementary Figures:

Fig. S1

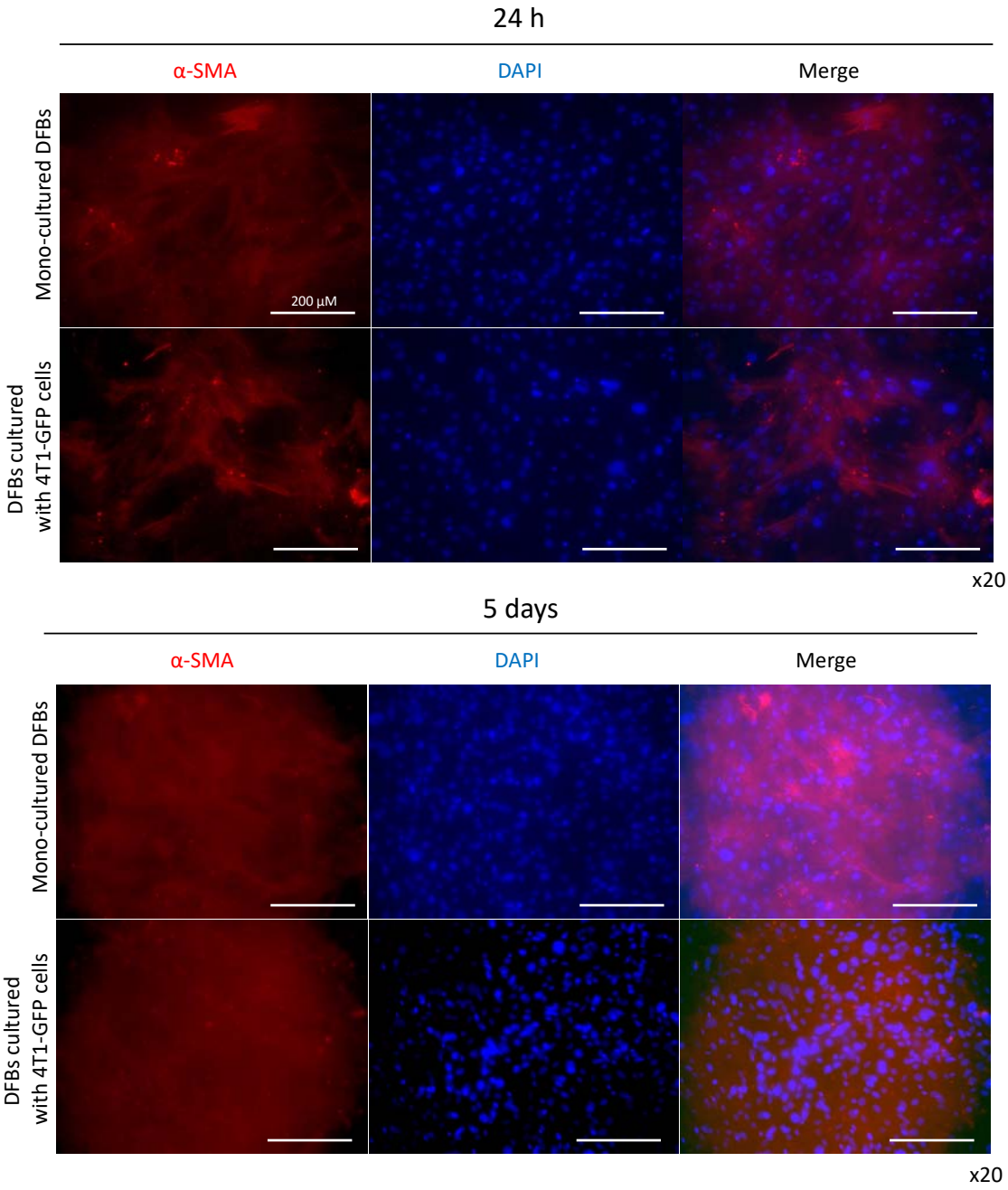


Figure S1. Effect of DIF-1 on the expression of α-SMA in CAF-like cells

Immunostaining of a-SMA (red) and DAPI (blue) for mono-cultured DFBs and DFBs cultured with 4T1-GFP cells at 24 h (upper panel) and 5 days (lower panel). Scale bar: 200 μm.

Fig. S2

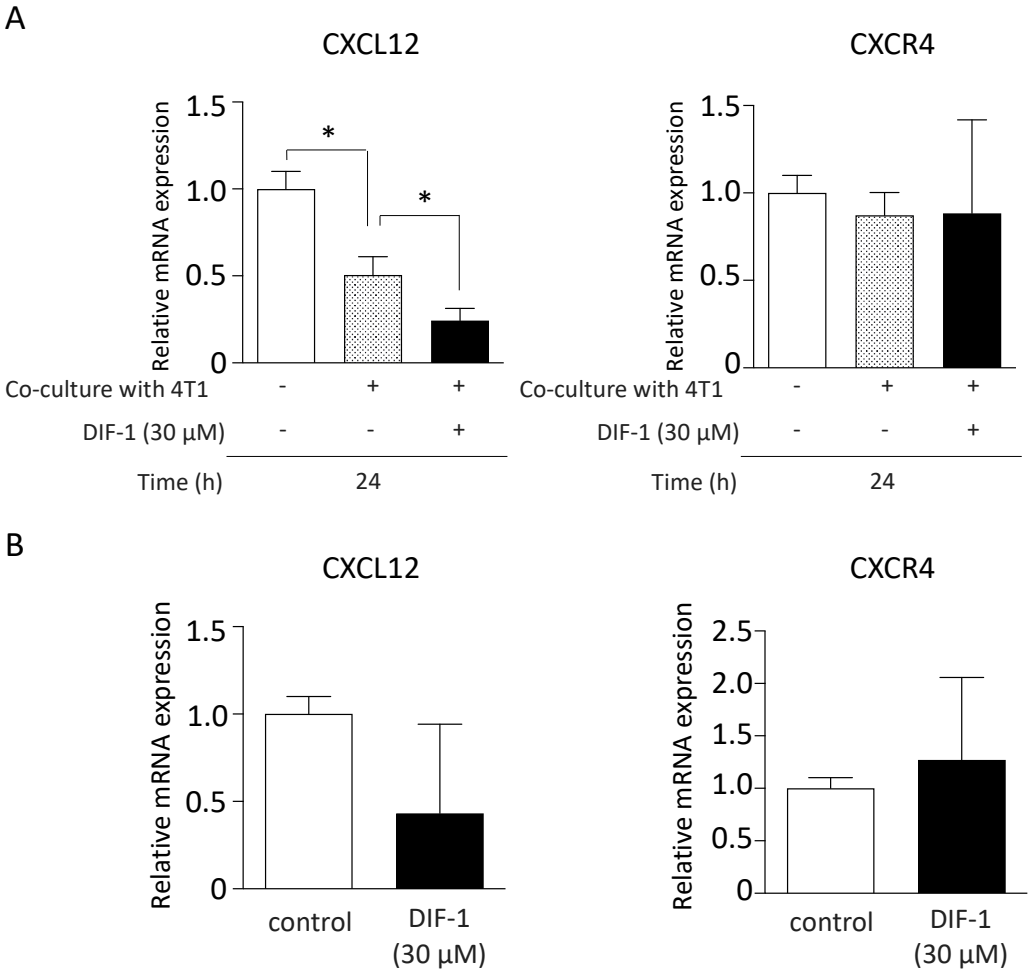


Figure S2. Effect of DIF-1 on the expressions of CXCL1 and CXCR4.

Effect of DIF-1 on CXCL12 and CXCR2 mRNA in (A) DFBs and (B) 4T1-GFP cells. The results are presented as mean ± SD of three independent experiments. **P* < 0.05.

Fig. S3

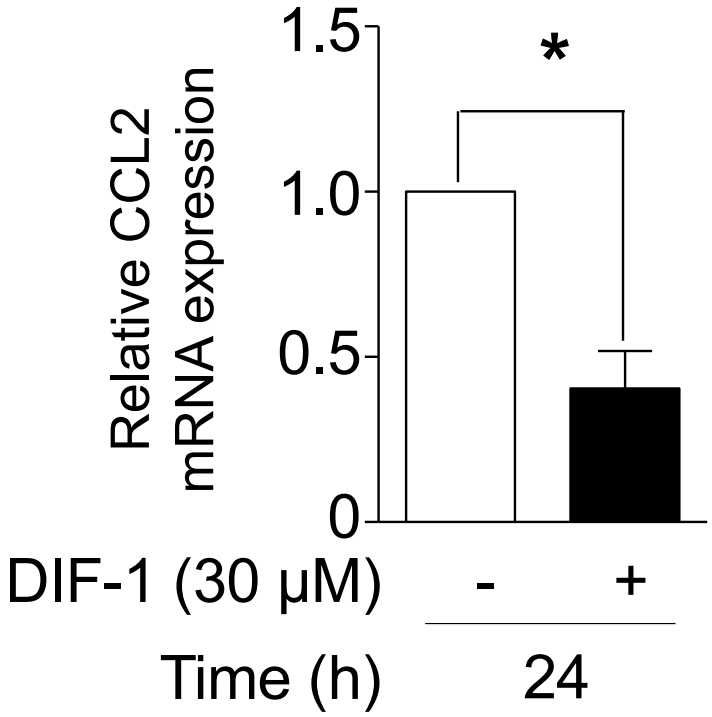


Figure S3. DIF-1 inhibited CCL2 mRNA expression.

Effect of DIF-1 on CCL2 mRNA expression. The results are presented as mean ± SD of three independent experiments. *$P < 0.05$.

Fig. S4

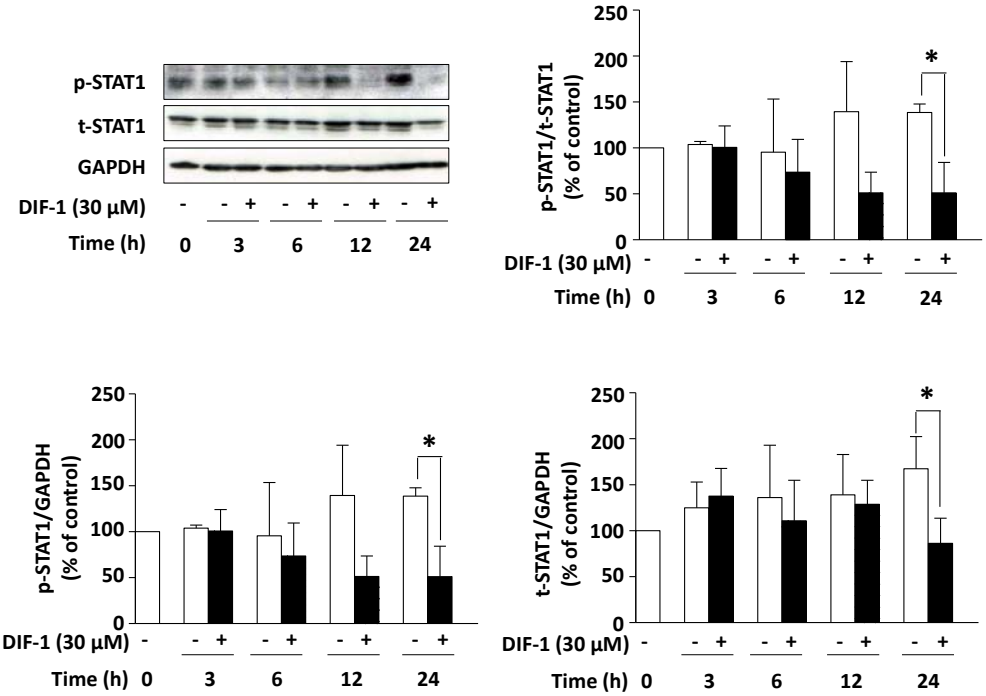


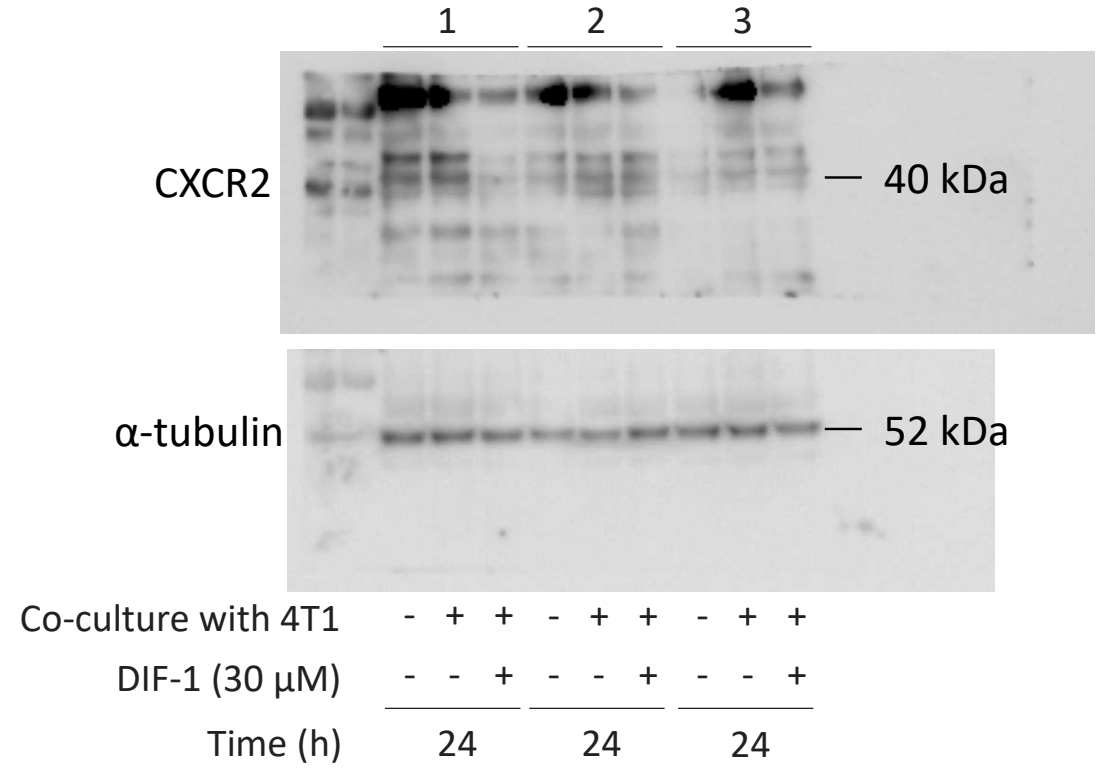
Figure S4. DIF-1 inhibited STAT1 protein expression.

Western blotting for t-STAT1 and p-STAT1 in 4T1-Luc cells. Cells were incubated with and without DIF-1 (30 μM) for 0, 3, 6, 12, and 24 h. Representative blots and quantitative analyses of p-STAT1, t-STAT1, and the ratio of p-STAT1 to t-STAT1 are shown in the figure. The results are presented as mean ± SD of three independent experiments. **P* < 0.05.

Supplementary data:

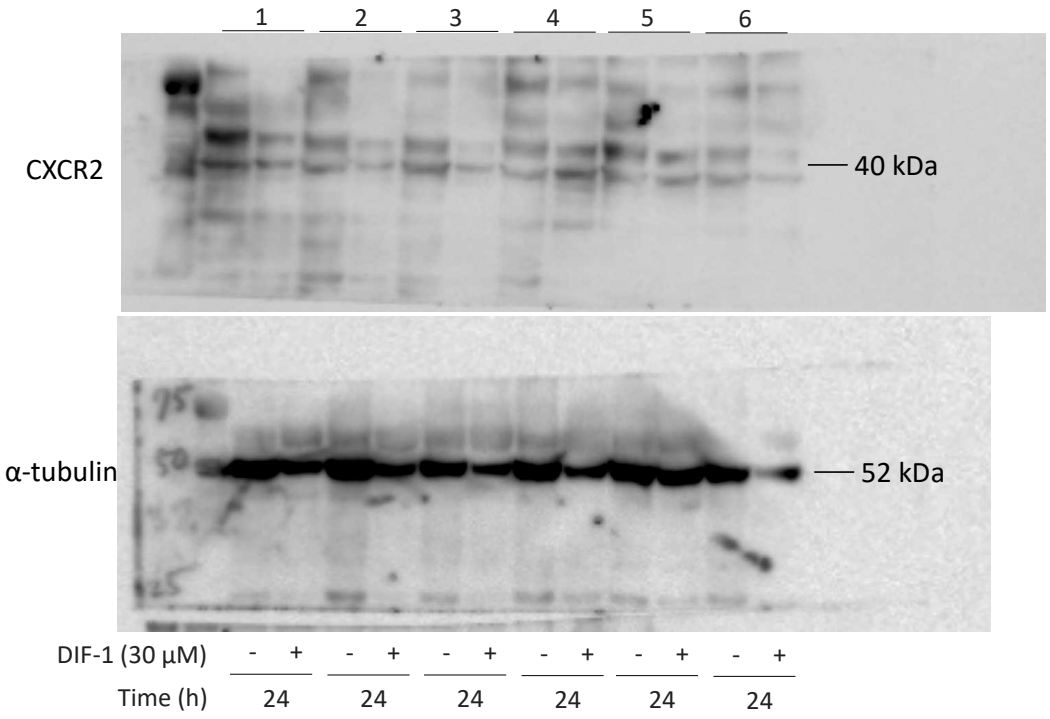
Western blots in Figure 2

Fig. 2



Western blots in Figure 3

Fig. 3



Western blots in supplemental figure 4

Fig. S4