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Soluble Factors Involved in Cancer Cell–Macrophage Interaction Promote Breast Cancer Growth

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Abstract. *Background/Aim: Recent studies have indicated the clinical significance of tumor-associated macrophages (TAMs) in breast cancer; however, the detailed mechanisms of cell–cell interactions between TAMs and cancer cells remain unclear. Materials and Methods: In vitro cell culture studies using human monocyte-derived macrophages and breast cancer cell lines were performed to test which cytokines would be involved in cell-cell interactions between cancer cells and macrophages. In addition, studies using human resected samples and animal breast cancer models were performed to examine the significance of TAMs in cancer development. Results: Osteopontin, HB-EGF, and IL-6 were suggested to be macrophage-derived growth factors for breast cancer cells. FROUNT inhibitor significantly blocked TAM infiltration and subcutaneous tumor growth in an E0771 mouse breast cancer model. Conclusion: TAMs express growth factors, such as osteopontin, for cancer cells, and targeting of TAM infiltration might be a promising approach for anti-breast cancer therapy.*

Breast cancer is the most common cancer in women, accounting for 23% of all cancers in women (1). Primary breast cancer exhibits many differences in morphology and novel classification dependent on estrogen receptor, progesterone receptor, human epidermal growth receptor 2 (HER2), and Ki67 is routinely performed (2). Such heterogeneity complicates pathological diagnosis and treatment protocols. Triple-negative (TNBC) subtypes that account for 10-20% of breast cancers are more aggressive than other subtypes (3). Anti-programmed cell death ligand 1 (PD-L1) therapy has shown a significant anti-cancer effect for TNBC in combination with chemotherapy; as such, there is increasing interest in the significance of the tumor immune microenvironment in breast cancer treatment (4).

Cancer progression is a complex process that depends on the interactions between tumor cells and host-derived cells in the tumor microenvironment, and the immune system can act to both promote and suppress tumor growth and invasion (5, 6). Several studies have indicated that high density of tumor-associated macrophages (TAMs) was linked to high malignant potential in several solid tumors including breast cancer. Immunohistochemical analysis using surgically resected samples showed that a high density of CD163-positive TAMs was linked to a poor clinical course in TNBC and HER2-positive breast cancer (7, 8). We previously demonstrated that the high number of infiltrating CD204-positive TAMs, but not that of CD163-positive macrophages, was a predictive marker for worse clinical course in breast cancer (9). Shimada *et al*. found that a high density of CD204-positive TAMs with a fibrotic focus was significantly associated with a worse clinical course in breast cancer, suggesting that the cell-cell interactions between TAMs and fibroblasts contribute to cancer progression (10). Activated TAMs are known to secrete several kinds of pro-tumor molecules and epidermal growth factor receptor (EGFR) ligands, including epidermal growth factor (EGF), IL-1β, IL-6, prostaglandin E2, TNF-α, and oncostatin M (11); however, the detailed molecular mechanisms related to the protumor functions of TAMs have yet to be uncovered in human breast cancer.

In the present study, we attempted to determine the kinds of soluble factors that are involved in cancer cell growth. In addition, we found that cancer cell-derived CCL5 is potentially associated with TAM infiltration in the tumor microenvironment (TME).

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Key Words: Breast cancer, macrophage, osteopontin, HB-EGF, IL-6, FROUNT.

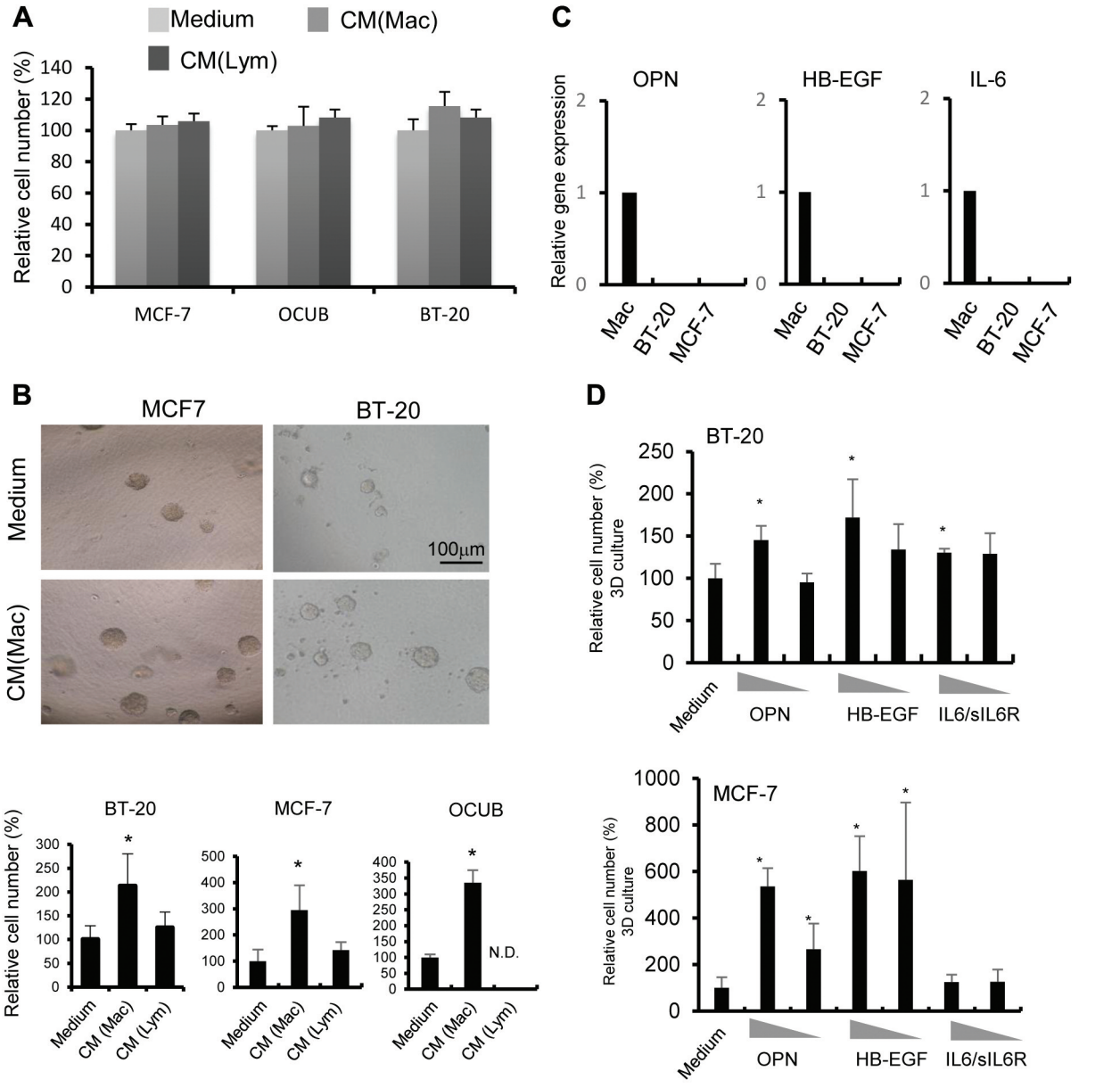

Figure 1. *Continued*

Materials and Methods

Cell lines and three-dimensional (3D) cell culture. Human breast cancer cell lines, BT-20, MCF-7, and OCUB-1, were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The murine breast cancer cell lines E0771 and 4T1 were obtained from CH3 BioSystem (Amherst, NY, USA) and Dr. Teizo Yoshimura (Okayama University, Okayama, Japan), respectively. Cells were cultured in DMEM/F12 or RPMI1640 (Wako, Tokyo, Japan) with 10% fetal bovine serum. The 3D cell culture was performed as described previously (12, 13). Recombinant osteopontin (OPN), heparin-binding EGF-like growth factor (HB-EGF), and interleukin (IL)-6 were obtained from Wako. In brief, cell lines (100 cells in 100 μl/well) were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum and SphereMax (Wako) using a 96-well ultra-low attachment plate (Corning, Corning, NY, USA) for 10 days. The total cell viability was evaluated by analyzing cellular ATP activity using a Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI, USA).

Cell culture of macrophages. Human monocyte-derived macrophages were obtained from healthy donors in accordance with protocols approved by the Kumamoto University Hospital Review Board (No. 1169), and cultured as described previously (14). In

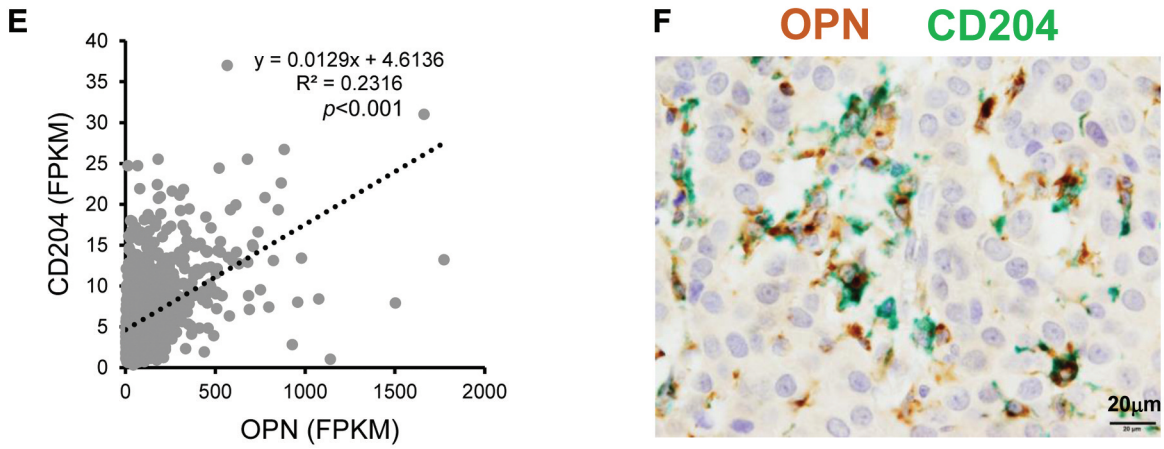

Figure 1. *Cell-cell interaction between macrophages and cancer cells. (A) Cancer cell lines (MCF-7, OCUB, and BT-20) were stimulated with conditioned medium (CM) of lymphocytes and macrophages for 2 days in a two-dimensional cell culture system (5,000 cells/well in 96-well plates), and cell viability was evaluated by the WST assay (n=5). (B) Cancer cell lines (MCF-7, OCUB, and BT-20) were stimulated with the CM of lymphocytes and macrophages in a three-dimensional (3D) cell culture system (200 cells/well in 96-well plates), and the total cell viability was examined by an ATP assay (n=5). (C) The expression of three genes encoding osteopontin (OPN), heparin-binding EGF-like growth factor (HB-EGF), and interleukin (IL)-6) was tested by quantitative real-time polymerase chain reaction (PCR). (D) BT-20 and MCF-7 cells were cultured with each recombinant protein in a 3D culture system. OPN, HB-EGF, and IL-6 were added at a final concentration of 10 ng/ml or 1 ng/ml. (E) The correlation between gene expression of CD204 and OPN from data of The Cancer Genome Atlas (TCGA) database was analyzed by Spearman's correlation test. (F) Representative figures of double immunohistochemistry (IHC) of Iba1 (green) and OPN (brown) are shown. *p-Value <0.05.*

brief, monocytes were isolated using RosetteSep Human Monocyte Enrichment Cocktail (STEMCELL Technologies, Vancouver, Canada). These monocytes were plated in UpCELL 6-well plates (CellSeed, Tokyo, Japan) and cultured in AIM-V medium (Thermo Fisher, Waltham, MA, USA) supplemented with 2% human serum, granulocyte macrophage-colony stimulating factor (1 ng/ml; Wako), and macrophage-colony stimulating factor (100 ng/ml; Wako) for 7 days to induce monocyte-differentiation into macrophage.

Cell culture of lymphocytes. Human T-lymphocytes were isolated from healthy donors using RosetteSep Human T-cell Enrichment Cocktail (STEMCELL Technologies). Mouse T lymphocytes were obtained from mouse spleen using the Pan T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). Cells were cultured in a cell-culture plate coated with anti-human CD3 antibody (OKT3; eBiosciences, San Diego, CA, USA), human CD28 antibody (BioLegend, San Diego, CA, USA), mouse CD3e (BD Biosciences, San Jose, CA, USA), and mouse CD28 (BD Biosciences).

Polymerase chain reaction (PCR). Total RNA was isolated from cells using RNAiso Plus (TAKARA Bio, Shiga, Japan). PrimeScript RT Reagent Kit (TAKARA Bio) was used for cDNA preparation. Pre-designed primer pairs for quantifying gene expression by SYBER Green PCR were purchased from TAKARA Bio. PCR amplification of the resulting cDNA was carried out with HotStartVersion (TAKARA Bio) using iCycler (BIO-RAD, Irvine, CA, USA).

Tumor models. For evaluating subcutaneous tumor growth, E0771 cells (8×10^5) in 50 μl of phosphate-buffered saline were inoculated subcutaneously into the left and right back of C57BL/6J female mice (CLEA, Shizuoka, Japan). To test the anti-tumor effect of disulfiram (DSF), mice were fed daily with or without DSF in combination with a CE-2 powder diet containing 5% sucrose as described previously (15). All animal procedures were planned according to the ARRIVE guideline (16), and approved by the Animal Research Committee at Kumamoto University (#A2020-089).

Immunohistochemistry (IHC). Paraffin-embedded samples obtained from patients diagnosed with breast cancer at the Izumi General Hospital (Kagoshima, Japan) were used for IHC. The study design was approved by the Institutional Review Board of Kumamoto University (#2059) and Izumi General Hospital (#05). The following antibodies were used: anti-CD204 antibody (SRA-E5; CosmoBio, Tokyo, Japan) and anti-OPN antibody (goat polyclonal, AF1433; R&D Systems, Minneapolis, MN, USA). Horseradish peroxidase-labeled secondary anti-mouse or anti-goat antibody (Nichirei, Tokyo, Japan) was used as the secondary antibody. The reaction was visualized using the diaminobenzidine system (Nichirei) and HistoGreen (Linaris, Heidelberg, Germany).

Murine subcutaneous tumors were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 3-mm were reacted with anti-Iba1 antibody (rabbit polyclonal; Wako) and anti-CD8 antibody (rabbit monoclonal; Cell Signaling, Danvers, MA, USA), then horseradish peroxidase-labeled anti-rabbit antibody (Nichirei) was used as the secondary antibody. The reaction was visualized using the diaminobenzidine system. Subsequently, the staining densities and positive cell counts were determined from ten randomly selected 400× fields using KEYENCE BZ-X800 software (Keyence, Itasca, IL, USA).

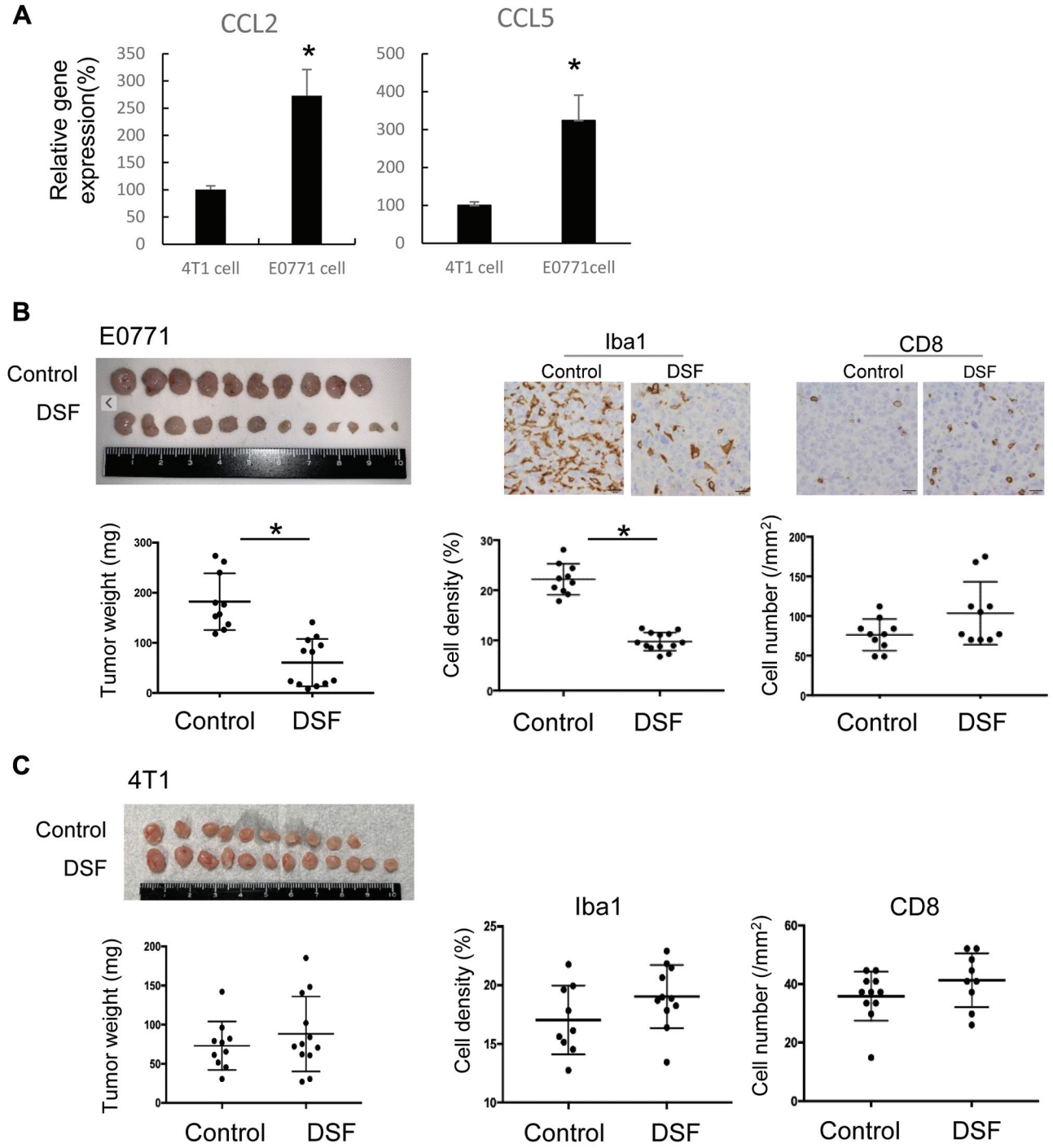

Figure 2. *Significance of tumor-associated macrophages (TAMs) in tumor development. (A) CCL2 and CCL5 gene expression in E0771 and 4T1 cells was tested by real-time PCR (n=3). E0771 cells (B) and 4T1 cells (C) were inoculated subcutaneously into mice, and DSF was fed to the mice with their diet as described in the Materials and Methods section. Subcutaneous tumors were resected after 14 days (E0771) and 10 days (4T1). The density of Iba1-positive TAMs and CD8-positive lymphocytes was evaluated by IHC. *p-Value <0.05.*

Statistical analysis. Statistical analysis was carried out using STATMate (ATOMS, Tokyo, Japan) and JMP7 software (SAS Institute, Chicago, IL, USA). Spearman's correlation test and the Mann-Whitney *U*-test were used to test for correlations between two groups. For all analyses, $p<0.05$ was considered to be statistically significant.

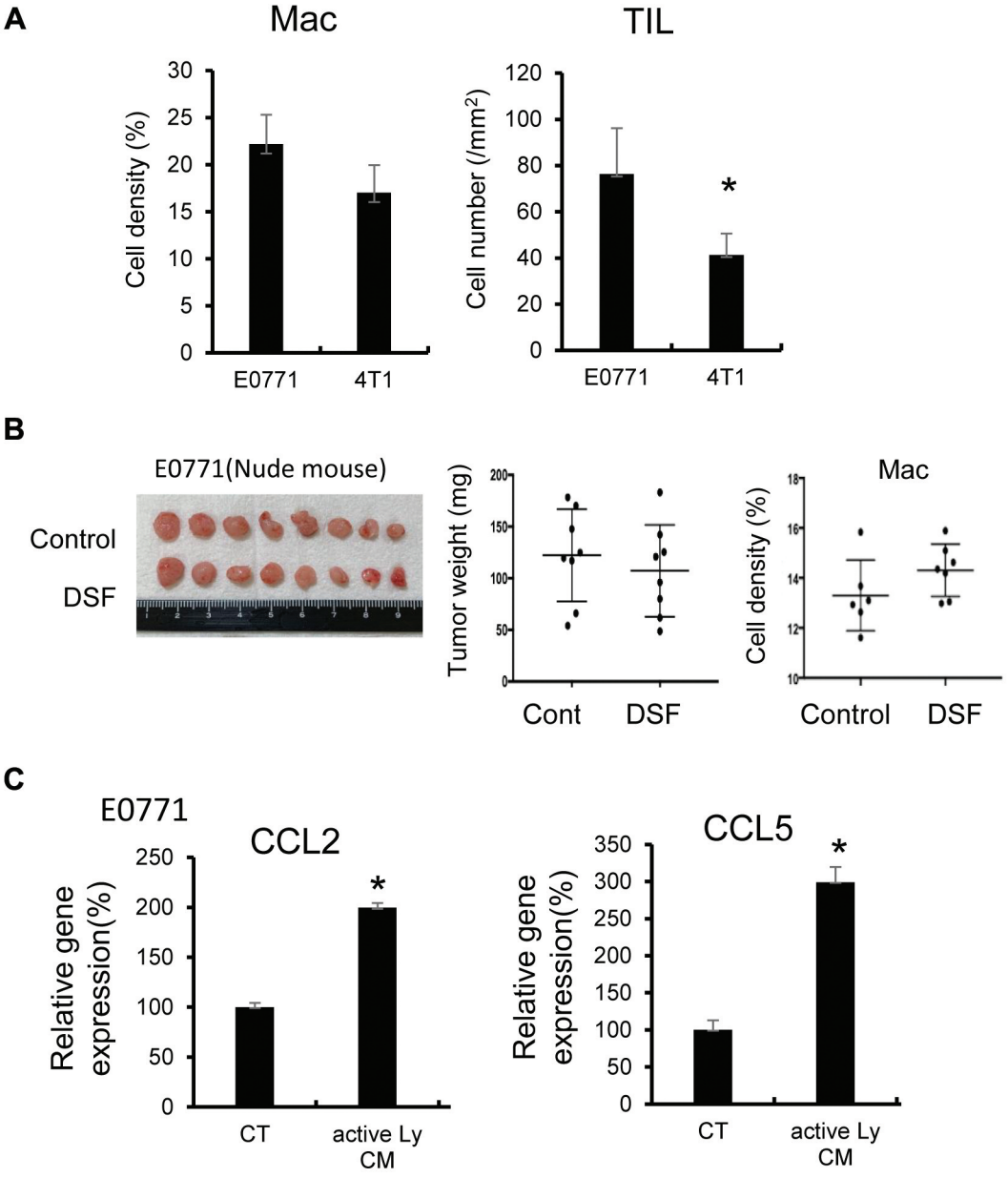

Figure 3. *The influence of lymphocytes on CCL expression. (A) The densities of TAMs and lymphocytes in tumor tissues were compared between E0771 tumors and 4T1 tumors. (B) E0771 cells were inoculated into nude mice, and the anti-tumor effect of DSF was tested by the same methods as in Figure 2C. (C) E0771 cells were co-cultured with the CM of activated lymphocytes for 1 day, and the mRNA expression of CCL2 and CCL5 was tested by real-time PCR.*

Results

Macrophage-derived OPN, HB-EGF, and IL-6 induced cancer cell growth in the 3D cell culture system. We previously suggested that TAM-derived factors influenced breast cancer cell growth based on an IHC study. To test this hypothesis, cancer cell lines (MCF-7, OCUB, and BT-20) were stimulated with conditioned medium (CM) of macrophages. The CM of lymphocytes was also used as control medium. As shown in Figure 1A, no significant difference was seen in the two-dimensional cell culture system. In contrast, in the 3D culture system, the CM of macrophages significantly induced cancer cell growth while the CM of lymphocytes did not (Figure 1B). Next, we focused on three macrophage-derived factors, i.e.,

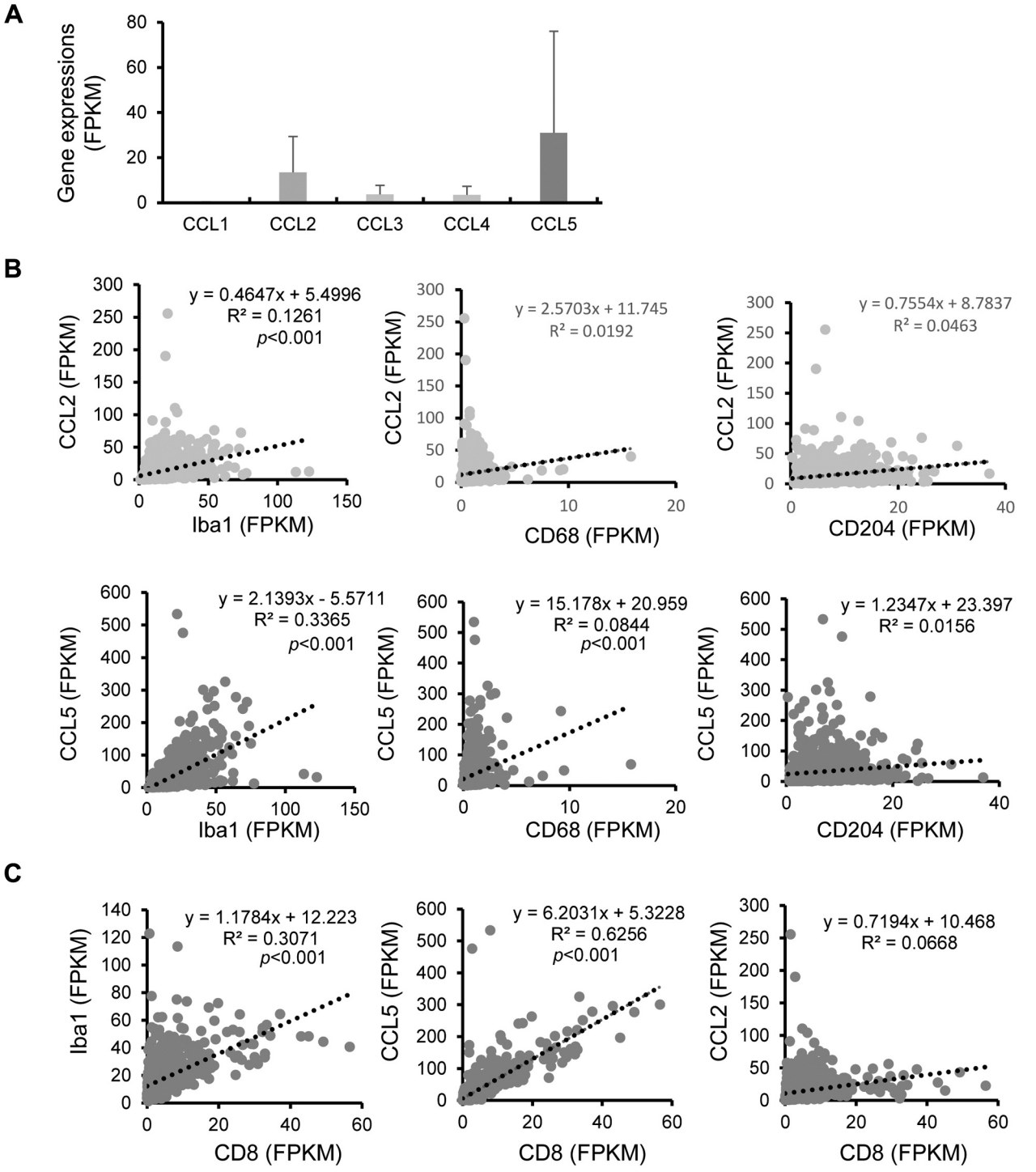

Figure 4. *Continued*

OPN, HB-EGF, and IL-6, as growth factors that induce cancer cell growth based on our previous study. PCR analysis showed that the three genes encoding these factors were expressed only in macrophages, and not in cancer cells (Figure 1C). BT-20 and MCF-7 cells were cultured with each recombinant protein in the 3D culture system. OPN and HB-EGF enhanced the growth of both cancer cell lines while IL-6 enhanced the growth of only the BT-20 cell line (Figure 1D). Based on the expression data

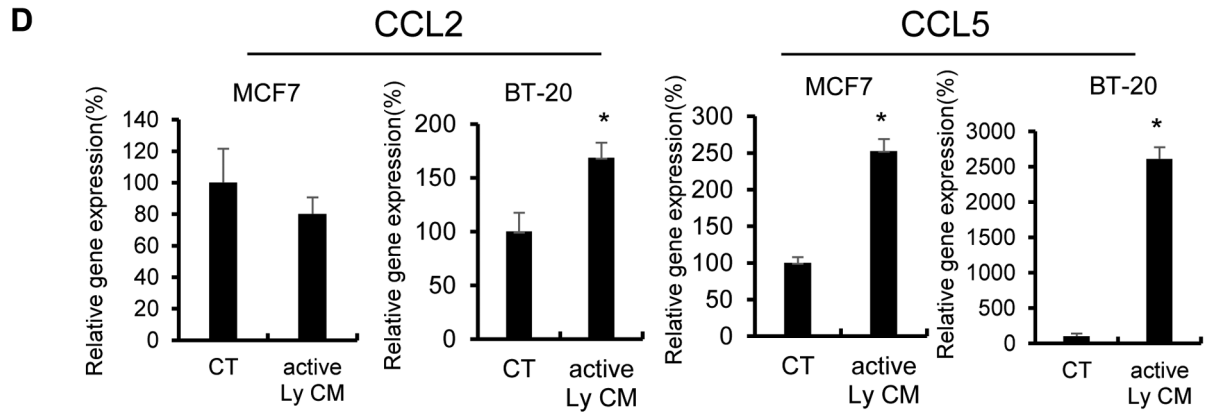

Figure 4. *Chemokines potentially involved in TAM infiltration. (A) Gene data from a cohort were obtained from TCGA database (n=1,075), and the bar graph shows the gene expression (fragments per kilobase of exon per million reads mapped; FPKM) of CCL1, 2, 3, 4, and 5. (B) The correlations between the gene expression of chemokines (CCL2 and CCL5) and macrophage markers (Iba1, CD68, and CD204) were tested by Spearman's correlation test. (C) The correlations between the gene expression of CD8 and the gene expression of Iba1, CCL2, and CCL5 were tested by Spearman's correlation test. (D) MCF7 and BT20 cells were co-cultured with the CM of activated lymphocytes for 1 day, and the mRNA expression of CCL2 and CCL5 was tested by real-time PCR.*

of a breast cancer cohort from The Cancer Genome Atlas (TCGA) database (https://www.proteinatlas.org/), there was a significant correlation between CD204 and OPN (Figure 1E); however, HB-EGF and IL-6 were not correlated with CD204 expression (unpublished data). Double IHC of OPN and CD204 indicated that TAMs express OPN in the TME (Figure 1F).

Blocking of macrophage migration into cancer tissue abrogated cancer cell growth in a mouse model. To investigate the significance of TAMs in *in vivo* cancer cell growth, the FROUNT inhibitor DSF was used in the E0771 and 4T1 murine cancer models. FROUNT is a co-activator of CCR2 and CCR5, and it is involved in macrophage chemotaxis. Before the *in vivo* studies, the mRNA expression of CCL2 and CCL5 in E0771 and 4T1 cell lines was examined by real-time PCR. CCL2 and CCL5 expression was detected in both cell lines; however, the levels were significantly higher in E0771 cells than in 4T1 cells (Figure 2A). This suggested that DSF might be more effective in the E0771 cancer model than in the 4T1 cancer model. As shown in Figure 2B, E0771 cell growth and TAM infiltration were significantly reduced by DSF administration. However, DSF was not effective in inhibiting cancer cell growth and TAM infiltration in the 4T1 model (Figure 2C). Although lymphocyte infiltration seemed to be increased by DSF administration, there was no statistically significant difference (Figure 2C).

Inflammatory reactions may have affected TAM infiltration via chemokine overexpression. In vivo studies using E0771 and 4T1 murine cancer models also revealed that the density of TAMs and lymphocytes in the TME was higher in E0771 tumors than in 4T1 tumors (Figure 3A). A previous study in melanoma demonstrated that anti-tumor immune reactions of lymphocytes induced TAM chemotaxis, which is potentially linked to resistance to immunotherapy (17). To test the effect of lymphocyte infiltration on TAM infiltration into the TME, the anti-tumor effect of DSF was examined in immune-deficient nude mice. Notably, no significant anti-cancer effect was observed when cancer cells were inoculated into the nude mice, and the TAM density was also not influenced by DSF in the nude mice (Figure 3B). This finding indicated that lymphocyte infiltration into the TME around E0771 tumors was associated with TAM infiltration. Therefore, CCL2 and CCL5 production from cancer cells appears to be induced by lymphocyte-derived factors. E0771 cells were stimulated with the CM of mouse lymphocytes activated by anti-CD3 and anti-CD28 antibody for 24 h, and gene expression was tested by real-time PCR. As expected, the expression of both CCL2 and CCL5 was significantly increased by the CM of activated lymphocytes (Figure 3C).

CCL5 may have promoted TAM infiltration into human tumor tissues. Next, we examined the mediators involved in TAM infiltration into the TME. We tested chemokines that are potentially associated with TAM chemotaxis based on TCGA gene expression data of a breast cancer cohort. Since CCL2 and CCL5 were highly expressed in breast cancer tissues (Figure 4A), the correlation between the gene expression of these three genes and the genes encoding macrophage markers were statistically tested. Iba1 and CD68 are markers for total TAMs, and CD204 is a marker for

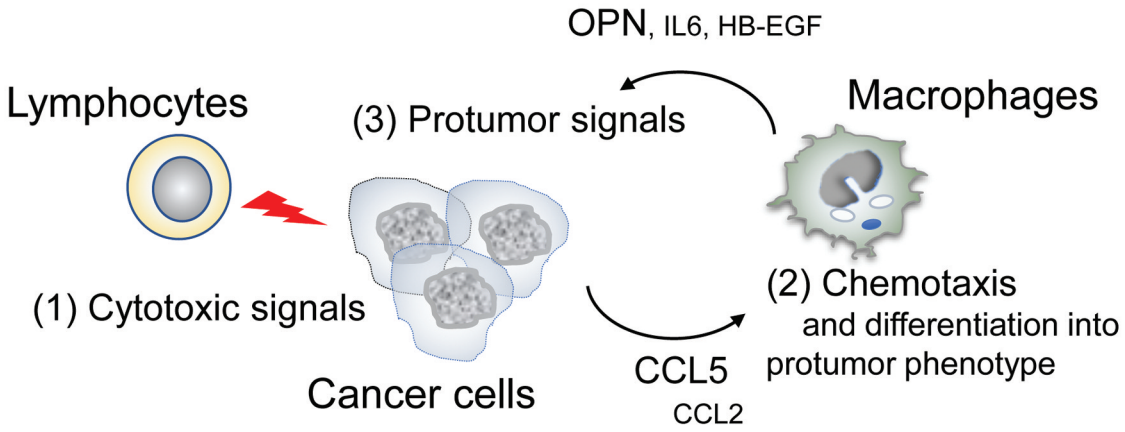

Figure 5. *Suggested scheme of cell–cell interaction of breast cancer cells and macrophages.*

protumor TAMs (18). Notably, the gene expression of CCL5 was significantly correlated to the gene expression of Iba1 and CD68; however, it was not associated with the gene expression of CD204 (Figure 4B). In addition, CCL2 was not linked to the expression of TAM markers (Figure 4B). We next investigated the correlation of TAMs and chemokines with lymphocyte infiltration using TCGA data, and we found a strong positive association between the gene expression of Iba1 and CD8 and between the gene expression of CCL5 and CD8 (Figure 4C). We also tested whether activated lymphocytes influenced CCL2 and CCL5 expression in MCF7 cells and BT-20 cells; the CM of activated human lymphocytes significantly increased CCL5 expression, but not CCL2 expression (Figure 4D).

Discussion

In the present study, we found that TAM-derived factors, including OPN, are potentially involved in breast cancer growth, and TCGA data and the double IHC results indicated that OPN might be one of the most critical factors for breast cancer growth among the cytokines tested in this study. EGF is listed as a growth factor derived from macrophages (19); however, our microarray data of human monocyte-derived macrophages (GSE11481, NCBI gene expression Omunibus) (20) showed that EGF gene expression is rarely seen in macrophages, whereas OPN, HB-EGF, and IL-6 expression is strong in macrophages. Since there are many genes that differ between human macrophages and murine macrophages (21), the importance of TAM-derived factors might differ between humans and mice.

By using the FROUNT inhibitor DSF, CCR2 and CCR5 signals were found to be critical for TAM infiltration in the E0771 breast cancer model. FROUNT was first identified as a CCR2-binding protein that facilitated monocyte chemotaxis (22), then it was found to be associated with CCR5-mediated chemotaxis (23). DSF was identified as a strong inhibitor of FROUNT. DSF administration suppressed tumor development in B16 and LLC murine models by inhibiting TAM infiltration, and it improved anti-tumor immune responses with anti-programmed death 1 therapy (15). TCGA data analysis suggested that CCL5, but not CCL2, is involved in TAM infiltration in human breast cancer. CCL5 expression was significantly up-regulated by lymphocyte-derived factors in human and mice breast cancer cells. In addition to these findings, TCGA data showed that there was a close correlation between gene expression of CCL5 and CD8 in breast cancer, suggesting that the inflammatory signal from activated lymphocytes induced CCL5 overexpression in cancer cells, which in turn accelerated TAM infiltration in the TME.

From TCGA data, CCL5 expression was closely associated with gene expression of Iba1 and CD68, but not that of CD204. Iba1 and CD68 are well-known useful markers for total macrophages. Iba1 was constitutively expressed on cells of the monocyte/macrophage lineage (24, 25), whereas CD68 expression seemed to be lower in TAMs than in resident macrophages in non-tumor areas (unpublished data). In fact, the fragments per kilobase of exon per million reads mapped (FPKM) values of CD68 gene expression were approximately 10% of the FPKM values of Iba1 gene expression. Therefore, Iba1 was suggested to be the most adequate marker for evaluating total TAMs in breast cancer. CD204 expression in TAMs in breast cancer was strongly up-regulated by breast cancer-derived factors, and the fact that the density of CD204-positive TAMs was positively associated with the Ki67-labeling index in breast cancer indicated that CD204 was a marker for protumor TAMs in breast cancer. After TAMs were

derived from circulating monocytes via the CCL5/CCR5 axis, they were differentiated into the protumor phenotype by means of yet-unknown cancer-derived factors.

DEK is an oncoprotein highly expressed in over 62% of breast cancer cases, and high DEK expression was correlated to high cancer cell proliferation and poor clinical course (26). CCL5 expression in breast cancer cells was suggested to be regulated by DEK, and DEK expression was associated to increased M2-like TAMs in murine breast cancer model (27). DEX was shown to induce epithelial to mesenchymal transition by activating PI3K/AKT/mTOR signaling pathway. DEK is suggested to be a key molecule for developing the protumor microenvironment in breast cancer.

In conclusion, our results suggest that the cell-cell interaction between cancer cells and TAMs occurs in the following steps: 1) cytotoxic stress from infiltrating lymphocytes induces CCL5 production in cancer cells; 2) the CCR5 signal recruits TAMs to infiltrate the TME, where cancer-derived factors induce them to differentiate into the protumor phenotype; and 3) TAM-derived soluble factors, such as OPN, HB-EGF, and IL-6, stimulate cancer cell growth (Figure 5). Blocking of TAM infiltration might be a promising approach for anti-breast cancer therapy.

Conflicts of Interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. All Authors have no financial competing interests to declare.

Authors' Contributions

K.M., Y.M., T.S., Y.S., and Y.K. carried out the experiment. K.M. and Y.K. wrote the manuscript with support from Y.Y., Y.F. and S.H. contributed to sample preparation.

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