

Collaboration of cancer-associated fibroblasts and tumour-associated macrophages for neuroblastoma development

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Abstract

Neuroblastoma is the most common extracranial solid tumour in children and is histologically classified by its Schwannian stromal cells. Although having fewer Schwannian stromal cells is generally associated with more aggressive phenotypes, the exact roles of other stromal cells (mainly macrophages and fibroblasts) are unclear. Here, we examined 41 cases of neuroblastoma using immunohistochemistry for the tumour-associated macrophage (TAM) markers CD68, CD163, and CD204, and a cancer-associated fibroblast (CAF) marker, alpha smooth muscle actin (α SMA). Each case was assigned to low/high groups on the basis of the number of TAMs or three groups on the basis of the α SMA-staining area for CAFs. Both the number of TAMs and the area of CAFs were significantly correlated with clinical stage, *MYCN* amplification, bone marrow metastasis, histological classification, histological type, and risk classification. Furthermore, TAM settled in the vicinity of the CAF area, suggesting their close interaction within the tumour microenvironment. We next determined the effects of conditioned medium of a neuroblastoma cell line (NBCM) on bone marrow-derived mesenchymal stem cells (BM-MSCs) and peripheral blood mononuclear cell (PBMC)-derived macrophages *in vitro*. The TAM markers CD163 and CD204 were significantly up-regulated in PBMC-derived macrophages treated with NBCM. The expression of α SMA by BM-MSCs was increased in NBCM-treated cells. Co-culturing with CAF-like BM-MSCs did not enhance the invasive ability but supported the proliferation of tumour cells, whereas tumour cells co-cultured with TAM-like macrophages had the opposite effect. Intriguingly, TAM-like macrophages enhanced not only the invasive abilities of tumour cells and BM-MSCs but also the proliferation of BM-MSCs. CXCL2 secreted from TAM-like macrophages plays an important role in tumour invasiveness. Taken together, these results indicate that PBMC-derived macrophages and BM-MSCs are recruited to a tumour site and activated into TAMs and CAFs, respectively, followed by the formation of favourable environments for neuroblastoma progression.

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Introduction

Neuroblastoma originating from neural crest-derived precursor cells is the most common extracranial solid tumour and is the cause of 15% of cancer-related deaths in children [1]. Such neuroblastomas are characterized by a broad spectrum of clinical behaviours including spontaneous regression, maturation into a benign tumour, and life-threatening aggressiveness. In association with their clinical behaviours, neuroblastomas exhibit a variety of histological types, namely ganglioneuroblastomas, differentiating neuroblastomas, and poorly differentiated neuroblastomas [2].

It is well known that non-tumour stromal cells have critical roles in various aspects of tumour development [3]. Cancer-associated fibroblasts (CAFs), which are the primary components of non-tumour stromal cell populations, are activated forms of fibroblasts that share similarities with the fibroblasts activated in wound healing [4]. These activated fibroblasts are distinct from fibroblasts in their expression of α SMA, and are known to promote tumour growth and progression [5,6]. Some studies showed that the population of α SMA-positive fibroblasts was associated with poor prognosis in several types of tumours [7,8].

Macrophages are also a major stromal component within tumours and are known as tumour-associated

macrophages (TAMs) [9]. Based on their functions, macrophages are broadly classified into two distinct types: the classically activated (M1) type and the alternatively activated (M2) type. M1 macrophages are characterized by the production of pro-inflammatory cytokines, which have the capacity for bactericidal and tumour-suppressive activities. M2 macrophages are characterized by the production of anti-inflammatory cytokines such as IL4, IL10, and TGF β [10]. TAMs are close in character to M2 macrophages; they express IL4 and IL10, and play significant roles in tumour progression [11]. Clinicopathological studies have shown that the number of CD163-positive TAMs in various tumours (including neuroblastomas) is associated with poor prognosis [12,13]. We recently reported that CD204-positive TAMs accumulated in progressive oesophageal squamous cell carcinoma [14].

Some reports have indicated that CAFs and TAMs are synergistically associated with prognostic significance [15–17]. However, the precise mechanisms invoked between these cells remain to be revealed. In the present study, we examined the clinicopathological association of CAFs and TAMs in neuroblastoma progression. We observed that PBMC-derived macrophages and bone marrow-derived mesenchymal stem cells (BM-MSCs) were activated into TAMs and CAFs by conditioned medium taken from a neuroblastoma cell line (NBCM) *in vitro*. Our findings also revealed that TAM-like macrophages enhanced the invasive abilities of surrounding cells and the proliferation of BM-MSCs, whereas CAF-like BM-MSCs increased tumour growth. Furthermore, it was clarified that CXCL2/CXCR2 signalling has a critical role in the invasive ability of neuroblastoma cells co-cultured with TAM-like macrophages.

Materials and methods

Tissue samples and immunohistochemical analyses

A total of 41 primary neuroblastoma and ganglioneuroblastoma (intermixed) tissue samples were obtained from Kobe Children's Hospital (Kobe, Japan) before the administration of chemotherapy or radiotherapy to the patients (see supplementary material, Table S1). Informed consent was obtained from all patients and/or their guardians, and both the Kobe Children's Hospital Institutional Review Board and the Kobe University Institutional Review Board approved this study.

All resected specimens were fixed in 10% formalin and embedded in paraffin wax. Each sample was categorized according to the International Neuroblastoma Pathology Committee (INPC) and staged using the International Neuroblastoma Staging System [18,19]. The tumours were further categorized into three groups – poorly differentiated, differentiating, and ganglioneuroblastomas – based on the population of Schwannian stromal cells. The risk of progression

in each case was assigned by the Children's Oncology group (COG) risk classification scheme [20,21].

Immunohistochemistry was performed with a Labelled Streptavidin-Biotin kit (Dako Japan, Kyoto, Japan) as described elsewhere [22]. Details of antibodies, antigen retrieval, antibody dilution, and secondary layers are given in the supplementary material, Table S2.

Macrophage count and CAF area calculation

Round cells positive for CD68 or CD163 or CD204 were identified by screening the entire tumour area in a low-power field (100 \times field) and selecting three areas with the highest density of macrophages. The mean number of macrophages counted in the three independent high-power fields (400 \times field) was calculated. The CAF area where macrophages were counted was assessed at 200 \times overall magnification. The area fraction of CAFs was defined as the ratio of the α SMA-positive and h-caldesmon-negative area to the total area on the microscopic field, using ImageJ software ver. 1.48 (National Institutes of Health, Bethesda, MD, USA).

Cell cultures

The neuroblastoma cell line BE(2)-C was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM and Ham's F-12 (DMEM/F-12; Wako, Osaka, Japan) supplemented with 10% FBS. We purchased human BM-MSCs from the ATCC and maintained them in low-glucose DMEM (Wako) supplemented with 10% FBS.

Recombinant human CXCL1 and CXCL2 were obtained from R&D Systems (Minneapolis, MN, USA; 275-GR and 276-GB). A CXCR2-specific antibody for neutralization was purchased from Abcam (ab10401; Cambridge, MA, USA).

Preparation of neuroblastoma-conditioned medium (NBCM)

We prepared the NBCM by plating 5×10^6 BE(2)-C cells in 10 ml of culture medium in 100-mm dishes for 24 h, thereafter changing the medium to DMEM supplemented with 10% human AB serum (Lonza, Walkersville, MD, USA). After 2 days, the supernatants were collected, centrifuged, and stored in aliquots at -80°C .

Preparation of TAM-like macrophages and CAF-like BM-MSCs

We obtained PBMCs from healthy volunteer donors. The CD14-positive PBMCs were purified using an autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany). After purification, the cells were plated at 1×10^5 cells/ml per 30 mm well and cultured with human M-CSF (hM-CSF) (25 ng/ml; R&D Systems) for 6 days to induce macrophage differentiation, regarded as M0 macrophages according to the recommendation [23].

In order to induce TAM-like macrophages, we then cultured M0 macrophages in 50% NBCM for 2 days. For the CAF-like BM-MSc preparation, human BM-MScs were plated in 1×10^5 cells/ml per well and cultured in 50% NBCM for 7 days. The medium was changed every 3 or 4 days.

Proliferation assays

For assessment of cell proliferation, 1×10^5 neuroblastoma cells or BM-MScs were cultured in the bottom chamber of a 24-well insert plate. Then 1×10^5 TAM-like macrophages, CAF-like BM-MScs, or neuroblastoma cells were seeded in the upper 0.4- μ m pore-size insert. There were no cells in the upper inserts of control wells. After co-culture in the serum-free conditions or treatment as indicated in the Results section for 48 h, we assessed cell proliferation by the increase in cell number, as estimated by using an MTS colourimetric assay (Promega, Madison, WI, USA). Absorbance was read at 405 and 492 nm according to the manufacturer's instructions.

In vitro invasion assays

The *in vitro* invasion assay was performed using a 24-well BioCoat Matrigel invasion chamber (BD Biosciences, Bedford, MA, USA). Prior to the tumour or BM-MSc invasion assay, 5×10^4 PBMCs were differentiated to TAM-like macrophages in the lower chambers. BM-MScs were differentiated to CAF-like cells in culture dishes and then 5×10^4 tumour cells or CAF-like cells were seeded into the lower chambers 24 h before the inserts were exposed. There was no cell in the lower chambers of control wells. At the same time, 5×10^4 tumour cells or BM-MScs were seeded in the upper inserts. When the inserts were exposed to the lower chambers, the conditioned media within the inserts and bottom chambers were changed to serum-free medium or treated as indicated in the Results section.

For the PBMC invasion assay, 5×10^4 PBMCs were separated to the upper inserts and 5×10^4 tumour cells or CAF-like BM-MScs were divided into the lower chambers. 24 h later, 5×10^4 PBMCs were distributed onto the upper inserts and then these inserts were exposed to the lower chambers. When the inserts were exposed to the lower chambers, the conditioned media within the inserts and bottom chambers were replaced with serum-free RPMI-1640 (Wako).

After the upper inserts were exposed to the lower chambers, the cells were incubated for 48 h at 37°C in a CO₂ incubator. The cells remaining in the upper inserts were then removed with a cotton swab. Thereafter, the cells that had invaded onto the lower surface of the Matrigel-coated membrane were counted using a Diff-Quik staining kit (Sysmex, Kobe, Japan).

Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was performed as described previously [22]. The primer sequences and detailed procedures are given in the supplementary material, Table S3 and Figure S1.

Immunofluorescence

The cells were grown on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA) and then fixed with 100% methanol at -20°C for 10 min. Immunofluorescence was conducted as described previously [22]. Details of the primary and secondary fluorescein conjugated antibodies are listed in Table S2 (supplementary material). The nuclei were stained with DAPI (Wako). All images were acquired by confocal microscopy (LSM 700; Carl Zeiss, Oberkochen, Germany).

Western blotting

Western blotting was performed as described previously [24]. Details of the primary antibodies and secondary horseradish peroxidase (HRP)-conjugated antibodies are provided in the supplementary material, Table S2.

Enzyme-linked immunosorbent assay (ELISA)

Human CXCL1 and CXCL2 concentrations were measured by human SimpleStep ELISA kits (Abcam) according to the manufacturer's instructions.

Statistical analysis

The relationships between clinicopathological factors and immunohistochemical results were estimated using Fisher's exact test. The results are expressed as mean \pm SD or SEM, and the statistical significance was analysed using two-sided Student's *t*-tests. A *p*-value less than 0.05 was considered significant. All statistical analyses were carried out using SPSS Statistics ver. 22 (IBM, Chicago, IL, USA).

Results

The number of TAMs was closely associated with clinicopathological features of the neuroblastomas

We examined the existence of macrophages positive for CD68, CD163, and CD204 in 41 neuroblastoma samples using immunohistochemistry. As reported before, there were more macrophages positive for CD68 and CD163 in the stage 4 (metastatic) samples compared with the samples of other stages (i.e. non-metastatic and stage 4s, spontaneously regressive stage) (Figure 1A, B, G, H). CD204-positive macrophages were also frequently detected in the aggressive-stage samples (Figure 1C, I).

Next, we divided the samples into high and low groups according to the median number of CD68-positive macrophages (26; range 9.3–92.3), CD163-positive macrophages (24.3; range 1.7–97.7), and CD204-positive macrophages (17.3; range 2.3–56.3), and then analysed the association with clinicopathological features (Table 1). A high number of CD68-positive and CD163-positive macrophages in

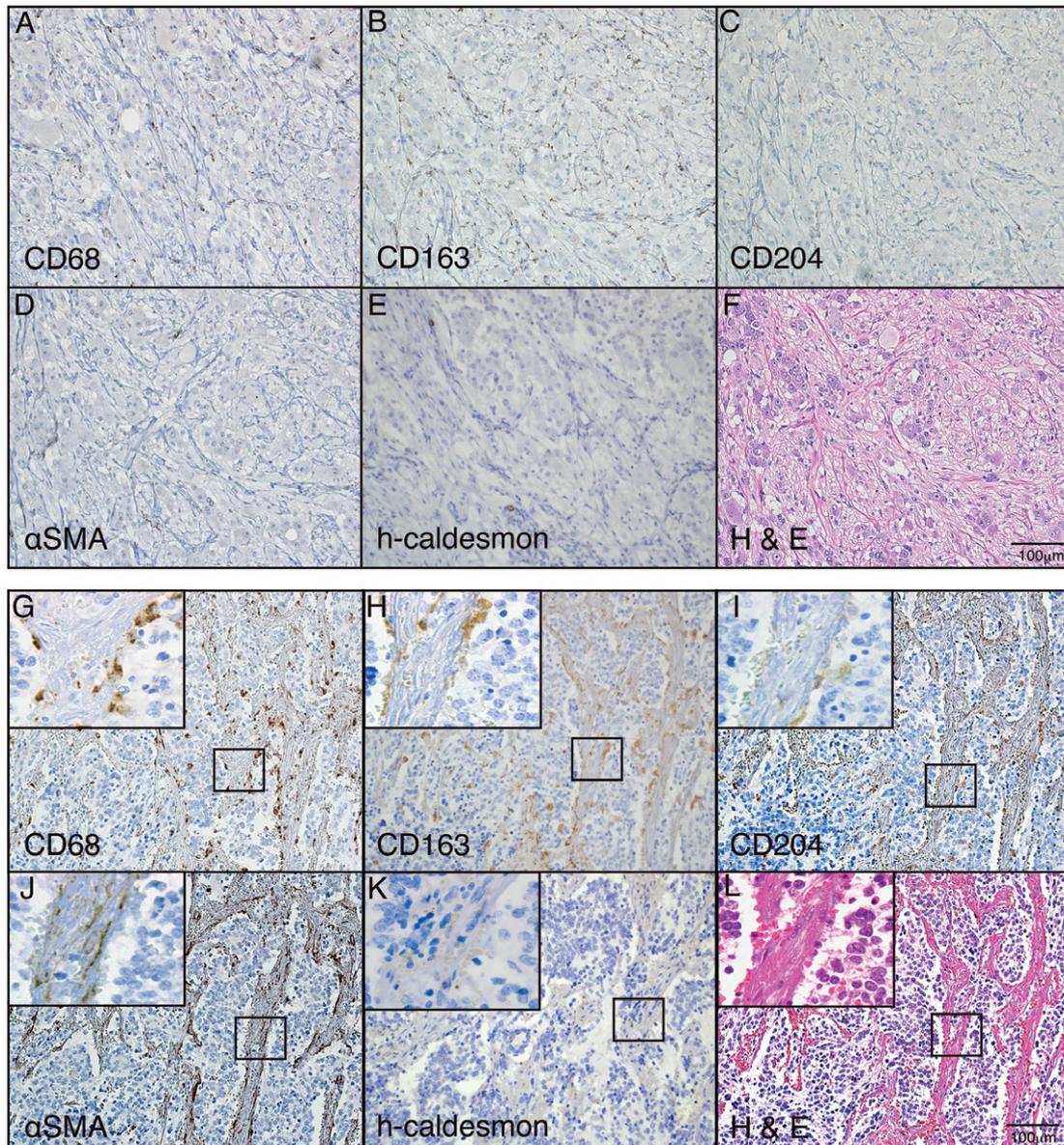


Figure 1. Immunohistochemical staining for CD68, CD163, CD204, α SMA, and h-caldesmon in neuroblastoma samples. Two representative cases are shown. (A–F) CD68 (A), CD163 (B), CD204 (C), α SMA (D), and h-caldesmon (E) were stained in a non-metastatic case, stage 1. (G–L) CD68 (G), CD163 (H), CD204 (I), α SMA (J), and h-caldesmon (K) were stained in a metastatic case, stage 4. The insets of G–L indicate high magnification views of the boxed area in each panel. Original magnification: 100 \times . Inset magnification: 400 \times .

the neuroblastoma were closely correlated with aggressive phenotypes including clinical stage ($p=0.001$ and $p<0.001$, respectively), *MYCN* amplification ($p=0.045$ and $p=0.045$, respectively), Ki-67 index ($p=0.041$ and $p=0.041$, respectively), INPC histological classification ($p=0.001$ and $p<0.001$, respectively), histological type ($p=0.001$ and $p=0.001$, respectively) and COG risk classification ($p=0.001$ and $p<0.001$, respectively).

Interestingly, a significant positive correlation between a high number of CD163-positive macrophages and bone marrow metastasis was observed ($p=0.028$). In contrast, a high number of CD204-positive macrophages was correlated only with Ki-67 index ($p=0.007$) and histological type ($p=0.006$). Our results also revealed that the neuroblastomas without

MYCN proto-oncogene amplification diagnosed at age ≥ 18 months showed significantly larger numbers of CD163-positive macrophages compared with those diagnosed at age < 18 months (ten 'high' and six 'low' patients diagnosed at age ≥ 18 months; four 'high' and 14 'low' children diagnosed at age < 18 months; $p<0.035$), whereas we observed that *MYCN* amplification was also correlated with the number of TAMs in the neuroblastomas.

The grades of CAFs were closely associated with clinicopathological features of neuroblastoma. We next examined the existence of α SMA-positive CAFs in 41 neuroblastoma samples by immunohistochemistry. As we observed for the TAMs,

Table 1. Correlation between the number of macrophages positive for CD68, CD163, and CD204 in tumour stroma with clinicopathological features of neuroblastoma

	No of cases	No of macrophages*								
		CD68 ⁺			CD163 ⁺			CD204 ⁺		
		Low (n = 21)	High (n = 20)	p value [†]	Low (n = 21)	High (n = 20)	p value [†]	Low (n = 21)	High (n = 20)	p value [†]
Age (months)										
<18	21	12	9	0.538	14	7	0.063	9	12	0.354
≥18	20	9	11		7	13		12	8	
Stage [‡]										
1, 2, 3, 4 s	25	18	7	0.001	19	6	<0.001	16	9	0.058
4	16	3	13		2	14		5	11	
MYCN										
Non-amplified	34	20	14	0.045	20	14	0.045	19	15	0.238
Amplified	7	1	6		1	6		2	5	
Ki-67 index [§]										
≤5%	19	13	6	0.041	13	6	0.041	14	5	0.007
>5%	22	8	14		8	14		7	15	
Bone marrow metastasis										
Negative	24	15	9	0.118	16	8	0.028	15	9	0.118
Positive	17	6	11		5	12		6	11	
INPC classification										
Favourable	25	18	7	0.001	19	6	<0.001	16	9	0.058
Unfavourable	16	3	13		2	14		5	11	
Histological type										
GNB	8	8	0	0.001	7	1	0.001	8	0	0.006
Differentiating	12	8	4		9	3		6	6	
Poorly	21	5	16		5	16		7	14	
COG risk										
Low	19	14	5	0.001	16	3	<0.001	12	7	0.054
Intermediate	7	5	2		4	3		5	2	
High	15	2	13		1	14		4	11	

*The median values of CD68⁺, CD163⁺ or CD204⁺ macrophage numbers in tumour nests and stroma within the areas were used to divide the patients into high and low groups.

[†]Data were analysed by Fisher's exact test and $p < 0.05$ was considered statistically significant.

[‡]According to the International Neuroblastoma Staging System [19].

[§]The presence of nuclear Ki-67 was counted in 1000 tumour cells in ten randomly selected fields.

^{||}Based on the population of Schwannian stromal cells. GNB = ganglioneuroblastoma.

α SMA-positive CAFs were also dominant in the stage 4 tumours (Figure 1D, J). Since it is known that α SMA is expressed in normal vascular smooth muscle cells, we used h-caldesmon antibody to detect them (Figure 1E, K). We then calculated the area of CAFs by subtracting the h-caldesmon-positive area from the α SMA-positive area. After the calculation, we divided the neuroblastoma samples into three groups on the basis of the CAF area and then analysed the association with clinicopathological features (Table 2). Intriguingly, the area of CAFs was significantly correlated with aggressive phenotypes including clinical stage ($p = 0.001$), MYCN amplification ($p = 0.045$), bone marrow metastasis ($p = 0.009$), histological classification ($p = 0.01$), histological type ($p < 0.001$), and COG risk classification ($p = 0.013$).

TAMs resided in the vicinity of CAFs in the neuroblastoma tissues

As TAMs were adjacent to α SMA-positive fibroblasts (Figure 1G–J), we investigated the association between the number of TAMs and the area of CAFs in the neuroblastomas. Interestingly, higher numbers of macrophages

expressing CD68, CD163, and CD204 were observed in higher CAF areas (Table 3).

NBCM induced TAM markers in PBMC-derived macrophages

To investigate whether macrophages are able to acquire TAM characteristics under a neuroblastoma tumour microenvironment, we evaluated the expression of TAM markers in PBMC-derived macrophages exposed to 50% NBCM (Figure 2A). The results confirmed an increase of CD163 and CD204 expression in PBMC-derived macrophages effected by 50% NBCM (Figure 2B, C). In addition, PBMC-derived macrophages treated with 50% NBCM significantly enhanced their expression of IL10, encoding an anti-inflammatory cytokine, but not IL12, encoding a pro-inflammatory cytokine (Figure 2D, E).

NBCM induced CAF markers in human BM-MSCs

We next investigated whether 50% NBCM would induce the expression of CAF markers in human neonate dermal fibroblasts and human BM-MSCs as examples of stromal cells. Although expression of the CAF marker α SMA (ACTA2) was not enhanced in the human neonate

Table 2. Correlation between CAF grades in tumour stroma with clinicopathological features of neuroblastoma

	No of cases	CAF grades*			<i>p</i> value [†]
		0 (n = 13)	1 (n = 14)	2 (n = 14)	
Age (months)					
<18	21	6	8	7	0.844
≥18	20	7	6	7	
Stage [‡]					
1, 2, 3, 4 s	25	13	8	4	0.001
4	16	0	6	10	
MYCN					
Non-amplified	34	13	12	9	0.045
Amplified	7	0	2	5	
Ki-67 index [§]					
≤5%	19	9	6	4	0.101
>5%	22	4	8	10	
Bone marrow metastasis					
Negative	24	12	7	5	0.009
Positive	17	1	7	9	
INPC classification					
Favourable	25	12	8	5	0.01
Unfavourable	16	1	6	9	
Histological type					
GNB	8	7	1	0	<0.001
Differentiating	12	5	7	0	
Poorly	21	1	6	14	
COG risk					
Low	19	9	6	4	0.013
Intermediate	7	4	2	1	
High	15	0	6	9	

*The grades of CAF area in tumour nests and stroma within the areas were used to divide the patients into three groups (grade 0, low; grade 1, intermediate; grade 2, high).

[†]Data were analysed by Fisher's exact test and *p* < 0.05 was considered statistically significant.

[‡]According to the International Neuroblastoma Staging System [19].

[§]The presence of nuclear Ki-67 was counted in 1000 tumour cells in ten randomly selected fields.

^{||}Based on the population of Schwannian stromal cells. GNB = ganglioneuroblastoma.

dermal fibroblasts (Figure 3A), the human BM-MSCs showed increased α SMA and FAP mRNA expression when in 50% NBCM, in a time-dependent manner (Figure 3B). We also confirmed that α SMA and FAP proteins were induced in human BM-MSCs cultured in 50% NBCM for 7 days (Figure 3C, D).

Effects of co-culture conditions on cell growth and invasive ability

To investigate the relationship between neuroblastoma cells and non-tumour stromal cells, we first analysed the effects of co-culture conditions on cell proliferation. The proliferation of tumour cells was enhanced by CAF-like BM-MSCs, but not TAM-like macrophages (Figure 4A). On the other hand, the proliferation of BM-MSCs was enhanced by both tumour cells and TAM-like macrophages (Figure 4B).

Second, we analysed the effects of co-culture conditions on invasive ability. The invasion of neuroblastoma cells was not enhanced under co-culture conditions with CAF-like BM-MSCs, but it was enhanced under co-culture conditions with TAM-like macrophages

(Figure 4C). On the other hand, the invasion of BM-MSCs and PBMCs was enhanced under both co-culture conditions with tumour cells and TAM-like macrophages or CAF-like BM-MSCs (Figure 4D, E). Interestingly, PBMCs invaded individually in the CAF-like BM-MSC co-culture conditions, while PBMCs formed clusters when they invaded in the tumour co-culture conditions (Figure 4E).

CXCL2 secreted from TAM-like macrophages enhanced tumour invasiveness through CXCL2/CXCR2 signalling

We assessed the mRNA levels of MMPs, CC/CXC chemokines, and growth factors that have been described as being involved in tumour growth and progression in TAM-like macrophages and CAF-like BM-MSCs (see supplementary material, Figure S1A, B). Levels of CXCL1 and CXCL2 were remarkably induced in TAM-like macrophages compared with M0 macrophages, and we confirmed the up-regulation of CXCL1 and CXCL2 secretion in the supernatants of TAM-like macrophages (Figure 5A). We then investigated the effects of CXCL1 and CXCL2 on proliferation and invasiveness in neuroblastoma cells and BM-MSCs. Neither CXCL1 nor CXCL2 had any effect on the proliferation of neuroblastoma cells and BM-MSCs (Figure 5B; see also supplementary material, Figure S2A). Although the invasiveness of BM-MSCs was not influenced even under CXCL1 and CXCL2 stimulation, CXCL2 stimulation enhanced the invasive ability of neuroblastoma cells in a dose-dependent manner (Figure 5C; see also supplementary material, Figure S2B). Furthermore, after we confirmed the expression of CXCR2 by neuroblastoma cells, it was revealed that neutralization of CXCR2 with the CXCR2 antibody attenuated the effect of TAM-like macrophages on the invasiveness of neuroblastoma cells (Figure 5D). As we could not detect the expression of CXCR2 in BM-MSCs, neutralization of CXCR2 did not have any effect on the invasiveness of BM-MSCs co-cultured with TAM-like macrophages (see supplementary material, Figure S2C).

Discussion

It is known that neuroblastomas form a tumour-promoting microenvironment by editing immune systems [25]. Using a tissue microarray, Asgharzadeh *et al* reported that metastatic neuroblastomas showed a higher degree of CD163-positive macrophage infiltration than non-metastatic neuroblastomas, and the number of CD163-positive macrophages was associated with the clinical stage. They also reported that neuroblastomas from children without MYCN proto-oncogene amplification at age ≥ 18 months showed greater expression of inflammation-related genes associated with macrophages compared with

Table 3. Correlation between macrophages positive for CD68, CD163, and CD204 with CAF grades in the stroma of neuroblastomas

CAF grade [†]	No of cases	CD68 ⁺			CD163 ⁺			CD204 ⁺		
		macrophages [‡]		<i>p</i> value [‡]	macrophages [‡]		<i>p</i> value [‡]	macrophages [‡]		<i>p</i> value [‡]
		Low (n = 21)	High (n = 20)		Low (n = 21)	High (n = 20)		Low (n = 21)	High (n = 20)	
0	13	13	0	<0.001	12	1	0.001	11	2	0.014
1	14	5	9		6	8		5	9	
2	14	3	11		3	11		5	9	

[†]The median values of CD68⁺, CD163⁺ or CD204⁺ macrophage numbers in tumour nests and stroma within the areas were used to divide the patients into high and low groups.

[‡]The grades of CAF area in tumour nests and stroma within the areas were used to divide the patients into three groups (grade 0, low; grade 1, intermediate; grade 2, high).

[‡]Data were analysed by Fisher's exact test and *p* < 0.05 was considered statistically significant.

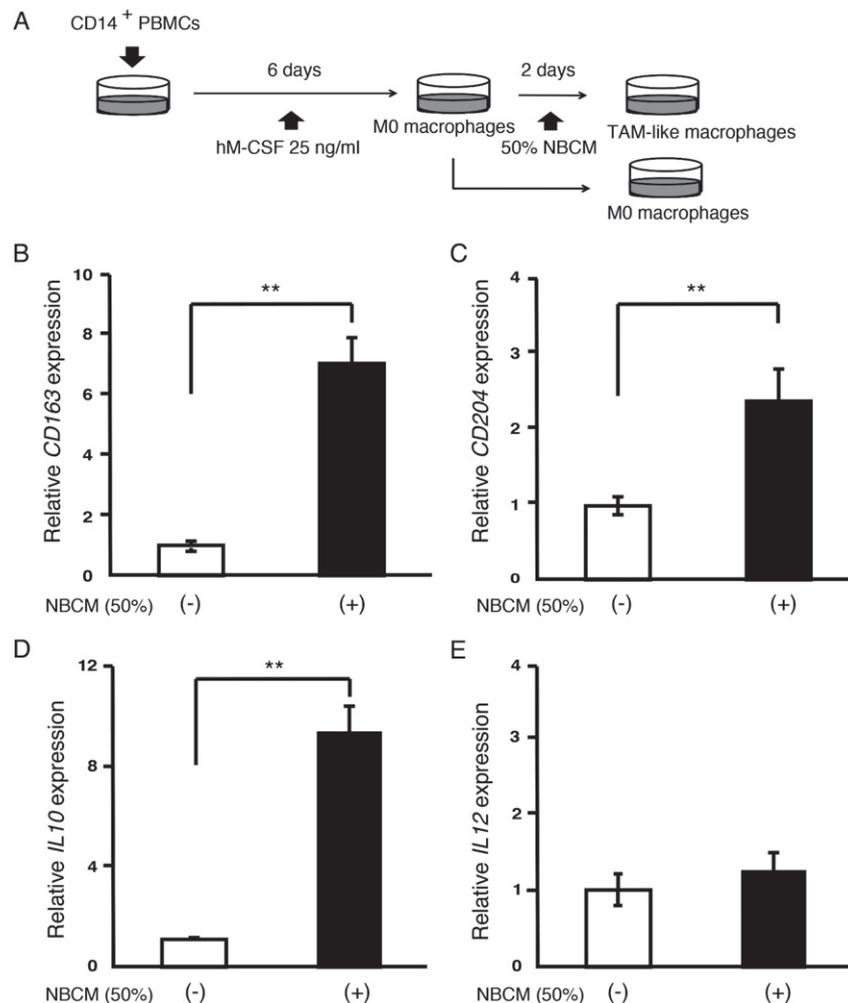


Figure 2. Acquisition of TAM characteristics in PBMCs treated with conditioned medium of the neuroblastoma cell line (NBCM). (A) CD14-positive PBMCs were selected with MACS and then treated with 25 ng/ml recombinant human M-CSF for 6 days to induce macrophage-like differentiation, and then exposed to 50% NBCM for 2 days. (B, C) The mRNA levels of the TAM markers *CD163* and *CD204* in PBMC-derived macrophages stimulated with 50% NBCM were determined by RT-qPCR, normalized to *GAPDH*. (D, E) The *IL10* and *IL12* mRNA expression patterns in PBMC-derived macrophages stimulated with 50% NBCM were analysed by RT-qPCR, normalized to *GAPDH* expression. Values represent the mean of three experiments in duplicate and are expressed as mean \pm SD (***p* < 0.01).

those from children diagnosed at age < 18 months [12]. In the present study, we selected the area with the highest density of macrophages within the entire tumour area of each case, and then we observed that the number of CD163-positive macrophages was significantly associated with other clinicopathological features. Although it is well known that amplification of *MYCN*

is associated with a high risk of neuroblastoma [26], the number of TAMs itself in *MYCN*-amplified neuroblastomas may contribute to the malignancy.

Neuroblastomas are histologically classified by the population of Schwannian stromal cells into diagnostic categories according to the INPC [27,28]. It was reported that the population of CAFs was inversely

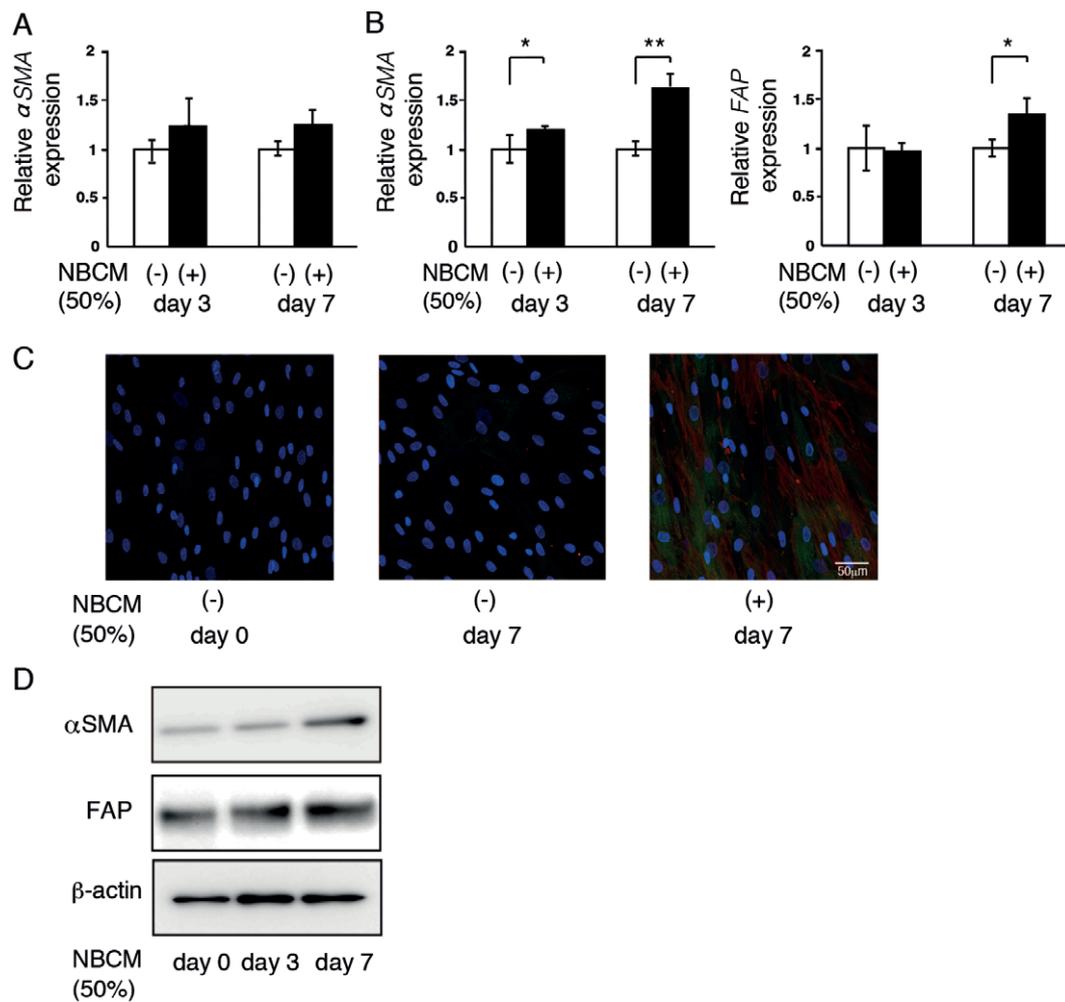


Figure 3. The induction of CAF markers, α SMA, and fibroblast activation protein (FAP) in human neonate fibroblasts and BM-MSCs treated with NBCM. Fibroblasts and BM-MSCs were exposed to 50% NBCM for 3 and 7 days. The medium was changed every 3 or 4 days. (A, B) The α SMA (*ACTA2*) mRNA expression in fibroblasts (A), and α SMA and FAP mRNA expression in BM-MSCs (B) stimulated with 50% NBCM for the indicated days were determined by RT-qPCR, normalized to *GAPDH* expression. Values represent the mean of three experiments in duplicate and are expressed as mean \pm SD (* p < 0.05; ** p < 0.01). (C) BM-MSCs were stimulated with 50% NBCM for 7 days. After the stimulation, double immunofluorescence was performed using anti- α SMA (red) and anti-FAP (green). DAPI (blue) provided nuclear counterstaining. Original magnification: 200 \times . (D) The protein levels of α SMA, FAP, and β -actin in BM-MSCs stimulated with 50% NBC for the indicated days were quantified by western blotting.

correlated with the population of Schwannian stromal cells [29]. In that study, a significant association between the CAF area and clinical features other than microvascular proliferation was not detected. However, our present findings revealed a significant association between the CAF area and clinical features, which was also shown by the results of our analysis of CD163-positive macrophages. This discrepancy in findings may be due to the differing methods used to measure the CAF area. We first defined the precise CAF area by subtracting the h-caldesmon-positive area from the α SMA-positive area in this study, and then we focused on the area where many macrophages had invaded the tumour. We speculated that the area formed a tumour microenvironment consisting of stromal cells including CAFs, based on a report that innate immune cells including macrophages were recruited to the tumour site at very early stages and then formed a tumour-progressive environment [30].

The combination of CAFs and TAMs within a tumour site has been reported to be a potential prognostic factor in oral cancer and colorectal cancer patients [16,17]. We also observed that there was a significant association between the number of TAMs and the area of CAFs in neuroblastomas, and both of these parameters were correlated with clinical features. It was recently reported that pro-inflammatory lipid mediators produced by CAFs contributed to tumour growth in high-risk neuroblastomas accompanied by a high infiltration of M2-polarized macrophages expressing CD163 [31]. In another report, low-dose aspirin, a dual COX-1/COX-2 inhibitor that blocks pro-inflammatory lipid mediators, inhibited the neuroblastoma progression by reducing the number of tumour-associated immune cells, including TAMs [32]. In keeping with our findings and these reports, the combination of TAMs and CAFs could be a potential prognostic factor and also a potential therapeutic target in neuroblastomas.

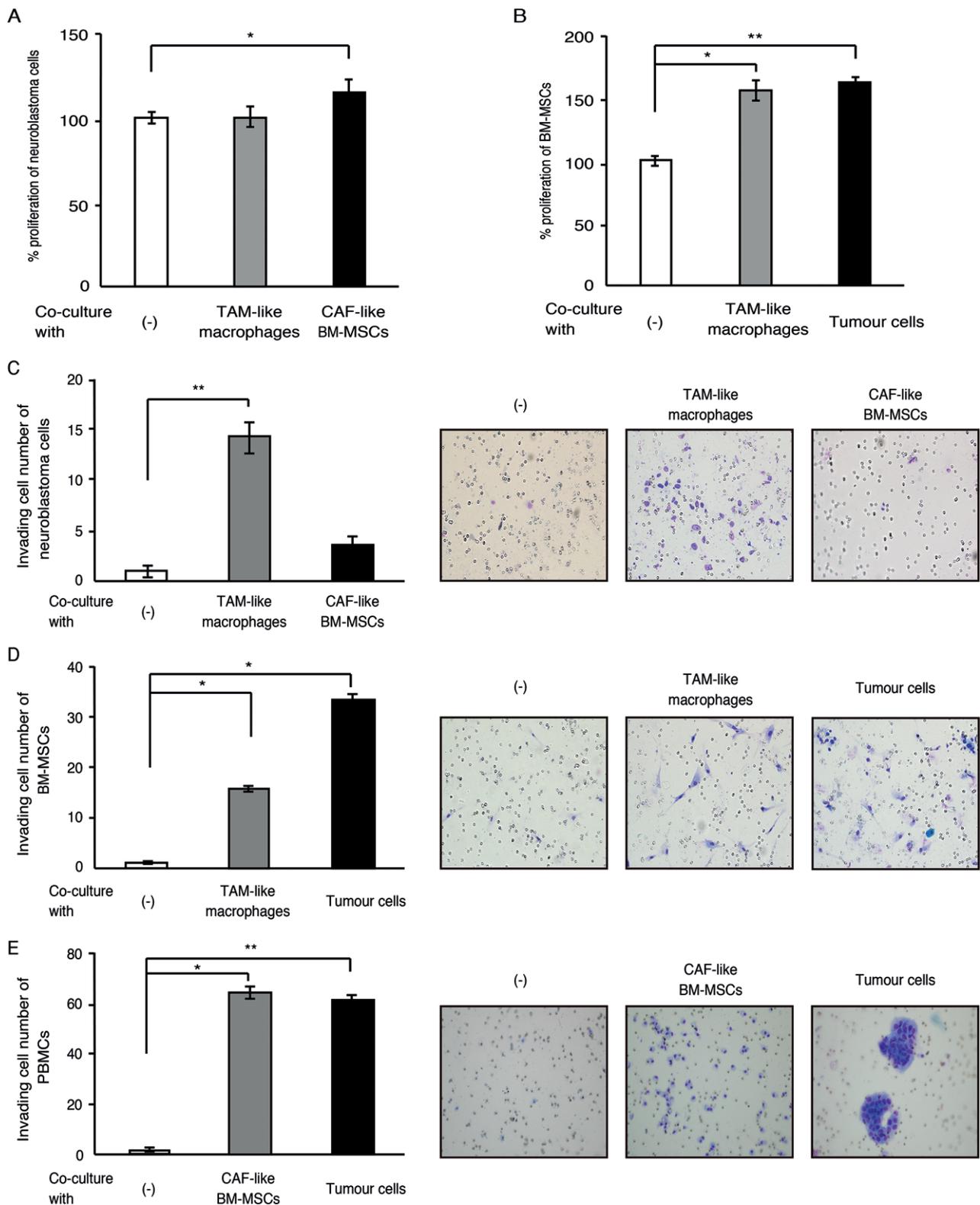


Figure 4. Induction of growth and invasive ability under co-culture conditions. The growth-promoting effects of co-culture conditions on neuroblastoma cells (A) and BM-MSCs (B) were assessed by the MTS assay. Values represent the mean of four experiments and are expressed as mean \pm SEM (* p < 0.05; ** p < 0.01). Effects of the co-culture conditions on the invasive ability of neuroblastoma cells (C), BM-MSCs (D), and PBMCs (E) were analysed by a BioCoat Matrigel invasion chamber assay. Invading cells were counted in five randomly chosen fields. Values are the mean of four experiments and are expressed as mean \pm SEM (* p < 0.05; ** p < 0.01).

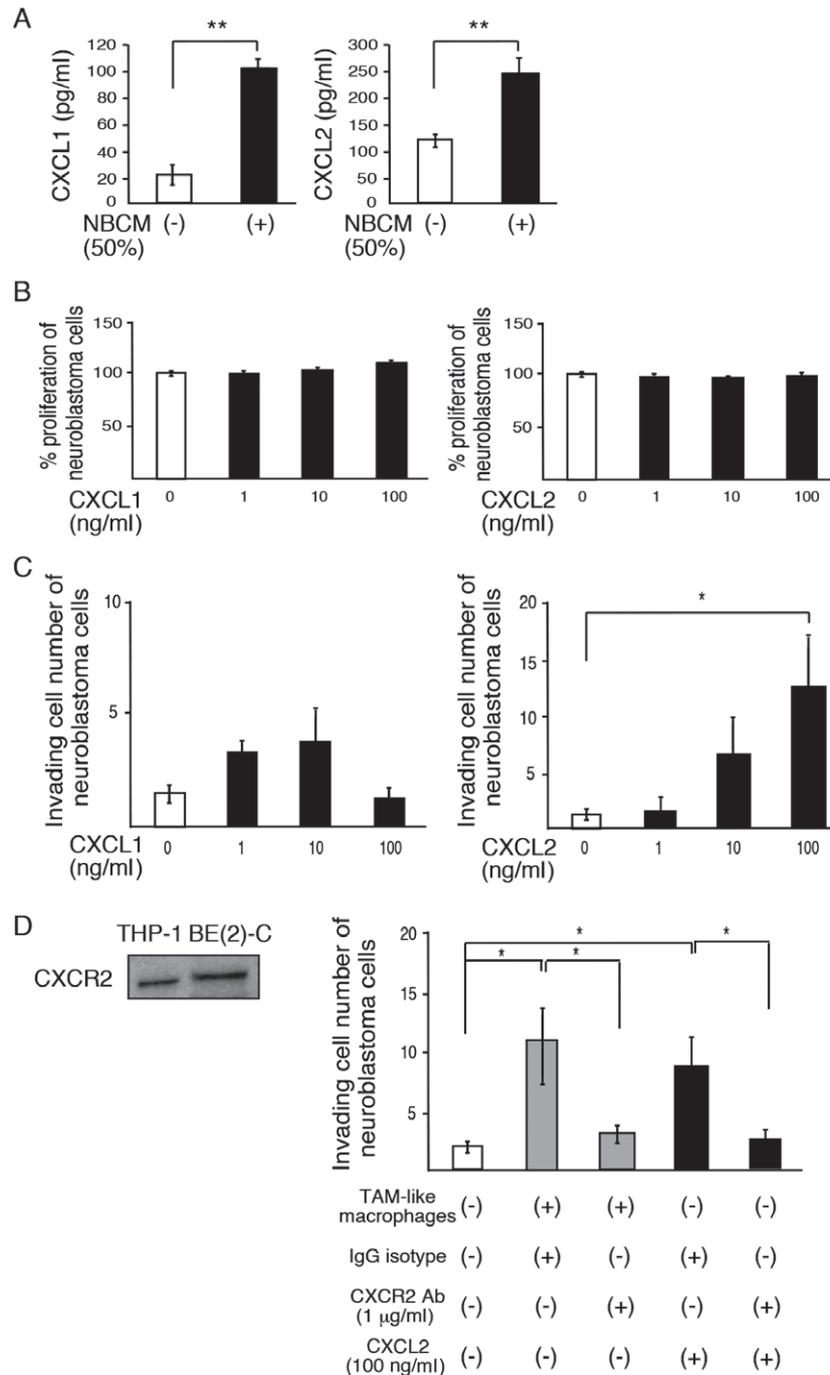


Figure 5. Effect of CXCL2 secreted from TAM-like macrophages on tumour invasive ability. (A) CXCL1 and CXCL2 concentrations on M0 and TAM-like macrophage supernatants were analysed by ELISA. Values represent the mean of three experiments in duplicate and are expressed as mean \pm SD (** $p < 0.01$). (B) The growth-promoting effects of human recombinant CXCL1 and CXCL2 in the indicated concentration on neuroblastoma cells were assessed by the MTS assay. (C) The effects of human recombinant CXCL1 and CXCL2 in the indicated concentration on the invasive ability of neuroblastoma cells were analysed by a BioCoat Matrigel invasion chamber assay. Invading cells were counted in five randomly chosen fields. (D) After expression of CXCR2 in neuroblastoma cells was confirmed by western blotting, the effect of CXCR2 Ab on the invasiveness of neuroblastoma cells co-cultured with TAM-like macrophages was investigated using a BioCoat Matrigel invasion chamber assay. THP-1 cells were used as a control of CXCR2 expression. Normal mouse IgG isotype was used as a control for CXCR2 Ab. Values are the mean of three experiments and are expressed as mean \pm SEM (* $p < 0.05$).

Several groups have reported that tumour cell line conditioned medium (TCM) induced macrophages to M2-like polarization with the expression of CD163, CD204, IL10, VEGF, and MMPs [33–36]. To the best of our knowledge, the present study is the first to report that PBMC-derived macrophages stimulated

with NBCM acquired M2 characteristics, along with the up-regulation of CD163, CD204, and IL10 expression and the suppression of IL12. Although some factors, including IL4, IL10, IL13, and glucocorticoids, are known to be inducers of M2 characteristics [37], a TCM varied the induction of the M2 markers CD163

and CD204 [14,33]. Interestingly, we observed that the NBCM from the BE(2)-C cell line with *MYCN* amplification induced the expression of *CD163*, *CD204*, and *IL10*, but not that of *IL12* in M0 macrophages *in vitro*. However, the NBCM from the SH-SY5Y cell line without *MYCN* amplification induced the expression of *CD163*, *IL10*, and *IL12*, but not *CD204* (data not shown). Mantovani *et al* proposed that M2 macrophages could be categorized into three defined forms, based on a variety of responses to different stimuli [10], suggesting that macrophages could be polarized to a heterogeneous character depending on the tumour type and tumour microenvironment. Further studies are needed to elucidate the fine mechanisms of TAM induction from naive macrophages under the tumour microenvironment.

A number of studies suggested that CAFs originate from a variety of cell types including resident fibroblasts, endothelial cells, and BM-MSCs [38]. In fact, fibroblasts and BM-MSCs showed similarities to CAFs when exposed to TCM over a prolonged period of time [39,40]. We first tried to induce CAFs from human neonatal dermal fibroblasts exposed to NBCM. However, the expression of α SMA (*ACTA2*) did not increase under NBCM even at 14 days, compared with that under a normal condition medium (data not shown). On the other hand, we observed that human BM-MSCs exposed to the NBCM showed increased *ACTA2* and *FAP* levels. As the increases of *ACTA2* and *FAP* expression were not significantly different between 7-day and 14-day NBCM exposure, we regarded the BM-MSCs exposed to NBCM for 7 days as CAF-like BM-MSCs. Although the effect of MSCs on neuroblastomas is still controversial [41–43], this is the first investigation to show that NBCM induces an BM-MSC transition to the CAF-like phenotype, indicating that BM-MSCs could be educated to support tumour progression under a prolonged neuroblastoma microenvironment. We found that CAF-like BM-MSCs significantly enhanced the proliferation of neuroblastoma cells, though the effect on the proliferation of the neuroblastomas was slight. This might be because the duration of exposure to the NBCM was too short to see evident effects of CAF-like BM-MSCs on neuroblastoma cells.

TAMs not only form an immunosuppressive environment where the activation of natural killer and T cells are suppressed; they also promote a variety of steps in tumour progression including tumour growth, invasion, motility, intravasation, and extravasation [44]. In the present study, we observed that TAM-like macrophages themselves did not enhance the growth of neuroblastoma cells but rather promoted their invasive ability. Neuroblastoma cells also attracted PBMCs to the tumour site, resulting in TAM-like macrophages in the tumour microenvironment. Intriguingly, when PBMCs were attracted to neuroblastoma cells, they formed clusters.

It was recently shown that circulating tumour cell (CTC) clusters in the bloodstream of tumour patients consisted of oligoclonal tumour cells from the primary tumour site and contributed to metastatic events compared with single CTCs [45]. Moreover,

TAMs cooperated in the tumour cell intravasation and co-existed with CTCs in the peripheral blood of patients [46,47]. Strikingly, our present study demonstrated that CD163-positive cells, but not α SMA-positive cells, co-existed with neuroblastoma cells in a bone marrow clot from a metastatic neuroblastoma patient (see supplementary material, Figure S3). This observation implies that TAMs are associated with CTCs and enhance the metastatic abilities of CTC clusters.

Although there are some reports that CAFs play an important role in forming an inflammatory environment to which monocytes/macrophages are recruited and in promoting tumour progression [7,48], the interplay of TAMs and CAFs leading to enhanced reactivity with each other was only recently identified [15]. Our present data revealed that TAM-like macrophages from PBMCs and CAF-like BM-MSCs from BM-MSCs acted not only on tumour cells but also on each other. The TAM-like macrophages induced both the proliferation and the invasive ability of BM-MSCs, and the CAF-like BM-MSCs enhanced the invasive ability of PBMCs. Both PBMCs and BM-MSCs are activated to TAMs and CAFs under a tumour microenvironment with the activation of signal pathways, including Akt, STAT, and WNK signals (see supplementary material, Figure S4), accompanied by the dynamic change of the gene expression profile demonstrated in the present study.

CXCL2, also known as macrophage inflammatory protein 2 (MIP-2) or growth-related gene product β (GRO β), belongs to the CXC chemokine family and specifically binds to the receptor CXCR2. CXCL2 was first identified as a potent chemotactic agent for leukocyte migration to sites of inflammation [49]. Recent reports showed that highly expressed CXCL2 was associated with poor prognosis in some tumours [50–52]. Furthermore, inhibition of CXCL2/CXCR2 signalling suppressed cell proliferation and metastatic ability in breast carcinoma and hepatocellular carcinoma [53,54]. In line with these studies, we found that TAM-like macrophages increased the expression of CXCL2 and enhanced the invasiveness of neuroblastoma cells through CXCL2/CXCR2 signalling.

Taken together, our present findings suggest that PBMCs and BM-MSCs are recruited to the tumour site and form the tumour microenvironment in which tumour cells and stromal cells cooperate with each other for tumour progression. For improved targeting therapies for tumours, further investigations of the mechanisms underlying cell–cell interactions in tumour microenvironments are necessary.

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Author contribution statement

OH and MY conceived and carried out the experiments. DH, TY, and YK prepared the tissue samples. NN provided the cells. HY, NN, and YK supervised the work and analysed the data. OH and HY wrote the manuscript. OH, MY, and HY performed the histological classification. All the authors had final approval of the submitted and published manuscript.

Abbreviations

α SMA, alpha smooth muscle actin; BM-MSC, bone marrow-derived mesenchymal stem cell; CAF, cancer-associated fibroblast; CTC, circulating tumour cell; FAP, fibroblast activating protein alpha; hM-CSF, human macrophage CSF; NBCM, conditioned medium from neuroblastoma cell line; TAM, tumour-associated macrophage; TCM, conditioned medium of tumour cell line

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary figure legends

Figure S1. Comparison of the gene expression patterns in TAM-like macrophages and CAF-like BM-MSCs to M0 macrophages and BM-MSCs

Figure S2. No effect of CXCL1 and CXCL2 secreted from TAM-like macrophages on BM-MSC proliferation and invasion

Figure S3. Immunohistochemical images of synaptophysin, a neuroblastoma marker, CD163, and α SMA

Figure S4. Screening of signalling pathways in BM-MSCs and PBMCs co-cultured with neuroblastoma cells

Table S1. Characteristics of the neuroblastoma patients

Table S2. Primary and secondary antibodies used in this study

Table S3. qPCR primer lists