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The role of myofibroblasts in upregulation of S100A8 and S100A9 and the differentiation of myeloid cells in the colorectal cancer microenvironment

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ABSTRACT

Background/aim: S100A8/A9 and myeloid cells in the tumor microenvironment play an important role in cancer invasion and progression, and the effect of tumor-infiltrated myofibroblasts on myeloid cells in the tumor microenvironment is relatively unknown. Accordingly, we investigated the role of myofibroblasts in the upregulation of S100A8/A9 as well as in the differentiation of myeloid cells in the colorectal cancer (CRC) microenvironment.

Materials and methods: To investigate the interactions among cancer cells, myofibroblasts, and inflammatory cells in the microenvironment of CRC, we used 10 CRC cell lines, 18CO cells and THP-1 cells, which were co-cultured with each other or cultured in conditioned media (CM) of other cells. Expression of S100A8/A9 was evaluated via Western blot, immunohistochemical staining and immunofluorescence. The secreted factors from the cell lines were analyzed using cytokine antibody array. Flow cytometry analysis was performed to analyze the differentiation markers of myeloid cells.

Results: 18CO CM induced increased expression of S100A8/A9 in THP-1 cells. Increased expression of S100A8/A9 was noted in inflammatory cells of the peri- and intra-tumoral areas, along with myofibroblasts in colon cancer tissue. S100A8/A9-expressing inflammatory cells also exhibited CD68 expression in colon cancer tissue, and 18CO CM induced differentiation of THP-1 cells into myeloid-derived suppressor cells (MDSCs) or M2 macrophages expressing S100A8/A9. Significant amounts of IL-6 and IL-8 were detected in 18CO CM, compared to those in both controls and THP-1 CM, and tumor-infiltrated myofibroblasts expressed IL-8 in colon cancer tissue. Finally, neutralizing antibodies to IL-6 and IL-8 attenuated 18CO CM-induced increased expression of S100A8/A9.

Conclusions: The upregulation of S100A8/A9 in tumor-infiltrated myeloid cells could be triggered by IL-6 and IL-8 released from myofibroblasts, and myofibroblasts might induce the differentiation of myeloid cells into S100A8/9-expressing MDSCs or M2 macrophages in the CRC microenvironment.

1. Introduction

S100A8 and S100A9 belong to the S100 protein family, a multigenic family of calcium binding proteins of the EF-hand homolog, and function mainly as heterodimers [1]. The overexpression of S100A8/A9 is associated with inflammatory and neoplastic disorders. S100A8/A9 are mainly expressed by myeloid cells, including neutrophils, monocytes and myeloid-derived suppressor cells (MDSC) [2–4], and are overexpressed in various cancers such as stomach cancer [2], pancreatic adenocarcinoma [3], lung cancer [4], and colorectal cancer (CRC) [5]. S100A8/A9 function in a concentration-dependent manner, demonstrating an apoptotic effect on tumor cells at high concentrations [6] and promotion of cell growth [7] and migration [8] at low concentrations. Recent studies reported that S100A8/A9 also induce MDSCs, which impair tumor immunity and facilitate tumor progression [9], and promote accumulation of MDSCs via an autocrine feedback loop in tumor microenvironments [10]. In addition, S100A8/A9 are also involved in the metastatic process and act as chemo-attractants to facilitate the homing of tumor cells to premetastatic niches [4]. Moreover, S100A8/A9 activate specific downstream genes associated with tumorigenesis and promote tumor growth and metastasis in colon cancer [11].

Interactions between tumor cells and their microenvironment such as inflammatory cells and fibroblasts play a central role in

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tumor growth, invasion and metastasis [12]. Macrophages are a major cellular component of immune cell infiltration in tumors [10] and are polarized into two subtypes: pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages [10]. Tumor-associated macrophages have been considered to be M2 macrophages, which play a role in the termination of inflammatory responses, immune escape of tumor cells and promotion of tumor growth [9,10]. Myofibroblasts, which are located in the pericryptal area of normal colon [13], increase in number throughout the course of adenoma–carcinoma sequences of colorectal cancer [14] as well as in inflammatory disease [15]. Tumor-infiltrated myofibroblasts, important cellular components of tumor-associated fibroblasts found in the colon tumor microenvironment, facilitate tumor growth, invasion and metastasis via the secretion of growth factors and cytokines, and produce extracellular matrix and matrix associated molecules [16–18]. Nevertheless, the network of interactions among tumor cells, fibroblasts and inflammatory cells is complicated. Most studies have focused on the interactions between tumor cells and inflammatory cells or fibroblasts in tumor microenvironments; however, the interactions between tumor-infiltrated inflammatory and myofibroblast cells are relatively unknown.

The aim of this study was to investigate the major contributing cells and factors involved in the upregulation of S100A8/A9 in tumor microenvironments, demonstrating the role of myofibroblasts in activating S100A8/A9 expression via IL-6 and IL8, as well as in inducing MDSC and M2 macrophage differentiation from myeloid cells in the CRC microenvironment.

2. Materials and methods

2.1. Reagents and antibodies

Antibodies against S100A8, CD68, CD33, arginase-1, CD163 and CD206 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against S100A9, α-smooth muscle actin (SMA) and β-actin were obtained from Abcam (Cambridge, UK). Anti IL-6 and anti IL-8 antibodies for neutralization were purchased from BioLegend (San Diego, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell lines, cell culture conditions and production of 18CO conditioned medium (CM)

The CRC cell lines (DLD-1, LOVO, HCT15, RKO, SW480, WiDr, HCT116, Caco-2, SW620 and HT29), THP-1 cells (inflammatory myeloid cell) and 18 CO cells (colonic myofibroblast cell) were obtained from the American Type Culture Collection. All cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C. Culture medium was changed every two days. The 18CO myofibroblast phenotype was verified by immunocytochemistry to confirm the expression of α-SMA and vimentin. For mixed co-culture experiments, the same number of cells for two cell lines (1×10^6 cells) were mixed and cultured in 10 cm dishes for 24 h.

To prepare 18CO CM, 18CO cells were gown to confluency in 75 cm^2 flasks in serum-supplemented culture medium. Cells were washed with phosphate-buffered saline (PBS) and starved overnight in serum-free medium. The following day, conditioned medium was prepared by incubating cells in fresh serum-free culture medium for two days. After two days, this medium was harvested, clarified by centrifugation (2000 rpm for 10 min) and stored frozen at −20 °C until ready for further use. 18CO CM was made by mixing 20% FBS-supplemented culture medium and serum-free conditioned medium from 18CO cells in equal amounts to make 10% FBS-containing 18CO CM. THP-1 CM and CRC cell CM were prepared in the same manner as 18CO CM, substituting THP-1 and CRC cells for 18CO cells, respectively. In the experiments described, the control medium was DMEM supplemented with 10% FBS.

2.3. Western blot

For protein extraction, cells were washed with PBS and lysed in ice-cold lysis buffer, PRO-PREP™ protein extraction solution (iNtRON Biotechnology Inc., Seongnam, Korea). After centrifugation at 13,000 rpm for 10 min, the supernatant was stored at −70 °C. Equal amounts of total cellular protein extracts were separated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with TBST (10 mM Tris–HCl, 0.09% NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h at room temperature. For immunodetection, membranes were incubated at 4 °C overnight with primary antibodies. Blots were then washed three times in TBST, followed by incubation with the HRP-conjugated secondary antibodies at room temperature for 45 min. Antibody binding was detected using enhanced chemiluminescence reagent.

2.4. Immunohistochemistry and immunofluorescence stain in colon cancer tissues

Sections of formalin-fixed paraffin-embedded human colon cancer tissue were deparaffinized and rehydrated through graduated changes of xylene and graded alcohol, then water. Endogenous peroxidase activity was blocked by incubating sections with 0.3% hydrogen peroxide for 20 min. Heat-mediated antigen retrieval was performed by heating sections (10 mM Tris-buffer, pH 10) in a microwave oven for 10 min. Sections were then washed with PBS before being exposed to 10% normal bovine serum for 1 h to block non-specific reactions. Sections were then incubated with primary antibodies at 4 °C overnight. Immunostaining was performed using the Dako REAL EnVision kit and diaminobenzidine visualization (Dako, Carpinteria, CA, USA). Sections were counterstained with hematoxylin.

For immunofluorescence stain, primary antibodies for S100A8, CD68, IL-8 and αSMA, as well as FITC- and Cy3-conjugated secondary antibodies were used. Samples were examined under a confocal laser-scanning microscope (LSM 710, Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

2.5. Migration assay

DLD-1 cells (5×10^5 cells/well) were seeded in each well of 12-well plate with an attached culture-insert (Ibidi, München, Germany) for 24 h. After removal of the culture-insert and washing with serum-free media, the cells were incubated in control media and each conditioned media for 15 h, respectively. Then, the cells on the plate were photographed, and the migration distances were measured.

2.6. Antibody array for selected growth factors and cytokines

Growth factors and cytokines in the THP-1 CM and 18CO CM were determined by a customized RayBio® human growth factor and cytokine antibody array (RayBiotech Inc., Norcross, GA, USA) according to the manufacturer's instructions. The array is able to

assess 20 growth factors and cytokines with three positive and three negative controls (Supplementary Table 1).

2.7. Flow cytometric analysis

To investigate the induction of MDSC and M2 macrophage differentiation as well as S100A8/A9 expression by 18CO CM, flow cytometric analysis of S100A8/A9 and markers for macrophages/monocytes (CD68), MDSCs (CD33 and arginase-1) and M2 macrophages (CD163 and CD206) was performed. After incubation of THP-1 cells in control media and 18CO CM for 72 h, the prepared cells were washed with PBS, and then resuspended in 1 ml of FACS buffer (1× PBS, 0.5% bovine serum albumin, 2 mM ethylene diamine tetraacetic acid). Then, after adding 100 μl of BD Cytofix/Cytoperm™ (BD Biosciences, Franklin Lakes, NJ, USA) in each tube, cells were incubated for 20 min at 4 °C. After washing in BD Perm/Wash™ (BD Biosciences), cells were incubated with primary antibodies for 15 min at 4 °C. After washing, FITC- and Cy3-conjugated secondary antibodies were used. Control samples were prepared with unstained cells, i.e., samples in which the primary antibody treatment was replaced with treatment of isotype-matched control antibodies. Samples were then washed and analyzed using a BD LSRII flow cytometer (BD Biosciences) coupled to a computer with BD FACS Diva software for data analysis.

2.8. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Differences between two groups were analyzed by Student's *t* test. *P*-values less than 0.05 were considered significant.

3. Results and discussion

3.1. Increased expression of S100A8 and S100A9 in 18 CO conditioned media (CM)-treated THP-1 cells

To mimic the three main cellular components of the tumor microenvironment, we used CRC cell lines, myofibroblast (18CO) and inflammatory myeloid cell lines (THP-1), which were co-cultured with each other or cultured in CM of other cells. S100A8/A9 were expressed in THP-1 cells, but not in 18 CO cells or ten CRC cell lines (Supplementary Fig. 1A). Then, we examined the interactions between THP-1 cells and 18CO cells. The expression of S100A8/A9 was greatly increased in THP-1 cells co-cultured with 18CO cells compared with THP-1 cells cultured alone (Fig. 1A). THP-1 cells which were stimulated with PMA did not express S100A8/A9, whereas PMA-stimulated THP-1 cells co-cultured with 18CO cells induced S100A8/A9 expression (Fig. 1A). Because the expression of S100A8/A9 is down-regulated during the early differentiation stages of macrophages and dendritic cells [19,20], S100A8/A9 were not expressed in THP-1 cells that differentiated into macrophages by PMA stimulation. However, even after macrophage differentiation of THP-1 cells, 18CO cells induced the expression of S100A8/A9. The expression of S100A8/A9 was also enhanced in THP-1 cells cultured in 18CO CM, compared to that in THP-1 cells cultured in control media (Fig. 1A). These findings suggested that the expression of S100A8/A9 in myeloid cells could be induced by factors secreted from myofibroblasts. Meanwhile, the expression of S100A8/A9 in various cancer cell lines co-cultured with 18CO cells was not detectable (Supplementary Fig. 1B). In addition, THP-1 CM was unable to induce the expression of S100A8/A9 in all 10 cancer cell lines (Supplementary Fig. 1C). However, the CM of each CRC cell line exerted various effects on the expression of S100A8/A9 in THP-1 cells, e.g., no change or some increase depending on the cell lines (Supplementary Fig. 1D), suggesting that factors from CRC cells possibly affect the induction of S100A8/A9 [4].

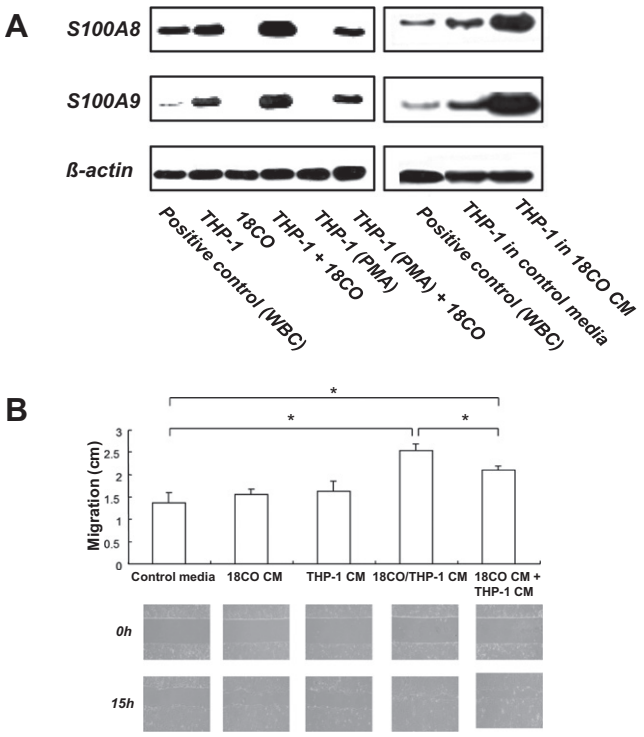

Fig. 1. 18CO conditioned media (CM) induced increased expression of S100A8 and S100A9 in THP-1 cells, and co-cultured CM of THP-1 and 18CO cells increased migration of CRC cells. (A) THP-1 cells, 18CO cells, and a mixture of THP-1 and 18CO cells in the same number (THP-1 + 18CO) were incubated for 24 h (lane 2, 3 and 4). After treatment of 100 nM PMA on THP-1 cells for 24 h, the same number of 18CO cells was added or not to THP-1 cells with fresh media, and incubated for 24 h (lane 5 and 6). THP-1 cells were cultured in media with or without 18CO CM for two days (lane 8 and 9). Western blotting for S100A8 and A9 was performed using cell lysates from each of the conditions. (B) After 15 h incubation in control media, 18CO CM, THP-1 CM, mixed co-cultured CM of 18CO and THP-1 cells, and a mixture of 18CO CM and THP-1 CM, the migration distances of the CRC cells (DLD-1) were measured using wound healing assay. Results are expressed as means of triplicate determinations and are representative of three independent experiments. A single asterisk (∗) denotes $P < 0.05$. CM, conditioned medium; PMA, phorbol 12-myristate 13-acetate; WBC, white blood cells; CRC, colorectal cancer.

3.2. Increased migration of CRC cells (DLD-1) by CM from co-culture of THP-1 and 18CO cells

We next investigated whether factors secreted from the interaction between THP-1 and 18CO play a role in cancer cell migration. The migration distances of DLD-1 cells in control media, 18CO CM, THP-1 CM, 18CO and THP-1 co-cultured CM, as well as a mixture of 18CO CM and THP-1 CM were measured. CM from a co-culture of 18CO and THP-1 cells induced significantly increased migration of colon cancer cells compared to the others ($P < 0.05$) (Fig. 1B), suggesting that factors secreted via an interaction between 18CO cells and THP-1 cells could increase the migration of CRC cells. However, because a neutralizing antibody to S100A8/A9 was not available, direct evidence for the role of S100A8/A9 in the above effect could not be estimated. Nevertheless, considering the dose-dependent effect of S100A8/A9, an apoptotic effect at high concentrations, and promotion of cell growth and migration at low concentrations, we anticipated that our experimental condition would be suitable for investigating tumor promotion conditions in our next experiments.

3.3. Increased expression of S100A8/A9 and CD68 in tumor-infiltrated inflammatory cells in human CRC tissue

To investigate which cells express S100A8/A9 in the human CRC microenvironment, immunohistochemical stain was performed. Various inflammatory cells accumulated in the margin of CRC tissue (Fig. 2A). The expression of S100A8/A9 was noted in inflammatory cells of peri- and intra-tumoral areas; the S100A8/A9-expressing cells showed macrophage-like morphology and were similar in shape to tumor-infiltrated CD68-positive myeloid cells, but not with CD45-positive lymphocytes (Fig. 2A). Myofibroblasts, which were identified by anti α-SMA antibody, infiltrated similar areas (Fig. 2B). Upon immunofluorescence staining, we identified both S100A8 and CD68 expressing inflammatory cells in peri- and intra-tumoral areas (Fig. 2C). These findings suggest that S100A8/A9 are expressed in tumor-infiltrated CD68 positive myeloid cells, which coexist with tumor-infiltrated myofibroblasts in the CRC microenvironment.

3.4. Increased fraction of MDSCs and M2 macrophages in 18CO CM-treated THP-1 cells

To elucidate the effect of 18CO CM on the differentiation of myeloid cells, which can express S100A8/A9, we determined the differentiation markers of 18CO CM-treated THP-1 cells. After treatment with 18CO CM for 24 h, we found that more THP-1 cells with podia-like processes were attached to the bottom of the plate, suggesting their differentiation into macrophage-like cells (Fig. 3A). We then examined the markers of macrophages/monocytes (CD68), MDSCs (CD33 and arginse-1) and M2 macrophages (CD163 and CD206). The fraction of THP-1 cells expressing CD68, CD33, arginase-1, CD163, and CD206 was increased after incubation in 18CO CM for 72 h, exhibiting an increased proportion of $CD68^{+}Arg\text{-}1^{+}$, $CD68^{+}CD163^{+}$, and $CD68^{+}CD206^{+}$ cells (Fig. 3B). Moreover, the MDSCs and M2 macrophages that differentiated from THP-1 cells by 18CO CM showed increased expression of S100A8/A9 (Fig. 3B).

In the CRC microenvironment, inflammatory mediators such as IL-6, IL-10, and prostaglandin E_2 promote the polarization of myeloid cells into M2 macrophages, and other mediators including IL-1β, IL-6, vascular endothelial growth factor, prostaglandin E_2 and S100A8/A9 proteins induce the differentiation of myeloid cells into MDSCs [21,22]. In addition to previous studies that showed these molecules could be produced by tumor cells or by tumor infiltrating macrophages, our findings suggested that myofibroblast-derived soluble factors might have the potential to modulate and differentiate myeloid cells into MDSCs and M2 macrophages with increased S100A8/A9 expression.

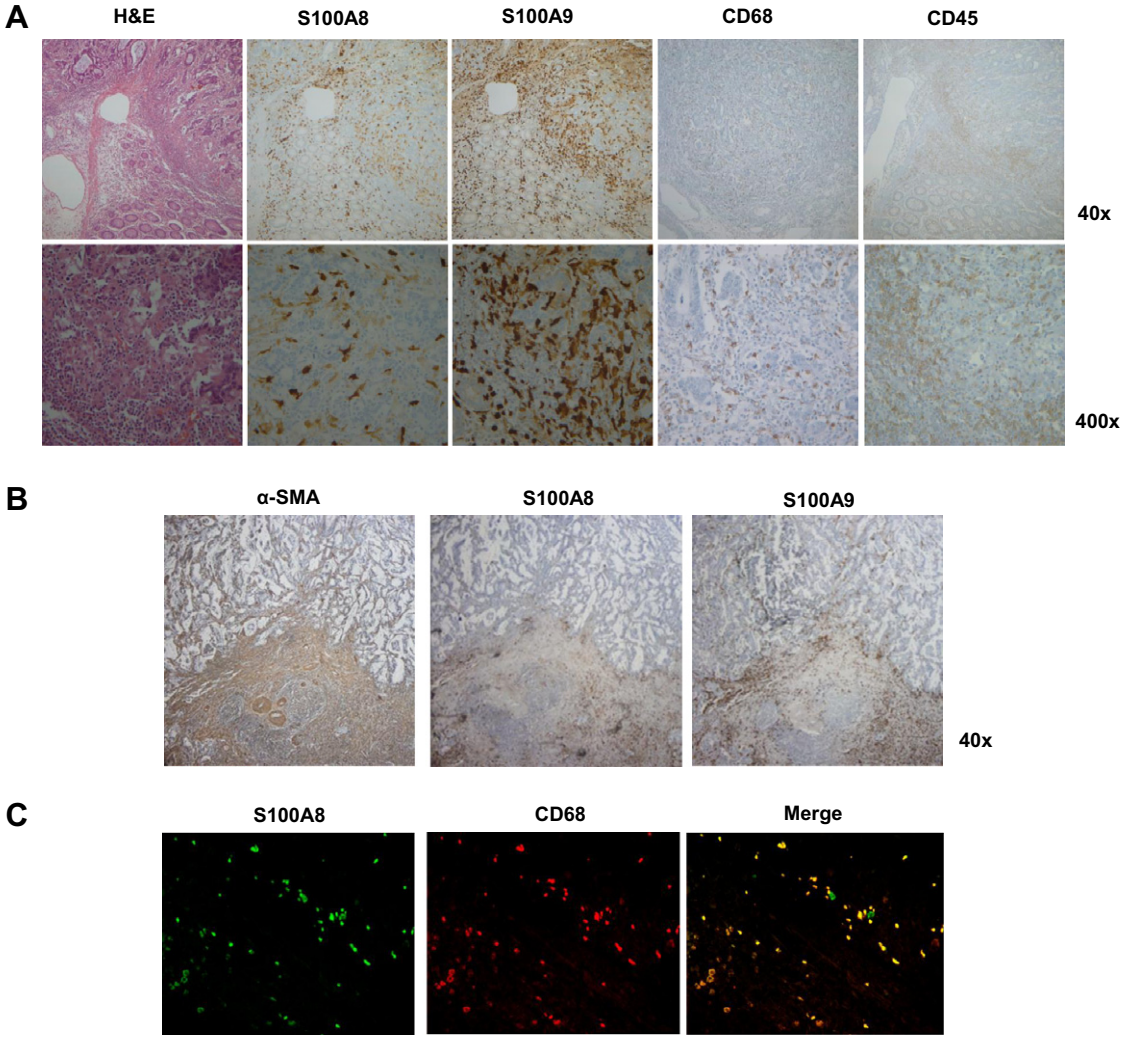

Fig. 2. S100A8/A9 was expressed in CD68-positive myeloid cells in colon cancer tissue. (A and B) Immunohistochemical studies of S100A8, S100A9, CD68, CD45 and α-SMA were performed in colon cancer tissue. (C) Human colon cancer tissue sections were analyzed by immunofluorescence studies using primary antibodies against CD68 and S100A8, as well as FITC- and cy3-conjugated secondary antibodies. Sections were visualized by confocal microscopy (200×). α-SMA, α-smooth muscle actin.

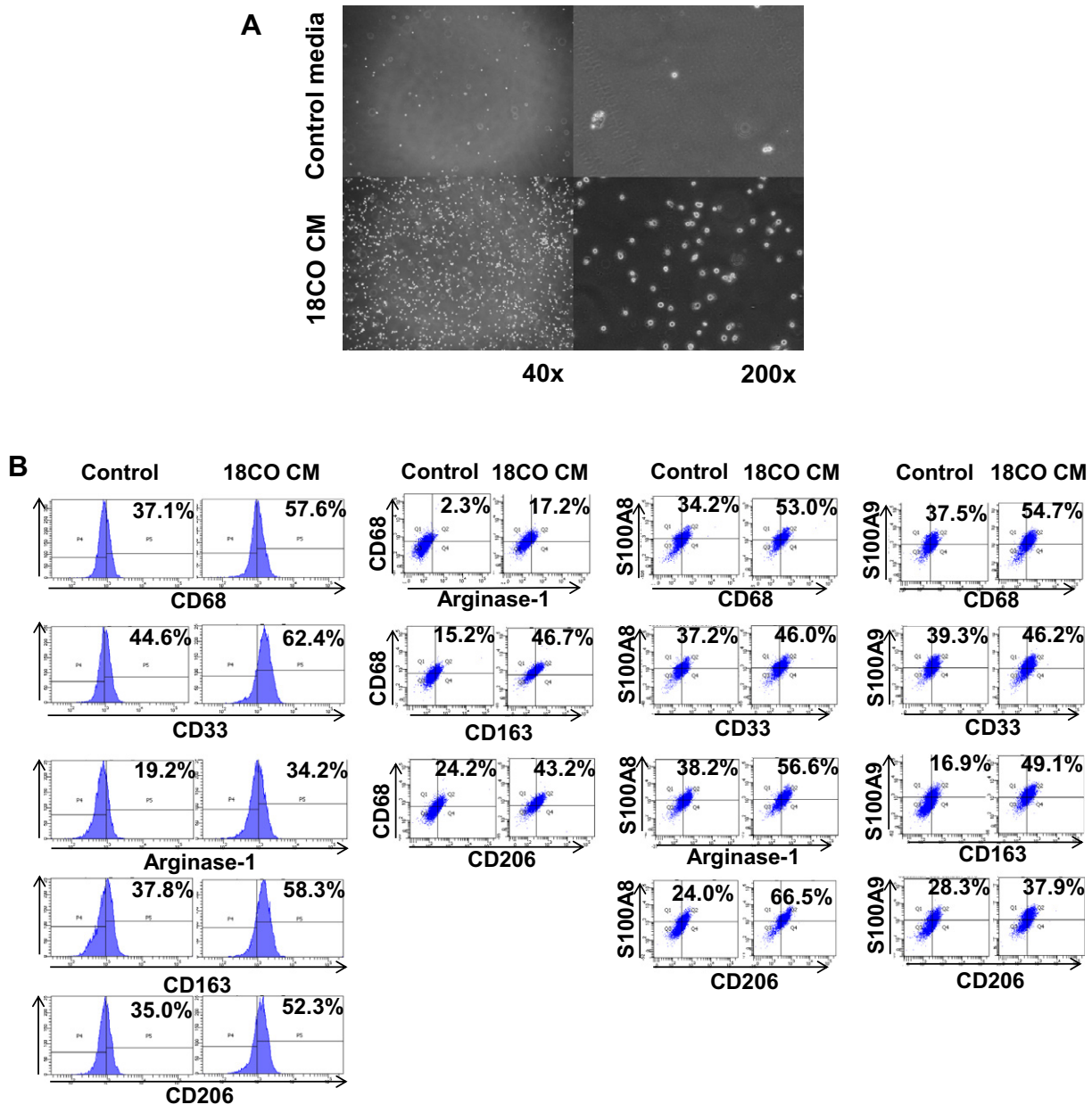

Fig. 3. 18CO CM induced differentiation of MDSCs and M2 macrophages from THP-1 cells. (A) After 24 h incubation in control media and 18CO CM, floating cells were removed and cells attached to the bottom of plate were photographed. (B) THP-1 cells were cultured in control media and 18CO CM for 72 h. Then, flow cytometric analysis was performed using markers for macrophages/monocytes (CD68), MDSCs (CD33 and arginase-1) and M2 macrophages (CD163 and CD206). Results are representative of three independent experiments. CM, conditioned medium; MDSC, myeloid-derived suppressor cells.

3.5. Induction of S100A8/A9 in THP-1 cells via IL-6 and IL-8 secreted from 18CO cells

To identify the factors secreted from 18CO cells that activated S100A8/A9 expression in THP-1 cells, antibody array was performed. The relative amounts of growth factors and cytokines in THP-1 CM and 18CO CM were measured using customized antibody array. By comparing signal intensities, the amounts of IL-6 and IL-8 in 18CO CM were significantly higher than those measured in control media and THP-1 CM (Fig. 4A). Moreover, we identified IL-8 expression in tumor-infiltrated myofibroblasts upon immunofluorescence staining of human colon cancer tissue (Fig. 4B). However, IL-6 expression in tumor-infiltrated myofibroblasts was not definite (data not shown), and, in antibody array, the secreted amount of IL-6 in 18CO CM was considerably less than that of IL-8, suggesting a relatively minor role for IL-6 in CRC tissue. Considering the well-known role of IL-6/IL-8 and the results of the antibody array, we tested the effect of neutralizing antibodies to IL-6 and IL-8 on 18CO CM-induced increase of S100A8/A9 expression in THP-1 cells. 18CO CM-induced expression of S100A8/A9 in THP-1 cells was significantly inhibited by treatment of neutralizing antibodies to IL-6 and IL-8, suggesting that two of the main contributing factors to 18CO CM-induced increase of S100A8/A9 expression in THP-1 cells might be IL-6 and IL-8 (Fig. 4C). As for the differentiation of MDSCs and M2 macrophages, we could not demonstrate a significant change in differentiation markers of MDSCs and M2 macrophages after the addition of neutralizing antibodies to IL-6 and IL-8 in 18CO CM (data not shown), suggesting a possible role for other unknown factors secreted from 18CO cells in the differentiation of THP-1 cells into MDSCs and M2 macrophages.

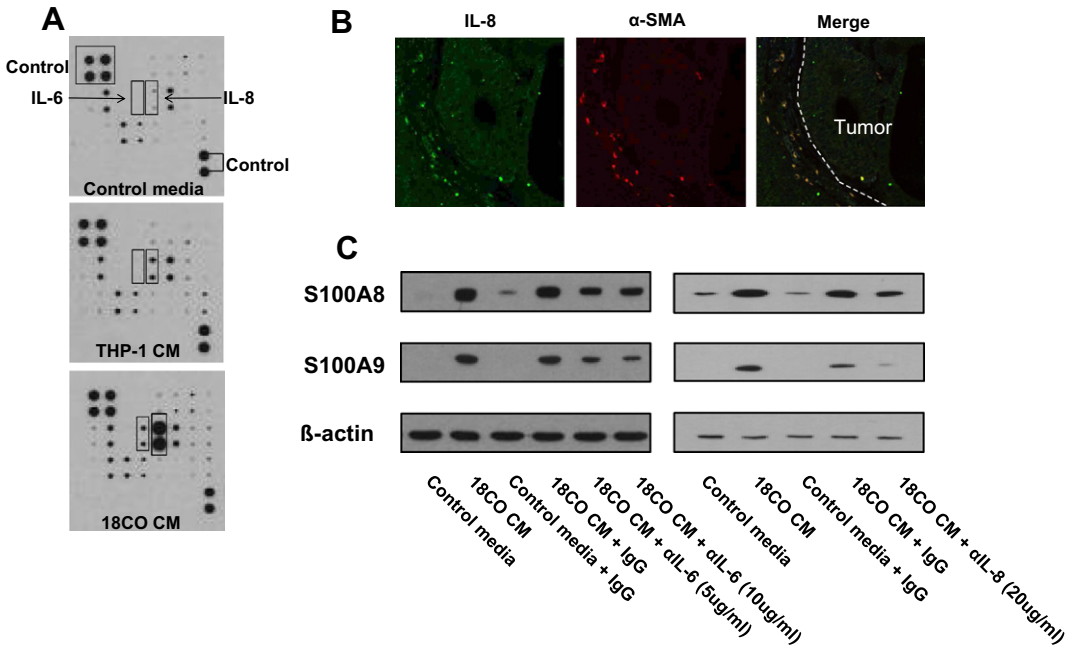

Fig. 4. 18CO CM-induced increased expression of S100A8/A9 was attenuated by neutralizing antibodies to IL-6 and IL-8. (A) Relative amounts of selected growth factors and cytokines in control media, 18CO CM, and THP-1 CM were measured using customized antibody array (Supplementary Table 1). (B) Immunofluorescence studies using primary antibodies against IL-8 and α-SMA, as well as FITC- and Cy3-conjugated secondary antibodies were performed in human colon cancer tissues. Sections were visualized by confocal microscopy (200×). (C) THP-1 cells were cultured as indicated with or without anti-IL-6 and anti-IL-8 neutralizing antibody or isotype-matched control IgG. Western blotting for S100A8/A9 was performed using cell lysates from each condition. α-SMA, α-smooth muscle actin; CM, conditioned medium.

As is well known, IL-6 and IL-8 function as significant regulatory factors within tumor microenvironments [23,24]. They are expressed in tumor cells and tumor-infiltrated inflammatory cells, and are associated with tumor progression and metastasis [25]. As for their relation to S100A8/A9, IL-6 has been shown to be associated with STAT3-dependent upregulation of S100A8/A9 in myeloid precursors [26], and IL-8 has been shown to be associated with positive feedback activation of S100A8/A9 [27].

Previous studies have mainly investigated the interaction between CRC cells and tumor infiltrating myeloid cells or fibroblasts [11,28,29]. In our study, we focused, however, on the interactions between tumor-infiltrating myeloid cells and myofibroblasts, and demonstrated that the release of IL-6 and IL-8 from myofibroblasts could upregulate the expression of S100A8/A9 in tumor-infiltrating myeloid cells, and myofibroblast could induce the differentiation of myeloid cells into MDSCs or M2 macrophages expressing S100A8/9. Further studies using tissue-derived cells or animal models would be needed to confirm our results and elucidate more detailed mechanisms *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.081.

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