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Combined GM-CSF treatment and M-CSF inhibition of tumor-associated macrophages induces dendritic cell-like signaling *in vitro*

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Abstract. Macrophages demonstrate plasticity, and tumor-associated macrophages (TAM) can function as immunosuppressive cells in the tumor microenvironment. Therefore, in this study, we aimed to reprogram TAM *in vitro* with cytokine signal alteration. Granulocyte macrophage colony stimulating factor (GM-CSF) treatment alone did not lead to changes in the expression of M1 (including IL-1β, TNFα and CXCL-10) or M2 (including CD36, CD206 and CCL17) molecules by TAM *in vitro*, although they adopted a round morphology and were less adhesive to the culture dish. When macrophage colony stimulating factor (M-CSF) signals were suppressed by siRNA against the M-CSF receptor (M-CSFR) in conjunction with GM-CSF treatment, the signal transduction pathway of TAM was altered, and the expression of STAT1, STAT5 and STAT6, which are usually expressed by dendritic cells, was increased. However, the same treatment did not alter the TAM expression pattern of M1/M2 marker molecules. With respect to the NF-κB pathway, GM-CSF and M-CSFR siRNA combination treatment significantly induced the expression of p65, which is usually not expressed by TAM, while p50 and p105 expression by TAM was not affected by the treatment. These findings indicate that our model could not redirect TAM to a monocyte-derived dendritic cell-like phenotype based on the analysis of M1/M2 marker expression, but it was able to modify cell signaling pathways toward a dendritic cell-like pattern. Therefore, the present data suggest that TAM demonstrate plasticity toward dendritic cell-like signal transduction patterns, and that the alteration of the tumor microenvironment has the potential to reverse the immunosuppressive properties of TAM.

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Introduction

Tumor-infiltrating myeloid cells are immature cells that are activated and matured under the influence of the tumor microenvironment and composed of two main subtypes, granulocytes and monocytes/macrophages (1,2), which have immunosuppressive properties (3). Among these cells, $CD11b^{+}Gr^{-}1^{+}$ granulocyte-lineage cells, also referred to as myeloid-derived suppressor cells (4,5), are present in bone marrow, spleen, and tumor tissue of the tumor-bearing host and suppress T-cell functions (6-9). In contrast, tumor-infiltrating monocyte/macrophage-lineage cells, referred to as tumor-associated macrophages (TAM), show immunosuppressive properties (10).

The number of infiltrating TAM, which is evaluated as the number of $CD68^{+}$ cells, seems to be inversely correlated with patient prognosis. With the exception of colon cancer and melanoma, the majority of tumors, including breast, kidney, prostate, and uterine corpus, show an increased number of TAM associated with poorer patient prognosis (11). In addition, Takeya's group clearly demonstrated that the number of tumor-infiltrating CD163 and CD204 positive cells, which are other useful markers of TAM, showed an inverse correlation with the prognosis of glioma patients (12). The relationship between TAM infiltration and poor prognosis may in part result from the secretion of epidermal growth factor (EGF) by TAM, which are distributed mainly at the infiltration border of the tumor tissue and perivascular areas at the tumor site. The tumor cells express EGF receptor (EGFR) and are attracted by TAM-secreted EGF, resulting in tumor infiltration into mesenchymal tissue or contact with blood vessels (13-16). Moreover, the fact that TAM infiltration and patient prognosis are not always inversely correlated indicated that various tumors activate TAM differently (11). Our group previously reported that the expression of cytokines, chemokines, and their receptors was tumor-type dependent, but that the site of

inoculation (skin, liver, or brain in our model) did not affect TAM characteristics (17). Our reports suggest that macrophages can be differentially activated and matured depending on tumor-derived factors.

Plasticity is an important characteristic of macrophages. Indeed, macrophages can be redifferentiated when the microenvironment of cells is changed (18,19). Both monocyte/macrophages and monocyte-derived dendritic cells (MoDC) originate from a common progenitor, $Ly6C^{high}$ monocyte progenitor cells, and macrophages have the potential to be re-directed toward a MoDC-like phenotype (20). Therefore, in this study, we aimed to redirect TAM toward a dendritic-cell-like phenotype utilizing cytokine and siRNA treatment.

Materials and methods

Mice. Male 6- to 8-week-old C57BL/6 and BALB/c mice were purchased from SLC (Wilmington, MA, USA). OT-II.2a microinjected/Rag1 knockout mice (OT-2 mice) were purchased from Taconic (Germantown, New York, USA).

Cell line. A murine colon carcinoma cell line (MCA38) was cultured in RPMI-1640 medium containing 10% fetal calf serum, L-glutamine, and penicillin-streptomycin (all from Invitrogen Life Technologies, Tokyo, Japan). Cells were maintained at 37˚C in a humidified 5% CO_2 atmosphere.

Implantation of tumor cells. Mice were inoculated with $3x10^6$ cells injected subcutaneously into the abdominal wall space.

Isolation of tumor-infiltrating cells. Mice were euthanized 14 or 21 days after tumor implantation, and the tumor-infiltrating cells were prepared. The tumors were collected and minced into small pieces before incubation for 15 min at 37˚C with the following enzymes dissolved in HBSS: collagenase type I (0.05 mg/ml), collagenase type IV (0.05 mg/ml), hyaluronidase (0.025 mg/ml, all from Sigma Chemical Co., St. Louis, MO, USA), DNase I (0.01 mg/ml), and soybean trypsin inhibitor (0.2 trypsin inhibitor unit/ml, both from Roche Diagnostics, Nutley, NJ, USA). Digested cells were harvested, and the red blood cells were lysed with hypotonic buffer (0.155 M NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$) for 1 min. $F4/80^+$ cells were isolated using biotin-conjugated anti-F4/80 antibody (clone BM8, Invitrogen, Carlsbad, CA), followed by treatment with anti-biotin magnetic immunobeads according to the manufacturer's instructions (MACS, Miltenyi Biotec, Berdish-Gladbach, Germany).

Multiple PCR analysis. Total RNA was purified using TRIzol (Invitrogen Life Technologies), and 600 ng total RNA was used for reverse transcription with Superscript III reverse transcriptase (Invitrogen Life Technologies). For multiplex PCR analysis, 100 ng cDNA samples or positive control DNA mixtures were mixed with a multiple primer pair mixture, buffer, and Taq polymerase, which were provided by the Multiplex PCR kits for mouse Chemokine Receptors (Sets 1 and 2), Chemokine Genes (Sets 1 and 2), Signaling Receptor (Set 1), Sepsis Cytokines (Set 2), Inflammatory Cytokine Genes (Set 1), CD Antigen (Set 1), and TH1/TH2 Cytokines (Set 2, all from Maxim Biotech, Inc, South San Francisco, CA, USA), and then subjected to PCR using the conditions indicated in the manufacturer's instruction manual. The amplified DNAs were analyzed by 5% acrylamide gel electrophoresis followed by ethidium bromide staining.

Real-time PCR analysis. Total RNA was purified using TRIzol (Invitrogen Life Technologies), and 600 ng total RNA was used for reverse transcription with Superscript III reverse transcriptase. For real-time PCR analysis, 5 μl cDNA samples diluted 20X, 1 μl each of the upper and lower primer, 3 μl PCR-grade water (Roche Diagnostics, Indianapolis, IN, USA), and 10 μl 2X concentrated SYBR Green and Taq enzyme-premixed reaction mixture (SYBR® Premix Ex Taq™, Takara Bio, Japan) were used. Some of the primer pair sequences used in this analysis are indicated in our previous papers (21,22). The sequences of the primer pairs used only in this report and not previously are: IFNγ, 5'-CGGGAC AGTCATTGAAAGCCTA-3', 5'-GTTGCTGATGGCCTG ATTGTC-3'; CCR2, 5'-GCAAGTTCAGCTGCCTGCAA-3', 5'-ATGCCGTGGATGAACTGAGGTAA-3'; CX3CR1, 5'-TGACCCTGCAAGCATCACGTA-3', 5'-CAATGTAA GCCTGCAAATGAGACC-3'; IL-4, 5'-TCTCGAATGTAC CAGGAGCCATATC-3', 5'-AGCACCTTGGAAGCCCTAC AGA-3'; IL-6, 5'-CCACTTCACAAGTCGGAGGCTTA-3', 5'-GCAAGTGCATCATCGTTGTTCATAC-3'. The reaction conditions consisted of one 5-min cycle at 95˚C, followed by 45 cycles at 95˚C for 10 sec, and 72˚C for 10 sec. The reaction and analysis were performed with a Light Cycler instrument (Roche Diagnostics, Indianapolis, IN, USA).

SDS-PAGE and immunoblotting. $F4/80^+$ cells isolated from tumor tissues were washed three times with PBS before being incubated on ice for 1 h with lysis solution [50 mM Tris-HCl, pH. 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 5 mM iodoacetamide, 1 mM Na_3VO_4, and protease inhibitor cocktail (Sigma Chemical Co., P-8340)]. The supernatants were collected and subjected to electrophoresis on 10% PAGE gels. After transferring the proteins onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA), the membranes were blocked with skim milk and then reacted for 1 h with 2 μg/ml of the following antibodies diluted in a primary antibody signal-enhancing solution (Can Get Signal™, Immunoreaction Enhancer Solution 1, Toyobo Co, Ltd., Osaka, Japan): anti-mouse stat1 (C-terminus, clone 42), anti-mouse phospho-specific stat1 (pY701, clone 14), anti-mouse stat3 (clone 84), anti-mouse phospho-specific stat3 (pY705, clone 4), anti-mouse stat4 (clone 8), anti-mouse phospho-specific stat4 (pY693, clone 38, all from BD Transduction Laboratories), anti-mouse stat5 (rabbit polyclonal), anti-mouse phospho-specific stat5 (Tyr694, rabbit polyclonal), anti-stat6 (rabbit polyclonal, all from Cell Signaling), anti-mouse phospho-specific stat6 (Y641, clone 16E12, Millipore Corporation), anti-mouse NF-κBp65 (rabbit polyclonal, Cell Signaling), anti-mouse NF-κBp105/p50 (clone 12A2F, Abcam), anti-mouse ERK (panERK, clone 16), anti-mouse ERK1 (clone MK12), anti-mouse pan-JNK/SAPK1 (clone 37), anti-mouse arginase I (clone 19), anti-mouse iNOS (clone 2), anti-mouse iNOS/NOS type II (clone 6, all from BD Transduction Laboratories). After washing, the membranes were stained with 16 ng/ml HRP-conjugated goat anti-rabbit

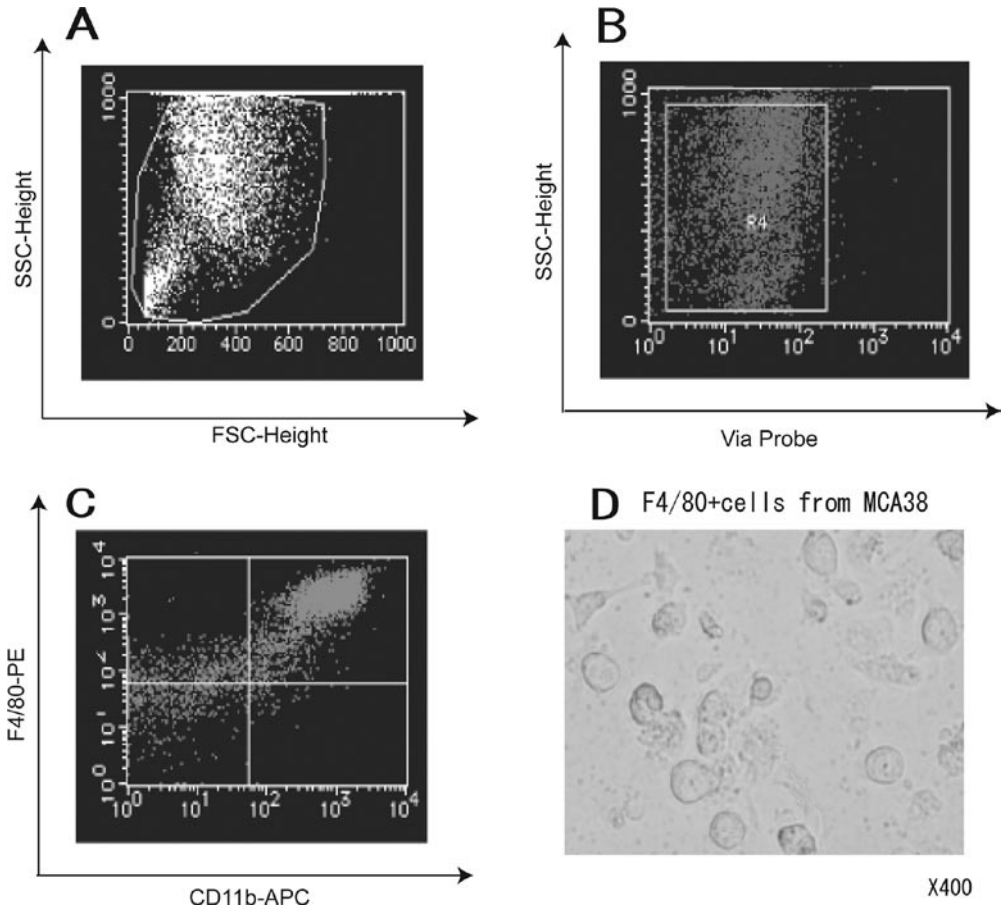

Figure 1. Characterization of tumor-infiltrating F4/80$^+$ cells. Mice were euthanized 14 or 21 days after tumor implantation, and tumor-infiltrating F4/80$^+$ cells were purified using an F4/80 positive immunobeading method (A-C). Dead cells were excluded by Via-Probe (B), and the CD11b and F4/80 2-D histogram is shown (C). Appearance of F4/80$^+$ cells after 24 h cell culture with medium is shown. Almost all cells adhered to the dish and demonstrated a short spindle shape (D). Representative data from three independent experiments are shown.

IgG or anti-mouse IgG (both from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted in Can Get Signal™ Immunoreaction Enhancer Solution 2 (Toyobo Co., Ltd.) for 1 h. The blots were developed with ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the images were captured by the Cool Saver Lumino capture system (Model AE-6955, ATTO, Tokyo, Japan) before analysis using CS Analyzer software (ATTO). The membrane was then stripped and reprocessed with 2 μg/ml anti-GAPDH (clone 9.B.88, United States Biological, Swampscott, MA, USA), and 16 ng/ml HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.).

siRNA silencing. Cells were transfected with 0.4 nmol/ml siRNA using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. The following siRNA sequences, designed by the manufacturer (Invitrogen, Carlsbad, CA), were used: M-CSFR1, 5'-UAGCCUUGCGG AUAAUGAACCCUCG-3' and 5'-CGAGGGUUCAUUAU CCGCAAGGCUA-3'; control siRNA, 5'-UAGCCCGUUCA GGCUAAUCAAGUCG-3' and 5'-CGACUUGAUUAGCC UGAACGGGCUA-3'.

Mitomycin C treatment of splenocytes. Splenocytes were prepared from male 6- to 8-week-old BALB/c mice. The collected splenocytes were treated with hypotonic buffer (0.155 M NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$) for 1 min to lyse red blood cells. The cells were then treated with 100 μg/ml mitomycin C (MMC) in RPMI-1640 medium for 30 min at 37˚C. After washing with PBS three times, the cells were used as allostimulator cells in mixed lymphocyte culture.

Analysis of antigen presentation by T-cell proliferation. Allo-antigen stimulation and ovalbumin antigen-specific T-cell systems were utilized. For the alloantigen stimulation system, responder cells were prepared as the negative population of magnetic immunobeading (Miltenyi Biotec, Germany) with anti-CD11b, CD11c, and B220 immunobeads. Responder cells were labeled with CFSE (Invitrogen) according to manufacturer's instructions for flow cytometric analysis. One million responder cells, 1x10^6 allostimulator cells, and 1x10^3 antigen-presenting cells (F4/80$^+$ cells treated with either control or M-CSFR1 siRNA for 5 days) were co-cultured in one well of a 24-well plate containing 2 ml medium for 5 days and then collected for analyses. For the ovalbumin-specific T-cell system, ovalbumin (OVA) was utilized instead of allostimulator cells, and OT-2 mouse-derived splenocytes were prepared by the same method used for responder-cell preparation in the alloantigen system. OVA (1 mg/ml), 1x10^6 CFSE-labeled responder cells, and 1x10^3 antigen presenting cells (F4/80$^+$ cells

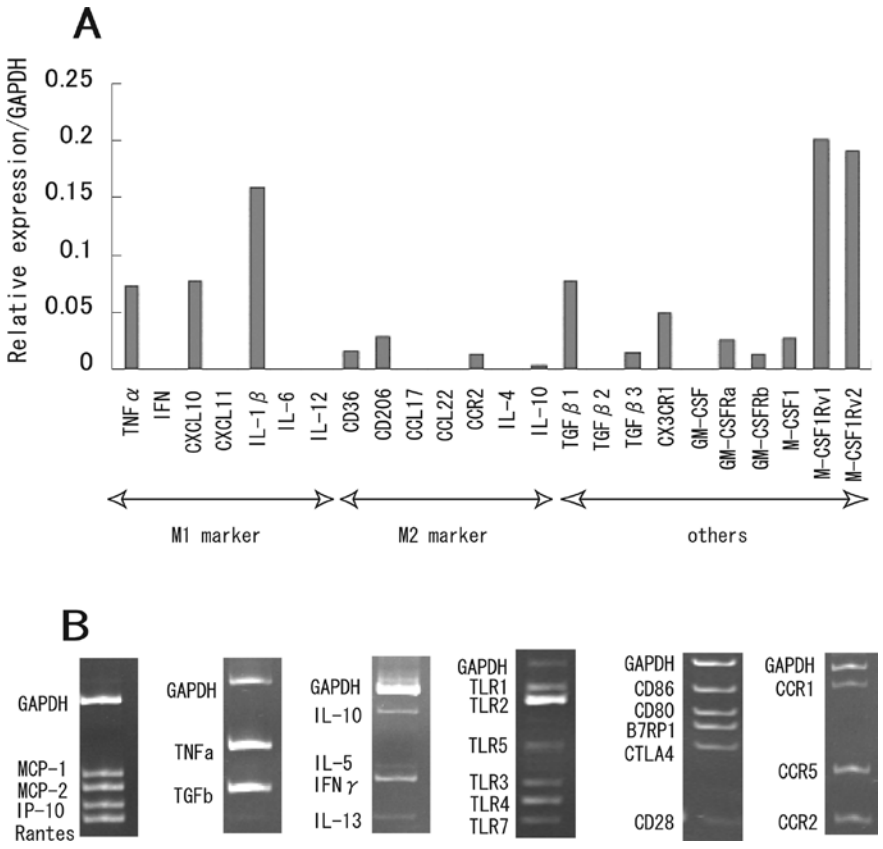

Figure 2. Characterization of freshly-isolated tumor-associated macrophages (F4/80$^+$ cells) based on M1/M2 classification. Real-time PCR analysis of TAM for the expression of cytokines, chemokines, and their receptors based on M1/M2 classification is shown as the relative expression level to GAPDH (A). The same cells were investigated with multiple PCR analysis (B). Representative data from two independent experiments are shown.

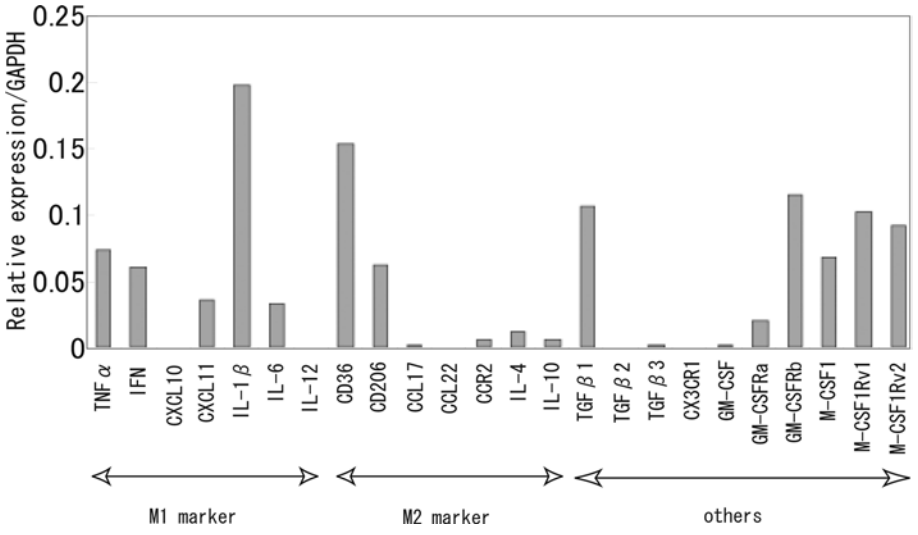

Figure 3. Effect of GM-CSF treatment on macrophage differentiation based on the M1/M2 classification of tumor-associated macrophages using real-time PCR. TAM were cultured with GM-CSF for 3 days. Real-time PCR analysis for the expression of cytokines, chemokines, and their receptors is shown relative to the expression level of GAPDH. Representative data from three independent experiments are shown.

treated with either control or M-CSFR1 siRNA for 5 days) were co-cultured in one well of a 24-well plate containing 2 ml medium for 5 days and then collected for analyses.

For flow cytometric analysis, the cells were preincubated with 10 μg/ml anti-CD16/32 (clone 2.4G2, BD Biosciences, Rockville, MD, USA) at 4˚C for 30 min prior to staining. Via-Probe (BD Biosciences) was used for dead cell exclusion according to the manufacture's instructions. The following antibodies were used at 10 μg/ml: APC-conjugated anti-CD3 (clone 145C11), PE-conjugated anti-CD4 (RM4-5), and

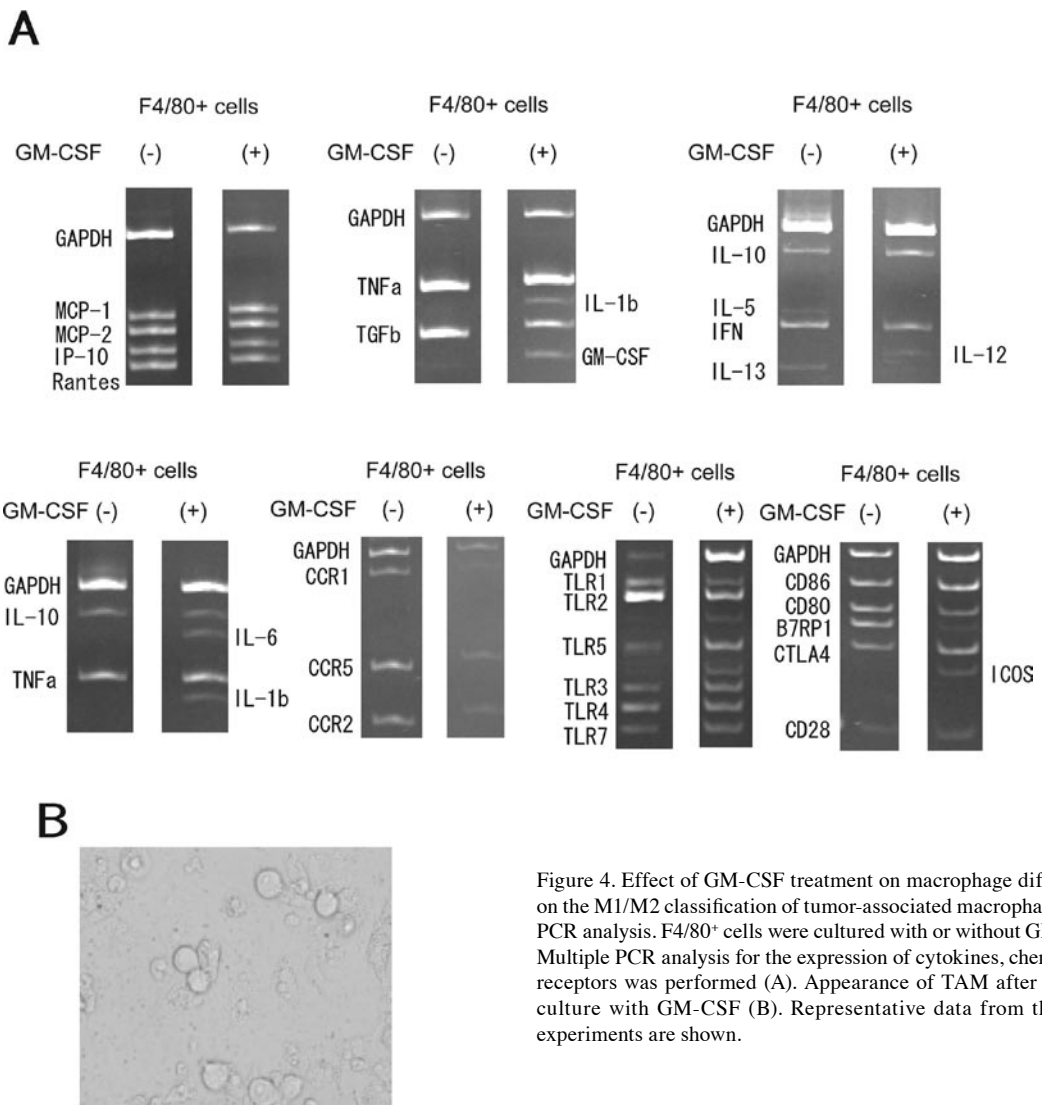

Figure 4. Effect of GM-CSF treatment on macrophage differentiation based on the M1/M2 classification of tumor-associated macrophages using multiple PCR analysis. F4/80+ cells were cultured with or without GM-CSF for 3 days. Multiple PCR analysis for the expression of cytokines, chemokines, and their receptors was performed (A). Appearance of TAM after 3 days of *in vitro* culture with GM-CSF (B). Representative data from three independent experiments are shown.

PE-conjugated anti-CD8b (clone 53-5.8, BD Biosciences). The cells were incubated with the antibodies for 30 min at 4˚C and washed with PBS. The samples were analyzed by a FACS Calibur flow cytometer and CellQuest software (Becton-Dickinson Japan, Tokyo). For cell proliferation assays, co-cultured cells were mixed well, distributed into a 96-well plate (180 μl cells/well), and 20 μl CellTiter 96 reagent (Promega Corp, Madison, WI, USA) was added to each well. The cells were cultured for 1 h at 37˚C, and the reaction was then measured by a microplate reader (Bio-Rad Laboratories) at 490 nm and analyzed using MPM-3 Microplate Manager software (Bio-Rad Laboratories).

Statistical analysis. The Student's-t test was used for statistical analysis. $P<0.05$ was considered as a significant difference.

Results

Tumor-associated macrophages bear characteristics of both M1- and M2-type macrophages. To characterize purified tumor-infiltrating F4/80+ cells, mice were euthanized 14 or 21 days after tumor implantation, and tumor-infiltrating F4/80+ cells were isolated. Purified cells were almost entirely F4/80^{high} and CD11b^{high}, which indicated that the population was suitable for consideration as tumor-associated macrophages (TAM) (Fig. 1). Macrophages were classified using the M1/M2 scheme (23) based on their expression pattern of M1/M2 factors using real-time PCR analysis. As shown in Fig. 2, TAM expressed not only M2-type markers such as CCL22, CCL17, IL-10, and CD206, but also M1-type markers, including IL-1β, TNFα, and CXCL10. The data indicated that the TAM utilized in this study simultaneously expressed both M1 and M2 factors.

Tumor-associated macrophages do not change character significantly when cultured with GM-CSF. Because macrophages have been reported to demonstrate plasticity, we investigated whether TAM can be redirected to dendritic cells using *in vitro* GM-CSF treatment because GM-CSF has been used previously to investigate DC differentiation by monocytes/macrophages (19). TAM were cultured with or without GM-CSF for 3 days (Fig. 3). The expression levels of M1-type markers (including IL-1β, TNFα, and CXCL10) decreased, but the levels of M2-type markers (including CD36 and CD206) were unchanged. The expression levels of CCR1, CCR2, CCR5, and IL-13 decreased, IL-1β was unchanged, and IL-6 and

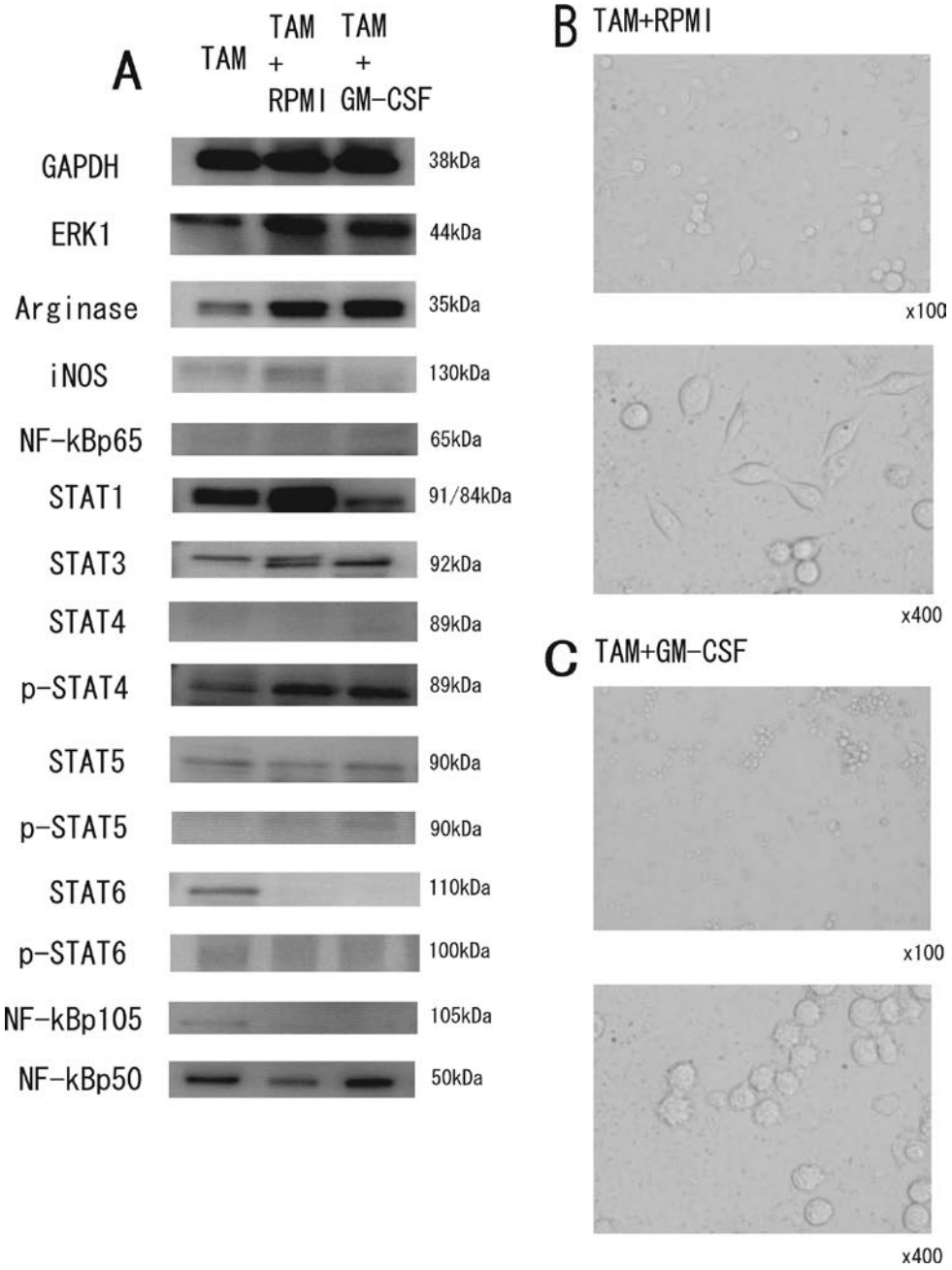

Figure 5. Tumor-associated macrophages (F4/80$^+$ cells) were cultured for 5 days in RPMI medium with or without GM-CSF. Immunoblot analysis of intracellular signaling pathways (A). Appearance of TAM from MCA38 tumors after *in vitro* culture without GM-CSF (B) and with GM-CSF (C). Representative data from three independent experiments are shown.

IL-12 were only detected after GM-CSF treatment (Fig. 4). When the expression of intracellular signal transduction proteins was compared between freshly isolated TAM and TAM cultured for 5 days with or without GM-CSF treatment (Fig. 5A), a few differed across groups (including STAT1, p-STAT5, STAT6, NF-κB), but others did not. In terms of morphology, GM-CSF treatment apparently decreased the adhesive ability of TAM; the cells were round and floated in the medium (Fig. 5B and C).

Blocking the tumor-associated macrophage M-CSF pathway in the presence of GM-CSF leads to altered intracellular signal transduction. Because the importance of M-CSF in macrophage activation has been demonstrated (24), the combined effect of M-CSF signal blockade and GM-CSF treatment on TAM was then investigated *in vitro*. No significant differences were observed in the expression pattern of M1/M2 factors analyzed using multiple PCR (Fig. 6). In terms of TAM intracellular signal transduction (Fig. 7A), the expression levels of all molecules were unchanged in TAM treated with control siRNA and GM-CSF compared to TAM cultured with GM-CSF alone, as shown in Fig. 5A. In contrast, M-CSFR knockdown by siRNA for M-CSFR1 led to increased expression levels of STAT5, STAT6, and NF-κBp65 (Fig. 7A). The morphology of cells treated with control siRNA and GM-CSF was similar to that of cells treated with GM-CSF alone (Figs. 5C

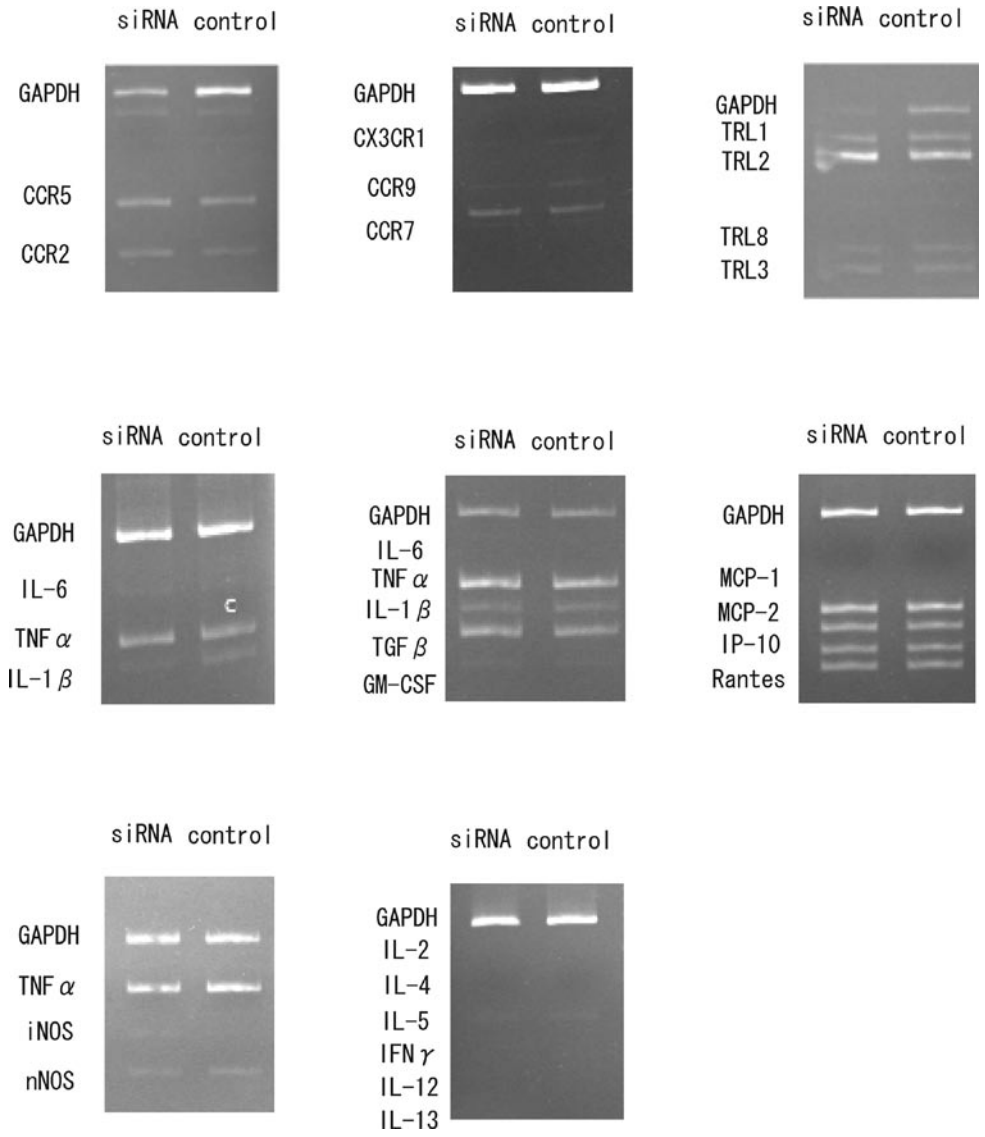

Figure 6. TAM M-CSFR knockdown by M-CSFR1 and control siRNA. Multiple PCR analysis of the expression pattern of M1/M2 factors. Representative data from three independent experiments are shown.

and 7B). However, M-CSFR knockdown by M-CSFR1 siRNA led to a change in cell morphology to a dendritic shape (Fig. 7C).

Blockade of the M-CSF pathway in conjunction with GM-CSF treatment sustains the antigen-presentation capability of tumor-associated macrophages. Finally, the antigen-presentation capability of TAM was examined. T cells were stimulated, but proliferation did not differ between control and M-CSFR1 siRNA conditions (both with GM-CSF treatment), as analyzed by two different antigen-responder combinations (alloantigen and ovalbumin systems) (Fig. 8).

Discussion

Macrophages have been classified into two subtypes based on the concept of Th1/2 T-cell subtype classification, because macrophage differentiation is strongly influenced by IFNγ and IL-4, which are secreted by Th1- and Th2-type T-cells, respectively (25). Based on this classification, TAM have been reported to fall into the M2 subtype (23). In this study, TAM (tumor-infiltrating F4/80$^+$ cells) were first assessed for their expression of cytokines, chemokines, membrane proteins, and intracytoplasmic signaling molecules related to macrophage activation and maturation. M1-type (IL-1β, TNFα, CXCL-10) and M2-type macrophage markers (CD206, CD36, CCL17) were found to be simultaneously expressed by TAM, indicating that our tumor-infiltrating F4/80$^+$ cells had the unique characteristic of expressing both M1 and M2 molecules (Figs. 1 and 2). These results are almost identical to previously reported findings that utilized tumor-infiltrating macrophages prepared by CD11b-positive immunobeading (22,26).

We previously reported that TAM strongly secrete TNFα, IL-1β, and TGFβ, and that inhibition of TGFβ blocked the immunosuppressive characteristics of TAM (22). We also reported that an immunogene therapy model utilizing IL-2 and sTNFR2 in combination suppressed TAM differentiation (21). These data suggested that macrophages have plasticity, and that different tumor microenvironments can differentially activate and differentiate macrophages to unique tumor-based

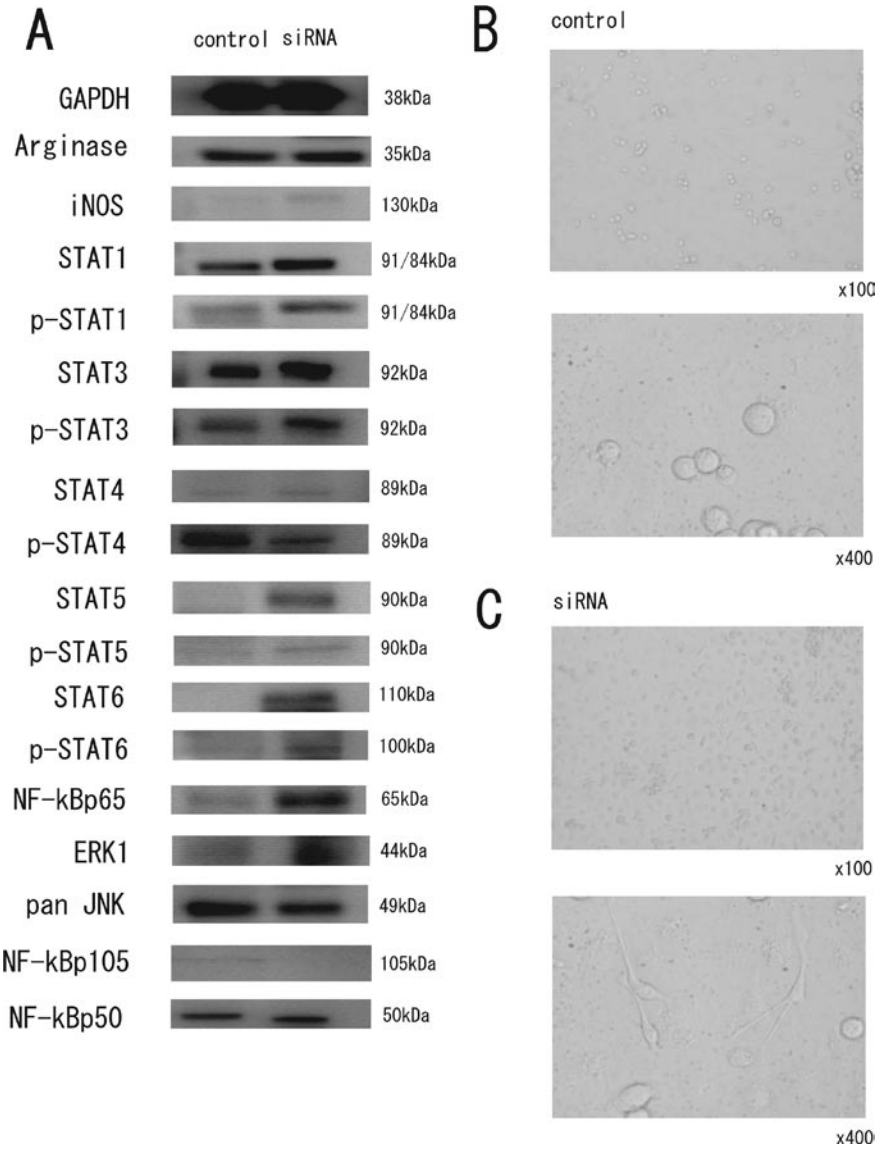

Figure 7. TAM M-CSFR knockdown by M-CSFR1 and control siRNA. Immunoblotting analysis of intracellular signal transduction (A). Appearance of TAM cultured with GM-CSF in RPMI medium (B). Appearance of TAM treated with siRNA (M-CSFR) in the presence of GM-CSF (C). Representative data from three independent experiments are shown.

phenotypes. This finding could be explained by the plasticity of macrophages for reprogramming as stated also previously (18,19,27). Therefore, in this study, we aimed to investigate the possibility that TAM could be redirected toward a dendritic cell-like phenotype by GM-CSF treatment *in vitro*, because GM-CSF was reported to be a strong monocyte differentiation factor for dendritic cells (28,29).

Contrary to our prediction, incubation of TAM with GM-CSF *in vitro* did not change the expression of M1/M2 molecules as determined and shown in Figs. 1 and 2 (Figs. 3, 4 and 5A), although the morphology of TAM changed to round cells that showed less adhesion to the culture dish (Fig. 5B and C). This could have been because TAM were more mature than monocytes previously differentiated by GM-CSF alone *in vitro* (28,30), suggesting that several factors were necessary for the redifferentiation of TAM. A single administration GM-CSF has also been shown not to be fully successful in tumor immunotherapy (31,32), which is supported by our present *in vitro* results concerning GM-CSF treatment alone. Therefore, in the case of GM-CSF tumor immunotherapy *in vivo*, TAM may remain at the tumor site and retain their immunosuppressive properties. Subsequently, we attempted to suppress M-CSF signaling, because M-CSF is essential for monocyte differentiation into macrophages (33,34). Indeed, several reports suggest that blocking M-CSF signaling by M-CSFR knock out, anti-M-CSF antibody, or anti-M-CSFR antibody can change the function or morphology of macrophages and induce dendritic cell-like characteristics (35-38). Therefore, in this study, M-CSFR siRNA was used in the presence of GM-CSF, with the expectation that M-CSF signal blockade could suppress TAM maturation, while GM-CSF could redirect immature TAM toward a dendritic cell-like phenotype.

Interestingly, combination treatment of TAM with GM-CSF and M-CSFR siRNA affected the signal transduction pathway of TAM but not the expression pattern of M1/M2 marker molecules (Fig. 7). A number of previous studies have investi-

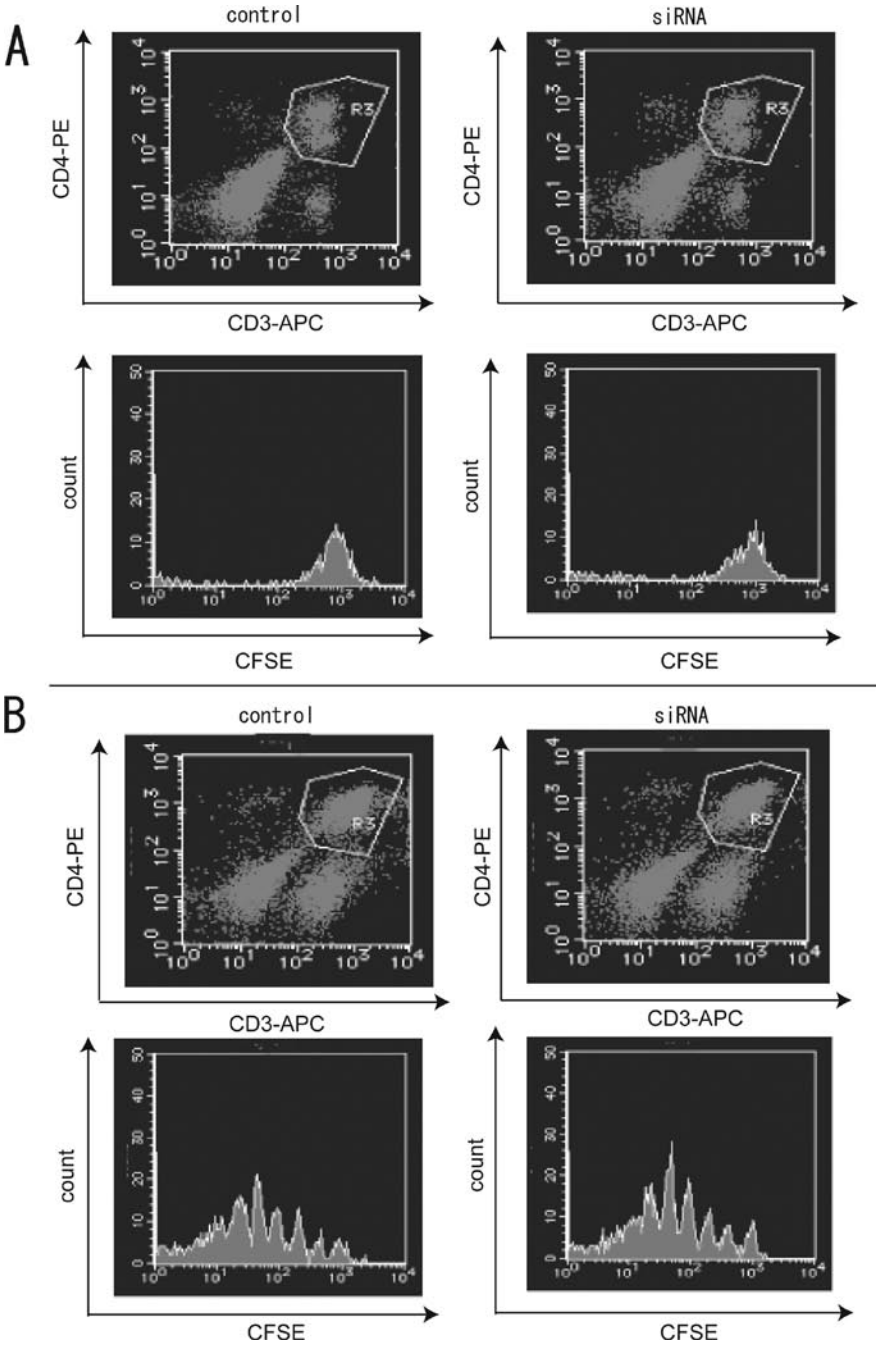

Figure 8. Analysis of the antigen presenting capability of TAM. Antigen responder T cells derived from BALB/c and OT-2 mouse splenocytes were labeled with CFSE. $CD4^+$ T cells among responder cells were analyzed by flow cytometry after stimulation with alloantigen (A) or ovalbumin antigen-specific systems (B). Representative data from two independent experiments are shown.

gated the STAT pathway in monocytes and dendritic cells. STAT5 was not induced in mature dendritic cells by GM-CSF treatment alone (39), although the same treatment of monocytes induced STAT5 and monocyte differentiation into dendritic cells (40). STAT6 was expressed by immature dendritic cells and was induced by treating monocytes with IL-4, which resulted in dendritic cell maturation (40). In contrast, GM-CSF treatment of macrophages did not induce STAT6 expression, but IL-4 treatment of the same cells did induce STAT6 (41). The IL-6/STAT3 pathway was essential for the redirection of monocytes from dendritic cell differentiation toward macrophage differentiation, and STAT3 was essential for macrophage survival (42). In terms of TAM, STAT3 did not induce, but spleen monocytes of tumor-bearing animals expressed STAT3 (43). STAT1 was consistently expressed at all steps of dendritic cell differentiation and was essential for the maturation of monocytes into dendritic cells (40). While STAT1 was expressed by TAM, monocytes from the spleens of tumor-bearing animals did not express STAT1 (43).

Previous findings and our present results indicate that treatment of tumor-infiltrating $F4/80^+$ macrophages with M-CSFR siRNA in combination with GM-CSF induced

STAT1, 5, and 6, all of which are essential for dendritic-cell maturation (40). Previous studies have shown that, in terms of the NF-κB pathway, p50 but not p65 are usually expressed by TAM (44-47). As shown in Fig. 7, GM-CSF and M-CSFR siRNA combination treatment significantly induced p65 expression, while p50 and p105 expression levels were not affected by the treatment. These results indicated that the combination of GM-CSF and M-CSFR siRNA could induce a different cell signaling pathway from that originally utilized by TAM. GM-CSF and M-CSFR siRNA treatment did not affect the capacity for antigen presentation (Fig. 8). This finding is understandable given that activated macrophages already have the ability for antigen presentation (48,49).

Taken together, our present data indicate that our treatment model could not redirect TAM to a MoDC-like phenotype based on the analysis of M1/M2 marker expression (Fig. 6), but could direct cell signaling pathways toward a dendritic cell-like pattern. Thus, treatment of tumor-bearing hosts by factors, such as GM-CSF, and blockade of M-CSF, as utilized in this study, could affect TAM differentiation via the plasticity of macrophages. Indeed, blockade of the NF-κB pathway has been shown to redirect TAM from an immunosuppressive phenotype to one with tumor-killing activity (M1 type) (27).

Tumor immunotherapy is a useful therapeutic strategy because it has fewer adverse effects than chemotherapy (50). However, because immune mechanisms are complicated, the effects of immunotherapeutic drugs have not been fully understood. Controlling the maturation and differentiation of tumor-infiltrating immune cells is critical for the development of effective immunotherapies. Macrophages remain at the sites of inflammation (such as vascular wall and soft tissue), while neutrophils die after degranulation (51). Considering that TAM are immunosuppressive (10,52) and remain at the tumor site due to their macrophage characteristics, suppressing the function and maturation of TAM, in addition to considering how to elicit anti-tumor immune responses, is very important.

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