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Siglec-F is induced by granulocyte–macrophage colony-stimulating factor and enhances interleukin-4-induced expression of arginase-1 in mouse macrophages

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Summary

Siglecs are cell surface lectins that recognize sialic acids and are primarily expressed in hematopoietic cells. Previous studies showed that some Siglecs regulate macrophage function. In the present study, we examined the induction and putative roles of mouse Siglec-F in bone-marrow-derived macrophages in mice. A quantitative RT-PCR analysis showed that the basal expression of Siglec-F was weak in bone-marrow-derived macrophages differentiated by macrophage colony-stimulating factor. However, a 24-hr stimulation with granulocyte–macrophage colony-stimulating factor (GM-CSF) enhanced Siglec-F expression. GM-CSF also enhanced Siglec-F expression in thioglycollate-induced peritoneal macrophages. The inhibition of signal transducer and activator of transcription 5 (STAT5), but not that of phosphoinositide 3-kinase or mitogen-activated protein kinase kinase, significantly reduced the induction of Siglec-F. Interleukin-3, which uses a common β-chain shared with the GM-CSF receptor to stimulate the STAT5 pathway, also enhanced Siglec-F expression. The knockdown of Siglec-F by a specific small interfering RNA enhanced GM-CSF-induced STAT5 phosphorylation, suggesting that Siglec-F down-regulates its own expression upon prolonged GM-CSF stimulation. Furthermore, the knockdown of Siglec-F reduced the STAT6 phosphorylation and expression of arginase-1 in interleukin-4-stimulated macrophages. These results suggest that Siglec-F fine-tunes the immune responses of macrophages.

Keywords: arginase; granulocyte–macrophage colony-stimulating factor; interleukin-4; macrophage; Siglec-F; signal transducer and activator of transcription 5.

Abbreviations: APC, allophycocyanin; Arg1, arginase-1; BMDM, bone-marrow-derived macrophage; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte–macrophage colony-stimulating factor; HPRT, hypoxanthine-guanosine phosphoribosyl transferase; IFN, interferon; IgG, immunoglobulin G; iNOS, inducible NO synthase; Jak2, Janus kinase 2; L929-BMDM, L929 supernatant-differentiated BMDM; LPS, lipopolysaccharide; M-BMDM, M-CSF-differentiated BMDM; M-CSF, macrophage colony-stimulating factor; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; siRNA, small interfering RNA; SOCS1, suppressor of cytokine signaling 1; STAT5, signal transducer and activator of transcription 5; STAT6, signal transducer and activator of transcription 6

Introduction

Macrophages are central players in inflammation and host defenses, and show considerable phenotype diversity and plasticity in response to environmental stimuli.[1,2] In many cases, macrophages have been categorized into two broad types – classical and alternatively activated states – as the extreme states of phenotypes. Experimentally, macrophages may polarize to classically activated macrophages (M1) in response to Toll-like receptor ligands and interferon-γ (IFN-γ), and to alternatively activated macrophages (M2) by interleukin-4 (IL-4) and IL-13. The former phenotype is characterized, for example, by the production of pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS), and the latter by the production of anti-inflammatory cytokines and arginase-1 (Arg1).[1,2]

Siglecs are sialic acid-recognizing immunoglobulin-like lectins primarily expressed in immune cells.[3–5] Five and 11 CD33-related Siglecs have been identified in mice and humans, respectively. Most Siglecs have immunoreceptor tyrosine-based inhibitory motifs in the cytoplasmic region of the protein, to which the phosphotyrosine phosphatases SHP1/2 bind. Siglecs exhibit potent immune-modulating activities including inflammation.[6–8] The expression patterns of Siglecs have been described,[3–5] and some Siglecs are conveniently used as cell-specific markers, including Siglec-H as a plasmacytoid dendritic cell marker[9] and Siglec-F as an eosinophil marker.[10] On the other hand, mouse macrophages have been reported to express several Siglecs upon activation. Siglec-G, originally reported as a specific inhibitor of B1 cells, is induced by an infection by the RNA virus, such as vesicular stomatitis virus.[11] Induced Siglec-G inhibits anti-virus responses by degrading the cytosolic pathogen sensor RIG-I.[11] Furthermore, the expression of Siglec-E is induced by several Toll-like receptor ligands such as lipopolysaccharide (LPS) or CpG DNA in bone-marrow-derived macrophages (BMDMs).[7] Siglec-E inhibits the production of pro-inflammatory cytokines *in vitro* in macrophages stimulated by Toll-like receptor ligands or group B Streptococcus,[7,12] and in microglia stimulated by neural debris.[13] Whether multiple Siglecs are regulated simultaneously on various stimuli has not been reported.

Siglec-F is a functional paralog of human Siglec-8, which is predominantly expressed in eosinophils.[10] Cross-linking of Siglec-F or Siglec-8 by antibodies results in the apoptosis of eosinophils through caspase activation.[14,15] Siglec-F$^{-/-}$ mice exhibited enhanced lung inflammation in an allergic model due to an increased number of eosinophils.[16] Hence, Siglec-F appears to reduce eosinophilic inflammation. However, the changes in expression level and the physiological role of Siglec-F in macrophages have not been clarified in detail, except that alveolar macrophages constitutively express a high level of Siglec-F.[17]

In the present study, we examined the inducible expression of CD33-related Siglecs by several stimuli in mouse BMDMs as a model system and found that Siglec-F was induced by granulocyte–macrophage colony-stimulating factor (GM-CSF) through the activation of signal transducer and activator of transcription 5 (STAT5). The knockdown experiments showed that Siglec-F reduced STAT5 signaling following GM-CSF stimulation, suggesting the existence of a regulatory loop of Siglec-F expression in which Siglec-F negatively controls its own expression through the inhibition of STAT5. We also showed that Siglec-F positively regulated the STAT6 signaling pathway as well as the expression of Arg1 in IL-4-stimulated macrophages. These results suggest that Siglec-F is induced by GM-CSF and fine-tunes macrophage responses.

Materials and methods

Mice

Eight-week-old male C57BL/6 mice were purchased from Slc Japan (Shizuoka, Japan). All animal experiments were approved by the Institutional Animal Experiment Committee of Nagoya University and conducted in accordance with the regulations on animal experiments in Nagoya University and the Guidelines for the Proper Conduct of Animal Experiments (Science Council of Japan).

Reagents

Mouse macrophage colony-stimulating factor (M-CSF), GM-CSF, IL-4, IFN-γ, IL-3, and IL-5 were purchased from PeproTech (Rocky Hill, NJ). LPS (*Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich (St. Louis, MO). Thioglycollate was obtained from Eiken Chemical (Tokyo, Japan). The mitogen-activated protein kinase kinase (MEK) inhibitor PD0325901 and phosphoinositide 3-kinase (PI3K) inhibitor wortmannin were purchased from Wako Chemicals (Osaka Japan) and Cayman Chemical (Ann Arbor, MI), respectively. The STAT5 inhibitor (CAS 285986-31-4) was obtained from Calbiochem (Darmstadt, Germany). The following antibodies were used in the analysis and purification of cells. A phycoerythrin-labeled rat anti-Siglec-F (E50-2440) antibody and an unlabeled rat anti-Siglec-F (238023) antibody were obtained from BD Biosciences (San Jose, CA) and R&D Systems (Minneapolis, MN), respectively. Fluorescein isothiocyanate (FITC) -labeled rat anti-F4/80 (BM8.1) and allophycocyanin (APC) -labeled rat anti-CD11b (M1/70) antibodies were from TONBO Biosciences (San Diego, CA). Fc block (anti-CD16/CD32 (Fc receptor) antibody, 2.4G2, 553141) was from BD Biosciences. Phycoerythrin-labeled control rat immunoglobulin G2a (IgG2a) (RTK2758) was obtained from Biolegend (San

Diego, CA). Antibodies were used at 1 μg/ml in the flow cytometric analysis. Antibodies towards phospho-p44/42 (Thr202/Tyr204) mitogen-activated protein kinase [phospho-extracellular signal-regulated kinase (pERK), #9101], phospho-Akt (Ser473, #9271), and phospho-STAT5 (Y694, C11C5, #9359) were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-phospho-STAT6 (Tyr641, sc-11762) and anti-IκB-α (C21, sc-371) antibodies were from Santa Cruz Biotechnology (Dallas, TX). A rabbit anti-Arg1 (GTX109242) antibody was from GeneTex (Irvine, CA). Rabbit anti-ERK (51068-1-AP) and anti-Akt (10176-2-AP) antibodies were from Proteintech (Rosemont, IL). Rabbit anti-STAT5 (AF2168) and anti-STAT6 (HPA001861) antibodies were obtained from R&D Systems and Sigma-Aldrich, respectively. A mouse anti-β-actin antibody (6D1) was from Medical and Biological Laboratories (MBL, Nagoya, Japan). Peroxidase-labeled goat anti-mouse IgG (#330) and anti-rabbit IgG (#458) antibodies were from MBL.

Preparation of macrophages

L929 cells were obtained from the Riken Cell Bank (Tsukuba, Japan), and maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/ml penicillin, and 100 μg/ml streptomycin. L929 cells were cultured for 7 days, and the supernatant containing M-CSF was harvested and filtered (0·45 μm), and aliquots were stored at −80°C until used.

Bone marrow cells were recovered from the femurs and tibias of the sacrificed mice by flushing out the bone cavities with phosphate-buffered saline (PBS). Cells were treated for 3 min with ACK solution (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0·1 mM EDTA) to lyze erythrocytes. To obtain L929-BMDMs, bone marrow cells were cultured for 7–9 days in the presence of 20% L929 supernatant, and to obtain M-CSF-differentiated (M-BMDMs), bone marrow cells were cultured in the presence of 10 ng/ml M-CSF. After non-adherent cells had been removed, macrophages were harvested. The purity of cells was routinely >90%, which was confirmed by staining with anti-F4/80 or anti-CD11b antibodies (data not shown). A quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis revealed that the expression levels of eosinophil-specific genes (IL-5 receptor α chain and GATA1[18]) were lower than 0·2% that of eosinophil freshly isolated from peritoneal cavity (data not shown). In some experiments, macrophages were purified based on the expression of CD11b and F4/80 using FACSJazz (BD Biosciences) as follows. Harvested M-BMDMs were initially incubated with Fc block, and stained with APC-labeled anti-CD11b and FITC-labeled anti-F4/80 antibodies. $CD11b^+$ $F4/80^+$ cells were sorted as macrophages (see Supplementary material, Fig. S1a).

In order to obtain thioglycollate-elicited peritoneal cavity-derived macrophages, C57BL/6 mice were injected intraperitoneally with 2·5 ml of 3% thioglycollate. After 72 hr, peritoneal cells were harvested by lavage with 0·67% EDTA/PBS. Cells were incubated with Fc block, and then stained with phycoerythrin-labeled anti-Siglec-F, APC-labeled anti-CD11b, and FITC-labeled anti-F4/80 antibodies. Siglec F^{low} $CD11b^+$ $F4/80^+$ cells were sorted as macrophages (see Supplementary material, Fig. S1b). In some experiments, Siglec-F^{high} cells were sorted as eosinophils to compare the expression of Siglec-F.

Cell stimulation

Macrophages ($4·0 \times 10^5$) were seeded in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin on 24-well plates and cultured for 2–4 hr to allow attachment. Medium was replaced with fresh medium to remove non-adherent cells. Cells were then stimulated in the absence of M-CSF. Unless otherwise stated, 10 ng/ml GM-CSF, 20 ng/ml IL-4, 10 ng/ml LPS, 10 ng/ml IFN-γ, and 20 ng/ml IL-3 were used.

Inhibitors were added at a predetermined concentration (ref.[19] and data not shown). As a control, the corresponding concentration of vehicle (dimethyl sulfoxide) was added to the culture. For MEK inhibition, cells were preincubated with PD0325901 (1 μM) for 30 min, washed with PBS, and then stimulated with GM-CSF in the absence of the inhibitor. To inhibit PI3K, cells were preincubated with wortmannin (100 nM) for 30 min, washed, and stimulated with GM-CSF in the absence of the inhibitor, and to inhibit STAT5, cells were pretreated with the STAT5 inhibitor (500 μM) for 24 hr and stimulated with GM-CSF in the presence of the inhibitor. In the case of the STAT5 inhibitor, the GM-CSF stimulation was performed for 8 hr to reduce cell damage, and gene expression was analyzed by qRT-PCR. For detection of the phosphorylation of ERK, Akt, and STAT5 by a Western blot analysis, cells were stimulated for 30 min.

qRT-PCR

Unless otherwise stated, cells were stimulated for 24 hr. Total RNA was extracted by Isogen II (Nippon Gene, Tokyo, Japan) and reverse-transcribed by ReverTra Ace (Toyobo, Osaka, Japan), as reported previously.[20] To obtain the cDNA of eosinophils, total RNA was extracted by the ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI) and reverse-transcribed by the ReverTra Ace qPCR RT Master Mix with the gDNA Remover (Toyobo). The qPCR was performed using the Thunderbird SYBR qPCR mix (Toyobo) using Light Cycler 96 (Roche Diagnostics, Basel, Switzerland) at 95°C for 60 seconds, followed by 45 cycles at 95°C for 5 seconds, at 58°C for

10 seconds, and at 72°C for 20 seconds. Data were normalized by hypoxanthine-guanosine phosphoribosyl transferase (HPRT) gene expression. To compare the expression levels of Siglecs, a purified plasmid DNA in which PCR fragments for the Siglecs and HPRT were inserted was used as standard after linearization. Normalization for other genes was by the $\Delta\Delta C_t$ method. The primers used are listed in the Supplementary material (Table S1).

Knockdown by small interfering RNA (siRNA)

Stealth small siRNAs were purchased from Invitrogen (Carlsbad, CA). Stealth RNAi™ siRNA-negative control med GC (Invitrogen) was used as control siRNA. The siRNAs were transfected to BMDMs by Lipofectamine 3000 (Invitrogen) or INTERFERin (Polyplus Transfection, Illkirch, France) according to the supplier's recommendations. In the knockdown of Siglec-F, a mixture of equal amounts of the two siRNAs (#1 and #2) or each siRNA (#1, #2 or #3) was used in experiments. BMDMs ($2{\cdot}0 \times 10^5$) were seeded on 24-well plates and knocked down for 48 hr in the presence of M-CSF (10 ng/ml), followed by a gentle wash with PBS, and were then stimulated in the absence of M-CSF. In some experiments, GM-CSF was used in the place of M-CSF. In the knockdown of STAT5, the siRNAs that targeted STAT5A and STAT5B were used. The sequences of siRNAs used are shown in the Supplementary material (Table S2).

Western blotting

Cells were washed with PBS and lyzed with sodium dodecyl sulfate sample buffer and subjected to Western blotting, as reported previously.[19] Band intensities were assessed using ImageJ software,[21] and compared after normalization by the band intensity of actin.

Statistical analysis

Data are presented as the mean ± standard error of at least three independent experiments. The significance of differences was analyzed by Student's *t*-test or a one-way analysis of variance followed by Tukey's *post hoc* test. A P value $< 0{\cdot}05$ was considered to be significant.

Results

Up-regulation of Siglec-F by GM-CSF in macrophages

The expression of CD33-related Siglecs in BMDMs activated by various stimuli was analyzed. Mouse bone marrow cells were cultured with M-CSF to induce M-BMDMs. These cells were confirmed to express iNOS (M1 marker) and Arg1 (M2 marker) mRNA by LPS plus IFN-γ and IL-4, respectively, as judged by qRT-PCR (data not shown). Hence, these cells were used as macrophages in subsequent analyses. Cells were challenged by several stimuli, such as LPS, IL-4, and GM-CSF, and the expression levels of Siglecs were analyzed by qRT-PCR (Fig. 1). The expression of CD33 and Siglec-G was induced by GM-CSF (3·4-fold and 4·5-fold, respectively). The expression of CD33 was slightly increased by LPS. Siglec-E was enhanced by LPS (8·3-fold), but not by LPS plus IFN-γ. Siglec-F was induced by GM-CSF (5-fold), but appeared to be repressed by LPS or LPS plus IFN-γ. Siglec-H was not induced by any of the stimuli tested. After the stimulation with GM-CSF, the expression level of Siglec-F appeared to be markedly higher than those of CD33 and Siglec-E, -G, and -H with all of the stimuli tested. In contrast to Siglec-E (Fig. 1 and ref. [7]), Siglec-F was not induced by LPS, suggesting that the expression of each Siglec is regulated in a different manner. To confirm the induced expression of Siglec-F by GM-CSF, the cell surface expression of Siglec-F was investigated using a flow cytometric analysis. A Siglec-F signal was detected by two different anti-Siglec-F antibodies in the absence of stimulation, whereas GM-CSF stimulation for 24 hr increased cell surface Siglec-F levels (Fig. 2a, b; see Supplementary material, Fig. S2).

We then assessed the dose dependency of GM-CSF to induce Siglec-F. M-BMDMs were stimulated with various concentrations of GM-CSF for 24 hr because the maximal induction of Siglec-F was observed after a 24-hr stimulation (data not shown). The expression of Siglec-F was up-regulated by GM-CSF in a dose-dependent manner (Fig. 2c), and maximal expression levels were achieved at approximately 10 ng/ml GM-CSF, which was similar to the concentration used in the *in vitro* culture to differentiate bone marrow cells to macrophages or dendritic cells by GM-CSF[22] and to stimulate eosinophils.[23]

To exclude the possibility that the up-regulation of Siglec-F was attributable to contaminated cells, we purified macrophages from M-BMDMs using a cell sorter (see Supplementary material, Fig. S1a). Although eosinophils express high levels of Siglec-F,[10,24] Siglec-F^{high} cells were not detected in the M-BMDM preparations. Therefore, CD11b$^+$ and F4/80$^+$ cells were collected without the removal of Siglec-F^{high} cells. The up-regulation of Siglec-F by GM-CSF was observed in purified M-BMDMs (Fig. 2d). The extent of the induction by GM-CSF (11·7-fold) was higher than that in M-BMDMs before purification (Fig. 1).

As the expression of Siglec-F was up-regulated by GM-CSF stimulation in M-BMDMs, we applied other conditions for macrophage differentiation. Mouse bone marrow cells were treated with an L929-cell culture supernatant (L929-BMDMs), which contains M-CSF and has been used for the differentiation of macrophages from bone marrow cells.[7] L929-BMDMs expressed less Siglec-F than M-BMDMs without stimulation (compare Fig. 1 with Fig. 2e); however, the expression of Siglec-F

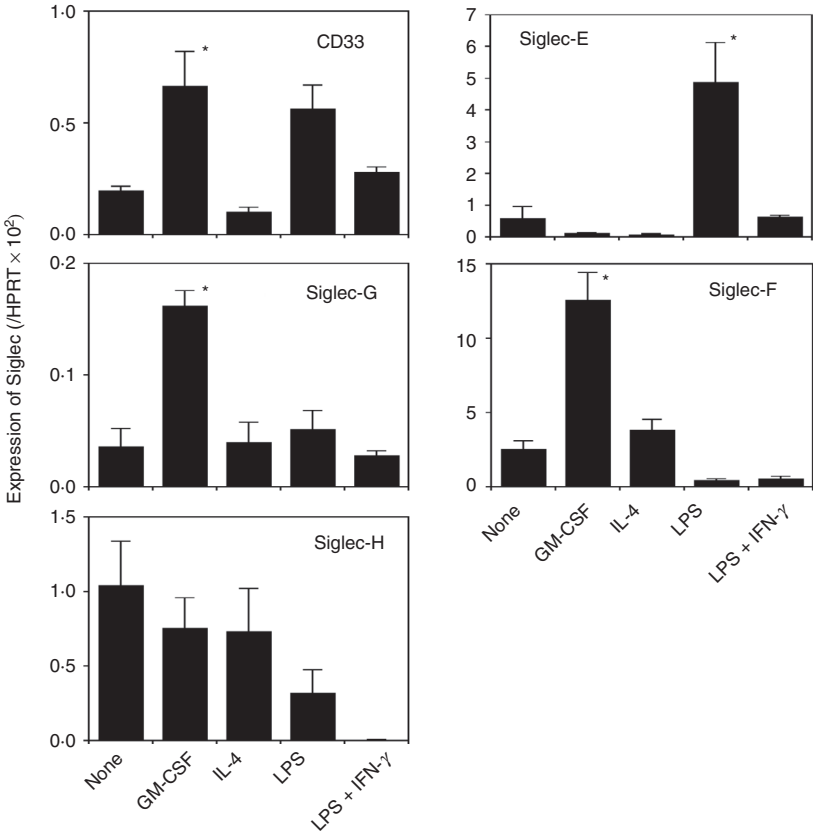

Figure 1. Induced expression of CD33-related Siglecs in macrophage colony-stimulating factor-differentiated bone-marrow-derived macrophages (M-BMDMs) by various stimuli. M-BMDMs were seeded on 24-well plates and stimulated as indicated for 24 hr. The expression of each Siglec was measured by qRT-PCR. Data are the mean ± SE of three to nine independent experiments. *$P < 0{\cdot}05$ versus none by a one-way analysis of variance followed by Tukey's *post hoc* test.

was induced by GM-CSF (approximately 14-fold) (Fig. 2e). Siglec-F was slightly induced by IL-4, but at a weaker magnitude than that by GM-CSF (Fig. 2e). When IL-4 was added in the presence of GM-CSF, further enhancements were not observed in the expression of Siglec-F (data not shown).

We then investigated whether similar up-regulation occurs in thioglycollate-elicited peritoneal cavity-derived macrophages. In thioglycollate-elicited peritoneal cavity cells, certain amounts of Siglec-F^{high} cells were detected by flow cytometry (see Supplementary material, Fig. S1b), which possibly corresponded to eosinophils.[24] Hence, macrophages were sorted as F4/80^{+} and CD11b^{+} cells after excluding Siglec-F^{high} cells from peritoneal cavity cells using a cell sorter (see Supplementary material, Fig. S1b). Basal Siglec-F mRNA levels were slightly lower in thioglycollate-elicited macrophages than in M-BMDMs (approximately 60–70%, compare Fig. 2f with Fig. 1), and a 3·9-fold increase in Siglec-F with GM-CSF and a 2·4-fold increase with IL-4 were observed in the qRT-PCR analysis of thioglycollate-elicited macrophages (Fig. 2f).

Siglec-F expression levels in GM-CSF-stimulated M-BMDMs were 0·12-fold to 0·15-fold of HPRT (Figs 1 and 2), whereas those in freshly isolated eosinophils were 2·5-fold of HPRT (data not shown): expression levels were approximately 17-fold to 21-fold higher in eosinophils than in GM-CSF-stimulated M-BMDMs.

Identification of the downstream signal of GM-CSF that drives Siglec-F expression

The binding of GM-CSF to its receptor activates Janus kinase 2 (JAK2),[25] which results in the activation of three signaling pathways: the STAT5, mitogen-activated protein kinase and PI3K pathways.[26] To clarify which pathway is involved in the up-regulation of Siglec-F by GM-CSF, downstream signaling pathways were blocked by their inhibitors. The MEK inhibitor PD0325901 did not affect GM-CSF-driven Siglec-F expression (Fig. 3a), although the phosphorylated form of ERK was reduced. The PI3K inhibitor wortmannin inhibited Akt phosphorylation (Fig. 3b). Wortmannin slightly reduced Siglec-F levels. In contrast, the STAT5 inhibitor strongly blocked GM-CSF-induced Siglec-F expression (Fig. 3c). Furthermore, STAT5 siRNA, by which the mRNA level of STAT5 decreased to 60%, reduced the induced expression of

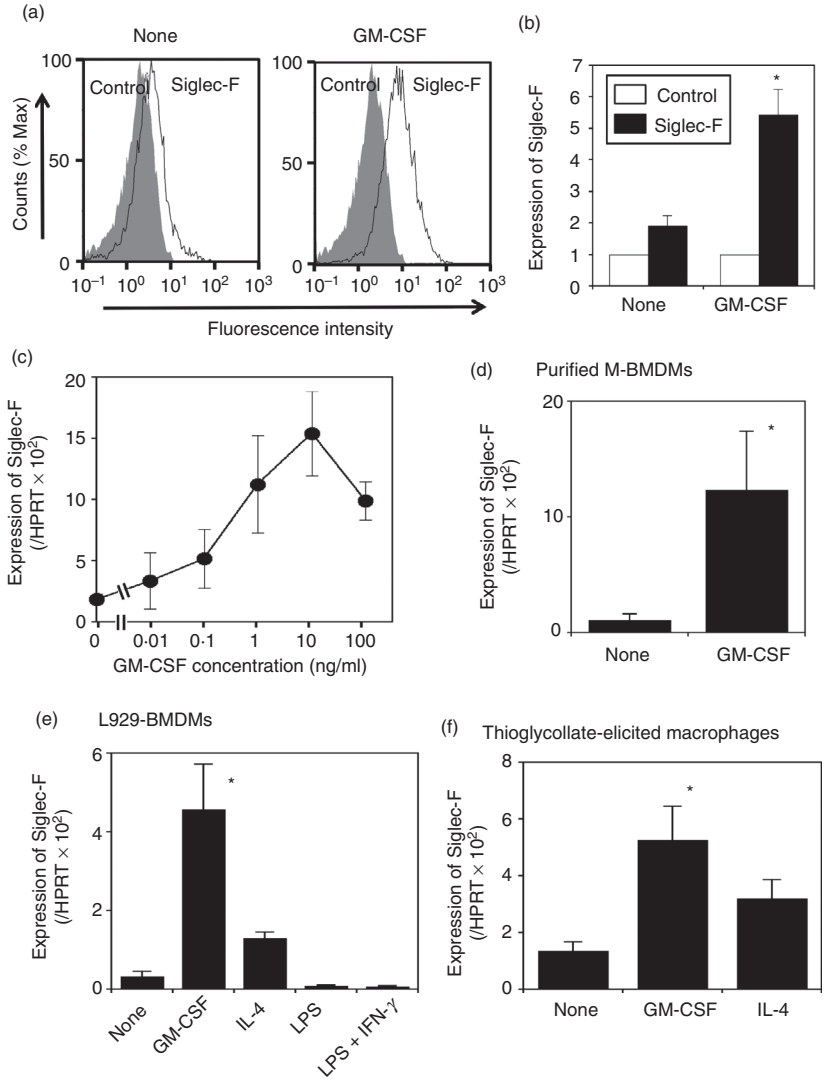

Figure 2. The expression of Siglec-F is enhanced by granulocyte–macrophage colony-stimulating factor (GM-CSF) on macrophages. (a) A flow cytometric analysis of Siglec-F on macrophages. Macrophage colony-stimulating factor-differentiated bone-marrow-derived macrophages (M-BMDMs) were cultured for 24 hr with or without GM-CSF, and stained by phycoerythrin-labeled anti-Siglec-F (BD Biosciences) or control antibodies. The black lines and shaded images show anti-Siglec-F and control staining, respectively. A representative result of three independent experiments is shown. (b) Quantification of the flow cytometric analysis. Relative expression was calculated by mean fluorescence intensity (control staining = 1). White and black bars indicate staining with control and anti-Siglec-F antibodies, respectively. Data are the mean ± SE of three independent experiments. *$P < 0{\cdot}05$ versus none by Student's *t*-test. (c) Dose dependency of Siglec-F expression induced by GM-CSF. M-BMDMs were stimulated with various concentrations of GM-CSF for 24 hr and the expression of Siglec-F was measured by qRT-PCR. Data are the mean ± SE of three to five independent experiments. (d) GM-CSF enhances Siglec-F expression in purified BMDMs. Sorted M-BMDMs were stimulated by GM-CSF for 24 hr and Siglec-F expression was examined by qRT-PCR. Data are the mean ± SE of three independent experiments. *$P < 0{\cdot}05$ versus none by Student's *t*-test. (e) Induced expression of Siglec-F in L929-BMDMs. L929-BMDMs were seeded on 24-well plates and stimulated as indicated for 24 hr. The expression of Siglec-F was measured by qRT-PCR. Data are the mean ± SE of three independent experiments. *$P < 0{\cdot}05$ versus none by a one-way analysis of variance followed by Tukey's *post hoc* test. (f) Induced expression of Siglec-F in thioglycollate-elicited macrophages. Purified macrophages were seeded on 24-well plates and stimulated as indicated for 24 hr, and Siglec-F expression was then examined by qRT-PCR. Data are the mean ± SE of four independent experiments. *$P < 0{\cdot}05$ versus none by a one-way analysis of variance followed by Tukey's *post hoc* test.

Siglec-F (Fig. 3d). These results indicate that the JAK2–STAT5 pathway was involved in Siglec-F expression induced by GM-CSF in macrophages.

The receptors for IL-3 and IL-5 share a common βc subunit with the GM-CSF receptor, and hence induce JAK2-dependent downstream signaling including

STAT5.[27,28] When M-BMDMs were stimulated with IL-3, Siglec-F expression was induced (approximately sevenfold, see Supplementary material, Fig. S3). Consistent with the lack of the expression of IL-5 receptor α chain in macrophages (data not shown), IL-5 did not induce Siglec-F expression in M-BMDMs (data not shown).

Siglec-F enhances Arg1 expression and STAT6 phosphorylation in IL-4-stimulated macrophages

To address the physiological roles of Siglec-F, the induction of Arg1 by IL-4 stimulation was analyzed in M-BMDMs after the knockdown of Siglec-F. Cells were simultaneously treated with siRNAs and GM-CSF for 48 hr, and the Arg1 induction by IL-4 was examined by Western blotting (Fig. 4a). By transfection of three different siRNAs against Siglec-F, Siglec-F expression decreased to approximately 25–50% of the control even after 24-hr stimulation with IL-4 (Fig. 4b). When the cells were stimulated with IL-4 for 24 hr, two isoforms of Arg1 were induced in both control- and Siglec-F-knockdown M-BMDMs (Fig. 4c), as reported previously.[29] However, the expression level was reduced to 50–60% of the control by the knockdown (Fig. 4c, d). The results suggest that Siglec-F enhanced Arg1 expression when macrophages were stimulated with IL-4.

As IL-4 responses require the activation of STAT6, the tyrosine phosphorylation of STAT6 was analyzed just after the IL-4 stimulation. We failed to detect short-term activation of STAT6 by IL-4 in M-BMDM that had been stimulated with GM-CSF (data not shown), possibly because of the interference of IL-4 signaling by prolonged GM-CSF activation including cross-activation of STAT6 by GM-CSF.[30] Therefore, Siglec-F in M-BMDMs was knocked down by the siRNA treatment for 48 hr in the absence of GM-CSF, and cells were then stimulated with IL-4 (Fig. 5a). Quantitative RT-PCR confirmed that Siglec-F mRNA was reduced to approximately one-seventh that of the control by transfection of two-siRNA mixture (Fig. 5b). This knockdown did not affect STAT6 protein levels (Fig. 5c). The phosphorylation of STAT6 was observed from 15 min after the addition of IL-4 and the knockdown of Siglec-F decreased the phosphorylation of STAT6 30 min after the stimulation (Fig. 5c, d). To confirm the effect of Siglec-F knockdown on STAT6 phosphorylation, three siRNAs were separately transfected and cells were stimulated with IL-4 (see Supplementary material, Fig. S4a). Knockdown efficiencies were 50–70% (see Supplementary material, Fig. S4b); slightly less than that by the mixture of siRNA#1 and #2. The knockdown of Siglec-F reduced the STAT6 phosphorylation induced by IL-4 (see Supplementary material, Fig. S4c, d). Similar results were obtained with L929-BMDMs (see Supplementary material, Fig. S5). The changes in phosphorylation of ERK were not evident in these experiments (data not shown). The phosphorylation of Akt was not observed (data not shown), suggesting that PI3K activation was weak under these conditions, as reported previously.[31] These results suggested that Siglec-F enhanced IL-4-induced phosphorylation of STAT6.

Siglec-F does not affect nuclear factor-κB activation induced by LPS plus IFN-γ

We then investigated whether the knockdown of Siglec-F modulates the responses induced by LPS plus IFN-γ. Cells were simultaneously treated with siRNAs and GM-CSF, and then washed and stimulated with LPS plus IFN-γ (see Supplementary material, Fig. S6a). The 4-hr stimulation strongly induced the expression of iNOS, IL-10 and tumor necrosis factor-α, which was not significantly changed by the knockdown of Siglec-F (see Supplementary material, Fig. S6b, c). The activation of nuclear factor-κB, which was inhibited by Siglec-E,[7] was then examined as the degradation of IκB. IκB was similarly reduced in control- and Siglec-F-siRNA-treated macrophages after the LPS plus IFN-γ stimulation for 1 hr (see Supplementary material, Fig. S6d–f). These results suggest that Siglec-F may not directly affect inflammation signals by LPS plus IFN-γ under these conditions.

Siglec-F reduces the STAT5 phosphorylation induced by GM-CSF

We also investigated whether Siglec-F affected GM-CSF responses in macrophages. M-BMDMs were transfected with Siglec-F siRNA, and cells were stimulated after 48 hr with GM-CSF (Fig. 6a; see Supplementary material, Fig. S7a). The amount of total STAT5 was not changed by the knockdown of Siglec-F (Fig. 6b; see Supplementary material, Fig. S7b), whereas the phosphorylation of STAT5 was enhanced (Fig. 6b, c; see Supplementary material, Fig. S7b, c). These results suggest that Siglec-F at least partly blocked the STAT5 activation in the GM-CSF stimulation. As the expression of class II MHC is controlled by GM-CSF,[32] the effect of the knockdown on the expression of this gene was then analyzed. M-BMDMs were transfected with three different siRNAs against Siglec-F and cultured for 48 hr, then stimulated for 24 hr with GM-CSF. Quantitative RT-PCR analysis indicated that the expression of class II MHC was induced by GM-CSF (approximately 10-fold), which was not changed by the knockdown of Siglec-F (data not shown).

Discussion

In the present study, we examined Siglec expression in BMDMs (Fig. 1). Siglec-E was strongly induced by LPS, but other CD33-related Siglecs were not, suggesting different regulatory mechanisms for the expression of each

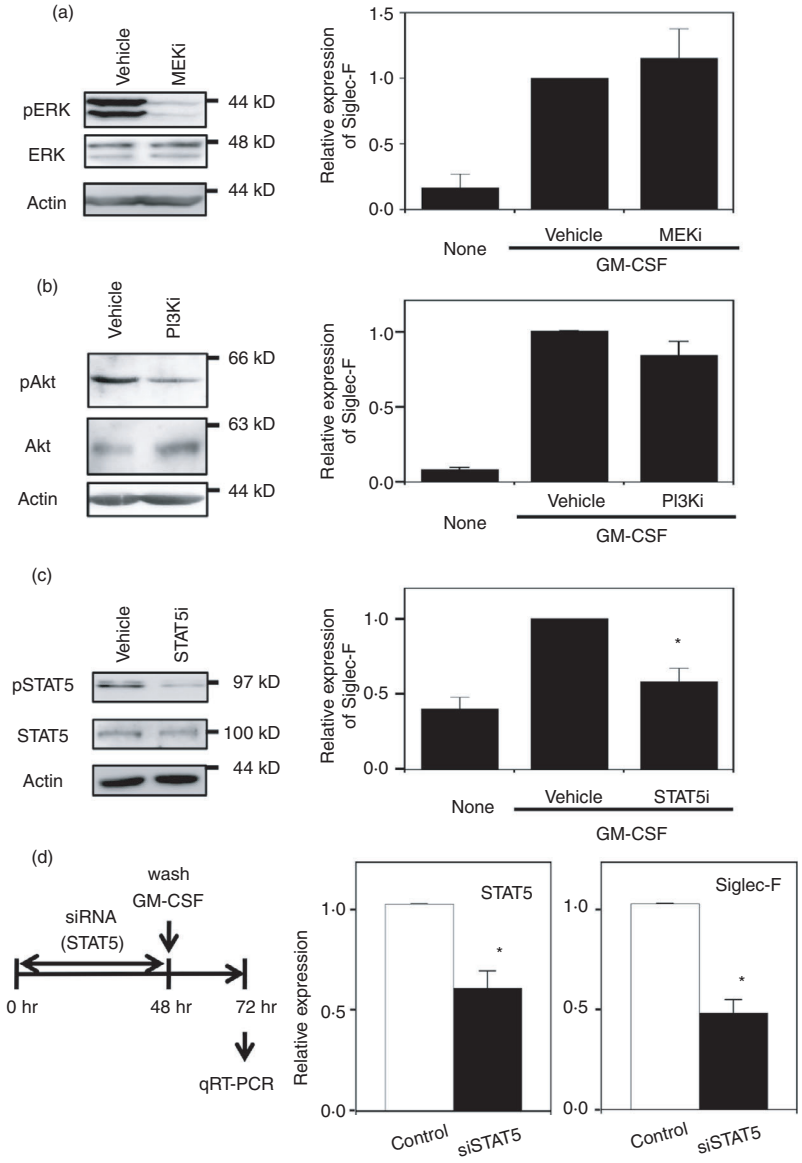

Figure 3. Enhancement of Siglec-F expression by granulocyte–macrophage colony-stimulating factor (GM-CSF) depends on the signal transducer and activator of transcription 5 (STAT5) pathway. (a) Effects of the mitogen-activated protein kinase kinase (MEK) inhibitor (PD0325901). (Left) Confirmation of MEK inhibition by a reduction in the phosphorylation of extracellular signal-regulated kinase (ERK). Macrophage colony-stimulating factor-differentiated bone-marrow-derived macrophages (M-BMDMs) were stimulated with GM-CSF for 30 min. A representative result of two independent experiments is shown. (Right) The lack of inhibition of Siglec-F expression by the MEK inhibitor. Siglec-F expression was measured by qRT-PCR after a 24-hr stimulation. The vehicle control [dimethyl sulfoxide (DMSO) 0·1%] was regarded as 1. Data are the mean ± SE of three independent experiments. (b) Effects of the phosphoinositide 3-kinase (PI3K) inhibitor (wortmannin). (Left) Confirmation of PI3K inhibition by a reduction in the phosphorylation of Akt. Cells were stimulated with GM-CSF for 30 min. A representative result of three independent experiments is shown. (Right) The lack of inhibition of Siglec-F expression by the PI3K inhibitor. Siglec-F expression was measured by qRT-PCR after a 24-hr stimulation. The vehicle control (DMSO 0·1%) was regarded as 1. Data are the mean ± SE of five independent experiments. (c) Effects of the STAT5 inhibitor. (Left) Confirmation of STAT5 inhibition by a reduction in phosphorylation. Cells were stimulated with GM-CSF for 30 min. A representative result of two independent experiments is shown. (Right) The inhibition of Siglec-F expression by the STAT5 inhibitor. Siglec-F expression was measured by qRT-PCR after an 8-hr stimulation. The vehicle control (DMSO 0·5%) was regarded as 1. Data are the mean ± SE of three independent experiments. *$P < 0·05$ versus the vehicle by Student's *t*-test. (d) Enhancing effects of GM-CSF on Siglec-F expression are inhibited by the knockdown of STAT5. (Left) Schematic presentation of the experiment. M-BMDMs were transfected with STAT5 or control small interfering RNA (siRNA). Cells were washed after a 48-hr culture and stimulated with GM-CSF for an additional 24 hr. The expression of STAT5 and Siglec-F was measured by qRT-PCR. (Middle) Knockdown efficiency of STAT5. (Right) Inhibition of Siglec-F expression by STAT5 siRNA. The mRNA level in cells transfected with control siRNA was regarded as 1. Data are the mean ± SE of three independent experiments. *$P < 0·05$ versus control siRNA by Student's *t*-test.

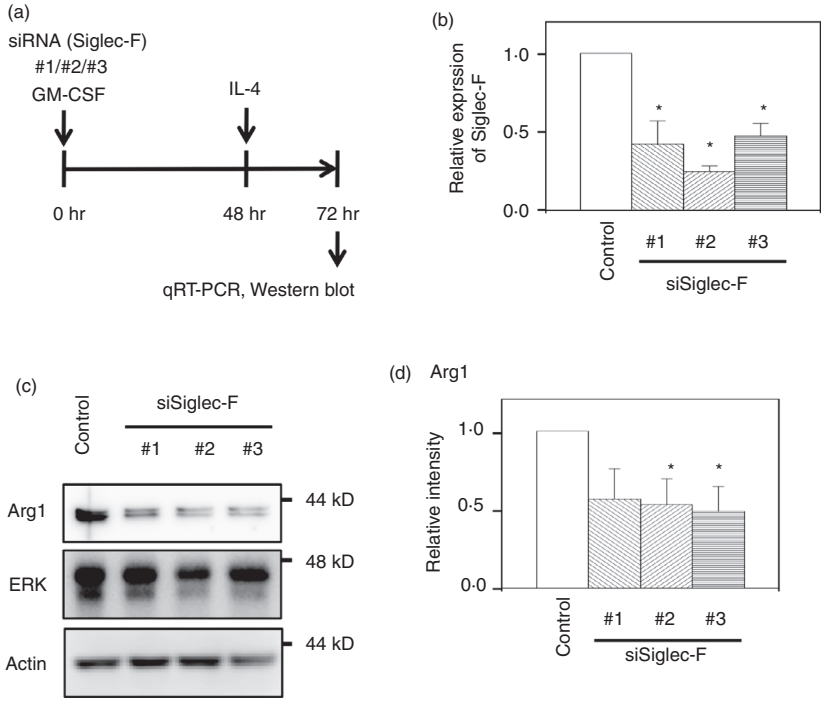

Figure 4. Siglec-F knockdown reduces arginase-1 (Arg1) expression induced by interleukin-4 (IL-4) in macrophage colony-stimulating factor-differentiated bone-marrow-derived macrophages (M-BMDMs). (a) Schematic presentation of the knockdown experiment. M-BMDMs were transfected with Siglec-F or control small interfering RNA (siRNA) in the presence of GM-CSF. Forty-eight hours later, cells were washed and stimulated with IL-4 for an additional 24 hr. The expression of Siglec-F and Arg1 was measured by qRT-PCR and Western blotting, respectively. (b) Confirmation of Siglec-F knockdown. Data are the mean ± SE of three independent experiments. $*P < 0{\cdot}05$ versus the control by Student's *t*-test. (c) Siglec-F knockdown reduced IL-4-induced Arg1 expression. Total extracellular signal-regulated kinase (ERK) and actin were measured as controls. A representative result of three independent experiments is shown. (d) Quantification of band intensity. The band intensity of Arg1 was normalized to that of actin. The band intensity of control siRNA-transfected cells was regarded as 1. Data are the mean ± SE of three independent experiments. $*P < 0{\cdot}05$ versus the control by Student's *t*-test.

Siglec. Furthermore, we found that several Siglecs, such as CD33 and Siglec-G and -F, were induced by GM-CSF simultaneously (Fig. 1). Among the Siglecs, we analyzed GM-CSF-driven Siglec-F expression in more detail because the results of the qRT-PCR analysis suggested that Siglec-F is a major Siglec expressed in BMDMs. Although macrophages show diversity, GM-CSF enhanced Siglec-F expression in two different BMDM preparations as well as thioglycollate-elicited macrophages (Figs 1 and 2). As GM-CSF is essential for the proper differentiation of alveolar macrophages,[33,34] the strong expression of Siglec-F in alveolar macrophages[17,35] may be related to the activity of GM-CSF.

Originally identified as a hematopoietic growth factor, GM-CSF is now known to be a potent cytokine that induces the proliferation, differentiation, and activation of macrophages.[36–39] Although the basal concentration of GM-CSF is low, its levels are often elevated during inflammatory reactions.[37,38] A number of different cell types including T lymphocytes, macrophages, and cancer cells produce GM-CSF.[37,40] In most cases, inflammatory stimuli induce the production of GM-CSF, including typical inflammatory mediators/inducers, such as tumor necrosis factor-α,[41] IL-1,[42] and LPS.[42] In addition to GM-CSF, IL-3 enhanced Siglec-F expression (see Supplementary material, Fig. S3). IL-3 is a major hematopoietic cytokine, and has been reported to play important roles in inflammation. A large amount of IL-3 was shown to be produced by BMDMs following stimulation with LPS plus IFN-γ.[43] Furthermore, GM-CSF and IL-3 were simultaneously produced in septic shock induced by LPS or cecal ligation and puncture.[44] These findings suggest that Siglec-F is indirectly induced by a number of inflammatory stimuli.

In an attempt to clarify the physiological significance of Siglec-F in BMDMs, we investigated whether Siglec-F modulates the activation of two JAK/STAT signaling pathways: STAT6 induced by IL-4 and STAT5 by GM-CSF. Siglec-F facilitated IL-4-induced STAT6 phosphorylation (Fig. 5; see Supplementary material, Figs S4 and S5), but inhibited GM-CSF-induced STAT5 phosphorylation (Fig. 6; see Supplementary material, Fig. S7). The latter result suggests the existence of a feedback loop for the regulation of Siglec-F expression. Siglec-F, which is

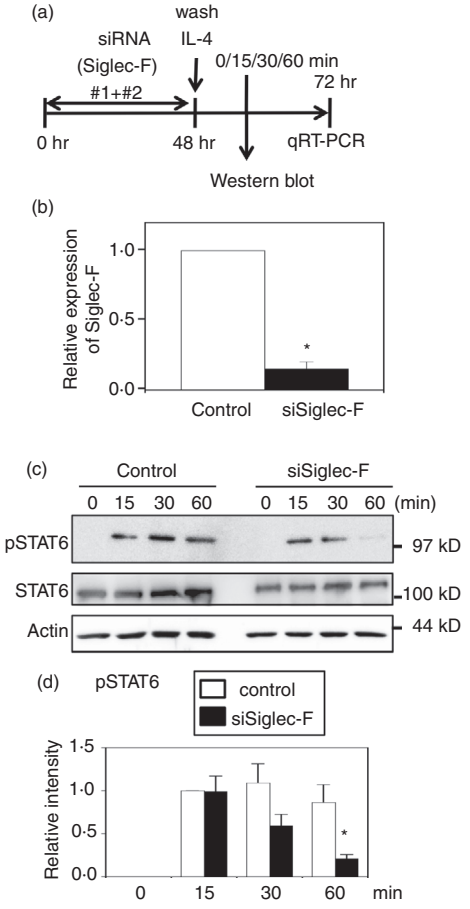

Figure 5. Siglec-F knockdown reduces signal transducer and activator of transcription 6 (STAT6) phosphorylation induced by interleukin-4 (IL-4) in macrophage colony-stimulating factor-differentiated bone-marrow-derived macrophages (M-BMDMs). (a) Schematic presentation of the knockdown experiment. M-BMDMs were transfected with Siglec-F or control small interfering RNA (siRNA). Cells were washed after a 48-hr culture and stimulated with IL-4 for the indicated periods. The phosphorylation of STAT6 was examined by Western blotting. (b) Confirmation of knockdown. Total RNA was collected 24 hr after the stimulation and subjected to qRT-PCR. Data are the mean ± SE of six independent experiments. $*P < 0{\cdot}05$ versus the control by Student's *t*-test. (c) Siglec-F knockdown reduced the phosphorylation of STAT6. Total and the phosphorylated form of STAT6 were examined. Actin was measured as a control. A representative result of six independent experiments is shown. (d) Quantification of band intensity. The band intensity of pSTAT6 was normalized to that of actin. The band intensity of control siRNA at 15 min was regarded as 1. White and black bars indicate control and Siglec-F siRNAs, respectively. Data are the mean ± SE of six independent experiments. $*P < 0{\cdot}05$ versus the control at the same time point by Student's *t*-test.

induced by GM-CSF (Figs 1, 2; see Supplementary material, Fig. S2), inhibited the downstream signaling of GM-CSF, suggesting that Siglec-F restricted its own expression through the inhibition of STAT5 when cells were continuously stimulated by GM-CSF.

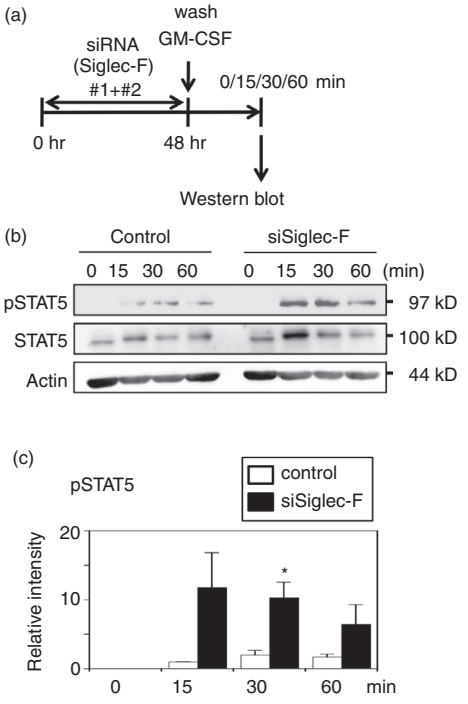

Figure 6. Siglec-F knockdown enhances signal transducer and activator of transcription 5 (STAT5) phosphorylation induced by granulocyte–macrophage colony-stimulating factor (GM-CSF) in macrophage colony-stimulating factor-differentiated bone-marrow-derived macrophages (M-BMDMs). (a) Schematic presentation of the knockdown experiment. M-BMDMs were transfected with Siglec-F or control small interfering RNA (siRNA). Cells were washed after a 48-hr culture and stimulated with GM-CSF for the indicated periods. Phosphorylation was assessed by Western blotting. (b) Siglec-F knockdown enhanced the phosphorylation of STAT5. Total and the phosphorylated form of STAT5 were examined. Actin was measured as a control. A representative result of three independent experiments is shown. (c) Quantification of band intensity. The band intensities of pSTAT5 were normalized to that of actin. The band intensity of control siRNA at 15 min was regarded as 1. White and black bars indicate control and Siglec-F siRNAs, respectively. Data are the mean ± SE of three independent experiments. $*P < 0{\cdot}05$ versus the control at the same time point by Student's *t*-test.

The mechanisms underlying Siglec-F activity to modulate signaling have not yet been elucidated in detail, but may be due to the binding proteins of immunoreceptor tyrosine-based inhibitory motifs in Siglec-F. One possibility is that immunoreceptor tyrosine-based inhibitory motifs of Siglec-F sequester cytosolic regulators from the receptors for GM-CSF or IL-4. As the common βc requires SHP-2 to initiate downstream signaling,[45] sequestration of SHP-2 from GM-CSF receptor by Siglec-F may reduce STAT5 signaling. Alternatively, sequestration of inducible suppressor by Siglec-F from IL-4 receptor may affect IL-4 signaling: when BMDMs that had been knocked down for Siglec-F were stimulated by IL-4,

the phosphorylation of STAT6 was observed just after the stimulation, but rapidly became weaker than that observed in control cells (Fig. 5; see Supplementary material, Fig. S5). Suppressor of cytokine signaling 1 (SOCS1), which is induced by IL-4 and suppresses STAT6 phosphorylation,[46] may be such a candidate, although binding between SOCS and Siglecs is not reported so far except for SOCS3 and Siglec-7 or CD33.[47,48] These possibilities remain to be investigated.

Although the knockdown of Siglec-F did not affect inflammatory gene expression or nuclear factor-κB activation induced by LPS plus IFN-γ (see Supplementary material, Fig. S6), Siglec-F knockdown reduced the expression of Arg1 in IL-4-stimulated macrophages (Fig. 4). On the other hand, the results of the qRT-PCR analysis suggested that other M2-specific genes, including Fizz1 or Mrc1, were not reduced by this knockdown (data not shown), indicating that Siglec-F specifically enhances Arg1 rather than generally promoting M2 phenotypes. Arg1 is known to participate in the inhibition of iNOS activity and in the production of polyamine and proline, which are required for tissue repair in inflammation,[49] while enhancing the growth of *Leishmania* parasites.[50] Our results imply the possibility that Siglec-F may participate in the regulation of macrophage activity by fine-tuning of Arg1 expression. Further studies, such as those using Siglec-F-knockout mice, are needed to clarify the exact role of Siglecs in the physiology of macrophages.

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Author Contribution

HT, YM, HH, HK, and YI designed and performed experiments. SI and KN conceived the study and wrote the manuscript. All authors analyzed the results and approved the final version of the manuscript.

Disclosures

The authors declare no financial conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Sorting strategy for macrophage purification. (a) Procedure for the purification of BMDMs (associated with Fig. 1d). M-BMDMs were sorted as F4/80$^+$ CD11b$^+$ cells. R1, sorted region. A representative result of 3 independent experiments is shown. (b) Procedure for the purification of thioglycollate-elicited macrophages (associated with Fig. 1f). Five days after the thioglycollate injection, macrophages were purified from peritoneal cavity cells as F4/80$^+$ CD11b$^+$ cells after excluding Siglec-F^{high} cells. R1, sorted macrophage fraction. Siglec-F^{high} cells were sorted as eosinophils for comparison. A representative result of 4 independent experiments is shown.

Figure S2. GM-CSF induces Siglec-F expression on M-BMDMs. Cells were stimulated for 24 h and cell surface expression of Siglec-F was examined by indirect staining. Anti-Siglec-F antibody (R&D Systems) and control rat IgG (Santa Cruz Biotechnology) were used for primary antibody. A representative result of 3 independent experiments is shown.

Figure S3. IL-3 enhances Siglec-F expression in BMDMs. M-BMDMs were stimulated with IL-3 for 24 h and Siglec-F expression was examined by qRT-PCR. Data are the mean ± SE of 5 independent experiments. *$P <$ 0·05 versus none by the Student's *t*-test.

Figure S4. Siglec-F knockdown reduces STAT6 phosphorylation induced by IL-4 in M-BMDMs. (a) Schematic presentation of the knockdown experiment. M-BMDMs were transfected with Siglec-F or control siRNA. Cells were washed after a 48-h culture, and stimulated with IL-4 for 60 min. The phosphorylation of STAT6 was examined by Western blotting. (b) Confirmation of knockdown. Total RNA was collected 24 h after the stimulation and subjected to qRT-PCR. Data are the mean ± SE of 3 independent experiments. *$P <$ 0·05 versus the control by the Student's *t*-test. (c) Siglec-F knockdown reduced the phosphorylation of STAT6. Total and the phosphorylated form of STAT6 were examined. Actin was measured as a control. A representative result of 7 independent experiments is shown. (d) Quantification of band intensity. The band intensity of pSTAT6 was normalized to that of actin. The band intensity of control siRNA was regarded as 1. Data are the mean ± SE of 7 independent experiments. *$P <$ 0·05 versus the control by the Student's *t*-test.

Figure S5. Siglec-F knockdown reduces STAT6 phosphorylation induced by IL-4 in L929-BMDMs. (a) Schematic presentation of the knockdown experiment. L929-BMDMs were transfected with Siglec-F or control siRNA. Cells were washed after a 48-h culture, and stimulated with IL-4 for the indicated periods. The phosphorylation of STAT6 was examined by Western blotting. (b) Siglec-F knockdown reduced the phosphorylation of STAT6. Total and the phosphorylated form of STAT6 were examined. Actin was measured as a control. A representative result of 3 independent experiments is shown. (c) Quantification of band intensity. The band intensity of pSTAT6 was normalized to that of actin. The band intensity of control siRNA at 15 min was regarded as 1. White and black bars indicate control and Siglec-F siRNAs, respectively. Data are the mean ± SE of 3 independent experiments. *$P <$ 0·05 versus the control at the same time point by the Student's *t*-test.

Figure S6. Effects of Siglec-F knockdown on the stimulation of LPS plus IFN-γ in M-BMDMs. (a-c) Effects of Siglec-F knockdown on gene expression induced by LPS plus IFN-γ. (a) Schematic presentation of the knockdown experiment. M-BMDMs were transfected with Siglec-F or control siRNA in the presence of GM-CSF. Forty-eight hours later, cells were washed and stimulated with LPS plus IFN-γ for an additional 4 h. The expression of Siglec-F, iNOS, IL-10, and TNF-α was measured by qRT-PCR. (b) Confirmation of Siglec-F knockdown at 52 h. (c) Effect of Siglec-F knockdown on the gene expression. Data are the mean ± SE. N=3 (iNOS), 4 (IL-10), 3 (TNF-α). *$P < 0{\cdot}05$ versus the control by the Student's *t*-test. (d-f) The effects of Siglec-F knockdown on IκBα degradation induced by LPS plus IFN-γ. (d) Schematic presentation of the knockdown experiment. M-BMDMs were transfected with Siglec-F or control siRNA in the presence of GM-CSF. Cells were washed after a 48-h culture, and stimulated with LPS plus IFN-γ for 60 min. The amount of IκBα was assessed by Western blotting. (e) Siglec-F knockdown did not affect IκBα degradation. A representative result of 3 independent experiments is shown. (f) Quantification of band intensity. The band intensity of IκBα was normalized by that of actin. The band intensity of control siRNA without a stimulation was regarded as 1. White and black bars indicate control and Siglec-F siRNAs, respectively. Data are the mean ± SE of 3 independent experiments.

Figure S7. Siglec-F knockdown enhances the STAT5 phosphorylation induced by GM-CSF in M-BMDMs. (a) Schematic presentation of the knockdown experiment. M-BMDMs were transfected with Siglec-F or control siRNA. Cells were washed after a 48-h culture, and stimulated with GM-CSF for 60 min. (b) Phosphorylation was assessed by Western blotting. A representative result of 7 independent experiments is shown. Total and the phosphorylated form of STAT5 were examined. Actin was measured as a control. (c) Quantification of band intensity. The band intensities of pSTAT5 were normalized to that of actin. The band intensity of control siRNA was regarded as 1. Data are the mean ± SE of 7 independent experiments. *$P < 0{\cdot}05$ versus the control by the Student's *t*-test.

Table S1. Primers used in this study.

Table S2. Small interfering RNAs used in this study.

