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Siglec-9 modulated IL-4 responses in the macrophage cell line RAW264

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Abbreviations: Arg1, arginase-1; C/EBP, CCAAT/enhancer-binding protein; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FITC, fluorescence isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL-4R, IL-4 receptor; IL-13R, IL-13 receptor; IRS, insulin receptor substrate; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; Jak, Janus kinase; LPS, lipopolysaccharide; MEK, mitogen-activated protein kinase kinase; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PI-3K, phosphatidylinositol 3 kinase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ROS, reactive oxygen species; SDS, sodium dodecylsulfate; SHP, SH2-containing cytoplasmic tyrosine phosphatase; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor.

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Abstract

Siglecs, an immunoglobulin-like lectin family that recognizes the sialic acid moiety, regulate various aspects of immune responses. In the present study, we investigated the effects of Siglecs on the macrophage cell line RAW264, which was stimulated with IL-4. The induction of arginase-1 (Arg1) by IL-4 was stronger in Siglec-9-expressing cells than in mock cells. Mutations in the cytoplasmic tyrosine-based inhibitory motifs in Siglec-9 markedly reduced the expression of Arg1. The phosphorylation of Akt by IL-4 and extracellular signal-regulated kinase (ERK) without IL-4 was stronger in Siglec-9-expressing cells, indicating the enhanced activation of the phosphatidylinositol 3 kinase (PI-3K) and mitogen-activated protein kinase kinase (MEK)/ERK pathways, respectively. The enhanced expression of Arg1 was inhibited by MEK inhibitors, but not by PI-3K inhibitor. These results indicate that Siglec-9 affects several different signaling pathways in IL-4-stimulated macrophages, which resulted in enhanced induction of Arg1 in Siglec-9-expressing RAW264 cells.

Keywords: lectin; macrophages; sialic acid, IL-4, RAW264

Introduction

Sialic acids cover the cell surface as the terminal residues of glycolipids or *N*- and *O*-glycans in glycoproteins and play various roles in the regulation of immune responses.[1)] Siglecs are sialic acid-recognizing immunoglobulin-like lectins that are primarily expressed in immune cells.[2-4)] Six and 12 CD33-related Siglecs have been identified in mice and humans, respectively, and typically have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that may down-regulate innate and acquired immune responses. The recruitment of SH2-containing cytoplasmic tyrosine phosphatase (SHP)-1 to ITIM has been suggested to inhibit immunoreceptor tyrosine-based activation motif (ITAM)-initiating signals.[2-4)]

Macrophages are ubiquitous cells that are known to be crucially involved in maintaining homeostasis under steady state conditions. Toll-like receptors (TLR) recognize a range of chemicals produced by bacteria, viruses, fungi, and protozoa in order to initiate the first defense against pathogens[5)] through the stimulation of macrophages, which results in the production of large amounts of proinflammatory cytokines and reactive oxygen species. Such macrophages are regarded as "classically activated" and induce inflammation. Recent findings demonstrated that gene expression by macrophages was heterologous when stimulated with different stimuli[6)] or even under stationary conditions in various organs.[7)] Type 2 cytokines such as IL-4/IL-13 have been shown to stimulate macrophages exhibiting different characteristics, referred to as "alternatively activated" macrophages, which mediate anti-inflammatory responses or wound healing as well as protection against some pathogens.[6,8)]

Siglecs are known to regulate macrophage activity. Siglec-9 or Siglec-E was previously shown to inhibit the production of proinflammatory cytokines in macrophages stimulated with several TLR ligands.[9,10)] Mouse Siglec-G inhibited the anti-viral responses of macrophages by degrading the cytosolic pathogen sensor RIG-I.[11)] These findings demonstrated the fundamental roles of Siglecs in regulating macrophage function. However, the effects of Siglecs on IL-4 signals have not yet been reported, in spite of the ability of IL-4 to strongly induce macrophage effector function. IL-4 binds to two types of IL-4 receptor (IL-4R) heterodimers: the shared IL-4R α chain, which also binds SHP-1 through ITIM, is associated with either the common γ chain (type I) or IL-13 receptor (IL-13R) α1 chain (type II). Both receptors typically activate

protein tyrosine kinase Janus kinase (Jak) to phosphorylate signal transducer and activator of transcription (STAT)6 and other molecules. The phosphorylation of STAT6 was previously reported to be inhibited by SHP-1 in some situations.[12-14] Since SHP-1 binds to the ITIM of Siglecs, Siglecs may have the ability to modulate the IL-4 signaling cascade. Therefore, we herein examined the effects of human Siglec-9 on the IL-4 induced responses of macrophages using mouse RAW264 cells as a model system.

Materials and methods

Reagents. Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was obtained from Sigma-Aldrich (St. Louis, MO, USA). IL-4, interferon (IFN)-γ, and IL-13 were purchased from Pepro Tech (Rocky Hill, NJ, USA). Goat anti-human Siglec-9 antibody was purchased from R&D Systems (Minneapolis, MN, USA). Anti-CCAAT/enhancer-binding protein (C/EBP)β (sc-150), anti-phospho-STAT6 (sc-11762-R), and fluorescence isothiocyanate (FITC)-labeled donkey anti-goat IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin (AC-15) antibody was obtained from Sigma-Aldrich. An anti-human CD14 antibody labeled with phycoerythrin (PE) was from Beckman Coulter (Brea, CA, USA). Anti-phospho-Akt (Ser473, #92715) and anti-phospho extracellular signal-regulated kinase (ERK) (#9101) antibodies were purchased form Cell Signaling Technology (Beverly, MA, USA). The Akt inhibitor GSK690693 and phosphatidylinositol 3 kinase (PI-3K) inhibitor wortmannin were purchased from Sigma-Aldrich. The ERK kinase (mitogen-activated protein kinase kinase (MEK)) inhibitors, U0126 and PD0325901, were from Cell Signaling Technology and Wako Pure Chemicals (Osaka, Japan), respectively. The Jak3 inhibitor Janex-1 and STAT6 inhibitor AS1517499[15] were obtained from Cayman Chemical (Ann Arbor, MI, USA) and Axon Medchem (Groningen, Netherlands), respectively.

Human peripheral blood mononuclear cells (PBMCs). Human PBMCs were purchased from DS Pharma Biomedical (Osaka, Japan). In order to examine the expression of Siglec-9 by flow cytometry, cells were sequentially incubated with the anti-Siglec-9 antibody, followed by anti-goat IgG-FITC and anti-CD14-PE. $CD14^+$ cells, corresponding to blood monocytes, were gated and analyzed for the expression of

Siglec-9 by EPICS Altra (Beckman Coulter).

Culture and stimulation of RAW264 cells. The mouse macrophage cell line RAW264 was obtained from the Riken BioResource Center (Tsukuba, Japan), and maintained in RPMI1640 containing 10% heat-inactivated fetal bovine serum, 0.03% L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U/mL penicillin G, and 100 μg/mL streptomycin. Mock (stably transfected with an empty pcDNA4 vector (Invitrogen, Carlsbad, CA, USA)) and Siglec-expressing RAW264 cells (Siglec-9, Siglec-9YFYF, Siglec-9RA, and Siglec-5) have been described previously.[10,16] RAW264 cells (4×10^5) were stimulated in 500 μl of medium in 24-well plates as indicated. In order to inhibit PI-3K or Akt, cells were pretreated for 30 min with wortmannin (100 nM), or GSK690693 (1 μM), gently washed twice, then stimulated with IL-4 (20 ng/mL) for 8 h. The expression of arginase-1 (Arg1) was compared with that by the control (dimethyl sulfoxide (DMSO) only). Cells were similarly pretreated with the indicated concentrations of U0126 or PD0325901 for 30 min to inhibit MEK. To inhibit STAT6, AS1517499 (1 μM) was added 30 min before IL-4 stimulation, and cells were further cultured for 8 h in the presence of AS1517499.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA was extracted using RNAiso plus (Takara Bio, Otsu, Japan), reverse-transcribed by ReverTraAce (Toyobo, Osaka, Japan), and subjected to real-time PCR using SYBR Green I dye (Thunderbird qPCR Mix, Toyobo) for detection, as previously described.[10] Expression levels were normalized by calculating the ratio of the mRNA of Arg1 to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The primers used were Arg1, direct: 5′-TTGGCAAGGTGATGGAAGAGACCT-3′ and reverse: 5′-CAAGGTTAAAGCCACTGCCGTGTT-3′; GAPDH, direct: 5′-CTACCCCCAATGTGTCCGTC-3′ and reverse: 5′-GCTGTTGAAGTCGCAGGAGAC-3′.

Western blotting. The phosphorylation of STAT6, Akt, or ERK, and the expression levels of C/EBPβ were confirmed by Western blotting of whole cell lysates as previously described.[10] Briefly, cells were lysed with sodium dodecylsulfate (SDS) sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene fluoride membranes and probed with a specific antibody, followed by a peroxidase-labeled anti-IgG antibody. The membrane was developed with ECL plus (Perkin Elmer, Yokohama, Japan), and analyzed by

LAS-3000 mini (Fuji Film, Tokyo, Japan).

Measurement of arginase activity. The total cell lysate was harvested 24 h after the stimulation, and arginase activity was assessed by the production of urea, which was generated by the arginase-dependent hydrolysis of L-arginine as previously described.[17)]

Results

Confirmation of Siglec-9 expression by transfected RAW264 cells at a similar level to that of PBMCs

We used the mouse macrophage cell line RAW264 because of its low expression of endogenous Siglecs. Expression levels on the cell surfaces of Siglec-9-transfected RAW264 cells were compared with those of $CD14^+$ human PBMCs, corresponding to circulating monocytes. Human monocytes constitutively expressed high levels of Siglec-9 (Fig. 1A), as reported previously.[18,19)] Siglec-9-expressing RAW264 cells showed similar fluorescence intensity (Fig. 1B). The results of the RT-PCR analysis confirmed that similar levels of Siglec-9 were expressed between $CD14^+$ PBMCs and Siglec-9-expressing RAW264 cells after normalization by GAPDH (data not shown).

Siglec-9 enhanced IL-4-induced Arg1 expression in RAW264 cells

IL-4 is a potent activator of macrophages and induces the expression of several genes including those related to the tissue repair or the suppression of inflammation. RAW264 cells have been used to examine IL-4 responses.[20-24)] Arg1 is one of the typical IL-4-inducing genes that have been detected at high levels in mice. Thus, we determined whether Siglecs modulated the expression of Arg1. IL-4 stimulated the induction of Arg1 expression in mock cells, as expected (Fig. 2A, right). Arg1 expression was strongly induced when Siglec-9-expressing cells were stimulated with IL-4 (Fig. 2A, left). The magnitude of induction was approximately 10-fold in mock cells and 1000-fold in Siglec-9-expressing cells compared to cells not stimulated with IL-4. IFN-γ and LPS, either alone or in combination, did not induce the expression of Arg1 in mock or Siglec-9-expressing cells. A dose dependency curve revealed that 20 ng/mL IL-4 was sufficient to induce the maximum level of expression of Arg1 in mock and Siglec-9-expressing cells (Fig. 2B). The expression of Arg1 in

Siglec-9-expressing cells increased after approximately 8 h and then decreased at 24 h (Fig. 2C). In contrast, its expression in mock cells gradually increased until 24 h. Enhanced expression was confirmed by the enzyme activity of the cell lysate at 24 h (Fig. 2D).

RAW264 cells that expressed lower levels of Siglec-9 were established to examine the relationship between the expression level of Siglec-9 and enhancement of Arg1 expression (Fig. 2E). The expression level of Siglec-9 was one third to one fourth that of original Siglec-9-expressing cells used in other experiments. The expression of Arg1 was strongly enhanced by this level of Siglec-9 expression (Fig. 2F), but the magnitude of enhancement was about one fifth that of the original Siglec-9-expressing cells (data not shown), roughly matched with the expression level. Therefore, the expression levels of Siglec-9 appeared to determine the magnitude of the enhancement in IL-4-induced Arg1 expression levels, thereby supporting the notion that Siglec-9 enhanced IL-4-driven Arg1 expression in RAW264 cells.

The full enhancing activity of Siglec-9 required ITIMs

Two tyrosine-based motifs, ITIM and ITIM-like, are present in the cytoplasmic domain of Siglec-9. These motifs appeared to be indispensable for Siglec activity including the inhibition of TLR-induced proinflammatory cytokine production.[10] Cells expressing mutant Siglec were studied to determine whether these motifs and their downstream signals were necessary to enhance IL-4-induced Arg1 expression. In a mutant, in which two tyrosine residues in the ITIM and ITIM-like motifs were replaced with phenylalanine (YFYF), Arg1 induction levels by IL-4 were similar to those of mock cells (Fig. 3), indicating the critical importance of these motifs in the enhancement of IL-4-induced Arg1 expression by Siglec-9.

We also examined the contribution of the lectin activity of Siglec-9 using a mutant in which a critical arginine for binding to sialic acid-containing sugar in the Siglec-9 lectin domain was replaced with alanine (RA). The lectin mutant strongly enhanced Arg1 expression (Fig. 3). The magnitude of this enhancement was 60-70% that of non-mutated Siglec-9. These results suggested that lectin activity was not indispensable for enhancements in IL-4-induced Arg1 expression by Siglec-9, although this does not completely exclude the possibility that Siglec-9 slightly affected immune responses in a lectin-dependent manner.

Siglec-9 modulated several signaling pathways upon the IL-4 stimulation

On IL-4 stimulation, STAT6 was shown to be activated by tyrosine phosphorylation induced by Jak1 and Jak2, which is important for the expression of various IL-4-inducible genes.[25,26] We investigated whether Siglec-9 enhanced the phosphorylation of STAT6 after the IL-4 stimulation, however, the phosphorylation appeared to be slightly lower in Siglec-9-expressing cells (data not shown).

In addition to the STAT6 pathway, IL-4 is known to activate the PI-3K pathway in various cell types. PI-3K/Akt signaling was an enhancement factor for the IL-4-driven proliferation of macrophages.[27] Furthermore, mouse Siglec-E has been suggested to enhance the production of reactive oxygen species (ROS) via the activation of Akt in fibrinogen-stimulated neutrophils.[28] In order to investigate the effects of Siglec-9 on the activation of the PI-3K pathway, the phosphorylation of Akt was examined as an indicator of PI-3K activation (Fig. 4A, B). The background phosphorylation level without the stimulation was similar or slightly higher in Siglec-9-expressing cells. When stimulated with IL-4, Akt phosphorylation was not induced in mock cells, as reported previously for bone marrow-derived macrophages.[29] However, Siglec-9-expressing cells showed the significant enhancement of Akt phosphorylation. The phosphorylation of Akt was already enhanced 15 min after the stimulation and was gradually increased by 60 min. These results suggested that Siglec-9 enhanced IL-4-induced PI-3K activation.

So far, it is reported that the expression of Arg1 is regulated by C/EBPβ as well as STAT6 in the downstream of IL-4.[26,30] We examined the expression level of C/EBPβ by Western blotting, and found that the levels were similar or slightly lower in Siglec-9-expessing cells (data not shown).

The expression of Arg1 by macrophages may be induced by serum amyloid P[31] or macrophage-stimulating protein (MSP, the ligand for RON kinase),[32] which was inhibited by the MEK inhibitor, suggesting that the activation of MEK, and its downstream kinase ERK, induced the expression of Arg1. Therefore, we examined the activation of MEK/ERK pathway. The phosphorylation of ERK was higher in Siglec-9-expressing cells than in mock cells, even in the absence of the stimulation (Fig. 4C, D). The strength of phosphorylation was not enhanced by the IL-4 stimulation in both mock and Siglec-9-expressing cells. This result indicated that the MEK/ERK

pathway was constitutively activated in Siglec-9-expressing cells.

Enhanced Arg1 expression required STAT6 and MEK/ERK pathways

To examine the role of STAT6 in Arg1 expression of Siglec-9-expressing cells, cells were stimulated with IL-4 in the presence of STAT6 inhibitor AS1517499. AS1517499 (1 μM) strongly inhibited IL-4-induced STAT6 phosphorylation (data not shown) and Arg1 induction in mock cells (Fig. 5A). Similarly, the expression of Arg1 was strongly inhibited by AS1517499 in Siglec-9-expressing cells. These results suggested that IL-4-induced Arg1 expression required STAT6 in both mock and Siglec-9-expressing cells.

In order to examine the role of PI-3K in the enhancement of IL-4-induced Arg1 expression by Siglec-9, cells were stimulated in the presence of wortmannin, which is specific inhibitor of classical PI-3K. The enhanced expression of Arg1 was not inhibited by 100 nM wortmannin (Fig. 5B, left panel). In mock cells, the inhibition of PI-3K did not affect the expression of Arg1 either (Fig. 5B, right panel). These results were not attributed to the insufficient inhibition of PI-3K because wortmannin strongly inhibited IL-4-induced and PI-3K-dependent FIZZ1 expression in mock cells (data not shown), as was reported for bone marrow-derived macrophages.[29] Furthermore, the Akt inhibitor GSK690693 did not reduce the expression of Arg1 either in mock or Siglec-9-expressing cells (Fig. 5B). IL-4 has been shown to stimulate macrophages via two types of receptors. While a shared α chain associates Jak1 and Jak2 to stimulate STAT6, only the type I receptor containing the γc chain associates with Jak3 to phosphorylate insulin receptor substrate (IRS) and activate the PI-3K pathway. In order to examine the role of Jak3 in the enhanced expression of Arg1 by Siglec-9, cells were stimulated with IL-4 in the presence of 50 μM Janex-1, a specific inhibitor of Jak3. As shown in Fig. 5C, the expression of Arg1 induced by IL-4 was not reduced by Janex-1. This result suggested that the enhanced expression of Arg1 by Siglec-9 did not require Jak3 activity. Totally, these results showed that PI-3K/Akt pathway did not contribute to enhanced expression of Arg1 in Siglec-9-expressing cells.

Next, cells were stimulated in the presence of MEK inhibitors. As shown in Fig. 6A, U0126 inhibited the expression of Arg1 induced by IL-4 in Siglec-9-expressing RAW264 cells. This inhibition was dose-dependent and as strong as 60% at 10 μM. In mock cells, the expression of Arg1 was also inhibited by U0126. Another MEK

inhibitor, PD0325901 strongly inhibited the expression of Arg1 in Siglec-9-expressing cells at 1 μM (Fig. 6B). In mock cells, the expression of Arg1 was reduced to one third by PD0325901. These results suggested that activation of the MEK/ERK pathway was involved in the expression of Arg1 in Siglec-9-expressing RAW264.

Siglec-9 enhanced Arg1 expression induced by IL-13

IL-13 is a cytokine that shares various activities with IL-4. IL-13 binds type 2 IL-4 receptors (composed of IL-4Rα and IL-13Rα1) to stimulate STAT6, but not PI-3K. When mock RAW264 cells were stimulated with 20 ng/mL IL-13, Arg1 was induced (Fig. 7), as expected. Siglec-9 strongly enhanced the expression of Arg1. Arg1 levels induced by IL-13 in Siglec-9-expressing cells were similar to those induced by the stimulation with the same concentration of IL-4, indicating that the signaling pathways initiated by the IL-4R γc chain, possibly PI-3K pathway, were dispensable for this enhancement.

Siglec-5 enhanced IL-4-induced Arg1 expression

Macrophages express several CD33-related Siglecs including Siglec-5.[33] In order to determine whether other Siglecs affected Arg1 expression, we introduced Siglec-5 into RAW264 cells, and examined the expression of Arg1 in this cell line. Siglec-5 strongly enhanced the expression of Arg1; the dose-response curve reached its maximum at 20 ng/mL IL-4 (Fig. 8A), and the expression of Arg1 in Siglec-5-expressing cells rapidly increased at 8 h and then decreased at 24 h (Fig. 8B). This dose dependency and the results of time course analyses showed similar results for Siglec-9-expressing cells, suggesting that Siglec-5 had similar immunoregulatory activities to those of Siglec-9.

Discussion

Macrophages mediate multiple roles depending on the environment. IL-4 is one of the potent activators of macrophages that induces an “alternative” activation state.[8] Although several common genes have recently been reported,[34] IL-4 responsive genes are generally different between species. For example, Arg1 is specific to mouse

macrophages in the alternative activation state, but not to human counterparts. In contrast, upstream signaling pathways that are induced by IL-4 appear to be limited to STAT6 or PI-3K in humans and mice. In the present study, we used RAW264, a widely used cell line to examine the activation mechanisms of macrophages,[20-24] as a model to examine the upstream pathways affected by Siglec-9. The obtained results suggested that Siglecs modified multiple signaling pathways in IL-4-stimulated RAW264 cells. However, in Siglec-9-expressing cells, IL-4-induced STAT6 phosphorylation did not increase compared with mock cells. Enhancements in the PI-3K/Akt pathway, another typical pathway downstream of the IL-4 receptor, were observed in Siglec-9-expressing cells stimulated with IL-4 (Fig. 4A, B). Consistent with our results, fibrinogen-induced Akt phosphorylation was shown to be reduced in Siglec-E$^{-/-}$ neutrophils.[28] A previous study reported that the PI-3K/Akt pathway was activated by IL-4 in peritoneal macrophages,[35] but not in bone marrow-derived macrophages[29] or mock RAW264 cells (Fig. 4). This difference may partly depend on the expression levels of Siglecs in these cells: The amounts of endogenous Siglecs are very low in RAW264 cells and bone marrow macrophages, whereas peritoneal macrophages express modest levels of Siglec E ([9,10] and data not shown).

In the present study, we selected Arg1 as the IL-4 responsive gene: Arg1 participates in ornithine synthesis, a precursor of collagen and polyamines required for tissue repair[36] and the regulation of its expression has been extensively examined among IL-4-inducible genes. We analyzed its expression in Siglec-9-expressing cells in relation to the modulation of IL-4 signals. However, the expression of Arg1 could not be simply explained by changes in the IL-4 signaling pathway in Siglec-9-expressing cells. The IL-4 stimulation was required for the induction of Arg1 in Siglec-9-expressing cells. In fact, the STAT6 inhibitor abrogated the expression of Arg1 (Fig. 5A). This result suggested that the STAT6 signal was indispensable for the expression of Arg1. SHP-1 is known to bind the ITIM of IL-4R and suppress STAT6 phosphorylation.[12,13,37] It may be possible that sequestration of SHP-1 from IL-4R by Siglec-9 strengthened Arg1 expression in RAW264. However, phosphorylation levels of STAT6 did not increase in Siglec-9-expressing cells. As for enhanced Akt phosphorylation, experiments using inhibitors for PI-3K did not support PI-3K enhancements promoting the expression of Arg1 (Fig. 5B). Consistent with this, Janex-1, an inhibitor of Jak3 that associates with IL-4 receptor γc and activates PI-3K,

did not abrogate Arg1 enhancement in Siglec-9-expressing cells stimulated with IL-4 (Fig. 5C).

The phosphorylation of ERK was higher in Siglec-9-expressing cells even in the absence of the stimulation (Fig. 4C, D). The background expression of Arg1 appeared to be higher in Siglec-9-expressing cells than in mock cells in the absence of the stimulation (data not shown), although quantitative analyses were difficult due to the low expression levels of Arg1. Since MEK inhibitors reduced Arg1 expression levels (Fig. 6), MEK/ERK plays an important role in the Siglec-9-dependent marked induction of Arg1. Although SHP-1 is considered to be a general inhibitor, the activating role of SHP-1 has also been reported for ERK phosphorylation in EGF signaling.[38] The ITIMs of Siglecs also bound to Cbl or SOCS3,[3] which were not reported to bind IL-4R ITIM. Cbl inhibited ERK phosphorylation[39] while SOCS3 modulated ERK either in the negative or positive manner depending on the condition.[40,41] These suggested that ITIMs may involved in ERK activation. Further study is needed to clarify the underlying mechanism for ERK activation by Siglecs.

Taken together, the results of the present study suggested that Siglec-9 strengthened multiple signaling pathways including MEK/ERK and PI-3K/Akt in IL-4-stimulated macrophages. The consequences of changes in multiple pathways may be variable for each gene and depend on the cellular context. For instance, Siglec expression level is high in blood monocytes[18,19] but low in bone marrow-derived macrophages.[9] Further studies will reveal the significance of Siglecs in immune regulation.

Conflict of interest

The authors have declared that no conflict of interests exists.

Authors' contributions

SI and KN designed the experiments; HH, TS, and YM performed the experiments; HH, TS, SI, and KN analyzed the data; SI and KN wrote the manuscript.

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Figure legends

Fig. 1. Transfected RAW264 cells expressed Siglec-9 at a similar level to that of PBMCs.

(A) PBMCs were stained with an anti-Siglec-9 antibody followed by anti-goat IgG-FITC and anti-CD14-PE antibodies. The expression of Siglec-9 in $CD14^+$ cells was analyzed by flow cytometer. The percentage of the positive fraction is shown. Numbers in parentheses indicate the mean fluorescence of $CD14^+$ cells. (B) RAW264 cells exogenously expressing Siglec-9 were indirectly stained with an anti-Siglec-9 antibody. A mock (empty vector)-transfected clone (Mock, top) and Siglec-9-transfected clone (Siglec-9, bottom) were stained as in A. Left, control IgG; right, anti-Siglec-9.

Fig. 2. Siglec-9 enhanced IL-4-induced Arg1 expression in RAW264 cells.

(A) Arg1 expression was measured by qRT-PCR. Cells were stimulated with LPS (10 ng/mL), IFN-γ (10 ng/mL), IFN-γ plus LPS, or IL-4 (20 ng/mL) for 24 h. Right, magnified graph for mock cells. The Arg1 level of unstimulated mock cells was regarded as 1. * $p < 0.05$ versus mock by Student's t test, ** $p < 0.05$ versus no stimulation (mock cells). (B) IL-4 dose-response curve for Arg1 expression. Cells were stimulated for 24 h. (C) Time course of Arg1 expression. Cells were stimulated with 20 ng/mL IL-4. (D) Arginase activity of cell lysates. Cells were stimulated for 24 h. (E) Confirmation of Siglec-9 expression in the clone with lower expression. Cells were analyzed as described in Fig. 1. (F) Lower expression of Siglec-9 enhanced Arg1 expression at a reduced magnitude. RAW264 cells expressing low levels of Siglec-9 were stimulated with IL-4 (20 ng/mL) for 24 h.

Fig. 3. Full Arg1 enhancement required cytoplasmic ITIMs of Siglec-9.

Cells expressing Siglec-9 (WT), Siglec-9 mutated with both cytoplasmic tyrosines (YFYF), and Siglec-9 lectin mutant (RA) were stimulated with IL-4 for 8 h. * $p < 0.05$ versus mock. The Arg1 level of cells expressing wild-type Siglec-9 that were stimulated with IL-4 was regarded as 1.

Fig. 4. Siglec-9 modulated IL-4 signaling pathways.

(A) Siglec-9 enhanced IL-4-induced Akt phosphorylation. Cells were stimulated with IL-4 for the indicated periods. p-Akt, phosphorylated Akt. (B) Estimation of Akt phosphorylation levels. The graph indicates the relative band intensity of p-Akt after normalization to β-actin intensity. * $p < 0.05$ versus mock. (C) Siglec-9 enhanced ERK phosphorylation in the absence of the stimulation. Cells were stimulated with IL-4 for the indicated periods. p-ERK, phosphorylated ERK. (D) Estimation of ERK phosphorylation levels. The relative band intensity of p-ERK after normalization to β-actin is shown.

Fig. 5. IL-4-induced Arg1 expression was inhibited by the STAT6 inhibitor but not by PI-3K or Jak3 inhibitors in Siglec-9-expressing cells.

(A) Cells were stimulated with IL-4 (20 ng/mL) for 8 h with or without 1 μM AS1517499 and Arg1 expression was measured by qRT-PCR. Right, a magnified graph for mock cells. Control, DMSO 0.01%. The Arg1 level of Siglec-9-expressing cells that were stimulated with IL-4 in the presence of DMSO (control) was regarded as 1. * $p < 0.05$ versus control (DMSO) (B) Effect of inhibitors against PI-3K (wortmannin) and Akt (GSK690693) on the expression of IL-4-induced Arg1. Cells were stimulated with IL-4 (20 ng/mL) for 8 h. Right, a magnified graph for mock cells. Control, DMSO 0.1%. (C) Cells were stimulated with IL-4 (20 ng/mL) for 8 h with or without 50 μM Janex-1. Right, a magnified graph for mock cells. Control, DMSO 0.5%.

Fig. 6. IL-4-induced Arg1 expression was inhibited by the MEK inhibitors.

(A) The MEK inhibitor U0126 inhibited Arg1 expression in a dose-dependent manner. Cells were stimulated with IL-4 (20 ng/mL) for 8 h. Bottom, magnified graph for mock cells. * $p < 0.05$ versus control (DMSO 0.1%). (B) Inhibition of Arg1 expression by the MEK inhibitor PD0325901 (1 μM). Cells were stimulated as in A. Bottom, magnified graph for mock cells. * $p < 0.05$ versus control (DMSO 0.1%).

Fig. 7. IL-13-induced expression of Arg1 was enhanced by Siglec-9.

Siglec-9 enhanced IL-13-induced Arg1 expression. Cells were stimulated with either IL-4 or IL-13 (20 ng/mL) for 8 h.

Fig. 8. Siglec-5 enhanced IL-4-induced Arg1 expression.

Mock and Siglec-5-expressing cells were stimulated with the indicated doses of IL-4 for 24 h (A) or with 20 ng/mL IL-4 for the indicated period (B). * $p < 0.05$ versus mock, ** $p < 0.05$ versus no stimulation (mock cells).