Constitutively expressed Siglec-9 inhibits LPS-induced CCR7, but enhances IL-4-induced CD200R expression in human macrophages

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Running head: Siglec-9 modulates human macrophage responses

Abbreviations: FITC, fluorescence isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; ITIM, immunoreceptor tyrosine-based inhibitory motif; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor; TNF, tumor necrosis factor.

ABSTRACT

Siglecs recognize the sialic acid moiety and regulate various immune responses. In the present study, we compared the expression levels of Siglecs in human monocytes and macrophages using a quantitative real-time reverse transcription-polymerase chain reaction analysis. The differentiation of monocytes into macrophages by macrophage colony-stimulating factor (M-CSF) or granulocyte macrophage colony-stimulating factor (GM-CSF) enhanced the expression of Siglec-7 and Siglec-9. The differentiated macrophages were stimulated by lipopolysaccharide (LPS) plus interferon (IFN)-γ or interleukin (IL)-4. The expression of Siglec-10 was enhanced by IL-4, whereas that of Siglec-7 was reduced by LPS plus IFN-γ. The expression of Siglec-9 was not affected by these stimuli. The knockdown of Siglec-9 enhanced the expression of CCR7 induced by the LPS or the LPS plus IFN-γ stimulation, and decreased the IL-4-induced expression of CD200R. These results suggest that Siglec-9 is one of the main Siglecs in human blood monocytes/macrophages and modulates innate immunity.

Keywords: human; lectin; macrophages; sialic acid; Siglec

Introduction

Sialic acids cover the cell surface as the terminal residues of glycolipids or of *N*- and *O*-glycans in glycoproteins and play roles in the regulation of immune responses.[1)] Siglecs are sialic acid-recognizing immunoglobulin-like lectins primarily expressed in immune cells.[2-4)] CD33-related Siglecs are rapidly evolving genes, with five and 11 members being identified in mice and humans, respectively. They typically have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that down-regulate innate and acquired immune responses. On the other hand, several new members of Siglecs lack ITIMs, but have positively charged residues in the transmembrane domain that recruit DNAX-activating protein 12 kDa to stimulate immunoreceptor tyrosine-based activating motif-dependent cellular responses. These two types of Siglecs have been suggested to antagonize each other. The overlapping expression of these two types of Siglecs on immune cells indicates the complex consequences of Siglec expression.

We previously used model mouse cells (RAW264) expressing human Siglec-9, and demonstrated that Siglec-9 reduced proinflammatory cytokine expression including that of tumor necrosis factor (TNF)-α and interleukin (IL)-6, when RAW264 cells were stimulated with various Toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS) and peptidoglycan.[5)] In addition, Boyd *et al.* reported that the crosslinking of Siglec-E inhibited the production of TNF-α and IL-6 by LPS-stimulated mouse macrophages.[6)] TLRs are receptors for a range of chemicals produced by bacteria, viruses, fungi, and protozoa that initiate the first line of defense against pathogens.[7)] They are key molecules in innate immunity that induce inflammation; therefore, Siglecs are considered to exhibit potent modulatory activity for TLR-mediated inflammatory reactions.

Macrophages are involved in various aspects of immune responses such as the induction and resolution of inflammation. Reflecting this wide variety of responses, macrophages exhibit various phenotypes, including classically activated macrophages called M1, which induce inflammation, and alternatively activated macrophages called M2, which mediate anti-inflammatory responses or wound healing.[8-10)] M1 and M2 macrophages are experimentally induced by interferon (IFN)-γ plus TLR ligands, such as LPS, and type 2 cytokines, including IL-4/IL-13, respectively. The molecular

mechanism underlying polarization toward M1 or M2 is an active research area and appears to be regulated by various signal transduction pathways.

Human blood monocytes constitutively express several Siglecs,[11] while mouse macrophages, especially bone marrow-derived macrophages, generally express low levels of Siglecs under unstimulated conditions, and mouse Siglecs are induced by several TLR ligands[6] or viral infections.[12] In the present study, we examined changes in the expression of CD33-related Siglecs in human monocytes and macrophages under M1- or M2-favored stimulating conditions in order to approach the possible role of Siglecs in human monocytes and macrophages. Furthermore, the results of knockdown experiments suggest roles for Siglec-9 in the control of genes related to inflammation in human macrophages.

Materials and methods

Reagents and antibodies. LPS from *Escherichia coli* 0111:B4 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Macrophage colony-stimulating factor (M-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4, and IFN-γ were purchased from Pepro Tech (Rocky Hill, NJ, USA). An anti-human CD14 antibody labeled with phycoerythrin (PE) was from Beckman Coulter (Brea, CA, USA). A goat anti-human Siglec-9 antibody was purchased from R&D Systems (Minneapolis, MN, USA). Fluorescence isothiocyanate (FITC)-labeled donkey anti-goat IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-human CD16 and control mouse IgG1 antibodies labeled with PE/Cy5 were purchased from Biolegend (San Diego, CA, USA).

Cells and cultures. Peripheral blood mononuclear cells (PBMCs) were prepared from the blood of healthy donors (Japanese) using Histopaque-1077 (Sigma-Aldrich). $CD14^+$ cells were separated by MACS (Miltenyi Biotec, Auburn, CA, USA) as recommended by the supplier. Briefly, PBMCs were incubated with the anti-CD14 antibody followed by anti-mouse IgG microbeads, and were then separated on a MACS column. In some experiments, PBMCs were seeded on tissue culture plates and non-adherent cells were discarded by extensive washing after a 24-h incubation.

The cells differentiated into macrophages by either M-CSF or GM-CSF. Cells were cultured for 6 days 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 in the presence of M-SCF (50 ng/mL) or GM-CSF (25 ng/mL). Adherent cells were stimulated in 500 μl of medium in 24-well plates. To induce M1 and M2 macrophages, cells were stimulated with LPS (100 ng/mL) plus IFN-γ (20 ng/mL) and IL-4 (20 ng/mL) for 24 h, respectively, as reported previously.[13] In some experiments, cells were stimulated by LPS alone. All experiments were performed with approval from the local Ethical Committee of Nagoya University.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was extracted using Isogen II (Nippon Gene, Tokyo, Japan), and subjected to qRT-PCR using SYBR Green I dye (Thunderbird qPCR Mix, Toyobo, Osaka, Japan) for detection, as previously described.[5] Expression levels were normalized by calculating the ratio of the mRNA of interest to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The expression levels of Siglecs were analyzed with the ΔΔCt method of qRT-PCR. The primers used were Siglec-5, direct: 5'-taccatcacctcgggttccag-3' and reverse: 5'-ggtccttaggctccctcgact-3'; Siglec-7, direct: 5'-ggcctgtatcaggagtgttgct-3' and reverse: 5'-gcagccaggccatggtg-3'; Siglec8, direct: 5'-agatccacaagcgagaaactg-3' and reverse: 5'-ggtgacctactgcatagcatg-3'; Siglec9, direct: 5'-gggtgctggagctgcctt-3' and reverse: 5'-gtcactcctgatgtggctttgc-3'; Siglec10, direct: 5'-atcaatgtggtcccgacg-3' and reverse: 5'-ggaaactgggcaactgatactg-3'; Siglec11, direct: 5'-ctcggagatcaagatccacac-3' and reverse: 5'-acttgctggtgtcctgttg-3'; Siglec14, direct: 5'-gccatcagcatcttcttcagaaatg-3' and reverse: 5'-gcttttccctcccggaacca-3'; Siglec16, direct: 5'-tcaaatggagcacgaaggag-3' and reverse: 5'-gatcttcatagccacctccc-3'; CD33, direct: 5'-tgtgcatgtgacagacttgaccc-3' and reverse: 5'-ttatgagcaccgaggagtgagtagtc-3'; CCR7, direct: 5'-ttcagtggcatgctcctacttc-3' and reverse: 5'-gctgagacagcctggacgat-3'; CD200R, direct: 5'-gagcaatggcacagtgactgtt-3' and reverse: 5'-gtggcaggtcacggtagaca-3'; mannose receptor C type 1 (Mrc1), direct: 5'-ggacgtggctgtggataaat-3' and reverse: 5'-acccagaagacgcatgtaaag-3'; GAPDH, direct: 5'-tccctccaaaatcaagtgggg-3' and reverse: 5'-gtccttccacgataccaaagttgtc-3'.

Knockdown of Siglec-9 expression. In order to knockdown the expression of

Siglec-9, Stealth RNAi oligonucleotide duplexes (Invitrogen, Carlsbad, CA, USA) were synthesized with the following sequences: sense: 5'-GGCACAGUAUCCACAGUCUUGGGAA-3', antisense: 5'-UUCCCAAGACUGUGGAUACUGUGCC-3'. Cells (1×10^5) were transfected with specific or control (Stealth RNAi negative control with medium GC; Invitrogen) siRNAs using INTERFERin (Polyplus Transfection, Illkirch, France) according to the supplier's recommendations. After a 48-h culture with siRNAs, cells were gently washed with phosphate-buffered saline and stimulated with LPS, IFN-γ and IL-4 for 24 h.

Flow cytometric analysis of Siglec-9 expression. PBMCs were incubated with 10 μg/mL BD Fc Block (BD Biosciences, San Diego, CA, USA) for 10 min at room temperature to prevent non-specific binding via Fc receptor, then, with either anti-Siglec-9 or control goat IgG antibodies (5 μg/mL) for 20 min on ice, followed by the incubation with FITC-anti-goat IgG, PE-anti-CD14 and PE/Cy5-anti-CD16 antibodies (5 μg/mL) for 20 min. The cells were analyzed with EPICS ALTRA cell sorter (Beckman-Coulter).

Statistical analysis. Data are shown as the mean and standard error of at least three independent experiments. The significance of differences was analyzed by Student's *t* test or one-way ANOVA followed by Tukey's post hoc test.

Results

Human CD14$^+$ monocytes express several Siglecs

Monocytes are known to express several CD33-related Siglecs. The findings of a previous flow cytometric analysis suggested that some Siglecs are expressed at high levels,[11)] but differences in the affinity of each antibody prevented direct comparisons of these levels. Therefore, we examined the expression of Siglecs by qRT-PCR. The CD14$^+$ fraction was collected using the anti-CD14 antibody and anti-IgG magnetic beads. Most cells were stained with the anti-CD14 antibody (LPS coreceptor, Fig. 1A). qRT-PCR revealed that monocytes expressed several ITIM-containing Siglecs such as

Siglec-5, Siglec-7, Siglec-9, and CD33 at similar levels (Fig. 1B), as reported previously using a flow cytometer.[11] Siglec-10 expression levels were approximately 50% those of these Siglecs. On the other hand, the expression levels of Siglec-8 and Siglec-11 were very low.

In addition to classical CD33-related Siglecs that contain ITIMs in the cytosolic domain, other types of Siglecs that have positive charges in the transmembrane domain have been identified.[2-4] Therefore, the "activating types" of Siglecs, namely, Siglec-14 and Siglec-16, were examined. The expression level of Siglec-16 was low, approximately 5% that of Siglec-9. Siglec-14 was not detected in most experiments. A large proportion of Asian species lacked both alleles of Siglec-14,[14] indicating that most of our blood donors lacked Siglec-14. These results suggest that blood monocytes constitutively express several Siglecs, mainly those containing ITIMs.

Expression of Siglecs in human macrophages

M-CSF and GM-CSF induced different characteristics in macrophages. Based on the differences observed in the expression of several cytokines such as IL-12 and IL-10, M-CSF-differentiated- and GM-CSF-differentiated macrophages are sometimes referred to as M2 and M1, respectively. However, a detailed analysis revealed that these two types of macrophages are not similar to M1 or M2 polarized by IFN-γ plus LPS and IL-4.[15] Thus, they are hereafter referred to as M-CSF-differentiated- and GM-CSF-differentiated macrophages.[8] Isolated human monocytes were cultured in the presence of M-CSF or GM-CSF in order to facilitate differentiation into macrophages. After a 6-day culture, adherent cells showed different morphologies depending on the cytokine used to induce differentiation (Fig. 2A). Cells treated with M-CSF showed irregular shapes including spindle shapes, while those by GM-CSF showed round "fried-egg" shapes, as reported previously.[16-18]

mRNAs for Siglecs were quantified by qRT-PCR. The expression of Siglec-7 was enhanced by M-CSF and GM-CSF (approximately 3 fold, Fig. 3A and 3B). The expression of Siglec-9 was modestly increased after the treatment with M-CSF and GM-CSF (approximately 2 fold, Fig. 3A and 3C). On the other hand, the expression of Siglec-5, Siglec-10, and CD33 generally decreased during differentiation (Fig. 3A). These results suggest that it is possible to divide Siglecs into three groups based on the

expression levels of monocytes and macrophages. One group is expressed in monocytes and enhanced by differentiation to macrophages, such as Siglec-7 and Siglec-9, another is expressed in monocytes and reduced by differentiation, such as Siglec-5, Siglec-10, and CD33, and the last includes those not expressed in these cells, such as Siglec-8, Siglec-11, Siglec-14, and Siglec-16.

Siglec repertoire is changed by stimuli with IFN-γ plus LPS or IL-4

Macrophages are heterologous and exhibit various phenotypes depending on the types of stimuli. LPS plus IFN-γ induces inflammatory M1 macrophages. We confirmed the expression of CCR7, a known marker of M1 macrophages,[8)] by LPS plus IFN-γ with M-CSF- and GM-CSF-induced macrophage preparations, and M-CSF induced higher expression levels than GM-CSF (Fig. 2B). IL-4 is another type of macrophage activator and induces anti-inflammatory M2 macrophages. The well-known M2-macrophage markers, mannose receptor Mrc-1 (Fig. 2C) and CD200R (Fig. 2D) were also induced by IL-4 as reported previously.[8)]

The M1- and M2-favored stimulation of differentiated PBMCs did not affect the expression of Siglec-9, whereas differentiation by M-CSF and GM-CSF increased expression levels (Fig. 3A and 3C). The expression of Siglec-7 was decreased by LPS plus IFN-γ either with M-CSF- or GM-CSF-differentiated macrophages (Fig. 3A and 3B). These results suggest that Siglec-7 expression was suppressed due to signaling by LPS and IFN-γ such as NF-κB, AP-1, and signal transducer and activator of transcription (STAT)1.[7,19)] In contrast, the stimulation with IL-4 strongly enhanced Siglec-10 expression in M-CSF- and GM-CSF-differentiated macrophages (Fig. 3A and 3D). The magnitude of the enhancement was 4.5 fold (M-CSF) and 8 fold (GM-CSF) on average, suggesting that an IL-4 signal such as STAT6 or PI-3K[20)] participated in the induction of Siglec-10. Expression levels increased or decreased slightly with CD33 and Siglec-5 (within two-fold) depending on the combination of CSFs and stimuli for M1 and M2 (Fig. 3A). The expression of Siglec-8, Siglec-11, Siglec-14, or Sigec-16 was not induced under these stimulating conditions. These results suggest that each Siglec is regulated in a different manner.

Knockdown of Siglec-9 in human macrophages enhances LPS-induced CCR7 expression

Siglec-9 is proposed to be a functional orthologue of mouse Siglec-E that suppressed monocyte inflammation,[6,21] and complemented some phenotypes of the Siglec-E knockout mouse.[22] In order to examine the role of Siglec-9 in human primary macrophages, Siglec-9 was knocked down by the transfection of siRNA after the M-CSF-induced differentiation. When cells transfected with the control siRNA were stimulated by LPS with or without IFN-γ, CCR7 was strongly induced by LPS alone (21 fold) and by LPS plus IFN-γ (150 fold) at 24 h (Fig. 4A). We then examined the effects of the knockdown of Siglec-9. Specific siRNA modestly reduced Siglec-9 levels to approximately 30-50% (Fig. 4B). Transfection of the siRNA of Siglec-9 enhanced the expression of CCR7 in LPS alone and the LPS plus IFN-γ stimulation (Fig. 4C). LPS-induced CCR7 expression was 3.7 fold higher in cells transfected with the siRNA of Siglec-9 than in those with control siRNA. In the presence of LPS plus IFN-γ, the expression of CCR7 was enhanced 2 fold by the knockdown of Siglec-9. These results suggest that Siglec-9 inhibits LPS-induced CCR7 expression.

Knockdown of Siglec-9 in human macrophages reduces IL-4-induced CD200R expression

We then investigated the role of Siglec-9 in the IL-4 response of human macrophages. After the IL-4 stimulation for 24 h, control siRNA-treated cells strongly expressed CD200R (Fig. 5). The knockdown of Siglec-9 reduced the expression of CD200R, of which level was reduced to approximately 60% that of control. These results suggest that Siglec-9 enhances IL-4-induced CD200R expression in human macrophages.

Siglec-9 is expressed on several different monocyte populations in human blood

Blood monocytes were classified based on the expression of CD14 and CD16.[23] We analyzed the expression levels of Siglec-9 in monocyte subpopulations (Fig. 6). Classical monocytes ($CD14^{hi}CD16^{-}$), a major monocyte population in blood corresponding to 70-80% of all monocytes, were intensely stained with the anti-Siglec-9 antibody, which is consistent with the expression pattern of all monocytes.[11] $CD14^{lo}CD16^{hi}$ non-classical monocytes, which have been shown to adhere to and crawl on the endothelium to patrol it,[24] and $CD14^{hi}CD16^{lo}$ intermediate monocytes also expressed similar levels of Siglec-9. These results suggest that Siglec-9 is expressed in

different subsets of blood monocytes.

Discussion

In the present study, we examined the expression levels of CD33-related Siglecs in human monocytes and macrophages. The results of qRT-PCR suggest that Siglec-7 and Siglec-9 are induced by M-CSF and GM-CSF in human macrophages. Although post-transcriptional regulation of Siglecs is not known except for the proteasomal degradation of CD33 and Siglec-7 after endocytosis due to the crosslinking by specific antibodies,[25,26] we can not exclude the possibility that our mRNA analysis did not exactly reflect the protein levels. However, the present results are consistent with previous findings by Lock et al. showing that monocytes and macrophages express high levels of Siglec-7 and Siglec-9, but not Siglec-8 or Siglec-11 using anti-Siglec antibodies.[11]

To date, various polymorphic changes have been detected in the human Siglec locus in chromosomes.[27] One of the biggest polymorphic differences identified in Siglecs is that some human populations lack Siglec-14. It was found that macrophages expressed Siglec-14, but not Siglec-5 in a Siglec-14 non-deleted population.[14] The deletion of the Siglec-14 locus resulted in the expression of Siglec-5 (sometimes referred to as Siglec-5/14 based on locus fusion) in macrophages under the control of the Siglec-14 promoter. Therefore, our results of the expression of Siglec-5, but non-detectable expression of Siglec-14 were reasonable.

The expression level of Siglecs was previously shown to be low in mouse monocytes and macrophages, while that of Siglec-E was induced by several TLR ligands including LPS.[6] Therefore, we examined the relationship between M1 or M2 polarization and Siglec expression. Siglec-10 was strongly enhanced by IL-4 in M-CSF- and GM-CSF-differentiated macrophages. Siglec-10 participated in enhancing the production of IL-10 when it bound to the flagellin of *Campylobacter jejuni* in a sialic acid-independent manner.[28] Therefore, IL-4 might enhance the production of IL-10 under certain conditions through Siglec-10. We also found that the expression of Siglec-7 was reduced by the LPS plus IFN-γ stimulation. By now, functions of Siglec-7 in macrophages have not been clear.

On the other hand, the expression of Siglec-9 was relatively unchanged under M1- and M2-favored conditions. We previously demonstrated that the expression of Siglec-9 in RAW264 cells modestly reduced LPS-induced TNF-α production (M1 marker in humans and mice)[5] and enhanced IL-4-induced Arg1 expression (M2 marker specific to mice).[29] However, the partial knockdown of Siglec-9 in human macrophages did not change the amount of TNF-α mRNA that was induced by LPS with or without IFN-γ, and the mRNA level of Mrc1 (M2 marker for both humans and mice) that was induced by IL-4 (data not shown). Recently, the numbers of M1- and M2-specific genes were identified,[8-10] but differed between mice and humans. Among the several genes tested, the LPS-induced expression of CCR7 mRNA was enhanced by the knockdown of Siglec-9. CCR7 is a chemokine receptor that mediates the localization of neutrophils, naive T cells, and dendritic cells to lymph nodes.[30,31] Our results also indicate that Siglec-9 enhances IL-4-induced CD200R mRNA expression in human macrophages. CD200R is a cell surface protein that is mainly expressed on myeloid lineages. Using knockout mice, the binding of CD200R to its ligand CD200, both of which are mainly expressed on macrophages, was found to prevent autoimmune diseases such as experimental autoimmune encephalomyelitis, uveoretinitis, and collagen-induced arthritis.[32,33] Since the results of flow cytometric analysis were correlated well with the mRNA amounts for CCR7 and CD200R,[34,35] protein levels may be also changed by Siglec-9 knockdown. Further studies are needed in order to establish the physiological functions of Siglec-9 in relation to the changes in the expression of CCR7 and CD200R. Macrophages express several ITIM-containing Siglecs, which supports the view that Siglecs function in redundant manners. However, our results that each Siglec was expressed under specific cellular environments suggest that they may also have unique role(s). This possibility is remained to be elucidated.

Authors' contributions

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

Conflict of interest

The authors have declared that no conflicts of interest exist.

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Figure legends

Fig. 1. Expression of Siglecs on human $CD14^+$ monocytes circulating in blood.
(A) Cells were purified by MACS and purity levels were examined by the expression of CD14. FS, forward scatter. (B) A qRT-PCR analysis revealed that several Siglecs were expressed in monocytes.

Fig. 2. Confirmation of differentiated macrophages.
(A) Monocytes were cultured in either M-CSF or GM-CSF for 6 days. Photographs after the removal of non-adherent cells are shown. (B-D) Confirmation of differentiation to M1 or M2 macrophages. Macrophages that had been cultured with M-CSF or GM-CSF were stimulated for 24 h by LPS plus IFN-γ and IL-4, which induced typical M1 and M2 responses, respectively. Confirmation of marker genes for the M1 specific-gene CCR7 (B) or M2-specific genes Mrc1 (C) and CD200R (D). Cells were analyzed by qRT-PCR. *, $p < 0.05$ versus none.

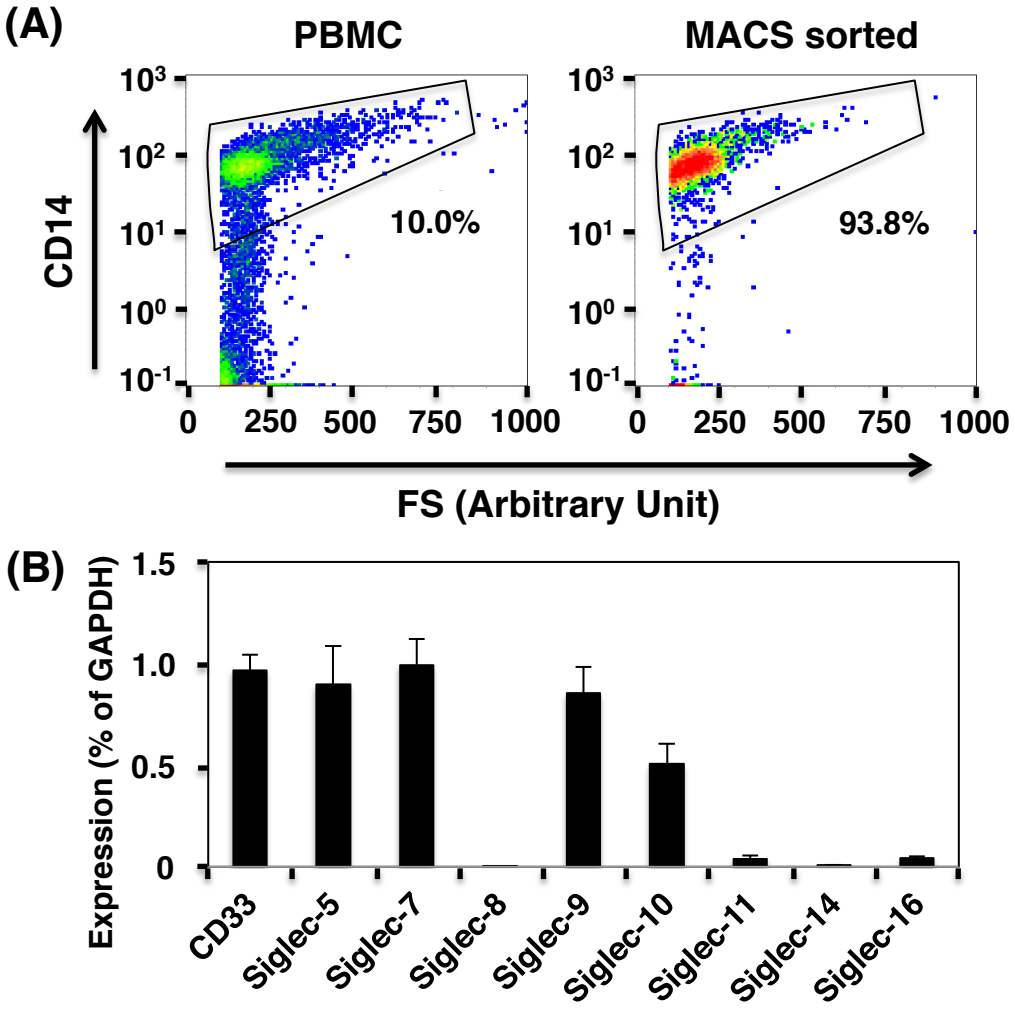
Fig. 3. Siglec expression in differentiated macrophages.
(A) Macrophages were stimulated for 24 h by LPS plus IFN-γ and IL-4, and subjected to a qRT-PCR analysis. Results for freshly isolated $CD14^+$ monocytes (Fig. 1B) were also included for comparison. (B-D) Changes in the expression of Siglec-7, Siglec-9, and Siglec-10 are shown separately. *, $p < 0.05$.

Fig. 4. Knockdown of Siglec-9 enhances LPS-induced CCR7 expression.
(A) CCR7 was induced by LPS with or without IFN-γ. CCR7 levels without stimulation (none) were regarded as 1. Macrophages were stimulated for 24 h. (B) Confirmation of knockdown. Macrophages were transfected by siRNA for Siglec-9 or control. The expression level of Siglec-9 was examined by qRT-PCR. Siglec-9 levels transfected with control siRNA were regarded as 1. (C) Effects of the Siglec-9 knockdown on the expression of CCR7. CCR7 levels with control siRNA were regarded as 1. *, $p < 0.05$.

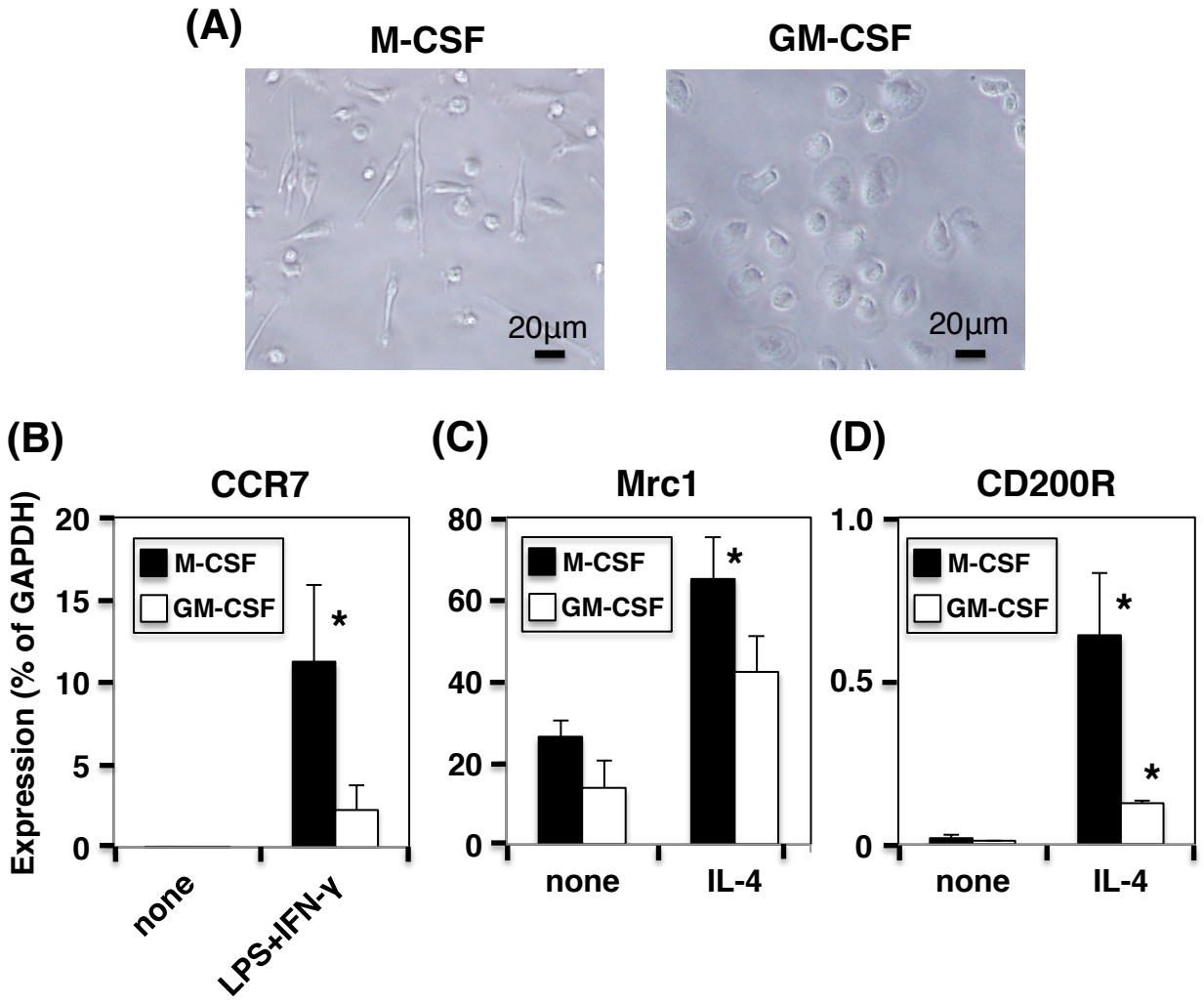
Fig. 5. Knockdown of Siglec-9 reduces IL-4-induced CD200R expression.
Siglec-9 was knocked down, similarly to Fig. 4. Cells were stimulated by IL-4 for 24 h. (A) The knockdown of Siglec-9 enhanced the expression of CD200R. *, $p < 0.05$. (B)

Confirmation of Siglec-9 knockdown.

Fig. 6. Several different monocyte populations in human blood express Siglec-9. (Top) PBMCs were stained with CD14 and CD16 and analyzed using a flow cytometer. Three different monocyte populations ($CD14^{hi}CD16^{-}$ classical (A), $CD14^{lo}CD16^{hi}$ non-classical (B), and $CD14^{hi}CD16^{lo}$ intermediate (C)) were discriminated. (Bottom) Three monocyte populations expressed Siglec-9. PBMCs were stained simultaneously by anti-CD14, CD16, and Siglec-9 antibodies and regions gated by the expression of CD14 and CD16 were separately analyzed for the expression of Siglec-9. Mean fluorescence intensity levels are shown in parenthesis.



Higuchi et al. Fig.1.



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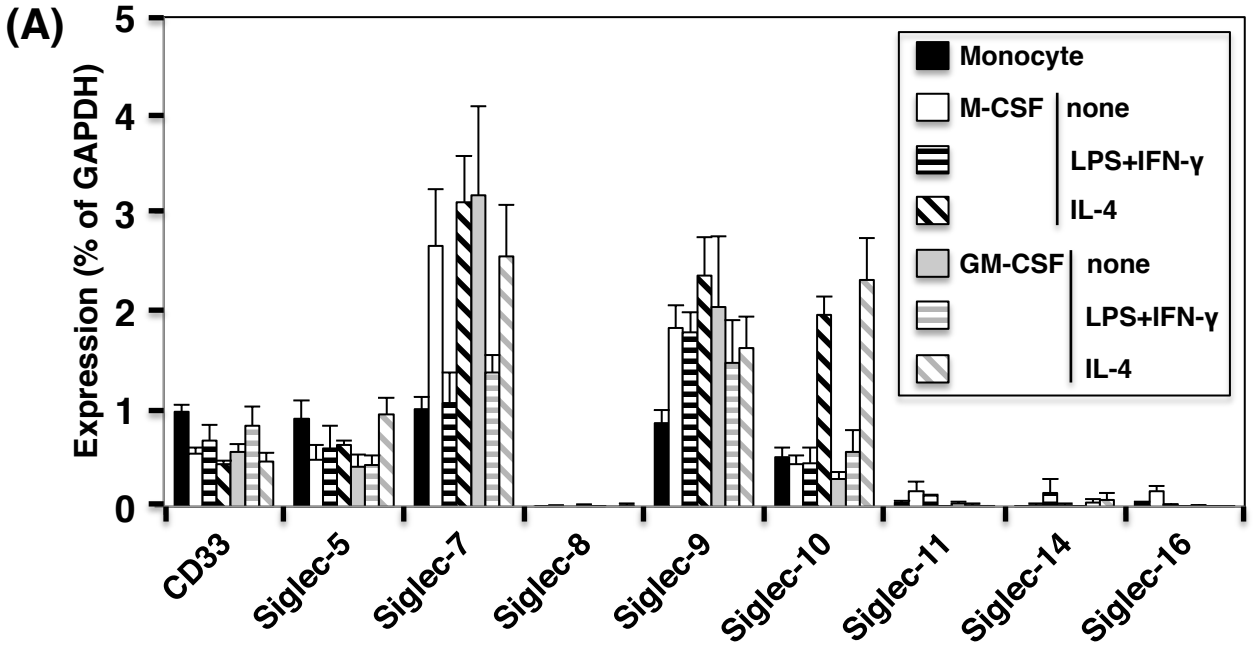

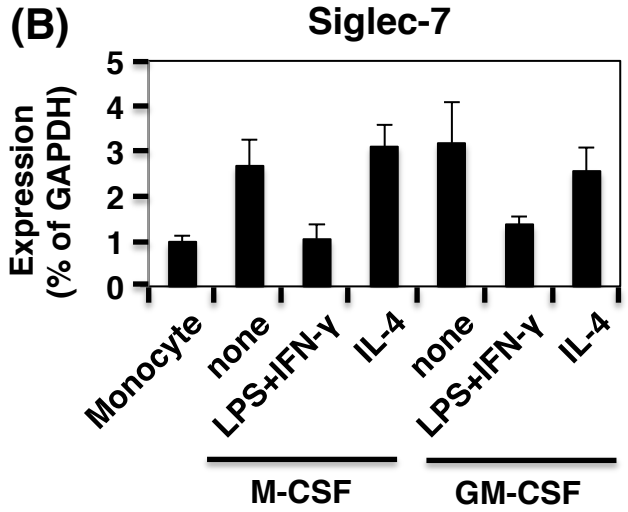

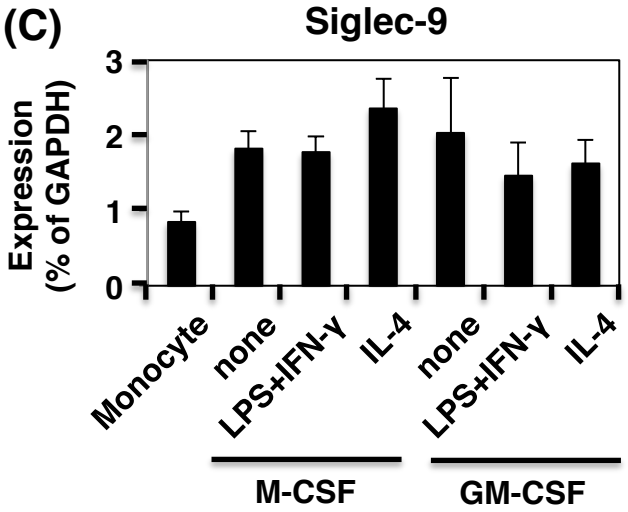

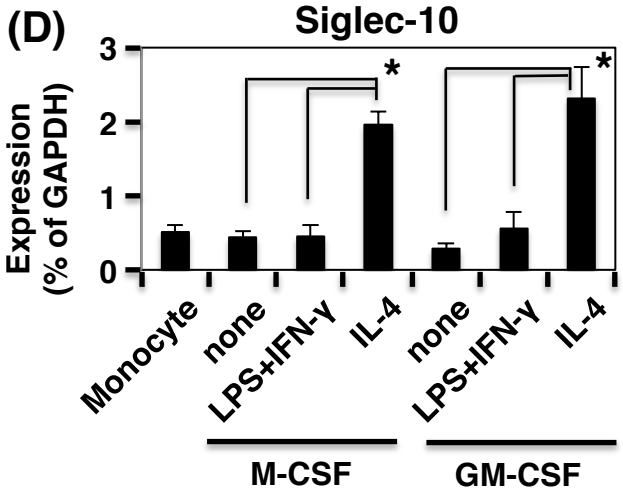

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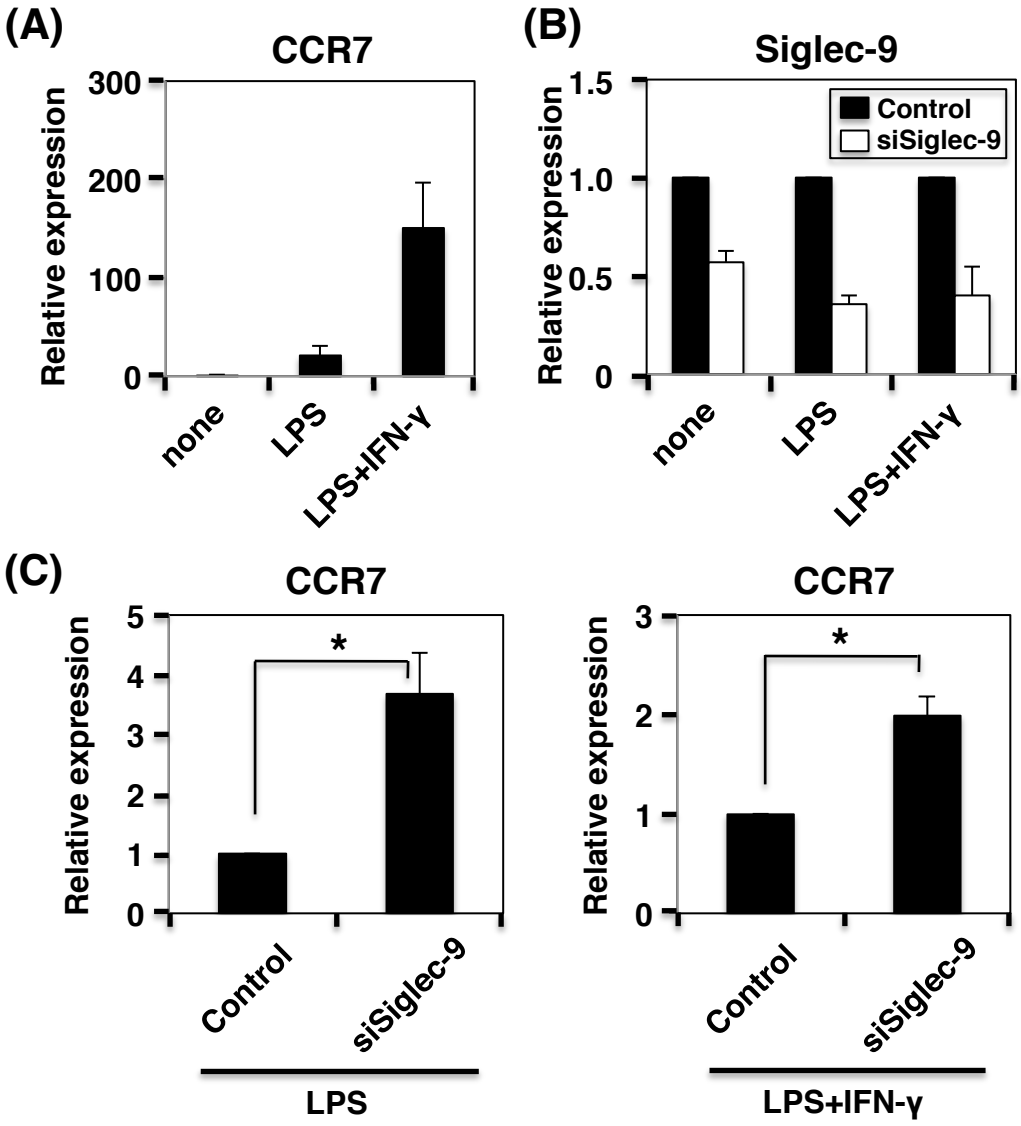

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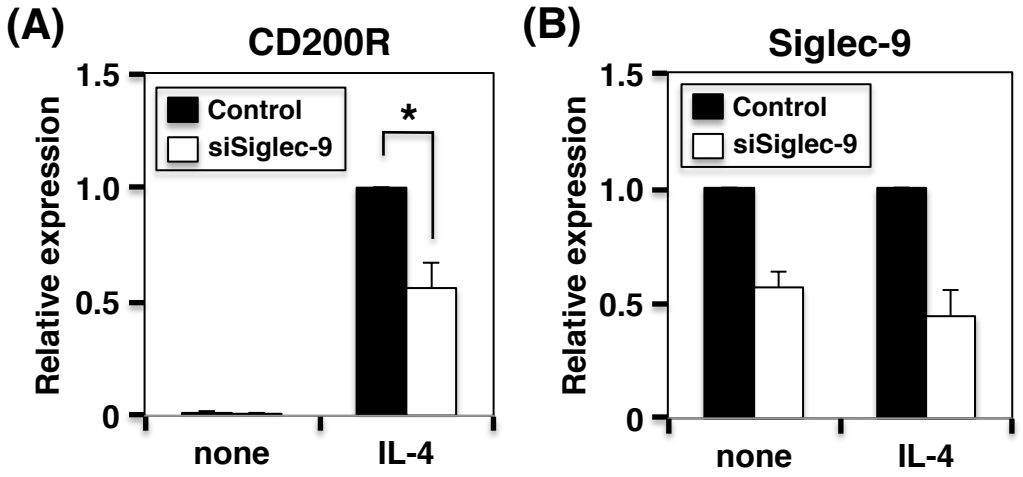

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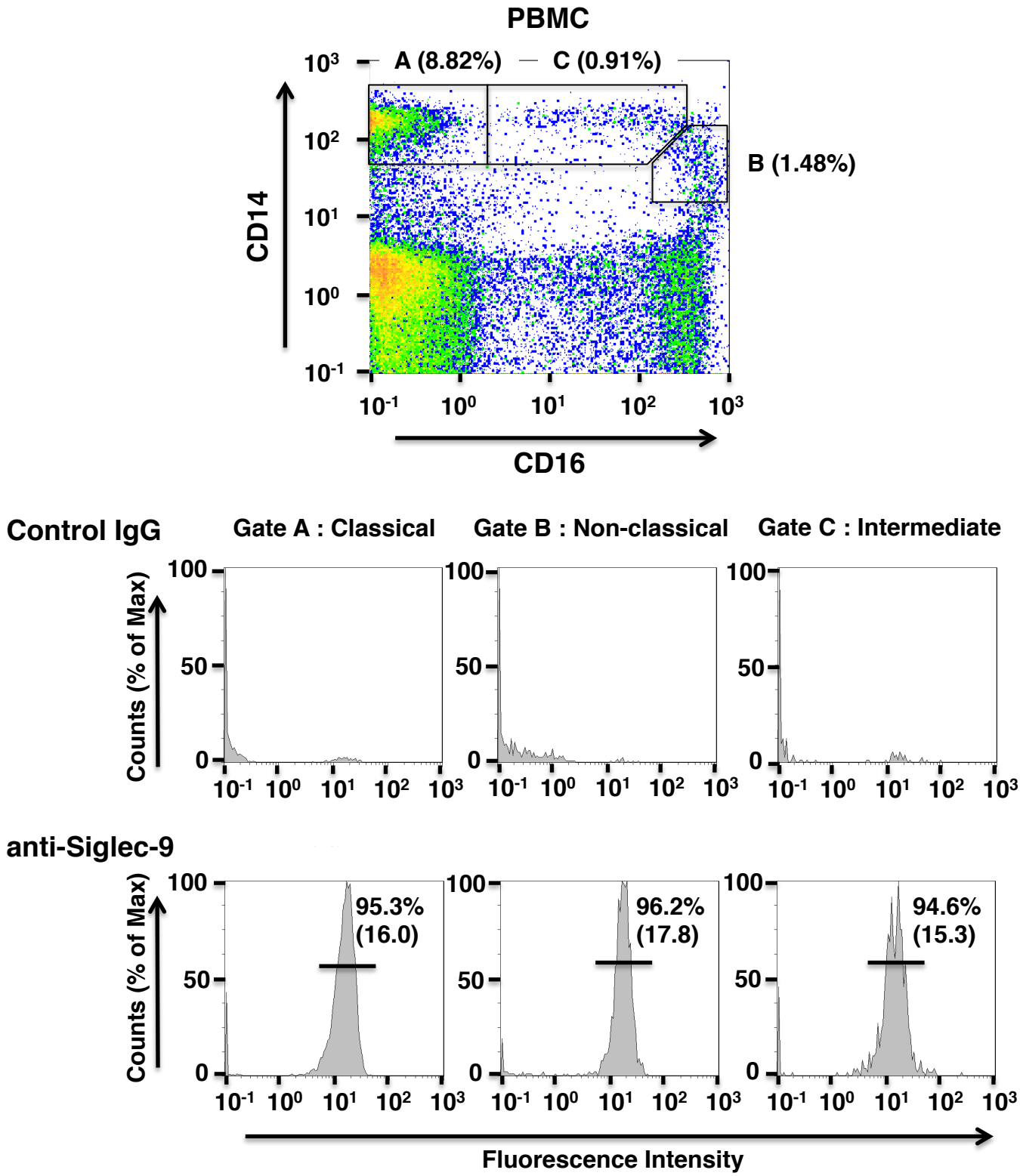

Higuchi et al. Fig.6.