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Deletion polymorphism of *SIGLEC14* and its functional implications

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Human Siglec-14, a member of the Siglec family of sialic acid-binding lectins, shows extensive sequence similarity to human Siglec-5. To analyze respective expression patterns of Siglec-14 and Siglec-5, we developed specific antibodies against each of them. We found that the former was expressed on granulocytes and monocytes, while the latter was on granulocytes and B-cells. Surprisingly, some individuals lacked the expression of Siglec-14, while they all expressed Siglec-5. We found that a fusion between *SIGLEC14* and *SIGLEC5* genes, resulting in the functional deletion of *SIGLEC14*, underlies this phenotype. The presence of the "*SIGLEC14* null" allele in all human populations we tested implies an ancient origin, while its allelic frequency is higher in Asians compared with Africans and Europeans. The forced expression of Siglec-14 in a monocytic cell line-enhanced TNF-α secretion elicited by lipopolysaccharide. These results imply that Siglec-14 may play some role in bacterial infection.

Keywords: DAP12/leukocytes/polymorphism/Siglec/TNF-α

Introduction

Siglecs are a family of mammalian lectins that recognize sialic acids (Varki and Angata 2006; Crocker et al. 2007). We recently discovered Siglec-14, a Siglec that shows extensive sequence similarity to Siglec-5 in the amino-terminal part (Angata et al. 2006). Siglec-14 has an arginine residue in the transmembrane domain, by which it associates with a signaling adapter molecule DNAX activating protein 12 (DAP12) (Lanier and Bakker 2000), and hence is expected to function as an activating molecule. This property contrasts with that of Siglec-5, which associates with protein tyrosine phosphatases and is considered to be an inhibitory molecule (Cornish et al. 1998; Avril et al. 2005). Based on these facts we proposed that Siglec-14 and Siglec-5 might counteract each other to fine-tune the balance between cell activation and dampening. For these two proteins to counteract each other, they would need to be expressed on the same cells. Thus, ascertaining their respective expression patterns is essential in order for us to gain insight into their individual and collective functions. However, the fact that most of the available anti-Siglec-5 antibodies recognized both Siglec-14 and Siglec-5 (Angata et al. 2006) makes it difficult to analyze their respective expression patterns.

To address this problem, we developed specific probes to recognize Siglec-14 and Siglec-5 and analyzed their expression patterns. We found that Siglec-14 was expressed on granulocytes and monocytes, while Siglec-5 was on granulocytes and B-cells. To our surprise, we found that some individuals lack the expression of Siglec-14 altogether, while the expression of Siglec-5 was intact. We discovered that a fusion between *SIGLEC14* and *SIGLEC5* genes, a common allelic variation of the *SIGLEC14–SIGLEC5* locus, underlies this phenotype.

We also demonstrate that the expression of Siglec-14 on a monocytic cell line enhances lipopolysaccharide-elicited tumor necrosis factor α (TNF-α) secretion and that this effect is dependent on the interaction of Siglec-14 with DAP12. Possible implications for the presence of this allele and its distribution in the human population will be discussed.

Results

Expression patterns of Siglec-14 and Siglec-5

We have previously reported that most of the available anti-Siglec-5 antibodies recognize both Siglec-14 and Siglec-5 (Angata et al. 2006). We therefore developed specific probes to recognize Siglec-14 (mouse monoclonal antibody) and Siglec-5 (goat polyclonal antibody), and used them to analyze the expression patterns of the two Siglecs on human peripheral blood leukocytes (Figure 1). We found that both Siglec-14 and Siglec-5 are expressed on granulocytes. In addition, Siglec-14 was expressed on monocytes, and Siglec-5 was expressed on B-cells.

Some individuals lack the expression of Siglec-14 because of a common genetic polymorphism

In our attempts to test the reproducibility of the above findings, we serendipitously found that some individuals lack the expression of Siglec-14, while the expression of Siglec-5 is intact (Figure 2A). Because all leukocytes were devoid of Siglec-14 in these individuals (not shown), we hypothesized that these individuals genetically lack the gene encoding for Siglec-14. As we have previously reported (Angata et al. 2006), *SIGLEC14* and *SIGLEC5* are located nearby in tandem, and the DNA sequence of the ∼1.5 kb segment of the *SIGLEC14* gene, consisting of the 5′untranslated region (∼0.3 kb) and the 5′-end of the coding region (∼1.2 kb), is >99% identical to that of the corresponding region of the *SIGLEC5* gene. These facts led us to hypothesize that unequal cross-over at the highly homologous regions of *SIGLEC14* and *SIGLEC5* resulted in generation of a *SIGLEC14/5* fusion gene, which results in the elimination of the genetic segment unique to *SIGLEC14* (and thus the

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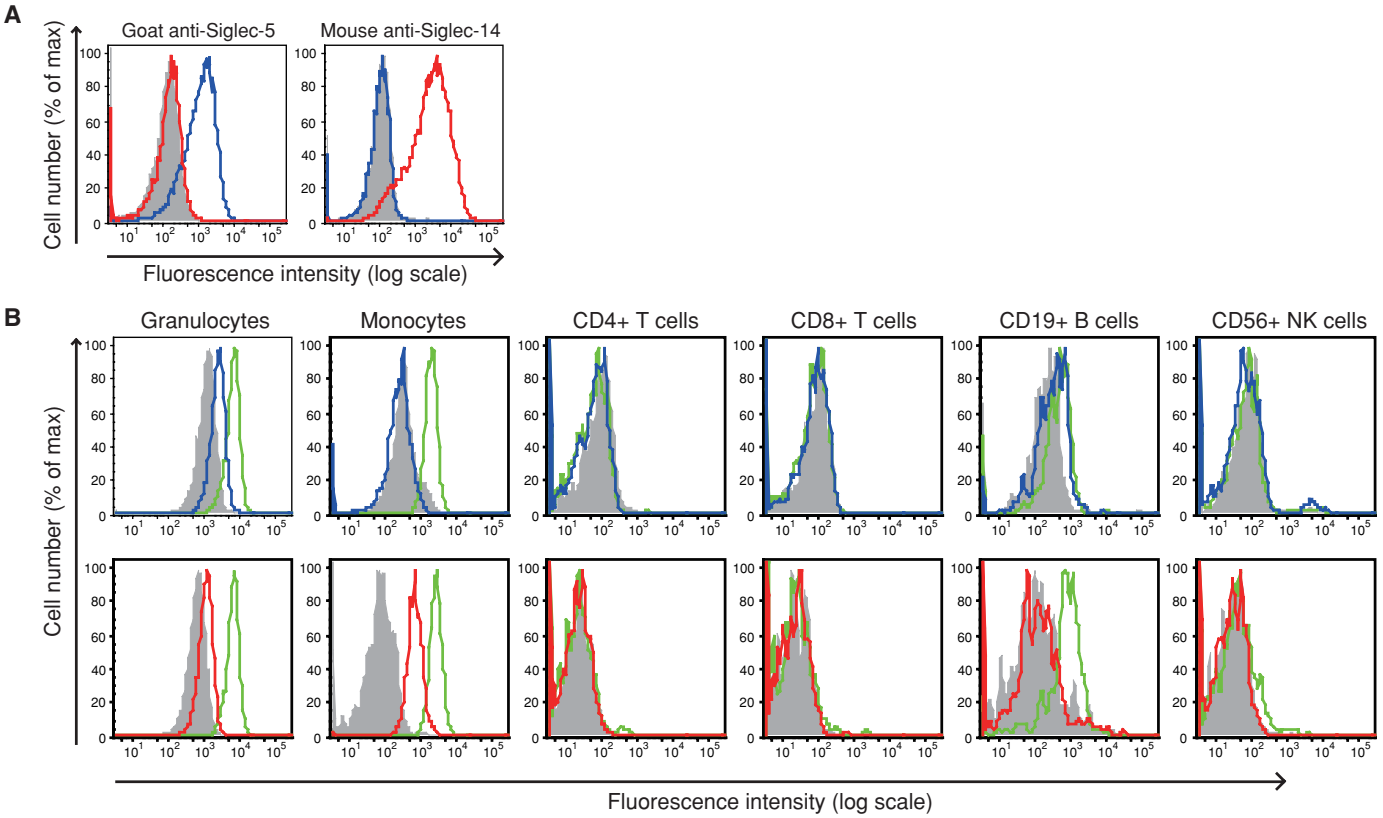

Fig. 1. Expression patterns of Siglec-14 and Siglec-5. (**A**) Specificity of the antibodies. THP-1 cells transduced with expression vectors for Siglec-5 (blue line), Siglec-14 (red line), or empty vector (gray shaded area) were stained with a goat anti-Siglec-5 polyclonal antibody (prepared as described in *Material and methods*; left panel) or a mouse anti-Siglec-14 monoclonal antibody (clone 40-1; right panel) and were analyzed by flow cytometry. (**B**) Staining of peripheral blood leukocytes (PBLs) with the specific anti-Siglec-5 and anti-Siglec-14 antibodies. Top panels: PBLs stained with a goat antibody recognizing only Siglec-5 (blue line), recognizing both Siglecs-5 and -14 (green line), or with a negative control antibody (gray shaded area). Bottom panels: PBLs stained with a mouse monoclonal antibody recognizing only Siglec-14 (clone 40-1; red line) or both Siglec-5 and Siglec-14 (clone 194128, R&D Systems; green line), or with a negative control antibody (MOPC-21; gray shaded area). Results are displayed for each leukocyte population, which was defined by scatter profiles (granulocytes and monocytes) or positive staining with the respective lineage marker (lymphocyte subpopulations).

elimination of the Siglec-14 protein; Figure 2B). An alternative assembly of human genome sequence in GenBank implies the presence of such an allele. To test this hypothesis, we designed 2 primer pairs that specifically amplify parts of *SIGLEC14* and *SIGLEC5*, respectively, and performed genomic PCR of individuals with or without Siglec-14 expression. As expected, we detected a *SIGLEC14*-specific PCR product from the genomic DNA of those who express Siglec-14, while we were unable to detect it from those who do not (Figure 2D). Moreover, in the latter individuals, genomic PCR using the primer pair that amplifies the hypothetical *SIGLEC14/5* gene yielded a PCR product of the expected size. DNA sequencing of the PCR product confirmed that these individuals do have a *SIGLEC14/5* fusion gene. The primer pair 14F + 5R did not give any product in some Siglec-14 positive individuals, who are presumably homozygous for the wild-type allele.

The SIGLEC14/5 fusion allele is widespread in the human population

To analyze the geographical distribution of the *SIGLEC14/5* fusion allele, we performed PCR analyses of genomic DNA samples from six populations (i.e., Sub-Saharan Africans, Northern Europeans, Middle Easterners, Indo-Pakistani, Southeast Asians, and Chinese; 9–10 individuals from each group) using the same set of primers. We found that the *SIGLEC14/5* fusion allele is present in all populations tested (Table I). We noted that the frequency of the *SIGLEC14/5* fusion allele was higher in Asians, and this skewed distribution was statistically significant ($P = 0.0026$, chi-square test).

Siglec-14 expression in THP-1 cells enhances TNF-α secretion

To gain insight into the functional outcome of the loss of Siglec-14, we introduced Siglec-14 into the THP-1 cell line and analyzed the cytokine response to lipopolysaccharide (LPS). THP-1 cells express Siglec-5 at a low level while they do not express Siglec-14 (data not shown). Retroviral transduction of Siglec-5 or Siglec-14 cDNA into THP-1 cells results in the surface expression of these proteins (Figure 3A). We differentiated THP-1 cells into macrophage-like cells (THP-1 macrophage) and stimulated with LPS. Upon LPS stimulation, THP-1 macrophages expressing Siglec-14 secreted approximately twice as much TNF-α as control or Siglec-5-expressing THP-1 macrophages (Figure 3B). Failure of the Siglec-14 R362A mutant, which is unable to interact with the adapter protein DAP12, to enhance TNF-α secretion implies that this effect

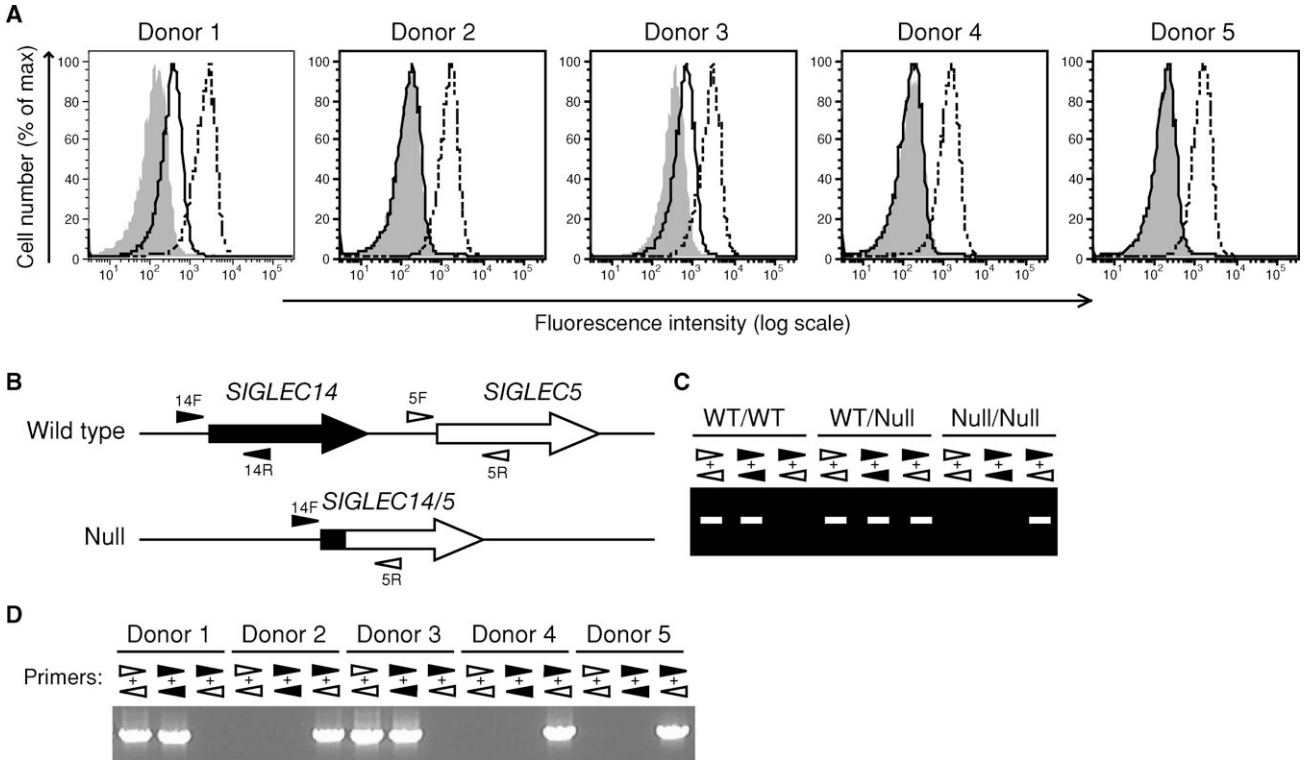

Fig. 2. The absence of Siglec-14 in some individuals and its correlation with their genotype. (**A**) Expression of Siglec-14 on granulocytes in multiple individuals. Peripheral blood leukocytes from five individuals were stained with an antibody recognizing only Siglec-14 (clone 40-1; solid line) or both Siglec-5 and Siglec-14 (clone 194128, R&D Systems; dotted line), or with a negative control antibody (MOPC-21; gray shaded area), and analyzed by flow cytometry. Some individuals (donors 2, 4, and 5 in this panel) lacked the expression of Siglec-14, while all individuals expressed an epitope shared by Siglec-5 and Siglec-14. (**B**) Schematic diagram for the genomic PCR experiment. The primer pair 5F + 5R specifically amplifies a part of the *SIGLEC5* gene, and the primer pair 14F + 14R a part of the *SIGLEC14* gene. Genomic PCR with the primer pair 14F + 5R yields a ∼1.6 kb product only when the *SIGLEC14/5* fusion gene is present. This pair can theoretically amplify a ∼17 kb fragment from the wild-type allele as well, but failed to do so under the conditions used. (**C**) Expected gel electrophoresis pattern of PCR products for each genotype. (**D**) Genomic PCR results of the same set of individuals as in **A**. Donors 2, 4, and 5 are homozygotes for the null allele, as expected.

Table I. Frequencies of wild-type and *SIGLEC14* null alleles in various populations. Nine to ten DNA samples from each population were analyzed by the genomic PCR as described in the *Material and methods*, and the frequency of each allele in each population was calculated

Population (number of samples)	Wild-type allele (frequency)	*SIGLEC14* null allele (frequency)
Sub-Saharan Africans (9)	0.67	0.33
Middle Easterns (10)	0.50	0.50
Indo-Pakistani (9)	0.61	0.39
Southeast Asians (10)	0.40	0.60
Chinese (10)	0.30	0.70
Northern European (10)	0.90	0.10

is dependent on the interaction between Siglec-14 and DAP12. Indeed, introduction of siRNA that targets DAP12 reversed the increase in TNF-α secretion from Siglec-14-expressing THP-1 macrophages to the baseline level (Figure 3C). These results imply that the absence of Siglec-14 results in attenuated cytokine response to some Gram-negative bacteria in null individuals.

Discussion

In this paper, we describe the individual expression patterns of Siglec-5 and Siglec-14 for the first time. The results show that the two proteins demonstrate partial overlap in their patterns of expression. Our results are essentially consistent with the previously reported expression pattern of Siglec-5 which used antibodies that simultaneously recognize both Siglec-5 and Siglec-14 (Cornish et al. 1998; Lock et al. 2004; Nguyen et al. 2006). Our findings also appear to provide a support to the original hypothesis that Siglec-14 and Siglec-5 are coupled, in that the expression patterns of the two proteins at least partially overlap with each other. However, we also observed apparently the unique expression of Siglec-14 on monocytes and Siglec-5 on B lymphocytes. These results may imply that these Siglecs also play unique roles independent of each other.

Our discovery of "Siglec-14 null" individuals was thus unexpected. We previously reported concerted evolution of Siglec-5 and Siglec-14 in all primate species we have tested, and we interpreted this fact as a sign of a selective pressure to maintain the functional balance between Siglec-5 and Siglec-14. However, selective pressure against the loss of the *SIGLEC14* gene is moderate, judging from the fact that the Siglec-14 null individuals are healthy. The biological meaning of the existence of the Siglec-14 null allele (i.e., *SIGLEC14/5* fusion gene) is elusive, but it is tempting to speculate that it protects the carrier against some infectious disease(s), based on the facts that (1) there is an apparent geographical distortion in the

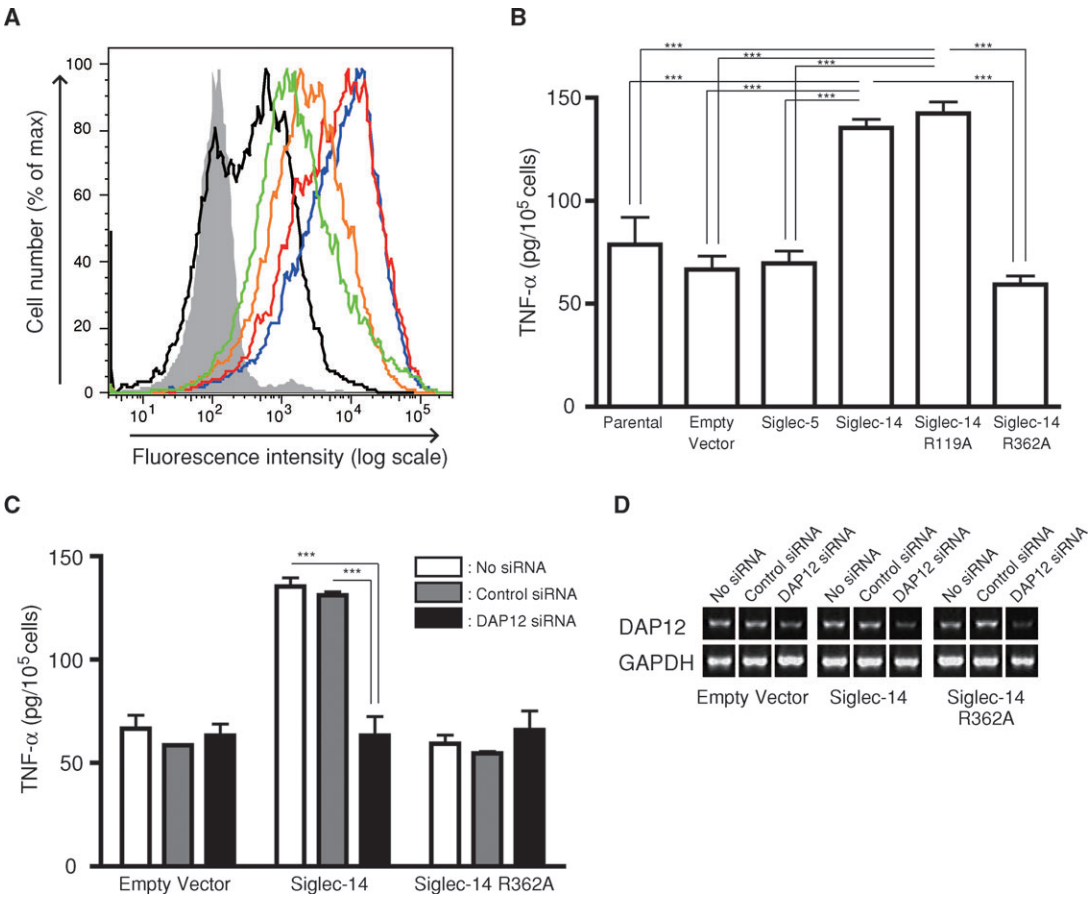

Fig. 3. Influence of Siglec-14 expression on TNF-α production from THP-1 macrophages. (**A**) Levels of Siglec-5 and Siglec-14 expression on THP-1 cells transduced with retroviral expression vectors. THP-1 cells transduced with the empty vector (black line), those expressing wild-type (red), R119A point mutant (orange), or R362A point mutant (green) of Siglec-14, and those expressing Siglec-5 (blue), were stained with an antibody that recognizes both Siglec-5 and Siglec-14 (clone 194128, R&D Systems). Gray shaded area represents staining of the parental THP-1 cells with a negative control antibody (MOPC-21). (**B**) Influence of Siglec-14 expression on TNF-α production. The expression of wild-type or R119A mutant Siglec-14 significantly enhanced TNF-α production, while the expression of R362A mutant failed to show any effect. Siglec-5 expression also showed no effect. *** $P < 0.01$ (Student's *t*-test). (**C**) Involvement of DAP12 in Siglec-14-induced enhancement of TNF-α production. A specific siRNA that suppresses DAP12 expression reversed Siglec-14-induced enhancement of TNF-α production to the control level, while it did not influence baseline TNF-α production in cells expressing Siglec-14 R362A mutant or mock-infected cells. Control siRNA did not show any effect. Statistical significance of the difference between means of the amount of TNF-α produced under two different treatments of the same cell type (e.g., DAP12 siRNA versus control siRNA treatment of Siglec-14-expressing THP-1) was tested. *** $P < 0.01$ (Student's *t*-test). (**D**) Suppression of DAP12 mRNA by siRNA. RT-PCR and agarose gel electrophoresis were performed as described in *Material and methods*.

distribution of this allele in the human population and that (2) the forced expression of Siglec-14 on THP-1 cells resulted in enhanced TNF-α response, which may lead to exaggerated or prolonged inflammation. Further studies are needed to test this hypothesis.

Biological functions mediated by the interaction of Siglec-14 with endogenous ligand(s), if any, should also be affected by the loss of Siglec-14. Although mutation of the arginine residue essential for sialic acid recognition (R119A) did not show any effect on TNF-α secretion, it should be noted that this experiment was performed in the absence of exogenous or *trans*-ligands. The presence of Siglec-14 *trans*-ligand containing sialic acids may further modify the TNF-α secretion.

In summary, we have discovered a deletion polymorphism of *SIGLEC14* that may have some consequences on human health. Identification of what aspect of human health is actually affected will be the subject of future studies.

Material and methods

Preparation of a Siglec-14-specific monoclonal antibody

Recombinant human Siglec-14-Fc proteins (containing Ig-like domains 1 through 3 or Ig-like domain 3 only) were prepared as described (Angata et al. 2006). BALB/c mice were repeatedly immunized with purified recombinant human Siglec-14-Fc, and their spleens were used for the generation of hybridomas by standard methodologies at Medical and Biological Laboratories (Nagoya, Japan). Hybridoma clones were screened by flow cytometry of COS-7 cells transiently transfected with Siglec-14 or Siglec-5 full-length protein expression construct, and further screened by flow cytometry using the THP-1 cells stably expressing Siglec-5 or Siglec-14 (preparation of which is described below). Clone 40-1, which produces a Siglec-14-specific antibody, was selected. A monoclonal antibody was purified from serum-free culture supernatant of the clone by absorption to Protein G-Sepharose (GE Healthcare, Chalfont St. Giles, UK).

Preparation of a Siglec-5-specific polyclonal antibody

The Siglec-14-Fc protein (containing Ig-like domains 1 through 3) was conjugated to Affigel-15 (Bio-Rad, Hercules, CA) at 1 mg/mL. Two hundred micrograms of commercially available goat anti-Siglec-5/14 antibody (AF1072, R&D Systems, Minneapolis, MN) was mixed with 1 mL of Siglec-14-Fc/Affigel and incubated overnight at 4°C. The antibody fraction, which was not adsorbed to the gel, was collected, concentrated, and buffer-exchanged to phosphate-buffered saline (PBS) using UltraFree-15 (cutoff = 30,000; Millipore, Billerica, MA), and used as a Siglec-5-specific polyclonal antibody preparation.

Flow cytometric analysis of peripheral blood leukocytes

The protocol involving human subjects was approved by the institutional review board, and informed consent was obtained from each donor. Peripheral blood (∼20 mL from each donor) was drawn by phlebotomy to collection tubes containing ethylenediaminetetraacetate as anticoagulant. Peripheral blood leukocytes (PBLs) were prepared by erythrocyte lysis of peripheral blood. PBLs were incubated on ice for 30 min with 1 mg/mL human IgG (Sigma, St. Louis, MO) to block Fc receptors, and then were incubated with the following primary antibodies (1 μg/10^6 cells, in 100 μL) on ice for 1 h: mouse monoclonal antibody against Siglec-14 (clone 40-1, described above), Siglec-5 and Siglec-14 (clone 194128, R&D Systems), or negative control (clone MOPC-21, Sigma); goat polyclonal antibody against Siglec-5 (described above), Siglec-5 and Siglec-14 (R&D Systems), or negative control (prepared in-house by affinity purification from goat serum using Protein G-Sepharose). The cells were washed with 1% bovine serum albumin (BSA) in PBS, and then incubated on ice for 30 min with 100 μL of 1:100 diluted allophycocyanine-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) or donkey anti-goat IgG (Jackson ImmunoResearch) antibody. The cells were washed again with 1% BSA in PBS, incubated with 10 μg of mouse IgG (for the cells stained with primary mouse monoclonal antibodies only, to block unoccupied mouse IgG-binding sites of the secondary antibody), and counter-stained with phycoerythrin (PE)-conjugated anti-CD4 (clone RPA-T4, Biolegend, San Diego, CA), PE anti-CD8 (clone RPA-T8, Biolegend), PE anti-CD19 (clone HIB19, Biolegend), or PE anti-CD56 (clone B159, BD Biosciences, San Jose, CA). The cells were washed, re-suspended in 1% BSA/PBS, and analyzed with FACSAria (BD Biosciences) equipped with Diva software. The data were processed with FlowJo software (TreeStar, Ashland, OR).

Genotyping

Genomic DNA was extracted from healthy donor PBLs (5×10^6 cells) using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, the Netherlands) or purchased from Coriell Institute for Medical Research (Camden, NJ). Primers used for specific amplification of *SIGLEC14*, *SIGLEC5*, and *SIGLEC14/5* fusion genes are as follows: 14F (5′-AGGATTTATTCTCCCATCTCGCT-3′), 14R (5′-GATGCTGATGGCGAGGTTCTG-3′), 5F (5′-GTGGTTCTGACATCTCACCTCATC-3′), and 5R(5′-CCTGAAGATGGTGATGGTCTG-3′). Primer pair 5F + 5R was used for amplification of a segment of *SIGLEC5*; primer pair 14F + 14R for *SIGLEC14*; and primer pair 14F + 5R for *SIGLEC14/5*. Each reaction tube contained the following (in 20 μL): genomic DNA, 100 ng; primers, 0.3 μM each; dNTP, 0.2 mM each; Expand High Fidelity enzyme (Roche Diagnostics, Basel, Switzerland), 0.49 U; in 1× PCR plus $MgCl_2$ buffer (Roche). Thermal cycling parameters were as follows: 94°C, 2 min; (94°C, 15 s; 56°C, 30 s; 72°C, 1.5 min) × 10 cycles; (94°C, 15 s; 56°C, 30 s; 72°C, 1.5 min + 5 s/cycle) × 20 cycles; 72°C, 7 min.

Retroviral transduction of Siglec-5 and Siglec-14 cDNA into THP-1 cells

THP-1 cells were obtained from Human Science Research Resources Bank, Osaka, Japan, and maintained in an RPMI1640 medium containing 10% fetal bovine serum. Siglec-5, Siglec-14, Siglec-14 R119A point mutant, or Siglec-14 R362A point mutant cDNAs were cloned into pMSCV-IRES-GFP (a bicistronic retrovirus vector kindly provided by Dr. Hiroaki Kume, Jichi Medical School) (Kume et al. 2000). PLAT-A cells (kindly provided by Dr. Toshio Kitamura, University of Tokyo (Kitamura et al. 2003)) were transfected with the constructs, and the culture supernatants containing retroviral particles were used to infect THP-1 cells on 6-well plates coated with Retronectin (Takara Bio, Otsu, Japan) for 24 h. After recovery culture for 2–3 days, successfully infected cells expressing the green fluorescence protein were sorted with FACSAria.

Macrophage differentiation of THP-1 cells and a TNF-α production assay

THP-1 cells were seeded in 96-well flat-bottom tissue culture plates at a density of 2×10^4 cells/100 μL per well and were allowed to adhere and differentiate for 4 days at 37°C in the presence of 50 nM phorbol 12-myristate 13-acetate (PMA) (Sigma). After repeated washing with the culture medium, PMA-differentiated THP-1 cells (THP-1 macrophages) were stimulated with LPS from *Escherichia coli* (O111:B4; 50 ng/mL culture media) for 24 h. The TNF-α concentrations in the culture supernatant were measured using OptEIA ELISA kits (BD Biosciences). The samples were assayed in triplicate.

RNA interference

A Stealth RNA targeting DAP12 mRNA (target sequence: 5′- UAGAGCAACUGCAAUCGCUCUGGGC-3′) and the control Stealth RNA (Negative Control Hi GC) were obtained from Invitrogen (Carlsbad, CA). THP-1 macrophages prepared as mentioned above were transfected with the specific or control Stealth RNA (100 nM) using LipofectamineRNAi MAX (Invitrogen), and cultured for 48 h. The cells were stimulated with LPS as mentioned above for 24 h and supernatants were collected for a TNF-α assay. RNA samples were isolated from these cells using the RNeasy Mini Plus Kit (Qiagen), and corresponding cDNA was synthesized using oligo dT primer and SuperScript II (Invitrogen). Primer pairs used for specific amplification of DAP12 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were: DAP12 Forward (5′-ATCCCACCGGCCCTTACACT-3′), DAP12 Reverse (5′-GGGGAGCGGTCTGGTCTCT-3′), GAPDH Forward (5′-CGGGAAACTGTGGCGTGATG-3′), and GAPDH Reverse (5′-AACTGTGAGGAGGGGAGATT-3′). Each reaction tube contained the following (in 20 μL): first-strand cDNA (equivalent of 50 ng RNA); primers, 0.2 μM each; dNTP, 0.2 mM each;

ExTaq DNA polymerase, 0.5 U (Takara); and 1× ExTaq PCR buffer (Takara). Thermal cycling parameters were as follows: 94°C, 1 min; (94°C, 1 min; 58°C, 1 min; 72°C, 1 min) × 35 cycles; 72°C, 7 min. The products were analyzed by agarose gel electrophoresis.

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Conflict of interest statement

None declared.

Abbreviations

BSA, bovine serum albumin; DAP12, DNAX adapter protein 12; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide; PBL, peripheral blood leukocyte; PBS, phosphate-buffered saline; PE, R-phycoerythrin; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor α.

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