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Concise report

Monocyte Siglec-14 expression is upregulated in patients with systemic lupus erythematosus and correlates with lupus disease activity

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Abstract

Objective. Siglecs are sialic acid-binding immunoglobulin-like lectins expressed on the surface of immune cells, which participate in the discrimination of self and non-self. We investigated myeloid CD33-related Siglec expression in a cohort of patients with SLE.

Methods. Cell surface expression of Siglec-5/14, Siglec-9 and Siglec-10 on peripheral myeloid subsets were analysed from 39 SLE patients using flow cytometry. Genotyping of the Siglec-5/14 locus was also performed. Clinical markers of SLE disease activity, including SLEDAI, serum complement concentrations and serum autoantibodies, were assessed and correlated with Siglec levels.

Results. Siglec-14 expression on SLE monocytes (median = 518, interquartile range: 411) was significantly higher when compared with healthy controls (median = 427, interquartile range: 289.3; $P < 0.05$) and correlated positively with SLEDAI scoring and anti-Sm and anti-SSB autoantibodies ($P < 0.05$). A negative correlation was determined with patient serum C3 concentrations ($P < 0.005$). Genotyping of the Siglec-5/14 locus revealed a high frequency of the Siglec-14 null allele across both groups, reflecting the incidence in Asian populations.

Conclusion. Our data suggest that the Siglec immunomodulatory molecules, in particular Siglec-14 expression on monocytes, may play an important role in the inflammatory events of SLE. No bias was found with regard to *SIGLEC14* genotype in our patient group compared with healthy controls. Larger comparisons of mixed ethnicity might, however, reveal an important role for Siglecs in the pathogenesis of autoimmune disease.

Key words: Siglec, systemic lupus erythematosus, monocytes

BASIC SCIENCE

Rheumatology key messages

- Siglec-14 expression is increased on SLE patient monocytes.
- Monocyte Siglec-14 expression correlates with SLEDAI and serological disease in SLE.
- No bias in the prevalence of *SIGLEC14* genotypes was determined for the SLE patient cohort.

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Introduction

SLE is a complex autoimmune disease of unknown aetiology characterized by the development of autoantibodies to self-antigens. SLE affects predominantly females and has a diverse clinical presentation with multi-organ involvement, which can lead to significant morbidity and mortality [1, 2]. The clinical and serological manifestations of SLE are attributed to both genetic and environmental factors, in which the involvement of the innate immune system, including mediators of apoptotic cell clearance

and the inflammatory response, such as monocytes, is crucial for disease pathogenesis [3, 4].

Sialic acid-binding immunoglobulin-like lectins (Siglecs) are cell surface transmembrane receptors predominantly expressed on immune cells. Siglecs recognize sialic acid moieties present on glycan structures on host proteins and lipids described as self-associated molecular patterns [5]. The CD33-related Siglecs (CD33rSiglecs) represent a rapidly evolving subfamily of these molecules, exhibiting high sequence similarity [6]. Upon recognition of sialoglycans, the majority of CD33rSiglecs signal negatively through immunoreceptor tyrosine-based inhibitory motifs to dampen the innate immune response. Siglec-14, a paired receptor with Siglec-5, serves as an activating signal transduction receptor, however, through association with the DAP12 adapter protein [7].

Siglecs play an important role in the regulation of the immune response and as such have been implicated in infectious and inflammatory diseases, autoimmunity and cancer (as reviewed by Macauley *et al.* [8]). The recognition of host sialic acids by Siglecs and the subsequent dampening of cellular activation has been suggested as a mechanism of preventing self-reactivity; furthermore, in mice, the CD33rSiglec gene cluster is located on chromosome 7, within the well-documented *Sle3* lupus-susceptibility locus [9]. In light of this, we set out to determine whether expression of the CD33rSiglecs on circulating innate immune cells might be altered in the context of SLE and thus potentially contribute to autoimmune pathogenesis. We therefore analysed the expression of CD33rSiglecs-5/14, -9 and -10 on the cell surface of peripheral myeloid subsets in a cohort of 39 SLE patients and correlated these with clinical and serological disease measures.

Methods

Patients

Thirty-nine adult patients who attended the Lupus Clinic at the National University Hospital, Singapore and fulfilled the ACR classification criteria for SLE were recruited [1, 10]. Peripheral blood was taken into a Vacutainer containing 3.8% EDTA (10% v/v; Becton Dickinson, Franklin Lakes, NJ, USA). Twenty-nine healthy control donors, matched to SLE patients for ethnicity and gender, were recruited for comparison (control and SLE donors were 19–64 years old). Peripheral blood from healthy control donors was taken within an hour of SLE patient peripheral blood sampling. SLE disease activity was assessed by SLEDAI. All subjects who had acute illness at recruitment were excluded. Demographic and clinical information, such as disease manifestation and drug use, were retrieved from clinical interview and electronic medical records of the hospital. Written informed consent was obtained from the participants before recruitment. The study was approved by the, National Healthcare Group (NHG) Domain Specific Review Board (#DSRB-E/11/061) and the National University of Singapore Institutional Review Board (NUS-IRB-09-256).

Serology

Serum ANAs were assessed by Luminex multiplexing technology (Luminex 200, Luminex Corporation, Austin, Texas, USA) using AtheNA Multi-Lyte ANA-III Test System (Zeus Scientific, Branchburg, NJ, USA) according to the manufacturer's instructions. ELISAs for anti-dsDNA and anti-snRNP autoantibodies were performed as previously described [11] using an alkaline phosphatase goat anti-human secondary antibody (Jackson ImmunoResearch). For anti-snRNP detection, the plates were coated with U1snRNP-68, U1snRNP-C and U1snRNP-A (Diarect AG, Freiburg, Germany). ANAs were measured by IIF staining of HEp-2 cells (positivity at 1:80 dilution). Serum C3 and C4 levels and anti-dsDNA were assayed by immunoturbidimetry or ELISA (BioRad, Hercules, CA, USA), respectively, at the National University Hospital laboratory.

Flow cytometry

Whole blood was stained with directly conjugated antibodies (supplementary Table S1, available at *Rheumatology* Online) and incubated for 30 min at 4 °C. Following red blood cell lysis (BD FACS Lysing solution, Becton Dickinson, Franklin Lakes, NJ, USA), myeloid subsets were identified using anti-CD45, CD14, CD11b (BD Pharmingen, San Jose, CA, USA), CD16 (eBioscience) and CD62L (Beckman Coulter, Brea, CA, USA). Siglec expression was analysed by cell surface staining with anti-Siglec-9 (RnD Systems, Minneapolis, MN, USA), anti-Siglec-10 (Biolegend, San Diego, CA, USA) and anti-Siglec-5 (RnD Systems, Minneapolis, MN, USA), which recognizes epitopes common to both Siglec-5 and Siglec-14. Acquisition and analysis was completed using a BD LSRFortess and FlowJo v10.1 for Windows (Treestar, Ashland, OR, USA).

Genotyping

DNA was isolated from whole blood, purified PBMCs or PMNs lysed in TRIzol reagent (ThermoFisher Scientific, Pittsburgh, PA, USA). For TRIzol samples, DNA was extracted according to the manufacturer's instructions, resuspended in 100 μl DNase-free water and incubated at 65°C for 30 min. DNA was isolated from whole blood or purified PBMCs using a PureLink Genomic DNA Mini kit (ThermoFisher Scientific, Pittsburgh, PA, USA) according to the manufacturer's instructions. PCR primers and conditions have been described previously [12].

PBMC isolation and stimulation

PBMCs were obtained from the blood of healthy donors by Ficoll-Hypaque centrifugation (Amersham Pharmacia Biotech, Little Chalfont, UK). Cells were seeded in 96-well plates at 2.5×10^5 cells/well and stimulated for 18 h with either cRPMI media alone [RPMI 1640 (Gibco, Thermo Fisher Scientific, Pittsburgh, PA, USA) supplemented with 10% FBS (Hyclone, Pittsburgh, PA, USA), 200 μM L-glutamine, 15 mM HEPES, 100 μg/ml streptomycin, 100 U/ml penicillin, 10 μM non-essential amino acids and 100 μM sodium pyruvate (all Gibco, Thermo Fisher

Scientific, Pittsburgh, PA, USA)] or cRPMI with LPS (Invivogen, San Diego, CA, USA) at 100 ng/ml or prednisolone (Sigma Aldrich, St Louis, MO, USA) at 1 μg/ml.

Statistics

The median fluorescence intensity values are reported for expression values using flow cytometry. Results are expressed as the median and interquartile range (IQR) or arithmetic mean (S.D.) for a given number of values (n). Normality was tested using the D'Agostino and Pearson normality test or Kolmogorov–Smirnov test. Data that did not pass were analysed using a Mann–Whitney *U*-test or Kruskal–Wallis non-parametric analysis of variance (ANOVA) followed by Dunn's multiple comparison test. Otherwise, a one-way ANOVA was used followed by Tukey's multiple comparisons test to analyse three groups. For correlation analysis, a non-parametric Spearman rank correlation was determined. Statistics and graphs were generated using GraphPad Prism v6.00 for Windows (GraphPad Software, Graphpad, La Jolla, CA, USA). Fisher's exact test was used to test for an association between *SIGLEC14*-null genotype and patient/control status in the R statistical language version 3.1.2.

Results

Myeloid Siglec expression in SLE patients and control donors

Thirty-nine patients with SLE were studied (see Table 1 for patient demographic and clinical information). Flow cytometry was performed on peripheral whole blood to assess the cell surface expression of Siglecs-5/14, -9 and -10 on monocytes, eosinophils and neutrophils (supplementary Fig. S1, available at *Rheumatology* Online). Siglec-5 and Siglec-14 have high sequence homology and are thus recognized by the same anti-Siglec-5 antibody; however, prior reports have demonstrated that while both of these receptors are expressed on neutrophils, only Siglec-14 is present on monocytes [12].

SLE patients were found to have increased expression of Siglec-14 on monocytes (median = 518, IQR: 411) when compared with healthy controls (median = 427, IQR: 289.3; $P < 0.05$). In contrast, PMN Siglec-5/14 levels were equivalent between the two populations (Fig. 1A). No differences were observed in monocyte or PMN Siglec-9 or Siglec-10 expression between SLE patients and controls; however, eosinophils from SLE patients had significantly lower surface Siglec-10 expression (supplementary Fig. S1, available at *Rheumatology* Online).

Monocyte Siglec-14 expression and SLE disease measures

Upon further analysis, SLE patient monocyte Siglec-14 expression was correlated positively with increasing SLEDAI score, whereas a negative correlation was determined with patient serum C3 levels (Fig. 1B and C). We did not observe a significant correlation between Siglec-14 expression and serum C4 levels, however, suggesting that increased Siglec-14 expression on SLE patient monocytes is associated with activation of the alternative complement pathway (Fig. 1C) [13]. Analysis of serum autoantibodies did not reveal any association of anti-dsDNA or anti-snRNP levels with monocyte Siglec-14 expression (Fig. 1D and supplementary Fig. S1, available at *Rheumatology* Online). Nevertheless, positive correlations were found between Siglec-14 expression and anti-SSB as well as anti-Sm autoantibodies in our patient cohort (Fig. 1E).

Table 1 Patient demographic, clinical and treatment characteristics

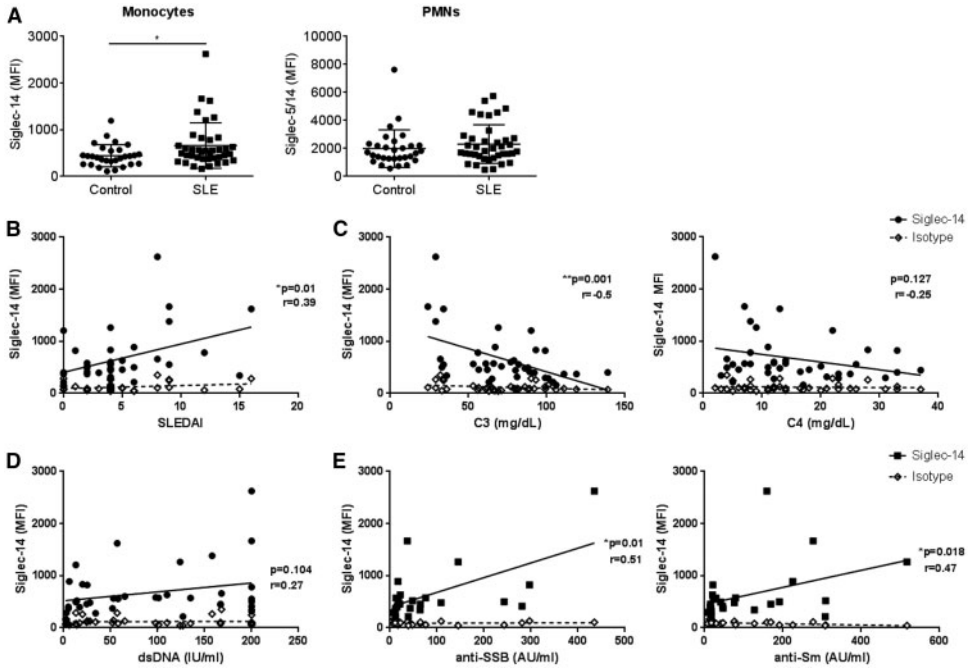
Characteristic	SLE patient cohort (n = 39)
Age, median (range), years	35 (20–64)
Female, %	88
Ethnicity, %	
Chinese	59
Malay	25.6
Indian	7.7
Filipino	7.7
Glucocorticoid treatment, %	97
Glucocorticoid dose, prednisolone, median (range), mg/day	7 (0–60)
MTX, %	3
HCQ, %	87
AZA, %	28
MMF, %	13
Ciclosporin, %	5
SLEDAI score, median (range)	4 (0–16)
Renal disease, %	13
CNS disease, %	8
Cutaneous disease, %	8
ANA positive, %	100
Serum creatinine, median (range),[a] μmol/l	52 (40–662)

[a]Data for 37 patients.

SIGLEC14 genotyping of SLE patients and control donors

A polymorphism in the gene encoding Siglec-14 (*SIGLEC14*-null) has been reported to have high prevalence in Asian populations and has been linked to preterm labour following Group B *Streptococcus* infection and decreased exacerbations in patients with chronic obstructive pulmonary disease (COPD) [12, 14, 15]. The *SIGLEC14*-null allele encodes an identical protein to Siglec-5 with an inhibitory immunoreceptor tyrosine-based inhibitory motif. Given the increased incidence and severity of SLE in Asian populations, we therefore sought to determine the Siglec-5/14 genotypes within our SLE patient cohort. We grouped patients and controls into those with the wild-type alleles, null individuals and heterozygous individuals (supplementary Table S2, available at *Rheumatology* Online). Our SLE patient and healthy donor control cohorts exhibited a similar distribution of Siglec-5/14 alleles, and we found no genotype bias associated with disease.

Fig. 1 Increased expression of Siglec-14 on SLE patient monocytes correlates with SLEDAI and serological disease



(**A**) Siglec-14 expression on monocytes and PMNs from SLE patients and controls was analysed by flow cytometry. (**B** and **C**) Correlation plots of Siglec-14 expression on patient monocytes with patient SLEDAI scoring or levels of complement activation (C3 and C4); dotted line shows no correlations were observed when staining with an isotype control antibody. (**D** and **E**) Correlation between patient autoantibody levels and Siglec-14 expression on monocytes. Bars represent mean (S.D.). *P $<$ 0.05, **P $<$ 0.01 (values were computed by non-parametric Spearman rank correlation (for P and r) or Mann–Whitney *U*-test (P only).

Prednisolone treatment does not upregulate monocyte Siglec-14 expression

It has been reported that inhaled CSs increase Siglec-5/14 expression in the sputum of patients with COPD [16]. Given that CSs are a mainstay of treatment for patients with SLE (Table 1), we sought to determine the effects of overnight prednisolone treatment on donor PBMC Siglec-14 expression. Treatment with prednisolone at a dose range comparable to therapeutic levels [17] did not affect monocyte Siglec-14 expression, despite upregulating CD62L and the CS-inducible CD163 (supplementary Fig. S2, available at *Rheumatology* Online). In contrast, LPS stimulation resulted in upregulation of Siglec-14 and the expected loss of surface CD62L (supplementary Fig. S2, available at *Rheumatology* Online). *SIGLEC14* genotype was found to have no effect on PBMC responses to treatment with either prednisolone or LPS.

Discussion

The CD33rSiglecs are a family of predominantly inhibitory transmembrane receptors that recognize host sialic acids and transmit signals to dampen the innate immune response. This study is the first to investigate the expression of these receptors on myeloid lineages in patients with SLE. We determined an increase in the expression of Siglec-14 on patient monocytes. Furthermore, we demonstrated an association between Siglec-14 expression on lupus monocytes and increasing SLE disease activity in terms of higher SLEDAI, lower serum C3 concentrations and positivity of anti-Sm and anti-SSB autoantibodies.

The increase in Siglec-14 is correlated with a decrease in serum C3 but not C4 concentrations, indicating that upregulation is associated with the inflammatory events of the alternative complement pathway rather than the classical or lectin pathways (in which antigen–antibody complexes or microbial particles lead to decreased C4 concentrations) [13]. The alternative pathway is stimulated by spontaneous and foreign particulate, such as LPS. This is congruent to our *in vitro* data showing that Siglec-14 is upregulated on the cell surface of monocytes in response to LPS. Alternatively, it is possible that some individuals may carry a C4 null mutation or have low C4 copy number, which would affect serum concentrations [13].

Although it has been reported that Siglec-5/14 is increased in the sputum of COPD patients as a result of inhaled CSs [16], our *in vitro* data indicate that monocyte Siglec-14 expression is unaffected by treatment with prednisolone. This would need to be confirmed using patient samples, however, by measuring monocyte Siglec-

14 expression levels before and after glucocorticoid treatment.

Alterations in the relative proportions of circulating monocyte subsets have been reported in SLE [18]. We find Siglec-14 expression to be significantly decreased in non-classical monocyte subsets from healthy donor controls (supplementary Fig. S3, available at *Rheumatology* Online). Although our whole blood analysis did not enable monocyte subpopulation analysis of Siglec expression, we did analyse subpopulation frequency in a number of individuals. These data showed a non-significant increase in non-classical $CD14^{lo}CD16^{+}$ monocytes in the patient cohort, eliminating this as a reason for the increased expression (supplementary Table S3, available at *Rheumatology* Online).

Siglec-14 is an activating receptor which, upon glycan ligand binding, sends activatory immune signals through association with DAP12 activator protein. A result of gene conversion of *SIGLEC5*, Siglec-14 may have evolved in response to pathogens evading immune surveillance [6, 7]. In agreement with this, it has been reported that *SIGLEC14*-null fetuses are more susceptible to prematurity following Group B *Streptococcus* infection than wild-type carriers [19]. Concurrent with published reports, we determined the *SIGLEC14*-null genotype to be highly prevalent in our SLE patient and control cohorts of Asian ethnicity [12, 14]. However, in contrast to a study investigating COPD in a cohort of Japanese patients, we observed no bias with regard to *SIGLEC14* genotype and SLE disease [14]. Genotyping of larger patient cohorts of mixed ethnicity would provide further insight into any potential associations between *SIGLEC14* genotype and autoimmunity.

Immune complex formation, resulting from ANAs in SLE patients, may be taken up by Fcγ receptors on monocytes, resulting in the release of pro-inflammatory cytokines and, ultimately, leading to tissue destruction in active disease. Therefore, it is possible that monocyte Siglec-14 expression is upregulated in active SLE in response to these inflammatory cytokines. Supporting this hypothesis, we found a non-significant increase in monocyte Siglec14 expression after Fcγ receptor ligation in healthy control donors (supplementary Fig. S4 and supplementary methods, available at *Rheumatology* Online).

Although no further correlations were found in this study with regard to Siglec-5/14 expression on other myeloid subsets from patients with SLE, it is possible that the results are confounding for cell types that express the highly homologous Siglec-5 and Siglec-14, such as PMNs, because both are recognized by the antibody used. There is currently no commercially available antibody that is specific for Siglec-14 alone, however.

A significant decrease in Siglec-10 expression was detected on SLE patient eosinophils. Although the mouse orthologue of Siglec-10, Siglec-G, has been implicated in B-cell tolerance [20] little is known regarding the role of this Siglec on this particular cell type, and additional studies are therefore required.

In summary, we have shown that in our cohort of SLE patients, monocyte Siglec-14 expression is upregulated and is correlated with clinical and serological disease. Furthermore, owing to Asian ethnicity, the majority of patients express the immunosuppressive Siglec-14/5 molecule. The biological functions of the CD33rSiglec family are still emerging, and further investigations are necessary to elucidate the full impact of the immunomodulatory Siglec-14 in autoimmunity.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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