Influence of *SIGLEC9* polymorphisms on COPD phenotypes including exacerbation frequency

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Summary at a Glance

A haplotype of *SIGLEC9* gene was associated with exacerbation frequency and emphysema in Japanese COPD patients (but not in ECLIPSE cohort). The Siglec-9 protein encoded by this haplotype was hypomorphic in its ability to suppress myeloid cell inflammatory responses. This study reinforces the connections between endogenous lectins and COPD phenotypes.

The first two authors contributed equally to this work.

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Abstract

Background and objective: The exacerbation-prone phenotype of chronic obstructive pulmonary disease (COPD) is particularly important, since exacerbations lead to poor quality of life and disease progression. We previously found that COPD patients who lack Siglec-14, a myeloid cell protein that recognizes bacteria and triggers inflammatory responses, are less prone to exacerbation. We hypothesized that the variations in other *SIGLEC* genes could also influence COPD exacerbation frequency, and investigated the association between *SIGLEC9* polymorphisms and the exacerbation-prone phenotype of COPD.

Methods: We examined whether *SIGLEC9* polymorphisms affect the frequency of COPD exacerbation in 135 subjects within our study population, and also analyzed the correlation between the genotypes and the severity of airflow obstruction and emphysema in 362 Japanese smokers including 244 COPD patients. The association between these SNPs and COPD phenotypes were also assessed in a Caucasian population of ECLIPSE study. The effects of these cSNPs on Siglec-9 protein functions were analyzed using in vitro assays.

Results: The G allele of rs2075803 and rs2075803 G/rs2258983 A (GA) haplotype in *SIGLEC9* was associated with higher frequency of exacerbations and the extent of emphysema in COPD. These results did not replicate in the ECLIPSE study. A myeloid cell line expressing the Siglec-

9 variant corresponding to GA haplotype produced more TNFα than the one expressing the variant corresponding to the other major haplotype.

Conclusions: The *SIGLEC9* rs2075803 G/rs2258983 A haplotype, which corresponds to a Siglec-9 variant less effective at suppressing inflammatory response, may be a risk factor for development of emphysema.

TRIAL REGISTRATION of ECLIPSE study: ClinicalTrials.gov NCT00292552.

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic disease characterized by respiratory symptoms including cough, sputum, and dyspnea on exertion, and functionally by airflow obstruction that is not fully reversible [1]. COPD causes a reduction in the activities of daily living and quality of life, and is projected to be the 3rd leading cause of mortality in the world by 2030 [2]. Exacerbations, one of the major phenotypes of COPD, promote the progression of irreversible airflow obstruction [3] and emphysema [4] and are a frequent cause of hospitalizations, increased patient mortality, accounting for a major proportion of the clinical and socio-economic burden of the disease [5-7]. As the current options to treat exacerbations are limited and often ineffective, there is an urgent need to understand the mechanisms which contribute to exacerbations in order to find new target molecules which may lead to new therapeutics and preventive strategies.

It is now evident that a subgroup of patients is particularly susceptible to exacerbations, and that this frequent-exacerbation phenotype is relatively stable over time [8]. This suggests the presence of persistent contributing factors, such as genetic factor(s). Since COPD exacerbations promote irreversible airflow obstruction [3] and emphysema [4], and COPD itself is also proved to be a disease influenced by multiple genes [9], genetic variants related to

susceptibility to exacerbations could also affect susceptibility to COPD and/or emphysema. However, reports correlating genetic polymorphisms and COPD exacerbations are limited [10-14].

Since COPD exacerbations are often triggered by bacterial or viral infections [15], it is reasonable to postulate that the immune response may be mechanistically involved in exacerbation susceptibility. The initial defense responses against these pathogens often involve endogenous glycan-recognition proteins, collectively called lectins. Indeed, polymorphisms in the *MBL2* gene, which encodes mannose-binding lectin that is found in plasma and bodily fluids and triggers the lectin pathway of the complement system, have been associated with susceptibility for frequent COPD exacerbations [11]. In addition, we previously demonstrated that Siglec-14, a member of the Siglec family of sialic acid-binding lectins, interacts with non-typeable *Haemophilus influenzae* (NTHi, a major cause of COPD exacerbations) to enhance pro-inflammatory cytokine production from myeloid cells. Loss of Siglec-14, due to *SIGLEC14*-null allele homozygosity, was associated with a reduced risk of COPD exacerbation in a Japanese patient population [16]. Our findings imply that, in the chronic inflammatory environment of the lungs in COPD patients, the balance of positive and negative regulation of immune cell activity may be easily tipped towards the direction of over-reaction. Thus, the

immune system that should protect the host might in fact trigger excessive inflammation that culminates in an exacerbation and subsequent tissue damage.

Siglecs are a family of endogenous membrane-bound lectins that recognize glycans containing sialic acid and modulate immune signals [17, 18]. While Siglec-14 triggers innate immune cell activation, most other Siglecs negatively regulate immune cell activities. Siglec-9 is a family member widely expressed on myeloid cells [19], and the engagement of Siglec-9 by its ligands suppresses innate immune responses [20, 21]. Therefore, we hypothesized that a genetic variant of *SIGLEC9* that attenuates the suppressive function of the Siglec-9 protein would promote more vigorous inflammatory responses in myeloid cells and would render COPD patients more susceptible to exacerbation. Since Siglec-9 is broadly expressed on myeloid cells and interacts with NTHi (similar to Siglec-14) [16], but transduces a suppressive signal (in contrast to Siglec-14), Siglec-9 is an ideal model to investigate whether more pro-inflammatory myeloid cells makes patients more susceptible to exacerbation, as was the case for Siglec-14.

SIGLEC9 gene has 2 common non-synonymous coding single nucleotide polymorphisms (cSNPs), rs2075803 and rs2258983, whose minor allele frequencies are relatively high among Asians and Europeans. To test our hypothesis, we investigated whether these two cSNPs were associated with COPD phenotypes including frequency of exacerbations

in a Japanese patient population. In addition, we also asked if the amino acid changes in Siglec-9 introduced by these cSNPs affect the inflammatory response by myeloid cells in an in vitro viral infection model. Finally, we tested whether the findings in the Japanese patients replicate in ECLIPSE study population.

Materials and Methods

PARTICIPANT RECRUITMENT AND SAMPLE COLLECTION

We used the Japanese population and also the ECLIPSE population. The ECLIPSE study is a clinical trial (TRIAL REGISTRATION: ClinicalTrials.gov NCT00292552.), and the information as a clinical trial is briefly described in the online supplement. Other details including COPD definition[1] are also in the online supplement.

ETHICAL CONSIDERATION

The current study was approved by the ethical committee of Nippon Medical School (Approval number: 18-11-31). The ECLIPSE study (NCT00292552; GSK code SCO104960) was approved by the ethical committees/institutional review boards of the participating clinical centers. Written informed consent was obtained from each subject.

STUDY DESIGN

We obtained 1-year follow-up records for exacerbations from a subset of Japanese COPD patients. First, we investigated whether the genotypes of *SIGLEC9* SNPs affected the

frequency of exacerbations in these subjects. In addition, to investigate the association of genetic variations of *SIGLEC9* with COPD phenotypes, we performed regression analyses and investigated the association between these genetic variations and airflow obstruction assessed by pulmonary function tests (PFT) or the severity of emphysema assessed by computed tomography in all Japanese patients (including those without COPD). This is because symptomatic current/ex-smokers without COPD also have exacerbations and evidence of airway disease [22-24], and the exacerbation could promote emphysema[4] or airflow limitation[3] in this population. Next, the effects of these cSNPs on the functions (i.e., glycan binding and suppression of inflammatory responses) of the Siglec-9 protein were analyzed by in vitro assays, as described in the following sections.

COPD-related parameters used to determine the association

Pulmonary function parameters and high-resolution computed tomography (HRCT) parameters were assessed on all patients. Exacerbation frequency, which was measured based mainly on patient diary, was assessed on a subset of Japanese COPD patients. Prospective data on exacerbations in the ECLIPSE study were assessed at each study visit and through monthly telephone calls. Other details including the definition of COPD exacerbations are in the online

supplement.

SNP selection and genotyping

Two coding SNPs of *SIGLEC9* (rs2075803: Lys100Glu and rs2258983: Glu315Ala) with a minor allele frequency (MAF) > 0.10 in Japanese were genotyped by using ABI TaqMan® SNP Genotyping Assays (Life Technologies Japan, Tokyo, Japan).

DATA ANALYSIS

The values are presented as means (SD). P-values < 0.05 were considered significant. We used the unpaired t-test or analysis of variance to compare continuous variables. The effects of genotypes or haplotypes on COPD phenotypes were determined with multivariate regression. The unobserved haplotype frequencies were estimated by the expectation-maximization (EM) algorithm and the haplotypes for each subject were selected as the most likely haplotype. The effect of genotypes or haplotypes on the frequency of exacerbation was determined by using Poisson regression with additive models. Adjustments for regression analyses was also performed using age, gender, FEV1%predicted, and usage of inhaled corticosteroid and/or long-acting bronchodilators in the analysis of exacerbation frequency and with age, gender,

and pack-years in the analysis of emphysema severity.

Details of genetic analyses including those with the ECLIPSE population and in vitro experiments are provided as Supplementary material.

Results

Characteristics of the study population

Our study population comprised 362 Japanese symptomatic smokers including 244 COPD subjects. We obtained 1-year follow-up records for exacerbations from 135 COPD patients from this population. The basic characteristics of this population are shown in Table 1. Hardy-Weinberg equilibrium was maintained in these SNPs among the control subjects (p = 0.61 [rs2075803] and p = 0.67 [rs2258983]). The two SNPs, rs2075803 and rs2258983, were in almost complete linkage disequilibrium (R2 = 0.93; p = 1.87e-25 by the test for linkage disequilibrium). Thus, we analyzed the association between various COPD phenotypes and only one SNP (rs2075803), whereas haplotype-based experiments were performed in the in vitro protein function analyses. The frequencies of the two major haplotypes, AC (rs2075803 = A, rs2258983 = C) and GA (rs2075803 = G, rs2258983 = A), were 0.38 and 0.60, respectively.

The association between SIGLEC9 genotypes and exacerbations

The G allele of rs2075803 was associated with higher frequency of exacerbations with nominal statistical significance (p = 0.0158) (Figure 1), and the association remained after adjustment

for age, gender, FEV1%predicted, and usage of inhaled corticosteroid and/or long-acting bronchodilators ($p = 0.0253$).

The G allele of rs2075803 corresponds almost completely to the GA haplotype (rs2075803 = G, rs2258983 = A). The GA haplotype was also positively associated with exacerbation frequency in multivariate models after similar adjustment ($p = 0.0132$).

The association between SIGLEC9 genotypes, airflow obstruction, and emphysema

Frequent exacerbation is associated with faster decline of lung function as measured by FEV1 [3] and also with progression of emphysema [4]. Thus, we tested the association between *SIGLEC9* rs2075803, airflow obstruction, and the extent of emphysema in a larger population. The number of G alleles of rs2075803 was significantly associated with LAA% at -940 HU, a quantitative measure of emphysema, in both univariate and multivariate models adjusted for age, gender, and pack-years in the subjects with COPD ($p = 0.0167$ without adjustment; $p = 0.0171$ with adjustment) (Figure 2). This SNP was not associated with FEV1% predicted, which reflects airflow limitation, in the whole population or in COPD subjects only (data not shown).

The frequency of the GA haplotype was also positively associated with the extent of

emphysema in the subjects with COPD after similar adjustment with significance ($p = 0.0433$).

Two major variants of Siglec-9 protein showed similar glycan binding

To investigate the functional consequence of the cSNPs, we prepared recombinant soluble Siglec-9 protein variants corresponding to the two major haplotypes, and analyzed their binding to synthetic glycans. The "reference" haplotype is AC (rs2075803 = A, rs2258983 = C), translating to $K^{100}A^{315}$ in terms of amino acids. The “risk” haplotype, GA, encodes the $E^{100}E^{315}$ variant. These protein variants showed similar glycan binding, both in structural preference and in binding intensity (Figure 3).

Siglec-9 $E^{100}E^{315}$ variant showed weaker suppression of TNFα response

We prepared THP-1 cell lines expressing full-length Siglec-9 variant proteins. An in vitro co-culture system using influenza virus-infected human airway epithelial cells and THP-1 cells was used as a model to investigate possible influence of the Siglec-9 sequence variations on inflammatory responses, which was assessed by quantifying IL-8 and TNFα secretion from THP-1 cells. The addition of anti-viral antibody was essential to elicit a robust response from

THP-1 cells, implying that the response was dependent on Fcγ receptor(s). Both variants of Siglec-9 significantly suppressed secretion of these cytokines from THP-1 cells (Figure 4). While the amount of secreted IL-8 was not influenced by the Siglec-9 sequence variations (Figure 4a), the amount of TNFα secreted from THP-1 cells that expressed "reference" form of Siglec-9 ($K^{100}A^{315}$) was significantly less than that from the cells expressing the risk variant ($E^{100}E^{315}$; Figure 4b).

The association between genetic variations of SIGLEC9 and COPD phenotypes in a Caucasian population

To explore whether the associations between *SIGLEC9* polymorphisms and exacerbation frequency and emphysema were generalizable to other ethnic populations, we examined subjects enrolled in the ECLIPSE study. Baseline characteristics of the cohort included in this analysis are summarized in Table 2.

There was no significant association between rs2075803 and exacerbation frequency during the first year of follow up in the ECLIPSE cohort. Similarly, we were unable to identify an association between this SNP and emphysema ($p = 0.5062$).

Discussion

In this study, we observed that the G allele of the cSNP rs2075803 and the GA haplotype (rs2075803 = G, rs2258983 = A) of *SIGLEC9* were associated with more frequent COPD exacerbations and more severe emphysema (assessed as LAA%) in a Japanese COPD population. The THP-1 cell line expressing the Siglec-9 $E^{100}E^{315}$ variant corresponding to the "risk" GA haplotype showed stronger TNFα response to viral infection than those expressing "reference" form (Siglec-9 $K^{100}A^{315}$). Thus, it appears likely that this *SIGLEC9* haplotype allows stronger pro-inflammatory responses by innate immune cells, making its carriers more vulnerable to exacerbation, which in turn may accelerate the development of emphysema.

Although our data demonstrates that the Siglec-9 $E^{100}E^{315}$ variant (GA haplotype) shows weaker inhibitory effects on the Fcγ receptor-mediated inflammatory response (TNFα production) as compared with the $K^{100}A^{315}$ variant (AC haplotype), it is not clear how these molecular events are connected. We initially hypothesized that the glycan binding property of Siglec-9 may be influenced by the amino acid substitutions and could modify the interactions between Siglec-9 and its ligands and thus affect downstream signaling. However, our data (Fig.

3) did not support this hypothesis. It is also possible that the interaction between Siglec-9 and its ligand is independent of sialic acids, as reported for CD22/Siglec-2 and B cell receptor [25], and is affected by the amino acid substitutions.

The relationships between *SIGLEC9* polymorphisms and COPD exacerbations in Japanese patients were not reproduced in the ECLIPSE cohort. Failure to replicate in the larger ECLIPSE cohort raises the possibility that the initial associations were false-positives. Additional possibilities for why the associations did not replicate include different criteria to define exacerbations (ECLIPSE did not use symptom diaries), and different distributions of mild/moderate/severe exacerbations between the study populations. While the allele frequencies of both cSNPs are similar in both populations, differences in environment and/or genetic background of the population may have influenced the exacerbation phenotype. Finally, it is possible that the cSNPs that we studied were actually markers for other functional variants in that genomic region, and the correlations with those potential functional variants could differ in our two study populations.

A recent paper reported that another cSNP in *SIGLEC9* gene (rs16988910) is

associated with emphysema in the African-American population [26]. Although this cSNP is rare among Asians or Europeans, the association of a functionally hypomorphic allele of *SIGLEC9* and emphysema in an independent human population appears to support our proposed model, in which excessive inflammatory response in innate immune cells leads to emphysema. However, we must acknowledge that the association between *SIGLEC9* cSNPs and emphysema in the Japanese population did not replicate in the larger ECLIPSE cohort. Resolving whether this discrepancy is due to false discovery in the Japanese population or pathophysiologically relevant differences between the two study populations would require a carefully designed replication study.

We speculate that both Siglec-9 and Siglec-14 are involved in COPD pathogenesis through modification of susceptibility towards exacerbations. The overall mechanism by which genetic polymorphisms in *SIGLEC9* (this study) and *SIGLEC14* [16] influences patient susceptibility toward exacerbation appears to be similar, in that the polymorphisms that allow stronger pro-inflammatory responses are associated with higher susceptibility to exacerbations. The exacerbation–susceptible genotypes of *SIGLEC9* and *SIGLEC14* were also associated with the extent of emphysema and airflow limitation, respectively. It was reported that frequent

exacerbation is associated with faster decline of lung function as measured by FEV1 [3] and also with progression of emphysema [4], which support our speculation.

In conclusion, we have observed that the $E^{100}E^{315}$ variant of Siglec-9 demonstrates reduced suppression of the inflammatory response induced by Fcγ receptor-mediated myeloid cell activation. Possibly through this mechanism, the combination of *SIGLEC9* genotypes which corresponds to $E^{100}E^{315}$ may be a risk factor for emphysema progression, possibly through promotion of exacerbations.

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1 Table 1. Basic characteristics of our study population

2

		Subset of COPD subjects with exacerbation data (n = 135)	All subjects (n = 362)
Age		69.3 (7.9)	67.8 (9.9)
Sex	M/F	127/8	323/39
Smoking status	Ex/current	130/5	298/64
	Pack-years	74.5 (47.6)	63.7 (43.2)
Pulmonary function tests			
VC	L	3.24 (0.85)	3.34 (0.88)
%VC	%	92.0 (18.6)	94.6 (17.2)
FEV1	L	1.61 (0.67)	1.98 (0.87)
FEV1/FVC	%	51.6 (12.3)	60.1 (15.8)
FEV1% predicted	%	57.8 (20.3)	70.5 (25.2)

RV/TLC	%	47.4 (9.2)	44.8 (9.4)
% DLCO/VA	%	60.2 (19.7)	66.6 (23.1)
COPD stages	At risk/I/II/III/IV	0/22/61/41/11	118/38/110/85/11
Computed tomography			
LAA% at -940 HU	%	34.4 (13.8)	28.8 (15.2)
Frequency of the exacerbations per year			
0	%	72	n.a.
1		21	
2		6	
3		1	
Minor allele frequency			
rs2075803 (A/G)	(A allele)	0.40	0.42
rs2258983 (A/C)	(A allele)	0.60	0.58

1 Note: All values are presented as means (SD). Computed tomography data were obtained from

355 subjects.

Abbreviations: VC, vital capacity; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; DLCO/VA, diffusing capacity divided by the alveolar volume; LAA%, percentage of the low-attenuation area; n.a., not applicable

Table 2 Characteristics of ECLIPSE subjects

N	1764
Age	63.6 (7.1)
Sex (M/F)	1182 / 582
Smoking Status (former/current)	1138 / 626
Pack-years	50.3 (27.4)
FEV_1 (L)	1.33 (0.51)
FEV_1 % predicted	47.6 (15.6)
FEV_1/FVC ratio	44.7 (11.6)
GOLD Stage (II/III/IV)	741 / 770 / 252
Percent emphysema (LAA% at -950 HU)	18.4 (12.2)
rs2075803 allele frequency (A allele)	0.41

rs2258983 allele frequency (A allele)	0.59
Number of exacerbations in Year 1 of follow-up	1.3 (1.5)

1 Data are presented as mean (SD) unless otherwise noted.

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Figure legends

Figure 1. Effect of *SIGLEC9* genotype on the frequency of COPD exacerbations.

The frequency of exacerbations in each genotype of rs2075803 in *SIGLEC9* is shown.

Figure 2. *SIGLEC9* genotype and emphysema severity.

The percentage of the low-attenuation area (LAA% at -940 HU), which indicates the severity of emphysema, in each genotype of rs2075803 in *SIGLEC9* is shown. Values are presented as mean +/- standard deviation.

Figure 3. Effect of SNPs on the Siglec-9 binding to glycans.

Recombinant soluble Siglec-9 proteins with amino acid variations ($K^{100}A^{315}$ and $E^{100}E^{315}$) corresponding to the two major haplotypes (AC and GA) were prepared, and their binding to synthetic glycan probes were analyzed as described in Materials and Methods. The structure of oligosaccharides attached to each probe is shown in the inset.

Assays were carried out in triplicate (i.e., 3 wells for each combination of protein and probe). Error bars represent standard error of means. ###: $p < 0.001$, ##: $p < 0.01$, #: $p < 0.05$, ns: not

significant, as compared with the control probe binding to the same protein by one-way ANOVA followed by Dunnett's post-test. ***: $p < 0.001$, ns: not significant ($p > 0.05$), comparing the Neu5Acα2-3Galβ1-4Glc probe binding to two proteins, by one-way ANOVA followed by Tukey post-test. The assay was repeated twice independently, with consistent results.

Figure 4. Effect of SNPs on the anti-inflammatory function of Siglec-9.

Full-length Siglec-9 proteins with amino acid variations ($K^{100}A^{315}$ and $E^{100}E^{315}$) corresponding to the two major haplotypes (AC and GA) were expressed on THP-1 cell line, and their effects on (A) IL-8 and (B) TNFα production elicited by incubation with influenza A (A/PR/8/34) virus-infected BEAS2B cells and anti-hemagglutinin antibody were evaluated as described in Materials and Methods.

Assays were carried out in octuplicates (i.e., 8 wells per cell line). Error bars represent standard error of means. ***: $p < 0.001$, *: $p < 0.05$, ns: not significant ($p > 0.05$), by one-way ANOVA followed by Tukey post-test. The assay was repeated 3 independent times, with consistent results.