LETTERS

Functionally defective germline variants of sialic acid acetylesterase in autoimmunity

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Sialic acid acetylesterase (SIAE) is an enzyme that negatively regulates B lymphocyte antigen receptor signalling and is required for the maintenance of immunological tolerance in mice[1,2]. Heterozygous loss-of-function germline rare variants and a homozygous defective polymorphic variant of *SIAE* were identified in 24/923 subjects of European origin with relatively common autoimmune disorders and in 2/648 controls of European origin. All heterozygous loss-of-function *SIAE* mutations tested were capable of functioning in a dominant negative manner. A homozygous secretion-defective polymorphic variant of *SIAE* was catalytically active, lacked the ability to function in a dominant negative manner, and was seen in eight autoimmune subjects but in no control subjects. The odds ratio for inheriting defective *SIAE* alleles was 8.6 in all autoimmune subjects, 8.3 in subjects with rheumatoid arthritis, and 7.9 in subjects with type I diabetes. Functionally defective *SIAE* rare and polymorphic variants represent a strong genetic link to susceptibility in relatively common human autoimmune disorders.

Our previous studies showed a defect in B-cell tolerance as evidenced by the spontaneous development of autoantibodies in *Siae* mutant mice on a C57BL/6 background[1]. Given this phenotype we sought to ask if this enzyme was linked to autoimmunity in human subjects. Although genome-wide association studies had not revealed altered frequencies of common variants of *SIAE* in patients with autoimmunity, the possibility that loss-of-function rare variants of this gene might be enriched in patients with autoimmune disorders was addressed by complete re-sequencing of all the exons of *SIAE* in patients with autoimmunity and in healthy controls.

In the first phase of our studies we completely re-sequenced the *SIAE* gene from 188 subjects with autoimmunity and 190 healthy controls as described below. Initially we analysed 19 subjects from Massachusetts General Hospital (MGH) selected only on the basis of their having high antinuclear antibody titres. Of these 19 subjects, 13 had defined autoimmune disorders and were included in our studies. In this initial set of 13 subjects of European ancestry, unique non-synonymous changes were observed in one subject with Crohn's disease and in one subject with rheumatoid arthritis. As a result of these preliminary observations, we next analysed 10 more subjects from MGH with rheumatic disorders (rheumatoid arthritis, systemic lupus erythematosus and Sjogren's syndrome), 76 subjects of European ancestry with rheumatoid arthritis from the NARAC (North American Rheumatoid Arthritis Consortium) collection, and 89 subjects with inflammatory bowel disease from MGH, making an initial total of 188 autoimmune subjects. The only criterion used in selection was ethnicity. The control DNAs in this initial phase were obtained from 190 healthy volunteers at MGH primarily of European ancestry. Re-sequencing of all 10 exons of *SIAE* revealed the existence of point substitutions in *SIAE* in both patients and controls. A number of known small nucleotide polymorphisms (SNPs) were identified as expected (Supplementary Table 1). A total of 19 out of 923 autoimmune subjects presented with one of 14 previously unidentified non-synonymous SNPs in the *SIAE* gene, whereas 8 other autoimmune subjects had a homozygous polymorphism resulting in a valine replacing methionine at position 89 (Table 1). Among control subjects, 17/648 presented with one of 8 non-synonymous SNPs in the *SIAE* gene. No controls presented with the homozygous 89V/89V polymorphic form of SIAE (Table 1). Functional analyses were performed on each SIAE variant as described below.

Because the initial analyses showed a marked enrichment of loss-of-function *SIAE* variants in autoimmune subjects compared with controls, a larger number of autoimmune subjects and controls were analysed. Power calculations showed that a sample size of 550 cases and 550 controls would be required to obtain a power of at least 0.80 (see power table and calculations in Supplementary Information). Autoimmune subjects in this second phase included more subjects with rheumatoid arthritis from NARAC, subjects from the MADGC (Multiple Autoimmune Disease Genetics Consortium) collection with systemic lupus erythematosus and juvenile idiopathic arthritis, and subjects from MGH with inflammatory bowel disease and rheumatic disorders including systemic lupus erythematosus, rheumatoid arthritis, mixed connective tissue disorder and Sjogren's syndrome. We also included subjects with multiple sclerosis from a collection at the

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Table 1 | *SIAE* variants identified in autoimmune subjects of European origin and controls

SIAE change	Esterase activity	Secretion	Dom. neg.	Disease	Source
Autoimmune patients (*n* = 923)					
T312M	Defective	Defective	Yes	RA	MGH
T312M	Defective	Defective	Yes	MS	BWH
Q309P	Defective	Defective	Yes	RA	NARAC
C196F	Defective	Defective	Yes	RA	NARAC
C196F	Defective	Defective	Yes	SjS	MGH
M89V/M89V	Normal	Defective	No	RA	NARAC
M89V/M89V	Normal	Defective	No	RA	NARAC
M89V/M89V	Normal	Defective	No	RA	NARAC
M89V/M89V	Normal	Defective	No	SLE	MADGC
M89V/M89V	Normal	Defective	No	MS	BWH
M89V/M89V	Normal	Defective	No	T1D	NIH
M89V/M89V	Normal	Defective	No	T1D	NIH
M89V/M89V	Normal	Defective	No	T1D	NIH
G212R	Defective	Defective	Yes	CD	MGH
F404S	Defective	Defective	Yes	JIA	MADGC
F404S	Defective	Defective	Yes	SLE	MADGC
F404S	Defective	Defective	Yes	UC	MGH
F404S	Defective	Defective	Yes	MS	BWH
Y349C	Defective	Reduced	Yes	SLE	MADGC
R479C	Defective	Defective	Yes	CD	MGH
W48X	Truncated/NT	Truncated/NT	NT	T1D	NIH
C266G	Defective	Defective	NT	T1D	NIH
R230W	Defective	Defective	NT	T1D	NIH
R393H	Defective	Defective	NT	SLE	MGH
K400N	Normal	Doublet	No	CD	MGH
A3G	Normal	Normal	NT	CD	MGH
N33S	Normal	Normal	NT	RA	NARAC
Ethnically matched controls (*n* = 648)					
R314H	Defective	Defective	NT	Control	NS/LIJ
T312M	Defective	Defective	Yes	Control	NS/LIJ
Q161K	Normal	Normal	NT	Control	MGH
G64S	Normal	Normal	NT	Control	MGH
G64S	Normal	Normal	NT	Control	MGH
G64S	Normal	Normal	NT	Control	MGH
G64S	Normal	Normal	NT	Control	MGH
G64S	Normal	Normal	NT	Control	MGH
G64S	Normal	Normal	NT	Control	NS/LIJ
G64S	Normal	Normal	NT	Control	BWH
G64S	Normal	Normal	NT	Control	BWH
G64S	Normal	Normal	NT	Control	BWH
G64S	Normal	Normal	NT	Control	BWH
Q462R	Normal	Normal	NT	Control	MGH
H447R	Normal	Normal	NT	Control	MGH
R62H	Normal	Normal	NT	Control	NS/LIJ
M456I	Normal	Normal	NT	Control	NS/LIJ

BWH, Brigham and Womens' Hospital; CD, Crohn's disease; JIA, juvenile idiopathic arthritis; MADGC, Multiple Autoimmune Disorders Genetics Consortium; MGH, Massachusetts General Hospital; MS, multiple sclerosis; NARAC, North American Rheumatoid Arthritis Consortium; NIH, National Institutes of Health; NS/LIJ, North Shore Long Island Jewish; NT, not tested; RA, rheumatoid arthritis; SjS, Sjogren's syndrome; SLE, systemic lupus erythematosus; T1D, type 1 diabetes; UC, ulcerative colitis.

Brigham and Womens' Hospital, and subjects with type 1 diabetes from the EDIC (Epidemiology of Diabetes Intervention and Complication study) collection of the NIDDK (National Institute of Diabetes, Digestive and Kidney Diseases). Additional healthy control DNAs, primarily from subjects of European ancestry, were obtained from the MGH Cancer Center, the Feinstein Institute and the phenogenetic collection at Brigham and Womens' Hospital.

To determine whether variants were functional or defective, we recreated the changes corresponding to all the coding *SIAE* variants that we had discovered in patients and controls into a carboxy-terminal Flag-tagged human *SIAE* cDNA cloned from MDA-MB 231 cells. Each cDNA was transfected into 293T cells, and lysates and supernatants were each divided into two equal aliquots. SIAE was immunoprecipitated with anti-Flag antibodies, and one aliquot was saved for a quantitative western blot assay whereas the other was used in an esterase assay using a fluorogenic substrate, 4-methylumbelliferyl acetate. Quantitative western blotting was performed using a near-infrared dye-labelled second antibody and detected using the LI-COR Odyssey system. Each cDNA was transfected three or more times and the entire assay performed on at least three occasions for each cDNA.

As described in Table 1 we have now identified 27/923 autoimmune subjects with either rare heterozygous non-synonymous substitutions in *SIAE* that do not represent known SNPs or a specific defective homozygous polymorphism. In 24 of these patients the *SIAE* variants were found to be functionally defective either because of a defect in catalytic activity to below 50% of wild type or because of a profound defect in secretion (in the absence of a catalytic defect). A group of missense variants that are severely catalytically defective include c.935C>T, c.587G>T, c.926A>C, c.634G>A, c.1435C>T, c.1178G>A, and c.688C>T and encode SIAE(T312M), SIAE(C196F), SIAE(Q309P), SIAE(G212R), SIAE(R479C), SIAE(R393H), and SIAE(R230W). The analysis of these severely catalytically defective variants by transfection, immunoprecipitation, enzyme assays and immunoblot assays are shown in Fig. 1 and Supplementary Fig. 3. These variants are also very poorly secreted presumably because they are grossly misfolded proteins that fail to egress the endoplasmic reticulum.

More modest, but reproducible catalytic defects were seen in the c.1046A>G variant that encodes SIAE(Y349C), and this variant also exhibits reduced secretion (see Fig. 1, bottom panels). The c.1211T>C variant encodes SIAE(F404S) that also seems to exhibit a less severe catalytic defect (but nevertheless below the 50% cutoff set), similar to that seen in SIAE(Y349C) (Fig. 1). SIAE(F404S) was found in four autoimmune patients including two blood relatives, one with systemic lupus erythematosus and the other with juvenile idiopathic arthritis. The c.796T>G variant found in one subject with type I diabetes encodes SIAE(C266G) that is also defective (Fig. 1).

In contrast to the catalytically defective *SIAE* variants seen in patients with autoimmune diseases, with the exception of two variants (R314H and T312M) observed once each in controls (Table 1), most of the new *SIAE* variants found in normal subjects did not exhibit reduced catalytic activity, as shown in Fig. 2. Interestingly, the protein encoded by the 89V polymorphic allele of *SIAE* is catalytically active but is not secreted (see Fig. 2, third set of panels). The 89V polymorphism is quite common in controls in the heterozygous state (9.7%, see Supplementary Table 1). To more precisely establish that SIAE(M89V) is secretion-defective, 293T cells transfected with wild type and *SIAE(M89V)* respectively were metabolically labelled with [^{35}S]methionine and chased for 10 min, 1 h, 2 h and 4 h. As seen in Fig. 3b, a striking defect in secretion of SIAE(M89V) was confirmed by this analysis.

Because SIAE exists as a dimer or higher order oligomer (Supplementary Fig. 2), we examined whether catalytically dead mutants from patients with autoimmunity on the one hand, and the catalytically active but secretion-defective M89V variant on the other, could function in a dominant interfering manner (Fig. 3a). The *K400N* allele (Fig. 1) was also tested as a representative catalytically normal *SIAE* allele. Because the ultimate test of dominant negative function would be to recreate a heterozygous animal with one mutant allele, mutations were recreated in a murine *Siae* cDNA for these studies. As shown in Fig. 3a and Supplementary Fig. 1, the murine equivalents of the C196F, G212R, Q309P, T312M, Y349C, F404S and R479C variants are capable of dominantly inhibiting wild-type SIAE whereas SIAE(M89V) and SIAE(K400N) are not. On the basis of this finding it was clear that only subjects with homozygous 89V/89V SIAE polymorphisms (as opposed to subjects with heterozygous *M89V* changes) should be considered to be of potential functional relevance for predisposition to autoimmunity.

Strikingly, eight autoimmune subjects (three with rheumatoid arthritis, one with systemic lupus erythematosus, one with multiple sclerosis, and three with type I diabetes) are homozygous for c.[265A>G]+[265A>G] polymorphic alleles, which encode 89V/89V SIAE variants, whereas these homozygous genotypes were not observed in a single control. Given the defect in secretion of this variant, we consider it likely that in subjects with homozygous 89V/89V SIAE this esterase is unlikely to be able to effectively access the post-Golgi compartment in which it would normally de-acetylate 9-*O*-acetylated sialoproteins that serve as CD22 ligands. Hardy–Weinberg equilibrium tests for the *M89V* polymorphism showed a deviation from equilibrium for the cases but not for the controls (Supplemen-

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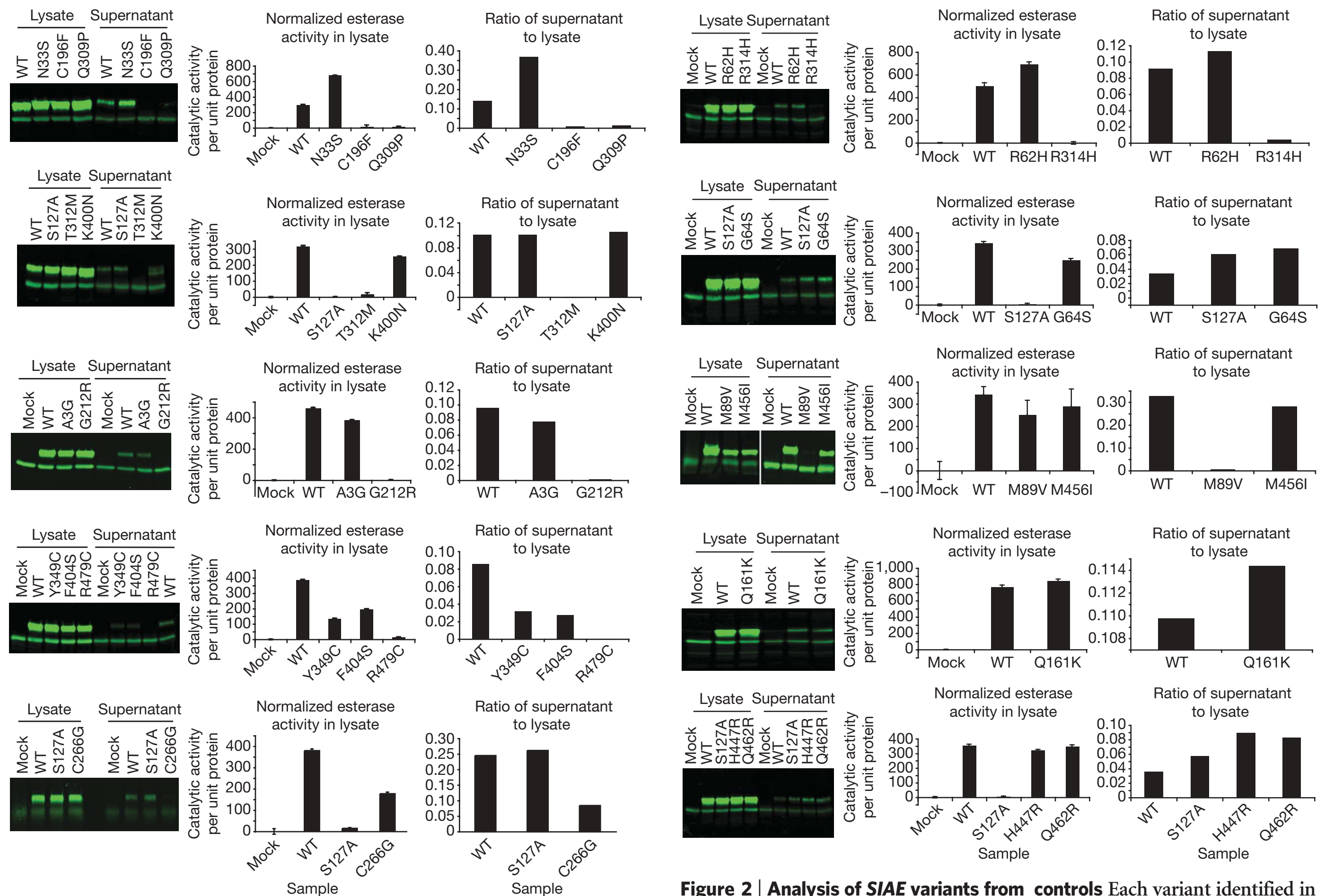

Figure 1 | Analysis of *SIAE* variants from subjects with autoimmunity. Each *SIAE* variant found in subjects with autoimmunity was re-created by site-directed mutagenesis in a human *SIAE* cDNA, that was then sequenced along its entire length. Wild-type (WT) *SIAE*, a known catalytic site mutant (*SIAE(S127A)*, ref. 12), and each *SIAE* variant that was unique to autoimmune subjects were transfected into 293T cells. Assays were performed for *SIAE(A3G)*, *SIAE(N33S)*, *SIAE(C196F)*, *SIAE(G212R)*, *SIAE(C266G)*, *SIAE(Q309P)*, *SIAE(T312M)*, *SIAE(Y349C)*, *SIAE(K400N)*, *SIAE(F404S)* and *SIAE(R479C)*. Quantitative western blot analysis (using anti-Flag antibodies) was performed on both the cell lysate and the culture supernatant, and a ratio of these two measurements is shown in the right hand panels of the figure. 'Mock' refers to cells that were not transfected but from which lysate and supernatant were analysed. Half of each lysate was immunoprecipitated with anti-Flag antibodies and examined for esterase activity, presented following normalization for lysate SIAE protein content. Each row shows results from one representative transfection. Each variant was tested in this manner on at least three or more occasions to ensure reproducibility. Data shown are mean ± s.e.m.

Figure 2 | Analysis of *SIAE* variants from controls Each variant identified in control subjects was recreated in an *SIAE* cDNA as described above for subjects with autoimmunity. Wild-type (WT) *SIAE*, *SIAE(S127A)* and each *SIAE* variant that was unique to controls (*SIAE(R62H)*, *SIAE(G64S)*, *SIAE(Q161K)*, *SIAE(R314H)*, *SIAE(H447R)*, *SIAE(M456I)* and *SIAE(Q462R)*) was transfected into 293T cells. Also shown are results from *SIAE(M89V)*, which was found in heterozygous form in both patients and controls and in homozygous form only in patients. *SIAE(T312M)* was observed in one control and in two patients. Results for this variant are included in Fig. 1. Analyses were performed as described in the legend for Fig. 1. Data shown are mean ± s.e.m.

tary Tables 2a–c). This deviation is statistically significant. Given the overall similarities in the *89V* and *89M* allele frequencies in cases and controls this clearly reflects an enrichment of *89V* homozygotes in autoimmune subjects, strongly supporting a role for this homozygous polymorphism in disease susceptibility.

A number of *SIAE* rare variants were found in patients with autoimmunity that are probably not involved in the genetic predisposition of these subjects to autoimmunity. For example, a c.98A>G variant encoding SIAE(N33S) was discovered in a patient with rheumatoid arthritis and was found to be functionally normal on the basis of the criteria used (Fig. 1, top panels). One patient with Crohn's disease inherited a c.8C>G variant encoding an A3G change in the signal peptide encoding portion of *SIAE*. The coding region of *SIAE* would be predicted to be intact in this variant though it is theoretically possible that SIAE(A3G) might not be readily translocated into the ER. We consider it unlikely that SIAE(A3G) is translocation-defective given the accumulation of SIAE in culture supernatants when *SIAE(A3G)* is transfected into 293 T cells (Fig. 1, third set of panels). A c.1200G>T variant that encodes SIAE(K400N) was discovered in a patient with Crohn's disease initially examined as part of a small subset of patients with high antinuclear antibody titres. This enzyme is active and is efficiently secreted but always appears in supernatants as a protein doublet (Fig. 1, second set of panels). Lysine 400 is immediately adjacent to a consensus N-linked glycosylation site, and it may be that a particular N-glycan is added inefficiently in this variant. This variant is however catalytically active and we classify it as a non-defective allele.

An absolute correlation was not found between conservation of amino acid residues of SIAE across species and a requirement for catalytic activity. Of the 11 heterozygous variants that were found to be defective in autoimmune patients only one (C266G) was not conserved between primates and rodents. One of three catalytically normal variants identified in autoimmune subjects (SIAE(N33S)) was also not conserved across species.

In the first phase of this study defective variants were identified in 7/188 autoimmune patients and 0/190 controls. The odds ratio could only be calculated as an estimate (Peto odds ratio), and this approach yielded an odds ratio of 7.71. In the second phase of the study 17/735 autoimmune patients and 2/458 controls inherited defective *SIAE* alleles, and the calculated odds ratio was 5.40. In summary, the total number of patients with autoimmune disorders analysed was 923,

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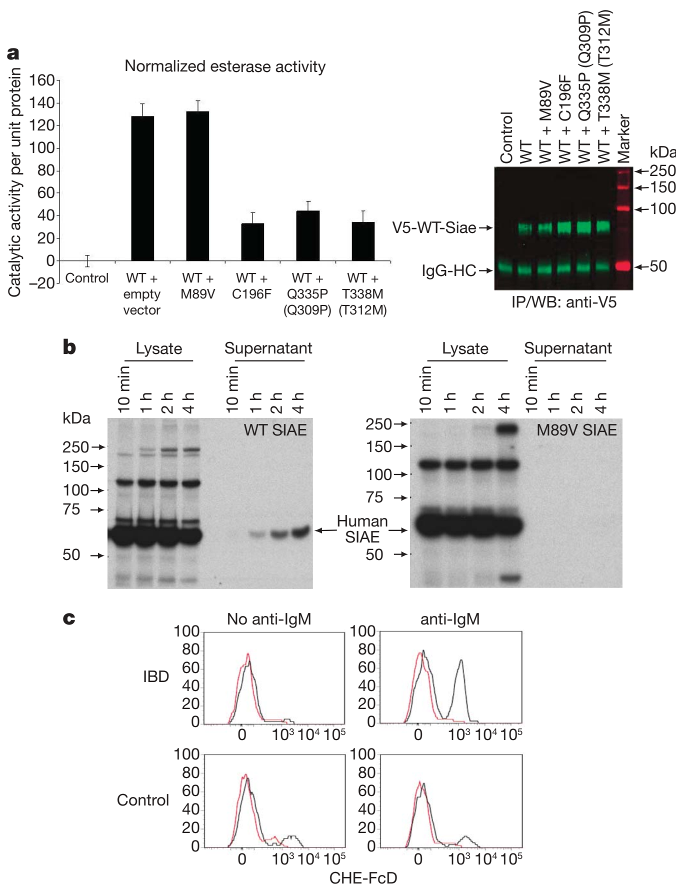

Figure 3 | Analysis of SIAE mutants in terms of secretion, *in vitro* dominant interfering activity, and effect on induced cell surface 9-*O*-acetylation of sialic acid a, Murine Siae(C196F), and the murine equivalents of SIAE(Q309P) and SIAE(T312M), (Q335P and T338M Siae), function in a dominant interfering fashion but Siae(M89V) does not. V5-tagged wild-type Siae was transfected along with Flag-tagged Siae(C196F) or Flag-tagged Siae(M89V) and the enzyme activity of V5-tagged wild-type Siae was assessed in transfectants as a function of its protein level. Expression of mutant Siae was monitored by an anti-Flag western blot of immunoprecipitated mutant proteins (IP/WB; see Supplementary Fig. 4). Data shown are mean ± s.e.m. **b**, Pulse-chase analysis comparing secretion of wild-type SIAE and SIAE(M89V). Transfected 293T cells were metabolically pulse-labelled with [^{35}S]methionine and lysates and supernatants were immunoprecipitated with anti-Flag antibodies after 10 min, 1 h, 2 h and 4 h of chase. Proteins were separated by SDS–PAGE and revealed by autofluorography. The position of molecular mass markers is indicated on the left in kilodaltons. **c**, Enhanced 9-*O*-acetylation of sialic acid following BCR ligation in B cells from a subject with a defective *SIAE* mutation. Naive ($CD19^{+}CD27^{-}$) B cells from the peripheral blood of a subject with Crohn's disease (labelled IBD) with a heterozygous *SIAE* mutation (G212R) and from a control subject were analysed for cell surface 9-*O*-acetylation with and without anti-IgM induced BCR ligation. Cell surface 9-*O*-acetylation was detected using CHE-FcD staining approach as described in Methods. The black tracing reflects CHE-FcD staining and the red represents staining with the second antibody alone.

with 24/923 inheriting defective *SIAE* alleles, and the total number of ethnically matched controls was 648, with 2/648 controls inheriting defective *SIAE* alleles. The calculated odds ratio for all autoimmune disorders was 8.62 with a two-sided *P*-value of 0.0002.

While care was taken to include only non-Jewish subjects of European origin in this study, an objective determination of shared ethnicity, a principal components analysis[3,4], was conducted on samples with defective *SIAE* alleles and on controls (see Supplementary Fig. 5). The novel *SIAE* variants that we have observed in subjects with autoimmunity cannot be ascribed to population stratification with respect to controls.

Seven of the 648 controls inherited a non-synonymous rare variant of *SIAE* but only 2/648 inherited defective alleles (Fig. 2 and Table 1). One of the rare variants (c.935C>T encoding SIAE(T312M)) found in one of the controls (all 10 exons were sequenced in 648 controls) was identical to a defective variant originally found in a patient with rheumatoid arthritis and also in a patient with multiple sclerosis. Another variant, (c.941G>A encoding SIAE(R314H)), was found in a single control and was also found to be defective (Fig. 2). The remaining rare variants found in controls (c.1340A>G, c.481C>A, c.185G>A, c.1368G>A, and c.1385A>G encoding the H447R, Q161K, R62H, M456I and Q462R versions of SIAE respectively) were completely normal as determined by the two assays described (see Fig. 2 and Table 1 for a summary). A polymorphic variant c.190G>A, encoding SIAE(G64S), exhibits normal catalytic activity and is readily secreted (Fig. 2 and Table 1). It was found in controls, but not in autoimmune subjects (Table 1 and Supplementary Table 1). It might in theory convey protection against disease, but a biochemical basis for such a possible protective role is unclear.

All the defective heterozygous *SIAE* alleles in patients that were tested function in a dominant interfering manner in the transfection assay used. While high odds ratios were observed in a number of autoimmune disorders, the results from rheumatoid arthritis subjects (odds ratio, 8.3; two-tailed $P = 0.0056$) and type I diabetes patients (odds ratio, 7.9; two-tailed $P = 0.0075$) were particularly significant. Although a dominant negative effect may contribute to disease susceptibility for all tested defective heterozygous variants, we also consider haploinsufficiency to be a possible mechanism for some of the defective variants which might result in the reduction of levels of catalytically active enzyme in B cells below a threshold. This would imply that the W48X alteration found in a patient with type I diabetes (Table 1) may be clinically relevant. We plan to examine the effects of haploinsufficiency in mutant mice both on a C57BL/6 background as well as in a lupus-prone background.

A number of susceptibility loci for human autoimmune disorders have been uncovered by genome-wide association studies, and the relative risks for these associations are generally modest[5,6]. A number of recent reports have supported the hypothesis that rare genetic variants can contribute to disease susceptibility. A pioneering study on rare variants in genes that are relevant to lipoprotein synthesis in patients susceptible to cardiovascular disease used a predictive algorithm (Polyphen) to determine which variants were probably non-functional[7]. Loss-of-function variants in the *TREX* gene, which has a single coding exon, have also been described in patients with systemic lupus erythematosus[8] and a recent re-sequencing study has revealed rare variants in the cytosolic helicase MDA5/IF1H1, which mediates innate immune responses to pathogen-encoded RNAs[9]. Our results provide important support for a role for rare variation in the predisposition to autoimmune diseases and strikingly illustrate the importance of performing functional assays for the variants being studied. All of the variants identified through re-sequencing are listed in Supplementary Table 1. There is clear enrichment of defective coding variants in autoimmune patients compared to controls (Table 2), and it is notable that these variants primarily involved residues that are highly conserved across evolution. While our initial studies strongly support a role for defective *SIAE* rare and polymorphic variants in rheumatoid arthritis and type 1 diabetes, a role for these variants in other autoimmune disorders including systemic lupus erythematosus, multiple sclerosis and inflammatory bowel disease seems likely (Supplementary Table 3). More extensive studies on these and other autoimmune disorders are called for.

The contribution of B cells to disease, with or without a role for autoantibodies, is recognized in a growing number of diseases including rheumatoid arthritis, multiple sclerosis, and type 1 diabetes. Mutant *Siae* in rodents results in enhanced B-cell activation and a break in B-cell tolerance[1], but it remains formally possible that SIAE may be required in cell types other than B cells in humans as well as in rodents. One type of inflammatory bowel disease, Crohn's disease, like multiple sclerosis, is generally considered to be aetiologically linked to T_H1 or T_H17 cells[10]. Although B-cell depletion can result in marked clinical

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Table 2 | Functionally defective SIAE coding variants in rheumatoid arthritis, type I diabetes and all autoimmune diseases combined compared with controls*

Disease group	Number of subjects	Odds ratio (95% CI**)	Two-tailed *P*-value***
Rheumatoid arthritis	234	8.31 (1.69–40.87)	0.0056
Type I diabetes	252	7.89 (1.58–39.30)	0.0075
All autoimmune disorders	923	8.62 (2.03–36.62)	0.0002

* Patients and controls ($n = 648$) were of European ancestry; Jewish subjects were not included in these analyses.
** 95% CI = 95% Confidence interval.
*** Two-tailed *P*-value was determined using Fisher's exact test.

improvement in patients with multiple sclerosis[11], B cells are not generally considered to be of etiopathogenic significance in Crohn's disease; it remains formally possible that autoantigen-specific B cells may function as critical antigen-presenting cells that secrete cytokines, driving helper T-cell polarization in certain disease situations. Interestingly, analysis of B cells from a Crohn's disease patient harbouring a catalytically defective heterozygous *SIAE* variant (SIAE(G212R); see Table 1) revealed a marked enhancement of cell-surface 9-*O*-acetyl sialic acid following B cell receptor (BCR) activation compared to B cells from a control subject (Fig. 3c). Enhanced 9-*O*-acetylation of sialic acid on B cells was also noted in a patient with Sjogren's syndrome with a heterozygous SIAE(C196F) variant, and in a patient with lupus with a SIAE(R393H) variant (Table 1; Supplementary Fig. 6). These analyses indicate that a defect in SIAE results in enhanced BCR-mediated expression of surface 9-*O*-acetylated sialic acid, whereas the presence of normal SIAE prevents this enhanced 9-*O*-acetylation event. This phenomenon is being explored further in a range of subjects with autoimmune disorders. Further analyses will be necessary to determine whether there is a role for B cells in disease pathogenesis in a subset of patients with inflammatory bowel disease.

SIAE contributes to a signalling mechanism that helps set a threshold for B-cell activation, presumably preventing weakly self-reactive B cells from moving towards the T-cell zone and consequently being at risk for somatic mutation and for the potential generation of high affinity self-reactive B cells; alternatively, SIAE may possibly help maintain tolerance in germinal centres[1,2]. The strong association of defective *SIAE* alleles to rheumatoid arthritis and type I diabetes may well represent only the tip of the iceberg for a pathway that includes Lyn, SHP-1, a sialic acid acetyltransferase, SIAE, CD22 and probably other sialic-acid-binding immunoglobulin-like lectins expressed in B cells[2]. The possibility that SIAE may be of functional relevance in innate immune cells and thus influence disease pathogenesis also deserves exploration.

METHODS SUMMARY

Sequencing. Each exon of *SIAE* was amplified from genomic DNA from individual subjects and subjected to automated sequencing as described[13,14].

Functional assays. Each *SIAE* variant (other than the W48X truncation) was recreated by site-directed mutagenesis as a C-terminal Flag-tagged human *SIAE* cDNA in an expression vector and transfected into 293T cells. Protein was quantified in lysates and supernatants by immunoprecipitation and western blotting to quantify secretion at steady state. Esterase activity of immunoprecipitated SIAE variants was analysed by a fluorometric assay[15] and normalized to protein levels.

For analysis of dominant-negative SIAE function, each defective disease-associated variant previously tested as a human cDNA was recreated in a murine *Siae* cDNA as a Flag-tagged *Siae* variant. Each Flag-tagged variant was co-transfected with wild-type murine V5-tagged *Siae*. The catalytic activity of wild-type Siae in the presence of each variant was normalized to wild-type Siae expression.

Surface 9-*O*-acetyl sialic acid on B lymphocytes was detected using flow cytometry and the CHE-FcD reagent[16].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.P. was responsible for overall study design and writing the manuscript. S.P. and A.C. initiated this study. S.P.P., H.L., J.M., D.R.D., D.W.B., S.L., T.G., M.E.M., K.N.T., R.E., A.C., E.D. and S.P. contributed to sequencing and sequence analysis. I.S. cloned the full-length human SIAE. I.S., V.C., S.D. and I.N. performed mutagenesis, and I.S., S.P.P., K.H., V.C., K.N.T. and A.C. performed functional analyses. V.C. performed association studies, dominant negative analyses and metabolic labelling studies. J.F., A.L. and P.K.G. performed the Principal Components Analysis, and M.M., P.K.G., J.H.S., T.W.B., B.S., D.K.P., J.K., D.H., P.L.D.J., D.C. and D.B. provided annotated clinical material. A.V. provided advice on enzymology. Statistical analyses were performed by Y.C., I.N. and S.P.

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METHODS

Analysis of the sequence of *SIAE*. Each exon of *SIAE* was amplified from genomic DNA from individual subjects and subjected to automated sequencing. Residue numbering was based on ENST00000263593 (Ensembl), corresponding to the GenBank accession number NP_733746. Genomic DNA was extracted from clotted blood specimens from patients with autoimmune disease using a QIAamp DNA blood mini kit (Qiagen) according to the manufacturer's instructions. All 10 exons of the human *SIAE* gene were amplified by PCR using intronic primers (Supplementary Table 4). Purification of the amplified products, bi-directional automated sequencing and sequence analysis were performed as described earlier[13]. All sequence variants were confirmed by sequencing at least two independent PCR amplicons. DNAs from controls were obtained from Epstein–Barr virus-immortalized lymphoblastoid cell-lines established from healthy blood donors[14]. All blood samples were collected with approval from the MGH/Partners Human Studies Institutional Review Board and from the Institutional Review Board at North Shore Long Island Jewish Health System.

Site-directed mutagenesis of human *SIAE* and assays for SIAE catalytic activity and secretion. A human *SIAE* cDNA was cloned from MDA-MB 231 cells and a full-length Flag-tagged human *SIAE* expression construct (in pcDNA3.1) was generated. This clone was targeted for mutagenesis using PfuTurbo DNA polymerase (Stratagene). Each *SIAE* variant (other than the W48X truncation) was recreated by site-directed mutagenesis as a C-terminal Flag-tagged human *SIAE* cDNA in an expression vector. Site-directed mutagenesis was used to create the S127A variant with a defect in the catalytic site as well as each of the variants listed in Table 1. The PCR products were digested overnight with DpnI (10 U; Stratagene) and transformed into TOP10 chemically competent cells (Invitrogen). Clones containing the mutants were verified by DNA sequencing. All mutant and wild-type cDNAs were transfected into HEK 293T cells. Lysates and supernatants were immunoprecipitated with anti-Flag antibodies and catalytic activity of the immunoprecipitated esterase was assayed by a fluorometric method[15]. Equivalent amounts of each lysate and supernatant were immunoprecipitated for the catalytic assay as well as for quantification of the Flag-tagged protein by an immunoblot assay on the LI-COR Odyssey, using a mouse monoclonal anti-Flag antibody (Sigma) and an IR Dye 800CW labelled Goat anti-mouse IgG (LI-COR) as a secondary antibody. Immunoprecipitation, metabolic labelling and pulse-chase studies were performed as described in ref. 1.

Assays for determining the dominant negative function of specific SIAE variants. These assays were carried out by cotransfecting cDNAs encoding V5-tagged wild-type murine Siae, together with Flag-tagged murine versions of *SIAE* mutants discovered in subjects with rheumatoid arthritis into 293T cells. The V5-tagged wild-type proteins in cell lysates were immunoprecipitated using mouse monoclonal anti-V5 antibody (Invitrogen), for quantitative immunoblot and esterase activity assays. Expression of Flag-tagged mutants was also monitored by immunoprecipitation and western blot assays.

Analysis of cell surface 9-*O*-acetylation of sialic acid on human B lymphocytes. The method used was described in murine lymphocytes in ref. 1 and is based on a method described previously[16]. Briefly, human B cells were stained with antibodies to CD19 and CD27 (BD Pharmingen) and incubated either with or without $F(ab')_2$, polyclonal rabbit anti-human IgM (Dako). Cells were also stained either with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$, goat anti-human IgG, Fcγ-specific (Jackson Immunoresearch) alone, or the CHE-FcD reagent (an influenza C haemagglutinin esterase fused to the Fc portion of human IgG, chemically treated with diisopropyl fluorophosphate) complexed with FITC-$F(ab')_2$ fragment goat anti-human IgG, Fcγ-specific. Cells were analysed by flow cytometry.

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