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## Functionally defective germline variants of sialic acid acetyltransferase in autoimmunity

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**Author Contributions** SP was responsible for overall study design and writing the manuscript. SPP, HL, JM, DRD, DWB, SL, TG, MEM, KNT, RE, AC, ED and SP contributed to sequencing and sequence analysis. Full length human SIAE was cloned by IS. IS, VC, SD, and IN performed mutagenesis, and IS, SPP, KH, VC, KNT, and AC performed functional analyses. Association studies, dominant negative analyses, and metabolic labeling studies were performed by VC. JF, AL, and PKG, performed the Principal Components Analysis, and MM, PKG, JHS, TWB, BS, DKP, JK, DH, PD, DC and DB provided annotated clinical material. AV provided advice on enzymology. Statistical analyses were performed by YC, IN and SP.

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## Abstract

Sialic acid acetyltransferase (SIAE) is an enzyme that negatively regulates B lymphocyte antigen receptor signaling and is required for the maintenance of immunological tolerance in mice<sup>1, 2</sup>. Heterozygous loss-of-function germline rare variants and a homozygous defective polymorphic variant of *SIAE* were identified in 24/923 Caucasian subjects with relatively common autoimmune disorders and in 2/648 Caucasian controls. 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 8 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 revealed a defect in B cell tolerance as evidenced by the spontaneous development of autoantibodies in *Siae* mutant mice on a C57BL/6 background<sup>1</sup>. 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 analyzed 19 subjects from Massachusetts General Hospital (MGH) selected only on the basis of their having high ANA titers. 13 of these 19 subjects had defined autoimmune disorders and were included in our studies. In this initial set of 13 Caucasian subjects, unique non-synonymous changes were observed in one subject with Crohn's disease and in one subject with rheumatoid arthritis (RA). As a result of these preliminary observations, we next analyzed 76 Caucasian subjects with RA from the NARAC (North American Rheumatoid Arthritis Consortium) collection, and 89 subjects with inflammatory bowel disease (IBD) 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 SNPs were identified as expected (Supplementary Table I). A total of 19 out of 923 autoimmune subjects presented with one of 14 previously unidentified non-synonymous SNPs in the *SIAE* gene, while 8 other autoimmune subjects had a homozygous polymorphism resulting in a valine replacing methionine at position 89 (Table I). 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 I). Functional analyses were performed on each *SIAE* variant as described below.

Since the initial analyses revealed a marked enrichment of loss-of-function *SIAE* variants in autoimmune subjects as compared with controls, a larger number of autoimmune subjects and controls were analyzed. Power calculations revealed 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 Notes). Autoimmune subjects in this second phase included

more subjects with RA from NARAC, subjects from the MADGC (Multiple Autoimmune Disease Genetics Consortium) collection with systemic lupus erythematosus (SLE) and juvenile idiopathic arthritis (JIA), and subjects from MGH with IBD and rheumatic disorders including SLE, RA, mixed connective tissue disorder (MCTD) and Sjogren's syndrome (SjS). We also included subjects with multiple sclerosis (MS) from a collection at the Brigham and Womens' Hospital, and subjects with Type 1 diabetes (T1D) 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.

In order 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 C-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 while the other was utilized in an esterase assay using a fluorogenic substrate, 4-methylumbelliferyl acetate. Quantitative Western blotting was performed using a near infrared-dye labeled 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 I 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 T312M *SIAE*, C196F *SIAE*, Q309P *SIAE*, G212R *SIAE*, R479C *SIAE*, R393H *SIAE*, and R230W *SIAE*. 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 Y349C *SIAE*, and this variant also exhibits reduced secretion (see Fig. 1, bottom panels). The c.1211T>C variant encodes F404S *SIAE* that also appears to exhibit a less severe catalytic defect (but nevertheless below the 50% cutoff set), similar to that seen in Y349C *SIAE* (Fig. 1). F404S *SIAE* was found in four autoimmune patients including two blood relatives, one with SLE and the other with juvenile idiopathic arthritis. The c.796T>G variant found in one subject with Type I diabetes encodes C266G *SIAE* 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 S1). In order to more precisely establish that M89V *SIAE*

is secretion defective, 293T cells transfected with wild type and *M89V SIAE* respectively were metabolically labeled with  $^{35}\text{S}$  methionine and chased for 10 min, 1h, 2h and 4h. As seen in Fig. 3b, a striking defect in secretion of M89V SIAE was confirmed by this analysis.

Since 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. Since the ultimate test of dominant negative function would be to re-create a heterozygous animal with one mutant allele, mutations were recreated in a murine *Siae* cDNA for these studies. As seen 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 while M89V SIAE and K400N SIAE are not. Based on 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 (3 with RA, 1 with SLE, 1 with MS, 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 (Supplementary Tables S2a, 2b, and 2c). 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 N33S SIAE was discovered in a patient with RA and was found to be functionally normal based on 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 A3G SIAE might not be readily translocated into the ER. We consider it unlikely that A3G SIAE is translocation-defective given the accumulation of SIAE in culture supernatants when *A3G SIAE* is transfected into 293 T cells (Fig. 1, third set of panels). A c.1200G>T variant that encodes K400N SIAE was discovered in a patient with Crohn's disease initially examined as part of a small subset of patients with high ANA titers. 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-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 3 catalytically normal variants identified in autoimmune subjects (N33S SIAE) 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 analyzed was 923, 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 Caucasian non-Jewish subjects in this study, an objective determination of shared ethnicity, a principal components analysis <sup>3,4</sup>, 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 I). One of the rare variants (c.935C>T encoding T312M *SIAE*) 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 RA and also in a patient with MS. Another variant, (c.941G>A encoding R314H *SIAE*), 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 I for a summary). A polymorphic variant c.190G>A, encoding G64S *SIAE*, exhibits normal catalytic activity and is readily secreted (Fig. 2 and Table I), was found in controls, but not found in autoimmune subjects (Table I and Supplementary Table S1). 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 employed. While high Odds Ratios were observed in a number of autoimmune disorders, the results from rheumatoid arthritis subjects (Odds Ratio, 8.3, 2-tailed p= 0.0056) and Type I diabetes patients (Odds Ratio, 7.9, 2-tailed p= 0.0075) were particularly significant. While 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 C57B/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 <sup>5, 6</sup>. 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 utilized a predictive algorithm (Polyphen) to determine which variants were probably non-functional<sup>7</sup>. Loss-of-function variants in the *Trex* gene, which has a single coding exon, have also been described in patients with SLE8 and a recent re-sequencing study has revealed rare variants in the cytosolic helicase MDA5/IFIH1, which mediates innate immune responses to pathogen encoded RNAs <sup>9</sup>. 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 S1. There is clear enrichment of defective coding variants in autoimmune patients as 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 RA and T1D, a role for these variants in other autoimmune disorders including SLE, MS and IBD appears likely (Supplementary Table S3). 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<sup>1</sup>, 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 etiologically linked to T<sub>H</sub>1 or T<sub>H</sub>17 cells<sup>10</sup>. Although B cell depletion can result in marked clinical improvement in patients with multiple sclerosis<sup>11</sup>, 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 harboring a catalytically defective heterozygous *SIAE* variant (G212R SIAE; see Table I) revealed a marked enhancement of cell surface 9-*O*-acetyl sialic acid following 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 C196F SIAE variant, and in a patient with lupus with a R393H SIAE variant (Table I; Supplementary Fig. 6). These analyses suggests that a defect in SIAE results in enhanced BCR mediated expression of surface 9-*O*-acetylated sialic acid, while the presence of normal SIAE prevents this enhanced 9-*O*-acetylation event. This phenomenon is being further explored 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 IBD.

SIAE contributes to a signaling 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 centers<sup>1-2</sup>. 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 likely other Siglecs expressed in B cells<sup>2</sup>. The possibility that SIAE may be of functional relevance in innate immune cells and thus influence disease pathogenesis also deserves exploration.

## 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, Valencia, CA) according to the manufacturer's instructions. All 10 exons of the human *SIAE* gene were amplified by PCR using intronic primers (Supplementary Table S4). Purification of the amplified products, bidirectional

automated sequencing and sequence analysis were performed as described earlier<sup>13</sup>. All sequence variants were confirmed by sequencing at least two independent PCR amplicons. DNAs from controls were obtained from EBV-immortalized lymphoblastoid cell-lines established from healthy blood donors<sup>14</sup>. 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, CA, USA). 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 I. The PCR products were digested overnight with DpnI (10 Units; Stratagene) and transformed into TOP10 chemically competent cells (Invitrogen, Carlsbad, CA). 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 fluorimetric method<sup>15</sup>. Equivalent amounts of each lysate and supernatant were immunoprecipitated for the catalytic assay as well as for quantitation 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 labeled Goat anti-mouse IgG (LI-COR) as a secondary antibody. Immunoprecipitation, metabolic labeling, 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 co-transfecting 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 reference<sup>1</sup> and is based on the method described originally by Krishna and Varki<sup>16</sup>. Briefly human B cells were stained with antibodies to CD19 and CD27 (BD Pharmingen) and incubated either with or without F(ab')<sub>2</sub>, polyclonal rabbit anti-human IgM (Dako). Cells were also stained either with FITC-F(ab')<sub>2</sub>, goat anti-human IgG, Fcγ specific (Jackson ImmunoResearch) alone, or the CHE-FcD reagent (an Influenza C hemagglutinin esterase-fused to the Fc portion of human IgG, chemically treated with diisopropyl fluorophosphate) complexed with FITC-F(ab')<sub>2</sub> fragment goat anti-human IgG, Fcγ specific. Cells were analyzed by flow cytometry.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

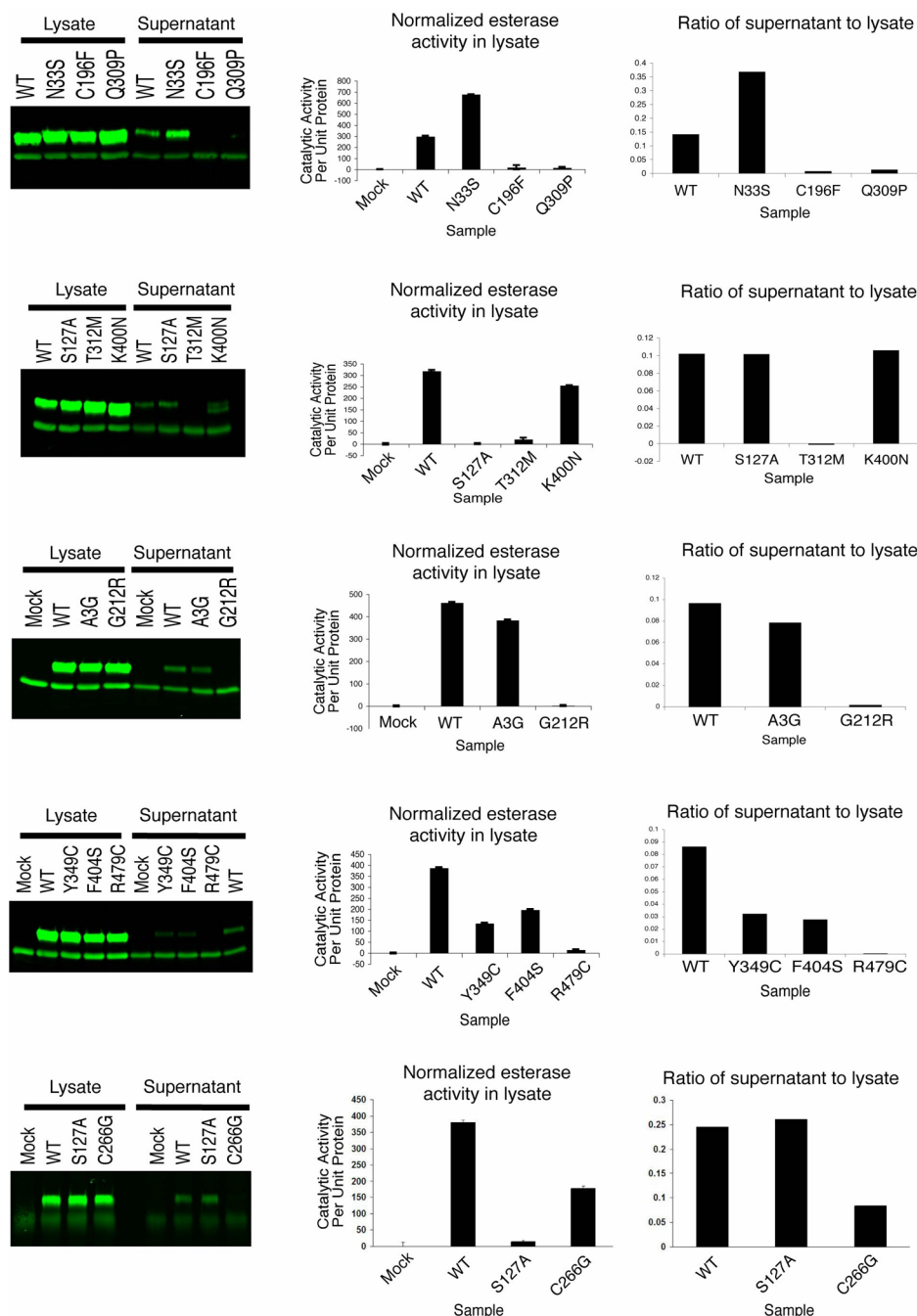
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## References

1. Cariappa A, et al. B cell antigen receptor signal strength and peripheral B cell development are regulated by a 9-O-acetyl sialic acid esterase. *J Exp Med*. 2009; 206:125–138. [PubMed: 19103880]
2. Pillai S, Cariappa A, Pirnie SP. Esterases and autoimmunity: the sialic acid acetyltransferase pathway and the regulation of peripheral B cell tolerance. *Trends Immunol*. 2009; 30:488–493. [PubMed: 19766537]
3. Nassir R, et al. An ancestry informative marker set for determining continental origin: validation and extension using human genome diversity panels. *BMC Genet*. 2009; 10:39. [PubMed: 19630973]
4. Tian C, et al. European Population Genetic Substructure: Further Definition of Ancestry Informative Markers for Distinguishing Among Diverse European Ethnic Groups. *Mol Med*. 2009
5. Altshuler D, Daly MJ, Lander ES. Genetic mapping in human disease. *Science*. 2008; 322:881–888. [PubMed: 18988837]
6. Gregersen PK, Behrens TW. Genetics of autoimmune diseases--disorders of immune homeostasis. *Nat Rev Genet*. 2006; 7:917–928. [PubMed: 17139323]
7. Cohen JC, et al. Multiple rare alleles contribute to low plasma levels of HDL cholesterol. *Science*. 2004; 305:869–872. [PubMed: 15297675]
8. Lee-Kirsch MA, et al. Mutations in the gene encoding the 3'–5' DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat Genet*. 2007; 39:1065–1067. [PubMed: 17660818]
9. Nejentsev S, Walker N, Riches D, Egholm M, Todd JA. Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. *Science*. 2009; 324:387–389. [PubMed: 19264985]
10. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*. 2007; 448:427–434. [PubMed: 17653185]
11. Hauser SL, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med*. 2008; 358:676–688. [PubMed: 18272891]
12. Jessani N, et al. Class assignment of sequence-unrelated members of enzyme superfamilies by activity-based protein profiling. *Angew Chem Int Ed Engl*. 2005; 44:2400–2403. [PubMed: 15765498]
13. Kwak EL, et al. Epidermal growth factor receptor kinase domain mutations in esophageal and pancreatic adenocarcinomas. *Clin Cancer Res*. 2006; 12:4283–4287. [PubMed: 16857803]
14. Bell DW, et al. Common nonsense mutations in RAD52. *Cancer Res*. 1999; 59:3883–3888. [PubMed: 10463575]
15. Shukla AK, Schauer R. Fluorimetric determination of unsubstituted and 9(8)-O-acetylated sialic acids in erythrocyte membranes. *Hoppe Seylers Z Physiol Chem*. 1982; 363:255–262. [PubMed: 7076126]
16. Krishna M, Varki A. 9-O-Acetylation of sialomucins: a novel marker of murine CD4 T cells that is regulated during maturation and activation. *J Exp Med*. 1997; 185:1997–2013. [PubMed: 9166429]



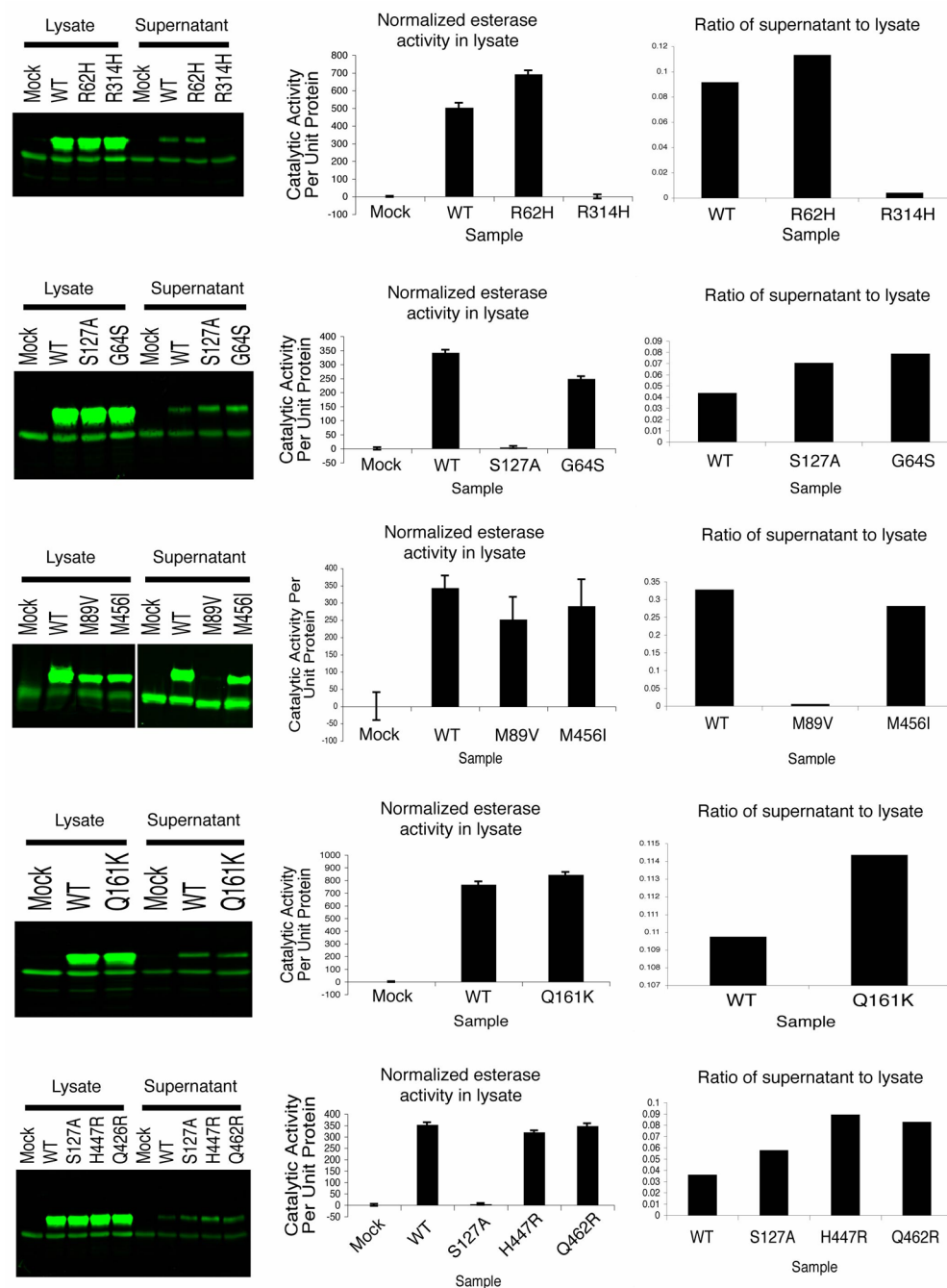


**Figure 1. Analysis of *SIAE* variants from subjects with autoimmunity**

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 (*S127A SIAE12*), and each *SIAE* variant that was unique to autoimmune subjects were transfected into 293T cells. Assays were performed for *A3G SIAE*, *N33S SIAE*, *C196F SIAE*, *G212R SIAE*, *C266G SIAE*, *Q309P SIAE*, *T312M SIAE*, *Y349C SIAE*, *K400N SIAE*, *F404S SIAE*, and *R479C SIAE*. 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

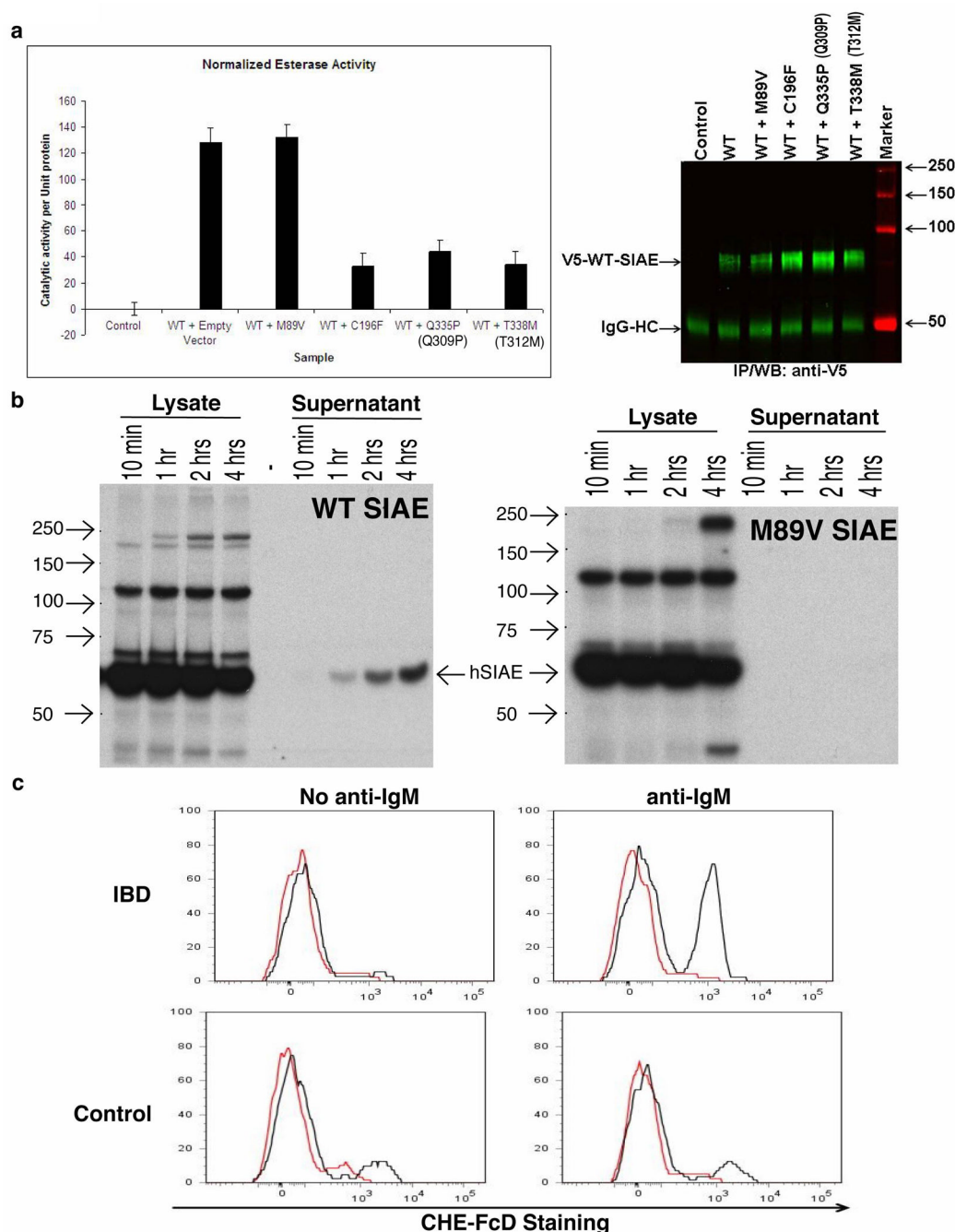
right hand panels of the figure. “Mock” refers to cells which were not transfected but from which lysate and supernatant were analyzed.

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.



**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*, *S127A SIAE* and each *SIAE* variant that was unique to controls (*R62H SIAE*, *G64S SIAE*, *Q161K SIAE*, *R314H SIAE*, *H447R SIAE*, *M456I SIAE*, and *Q462R SIAE*) was transfected into 293T cells. Also shown are results from *M89V SIAE*, which was found in heterozygous form in both patients and controls and in homozygous form only in patients. *T312M SIAE* 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.



**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 C196F Siae, and the murine equivalents of Q309P SIAE and T312M SIAE, (Q335P and T338M Siae), function in a dominant interfering fashion but M89V Siae does not. V5-tagged wild type Siae was transfected along with FLAG-tagged C196F Siae or FLAG-tagged M89V Siae 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 (see Supplementary Fig. 4).

- b. Pulse-chase analysis comparing secretion of wild type SIAE and M89V SIAE. Transfected 293T cells were metabolically pulse-labeled with  $^{35}\text{S}$  methionine and lysates and supernatants were immunoprecipitated with anti-FLAG antibodies after 10 minutes, 1 hour, 2 hours and 4 hours of chase. Proteins were separated by SDS-PAGE and revealed by autoradiography. The position of molecular weight 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. Naïve ( $\text{CD19}^+\text{CD27}^-$ ) B cells from the peripheral blood of a subject with Crohn's disease (labeled IBD) with a heterozygous *SIAE* mutation (G212R) and from a control subject were analyzed 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.



Table 1

SIAE variants identified in Caucasian autoimmune subjects 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

SIAE Change	Esterase Activity	Secretion	Dom. Neg.	Disease	Source
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

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

**Table II**

Functionally defective SIAE coding variants in rheumatoid arthritis, Type I diabetes and all autoimmune diseases combined compared with controls \*

Disease group	# of subjects	Odds Ratio (95% CI <sup>**</sup> )	Two-tailed p-value <sup>***</sup>
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

\*\*\* 2 tailed p-value was determined using Fisher's exact test