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African-Derived Genetic Polymorphisms in TNFAIP3 Mediate Risk for Autoimmunity

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The TNF-α-induced protein 3 (TNFAIP3) is an ubiquitin-modifying enzyme and an essential negative regulator of inflammation. Genome-wide association studies have implicated the TNFAIP3 locus in susceptibility to autoimmune disorders in European cohorts, including rheumatoid arthritis, coronary artery disease, psoriasis, celiac disease, type 1 diabetes, inflammatory bowel disease, and systemic lupus erythematosus (SLE). There are two nonsynonymous coding polymorphisms in the deubiquitinating (DUB) domain of TNFAIP3: F127C, which is in high-linkage disequilibrium with reported SLE-risk variants, and A125V, which has not been previously studied. We conducted a case–control study in African-American SLE patients using these coding variants, along with tagging polymorphisms in TNFAIP3, and identified a novel African-derived risk haplotype that is distinct from previously reported risk variants (odds ratio = 1.6, p = 0.006). In addition, a rare protective haplotype was defined by A125V (odds ratio = 0.31, p = 0.027). Although A125V was associated with protection from SLE, surprisingly the same allele was associated with increased risk of inflammatory bowel disease. We tested the functional activity of nonsynonymous coding polymorphisms within TNFAIP3, and found that the A125V coding-change variant alters the DUB activity of the protein. Finally, we used computer modeling to depict how the A125V amino acid change in TNFAIP3 may affect the three-dimensional structure of the receptor-associated factor; YRI, Yoruba population in Ibadan, Nigeria; WT, wild-type.

The online version of this article contains supplemental material.

Abbreviations used in this paper: CAD, coronary artery disease; CD, Crohn’s disease; CED, celiac disease; CI, confidence interval; dbSNP, Single Nucleotide Polymorphism Database; DUB, deubiquitinating; IB, immunoblotted; IB, inflammatory bowel disease; IP, immunoprecipitation; K48R-Ub, K48-ubiquitin; LD, linkage disequilibrium; OR, odds ratio; PVDF, polyvinylidene fluoride; RA, rheumatoid arthritis; RIP1, receptor-interacting protein 1; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; T1D, type 1 diabetes; TNFAIP3, TNF-α-induced protein 3; TRAF, TNF receptor-associated factor; YRI, Yoruba population in Ibadan, Nigeria; WT, wild-type.

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latter highlighted by the observation that siblings of affected individuals have a 20- to 30-fold increased risk of developing SLE (9).

In addition, compared with European-Americans the incidence of SLE is three to four times higher in African-Americans (10, 11). Genome-wide association studies have begun to identify genes that contribute to SLE susceptibility (12–17). Most of these studies have been conducted exclusively in European cohorts; thus, little is known about whether variants in these genes are risk factors in other ancestral backgrounds. Differences in autoimmune disease risk variants exist across different continental populations. For example, the autoimmune disease risk variant of the protein tyrosine phosphatase type 22 gene (PTPN22) is almost absent in African-derived populations (18).

The TNFAIP3 gene region has been implicated in susceptibility to multiple autoimmune diseases in populations of European ancestry. Single nucleotide polymorphisms (SNPs) ~185 kb upstream of the TNFAIP3 gene are associated with risk for rheumatoid arthritis (RA) (19, 20), type 1 diabetes (T1D) (21), and celiac disease (CeD) (22). The Wellcome Trust Case Control Consortium identified a SNP upstream of TNFAIP3 in 6q23 that is associated with risk for Crohn’s disease (CD) (23). In addition, this same chromosomal region was found as a candidate for susceptibility in a study of 260 inflammatory bowel disease (IBD)-affected relative pairs (24). Intronic TNFAIP3 SNPs are associated with psoriasis (25) and coronary artery disease (CAD) in type 2 diabetes patients (26).

Two independent studies have found an association between TNFAIP3 and SLE in European ancestry. The same SNP identified in RA and CeD that falls ~185 kb upstream of TNFAIP3 was also associated with SLE risk, and a second SLE risk haplotype was identified within the TNFAIP3 gene (15). Another study suggested a complex contribution of three individual variants, including a nonsynonymous F127C coding SNP (rs2230926) (17). Meta-analysis and imputation has narrowed down the SLE-associated region of TNFAIP3 in patients of European ancestry to a single 109 kb haplotype centered on the TNFAIP3 gene, which includes the F127C SNP (27). In functional studies, Musone et al. demonstrated decreased activity of the TNFAIP3 protein with the SLE-associated F127C amino acid change (rs2230926) (17). The identification of a functionally defective risk variant may explain how TNFAIP3 contributes to SLE pathogenesis. In people of European ancestry, the minor allele frequency of rs2230926 is relatively low (0.05 in SLE cases and 0.02 in controls) compared with the reported frequency of 0.447 in the Yoruba population in Ibadan, Nigeria (YRI) (28). In addition, the association between SLE and the TNFAIP3 gene region appears to be complex, and not completely accounted for by the F127C polymorphism. Overall, these studies demonstrate that SNPs within or near TNFAIP3 are associated with autoimmunity, and significant differences in TNFAIP3 SNP frequencies between European and African populations suggest that disease-associated haplotypes in these two ancestral backgrounds may be dissimilar.

We therefore assessed the contribution of TNFAIP3 variants to autoimmune risk in African-Americans. To better understand how alterations in TNFAIP3 affect autoimmune susceptibility, we conducted a case–control study to look at the association between haplotype-tagging and coding-change SNPs within the TNFAIP3 gene and SLE in African-Americans. We identified a novel African-derived SLE-risk haplotype and a protective haplotype for SLE defined by the A125V polymorphism. We genotyped the A125V and F127C coding variants in African-American subjects with IBD and found that the A125V variant was associated with risk for IBD. Next, we tested the functional effect of four nonsynonymous coding polymorphisms in TNFAIP3. The A125V SNP demonstrated a dramatic functional effect, greatly decreasing TNFAIP3-mediated deubiquitination. Finally, we used computer modeling to examine how these amino acid changes may be affecting the predicted three-dimensional structure of the TNFAIP3 DUB domain.

Materials and Methods

Patient samples

Patient genomic DNA samples were obtained from the Translational Research Initiative in the Department of Medicine registry at the University of Chicago (n = 1222) and from the Rush University Medical Center (n = 53). A total of 217 self-identified African-American SLE patients, 24 African-American IBD patients, 272 African-American nonautoimmune controls, and 615 self-identified white or European-American samples of mixed ancestry status were studied. All SLE patients met four or more of the American College of Rheumatology criteria for diagnosis (29). Diagnosis of IBD was made historically using intestinal biopsies. The control population consisted of adults recruited from outpatient clinics at the University of Chicago who were either normal healthy or had an unrelated condition diagnosed, such as cancer, type 2 diabetes, or hypertension. The medical records of the control population were examined to exclude those with autoimmune or inflammatory disease diagnoses, including SLE, CD, sarcoidosis, autoimmune thyroid disease, psoriasis, CeD, RA, and T1D. Subjects with documented CAD were also excluded given the results of a previous study suggesting an association between TNFAIP3 variants and CAD (26). The institutional review boards at both institutions approved this study, and informed consent was obtained from all participants.

Genotyping

Individual patients were genotyped at the coding SNPs A125V (rs5029941) and F127C (rs2230926) using direct sequencing. The close proximity of these two SNPs made probe-based genotyping difficult, and so direct sequencing was used instead. Briefly, a 303 bp fragment was amplified from genomic DNA using PCR (forward primer 5′-GAAAACCTTTGCTGGCTTCT-ACT-3′, reverse primer 5′-CCATGGAGCTCTGTTAGTAGATAA-3′). PCR products were treated with Exonuclease I and Shrimp Alkaline Phosphatase (USB, Cleveland, OH) prior to submission for ultra high-throughput sequencing at the University of Chicago Cancer Research Center DNA Sequencing Facility. Sequencing was performed on an Applied Biosystems 3730XL capillary sequencer (Applied Biosystems, Foster City, CA). Genotype calls were determined by visual inspection of individual base pair peaks in each sequence trace file. The haplotype-tagging SNPs rs5029939, rs5029953, rs2230926, rs610604, rs3577173, rs5029938, and rs582757 were chosen by focusing on the TNFAIP3 gene and the regions 10 kb upstream and downstream of the coding sequence. A two and three marker-tagging algorithm was used with a correlation coefficient (r2) threshold of 0.8 to choose SNPs with an allele frequency >10% in the International HapMap Project YRI population. Haplotype-tagging SNPs were genotyped using ABI TaqMan primers and probes on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) per manufacturer’s protocol. All scatter plots were examined visually to verify the accuracy of the genotype calls. None of the SNPs significantly deviated from predicted Hardy-Weinberg equilibrium in African-American control subjects (lowest p value = 0.0680) (Table I). Two tagging SNPs (rs5029938 and rs582757) were genotyped in only 25% of cases and 48% of controls due to either low allele frequency and/or low information content, whereas the remaining six SNPs were successfully genotyped in >93% of controls and >99% of cases (Supplemental Table I).

Statistical analysis

Odds ratios with 95% confidence intervals and p values using two-tailed χ2 tests were calculated with GraphPad Prism software (GraphPad, San Diego, CA). None of the reported p values were corrected for multiple testing. The odds ratios (ORs) were adjusted to account for the “winner’s curse” using WINNER version 1.1 (http://csd.sph.umich.edu/boehnke/win-ner/) written by Rui Xiao and Michael Boehnke (30). Ascertainment-corrected ORs are listed in Supplemental Table I. In addition, the minimum genetic effect that could be significantly (p = 0.05) detected in this study with 80% power was calculated using an additive model in the CaTS Power Calculator written by Andrew Skol (www.sph.umich.edu/csg/abecasis/CaTS/) and Power for Association with Error, version 1.2, written by Derek Gordon was used for the smaller cohorts that cannot be calculated using the CaTS program. Power for Association with Error can be found at https://cancer.genome.gov/powercalc. In the African-American SLE cohort, this study had 80% power to detect an OR of ≥1.53 with a p value of 0.05 using an additive genetic model over the allele frequency range represented by the SNPs studied, except the rare
variant A125V (Supplemental Table I). In the case of A125V, we could detect an OR of 0.33 or lower for A125V using the same parameters as above. In the African-American IBD cohort, we had 75% power to detect an OR of ≥3.7 for association of A125V using the same parameters as above. The degree of pairwise linkage disequilibrium (LD) between SNPs (r² values) and haplotype frequencies were determined with Haploview 4.1 software. Linkage blocks were defined using the solid spine of LD method implemented in the Haploview software. Multivariate and conditional logistic regression models were performed using an online tool written by John C. Pezzullo (http://statpages.org/logistic.html).

Expression plasmids

The wild-type (WT) human TNFAIP3 cDNA was PCR cloned from HCT116 epithelial cell cDNA into a pENTR entry vector (Invitrogen, San Diego, CA). The C103A, A125V, F127C, R706Q, and A766P point mutants, as well as the A125V/F127C double mutant and the E3 ligase-deficient mutant (C607A, C612A, C624A, and C627A) were generated from WT TNFAIP3 according to the QuickChange Site-Directed Mutagenesis Kit instructions (Stratagene, La Jolla, CA). The V5-tagged TNFAIP3 constructs were each generated by shutting the gene into pcDNA3.1/V5 using the Gateway Clonase LR II enzyme kit (Invitrogen). Cytokine response modifier A and pCMV-Myc-ubiquitin expression plasmids were gifts from M. Peter and C. Maki, respectively (University of Chicago, IL). WT Myc-ubiquitin was generated from Myc-K48R-ubiquitin (K48R-Ub) using site-directed mutagenesis (Stratagene). All plasmids were confirmed by sequencing through the University of Chicago Cancer Research Center DNA Sequencing Facility.

Cell culture and Abs

HEK293 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% FBS, penicillin 100 U/ml, streptomycin 100 μg/ml, and streptomycin 100 μg/ml (Life Technologies, Rockville, MD) and incubated at 37°C in a humidified, 5% CO₂ atmosphere. Polyonal rabbit anti-HA Ab (Y-11) and goat anti-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal mouse anti-Myc and anti-V5 Abs were purchased from Invitrogen. Species-specific secondary Abs conjugated to IRDye or Alexa Fluor infrared dyes (680 nm or 800 nm) were used at a 1:20,000 dilution (Rockland, Gilbertsville, PA, and Invitrogen).

Transfections, immunoprecipitation, and immunoblot analysis

For transient transfection assays, HEK293 cells were seeded at 2 × 10⁶ cells per 1 cm² plate (BD Falcon, Franklin Lakes, NJ) 18 h prior to transfection. Immediately before transfection, cells were rinsed in 1× PBS (Life Technologies) and supplemented with serum-free media. Plasmid DNA (7.5 μg total) and a 2 mg/ml polyethylenimine solution (Polysciences, Warrington, PA) were each diluted in 150 μl final volume of 150 mM NaCl and combined by adding the polymer solution dropwise to the plasmid solution while vortexing. All transfections included 0.5 μg cytokine response modifier A expression vector to protect against cell death. After 30 min the transfection mixture was added to the cells. The following day, cells were supplemented with complete media. Forty hours after transfection, samples were washed in PBS and lysed in 1% Triton lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 10% glycerol with 20 mM N-ethylmaleimide, 10 mM iodoacetamide, 100 mM 1,10-phenanthroline monohydrate, and 1% protease inhibitor mixture) (Roche, Basel, Switzerland). For immunoprecipitation (IP) studies, lysates were incubated overnight at 4°C with anti-HA Ab and for 2 h at 4°C with a 50% slurry of Protein G (Pierce, Rockford, IL). Beads were recovered, washed 3 times with lysis buffer, and mixed with equal volume 2× Laemmli sample buffer (with 50 mM DTT). For SDS-PAGE, whole cell lysates and immunoprecipitated protein were resolved on polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were blocked for 45 min at room temperature with blocking buffer for near infra-red fluorescent Western blotting (Rockland) diluted in PBS (1:1) before overnight incubation at 4°C with primary Ab. The following day membranes were incubated for 45 min with infrared secondary Abs and imaged using IR fluorescence detection on the LI-COR Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). The intensity of the bands was quantified by using the area under the curve method in ImageJ (http://rswebweb.nih.gov/ij/).

Results

Polymorphisms in the TNFAIP3 locus are associated with SLE risk in African-Americans

A case–control study was conducted using a cohort of African-American subjects to examine the association of TNFAIP3 variants with altered SLE risk. SLE cases (n = 217) and nonautoimmune controls (n = 272) were genotyped with real-time PCR using TaqMan probe chemistry at six tagging SNPs within the TNFAIP3 gene region. In addition, the Single Nucleotide Polymorphism Database (dbSNP) lists two SNPs within the DUB domain of TNFAIP3 (aa 92–263) that result in nonsynonymous coding changes in the protein sequence. These include rs5029941 (A125V) and rs2230926 (F127C), the latter associated with SLE risk in European populations (15, 17). Thus, in addition to the six tagging SNPs, we also genotyped these two coding polymorphisms in our patient samples using sequencing.

We tested each of the eight TNFAIP3 SNPs for association with SLE and found that two tagging SNPs (rs5029939 and rs5029953), along with the F127C coding SNP, were associated with SLE susceptibility (Table I). In Europeans, the tagging SNP rs5029939 (p = 0.0453, OR = 1.3) and the coding variant F127C (p = 0.0279, OR = 1.3) were identified as SLE risk variants, and similar to the European studies, we find these two variants in very high LD (r² = 0.99) (15, 17). The most significant SLE risk-associated SNP we observed was not either of these two previously associated SNPs, but was instead the African-derived SNP rs5029953 SNP (p = 0.008, OR = 1.6). Conditioning on rs5029953 abolished the association signals from the other SNPs (rs5029939 and F127C), indicating that they are not independently contributing to risk (Table I). No SNP-SNP interactions were observed, and thus the simple model of a single risk variant marked by rs5029953 was the best fit (data not shown). In the dbSNP database the minor allele of rs5029953 has a reported p value of 0.10 were conditioned on rs5029953 using logistic regression. The two predictor variables were the rs5029953 genotype and the genotype of the SNP being tested with the outcome variable being SLE.

Table I. Single marker and conditional association analysis of TNFAIP3 SNPs in African-American SLE cases

<table>
<thead>
<tr>
<th>SNP</th>
<th>Minor Allele</th>
<th>Allele Ratio Counts (Cases, Controls)</th>
<th>Minor Allele Freq (Cases, Controls)</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>OR (95% CI)</th>
<th>p value (Conditioned on rs5029953)</th>
<th>HWE p value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3757173</td>
<td>G</td>
<td>210:220, 262:302</td>
<td>0.488, 0.428</td>
<td>0.0621</td>
<td>1.276</td>
<td>(0.9875–1.648)</td>
<td>0.5069</td>
</tr>
<tr>
<td>rs5029939</td>
<td>G</td>
<td>15:195, 16:124</td>
<td>0.071, 0.114</td>
<td>0.1686</td>
<td>0.5962</td>
<td>(0.2845–1.4249)</td>
<td>0.7609</td>
</tr>
<tr>
<td>A125V</td>
<td>T</td>
<td>5:429, 15:491</td>
<td>0.0115, 0.0296</td>
<td>0.0549</td>
<td>0.3815</td>
<td>(0.1375–1.059)</td>
<td>0.3848</td>
</tr>
<tr>
<td>F127C</td>
<td>G</td>
<td>166:268, 172:364</td>
<td>0.382, 0.321</td>
<td>0.0453</td>
<td>1.311</td>
<td>(1.005–1.709)</td>
<td>0.5667</td>
</tr>
<tr>
<td>rs582757</td>
<td>C</td>
<td>66:144, 51:87</td>
<td>0.314, 0.370</td>
<td>0.2856</td>
<td>0.7819</td>
<td>(0.4975–1.472)</td>
<td>0.2606</td>
</tr>
<tr>
<td>rs101604</td>
<td>T</td>
<td>156:278, 197:325</td>
<td>0.359, 0.377</td>
<td>0.0560</td>
<td>0.9258</td>
<td>(0.7108–1.206)</td>
<td>0.7017</td>
</tr>
<tr>
<td>rs5029953</td>
<td>A</td>
<td>80:354, 66:470</td>
<td>0.184, 0.123</td>
<td>0.0080</td>
<td>1.699</td>
<td>(1.130–2.293)</td>
<td>0.1297</td>
</tr>
</tbody>
</table>

The eight TNFAIP3 SNPs genotyped in this study are listed, along with the identity of the minor allele. Minor allele frequency in cases and controls is shown. The p value (from χ² analysis) and OR with 95% confidence interval (CI) was calculated with GraphPad Prism software. SNPs with a p value < 0.10 were conditioned on rs5029953 using logistic regression. The two predictor variables were the rs5029953 genotype and the genotype of the SNP being tested with the outcome variable being SLE.

<sup>a</sup>p Values are uncorrected for multiple testing.

<sup>b</sup>Calculated using the control population.
frequency ranging from 0.077 to 0.161 in African populations, and this SNP has not been identified in Asian or European populations. We genotyped a total of 324 white or European-American samples, and all carried the major allele of rs5029953, supporting the idea that this SNP is indeed of African origin.

Surprisingly, we found that the rare minor allele of A125V was associated with protection from SLE in African-American subjects \( (p = 0.05, OR = 0.38) \) (Table I). We did not detect this variant in self-reported white or European-ancestry subjects \( (n = 291) \) (Supplemental Table II), and this variant was present in our nonautoimmune African-American controls at a frequency of \( 0.03 \) (Table II), which is comparable to the frequency reported in African populations in public databases. The minor allele of A125V is in high LD with the minor allele of F127C, as the two patients identified as homozygous for the minor allele of A125V were also homozygous for the minor allele of F127C, and every A125V heterozygote was either heterozygous or homozygous for the minor allele of F127C. The complete distribution of genotypes by ethnicity and autoimmune disease for both A125V and F127C can be found in Supplemental Table II.

Haplotype analysis in African-American subjects detected eight haplotypes with a frequency of \( \geq 1\% \) in cases and controls (Fig. 1A). The rs5029953 SNP associated with SLE risk is unique to one haplotype, and thus marks the SLE risk-associated haplotype (haplotype 2, \( OR = 1.6 \), \( p = 0.008 \)). A linkage disequilibrium plot of the SLE cases or controls shows that among the SNPs analyzed, rs5029953 is most strongly associated with rs5029939 and F127C (Fig. 1B, Supplemental Fig. 1). In addition, the A125V rare variant is only found on one haplotype, identifying the protective haplotype (haplotype 8, \( OR = 0.3, p = 0.027 \)), which does not include the SLE-risk-associated allele. These findings taken together suggest that the F127C polymorphism is not a major determinant of SLE risk in African-Americans, and that instead a model of risk, neutral, and protective haplotypes seems to apply. Interestingly, the protective haplotype contains a rare coding polymorphism (A125V) that could alter TNFAIP3 activity; whereas, the functional implications of the risk haplotype are not currently clear.

A key clinical feature of SLE is the production of autoantibodies against nucleic acids (i.e., anti-dsDNA) and small nuclear proteins that stably interact with RNA (RNA binding proteins or RBPs). Other genes that confer risk to SLE, such as IRF5, are associated with higher interferon responses in patients with either anti-dsDNA or anti-RBP autoantibodies (34). We tested whether any of the TNFAIP3 SNPs studied could predict the production of any SLE-related autoantibodies, and found no significant associations (data not shown).

The F127C and A125V TNFAIP3 coding SNPs are associated with autoimmunity in African-Americans

We next assessed the contribution of the A125V and F127C polymorphisms to inflammatory disease susceptibility. The patient samples were grouped according to diagnosis (IBD, SLE, or nonautoimmune controls), and the A125V and F127C genotypes were analyzed together as predictor variables in a logistic regression model. Strikingly, the minor allele of A125V is significantly associated with susceptibility to IBD (Table II) \( (OR = 3.73, p = 0.027) \). As noted previously, this same allele is associated with protection from SLE \( (OR = 0.33, p = 0.032) \). Thus, the rare variant A125V has opposite effects on risk of IBD and SLE in African-Americans. Despite the low number of African-American IBD case samples, this study had 75% power to detect the large genetic effect identified \( (OR = 3.7) \) with a \( p \) value \( = 0.05 \) (Supplemental Table I). Furthermore, correcting for the “winner’s curse effect” does not significantly decrease the estimated OR for this association (Supplemental Table I). Although the low number of patients limits our confidence in the IBD data to some degree, these preliminary data suggest a strong effect, which is almost directly opposite of that observed in SLE.

### Diminished functional activity of A125V TNFAIP3

The observation that coding SNPs in TNFAIP3 are associated with autoimmunity in African-Americans suggests that these amino acid substitutions may be altering the enzymatic function of the protein. Because the A125V and the F127C polymorphisms occur together (A125V is never found without F127C; whereas, F127C is often found without A125V), we tested the functional activity of A125V/F127C TNFAIP3. In addition to the A125V/F127C double mutant, four single nonsynonymous coding mutations (A125V, F127C, R706Q, and A766P) were made in the TNFAIP3 cDNA and cloned into mammalian expression vectors. R706Q and A766P were chosen because they are listed in the dbSNP database as validated, nonsynonymous coding SNPs located near the ubiquitin ligase domain of TNFAIP3. These variants were not studied in the genetic association study, as their probable low frequency in human populations based on HapMap data would likely prevent significant conclusions given our sample size. A previous study demonstrated that mutating the cysteine at amino acid position 103 to alanine inhibits the DUB activity of TNFAIP3 (3); thus, C103A TNFAIP3 was used as a negative control. TRAF2 is a key signaling molecule in several inflammatory pathways, is known to be ubiquitinated with K63-linked chains, and has been shown to be associated with TNFAIP3 (35–37). TNFAIP3 is a dual functioning enzyme that both deubiquitinates and induces the degradation of its targets. The two activities of this enzyme are linked, in that TNFAIP3 must deubiquitinate targets before it can induce their degradation. The ability of these TNFAIP3 mutants to induce the degradation of TRAF2 was tested to evaluate the functional consequence of nonsynonymous coding variants in TNFAIP3. WT TNFAIP3 induces the degradation of TRAF2 in a dose-dependent manner; whereas, the catalytic mutant C103A fails to do so (Fig. 2). We found that the A125V/F127C double mutant TNFAIP3 is less efficient at inducing the degradation of TRAF2 when compared with WT TNFAIP3 (Fig. 2A). Of the four single polymorphisms, the A125V SNP appears to have a diminished ability to

### Table II. Frequency of TNFAIP3 SNPs A125V and F127C in African-American autoimmune cases and association with disease

<table>
<thead>
<tr>
<th>SNP</th>
<th>Minor Allele</th>
<th>MAF</th>
<th>p Value</th>
<th>OR (95% CI)</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>A125V</td>
<td>T</td>
<td>0.104</td>
<td>0.027</td>
<td>3.73 (1.16–11.97)</td>
<td>0.012</td>
</tr>
<tr>
<td>F127C</td>
<td>G</td>
<td>0.354</td>
<td>0.932</td>
<td>0.97 (0.49–1.92)</td>
<td>0.385</td>
</tr>
</tbody>
</table>

African-American DNA samples were genotyped by sequencing the region centered on the A125V and F127C nonsynonymous SNPs in TNFAIP3. Allele frequencies are compared between patient groups and nonautoimmune controls using \( \chi^2 \) analysis, and ORs with 95% CI are shown for each comparison.
induce degradation of TRAF2 (Fig. 2B). This defect is not a complete loss of function, because high amounts of A125V TNFAIP3 lead to loss of TRAF2, but not to the same extent as WT TNFAIP3. In addition, the A766P mutation may have a subtle effect on the functional activity of TNFAIP3 and future genetic and biochemical studies will be needed to determine the significance of this observation. Thus, the A125V polymorphism is likely the main cause of the functional deficiency in the A125V/F127C double mutant. However, the A125V/F127C mutant may have a more profound functional defect (compare levels of TRAF2 degradation of A125V/F127C TNFAIP3 in Fig. 2A with A125V TNFAIP3 in Fig. 2B). Nevertheless, the F127C mutation does not appear to correct or significantly enhance the A125V TNFAIP3 enzymatic defect.

To further explore the effect of the A125V mutation, we tested the DUB activity of the A125V TNFAIP3 single mutant on ubiquitinated TRAF2 (Fig. 3). To reduce the amount of TRAF2 substrate degraded by TNFAIP3, this experiment was performed with ubiquitin ligase domain mutant constructs lacking the E3 ligase activity of TNFAIP3. The WT TNFAIP3/E3 ligase mutant had reduced TRAF2 degradation in the whole cell lysates prior to immunoprecipitation (pre-IP). After cotransfection of V5-tagged TNFAIP3 (WT or mutant), HA-tagged TRAF2, and Myc-tagged K48R-Ub, the lysates were immunoprecipitated with Ab against HA-TRAF2. By using the K48R ubiquitin construct, polyubiquitin chains formed with this protein cannot be linked via the lysine at position 48. Thus, the Myc tag marks monoubiquitinated and polyubiquitinated proteins linked at a position other than K48 (e.g., the K63 signaling form of polyubiquitin). The amount of non–K48-linked polyubiquitinated TRAF2 (IP) or total non–K48-linked polyubiquitinated proteins (pre-IP) present with each TNFAIP3 construct was determined by immunoblotting for Myc-K48R-Ub. Consistent with the reduced degradation of TRAF2 by the A125V variant (Fig. 2B), the A125V mutant had decreased DUB activity against immunoprecipitated TRAF2, as well as total non–K48-linked polyubiquitinated proteins in the pre-IP compared with WT TNFAIP3. Thus, the A125V mutation decreases the ability of TNFAIP3 to cause the deubiquitination and subsequent degradation of the target signaling protein TRAF2.

**Computer modeling of TNFAIP3 structure**

Of the four naturally occurring TNFAIP3 polymorphisms tested, we were surprised to find such a strong functional defect in the A125V mutant. Although both the A125V and F127C are located within the N-terminal DUB domain of TNFAIP3, it was not initially obvious why an alanine to valine amino acid change would have a greater effect on protein function compared with a phenylalanine to cysteine change. The DUB domain of TNFAIP3 has been crystallized, so we used computer modeling to determine why these changes may be affecting enzymatic activity (38). We found that after making the alanine to valine change, the R-group of the valine was pointing into the center of protein and potentially causing steric interference with isoleucines 29 and 202 (Fig. 4A). This could result in a conformational change that affects the nearby catalytic core of the DUB domain. The F127C substitution pointed away from the protein (Fig. 4B). Such a change may not directly interfere with the enzymatic activity of the protein, but could influence the ability of target proteins to bind with TNFAIP3. This could also explain why we failed to see a significant functional consequence of F127C in our assay; whereas, Musone et al. observed decreased regulation of NF-κB activation by this variant (17).

**Discussion**

Our results demonstrate the first association of TNFAIP3 with autoimmunity in African-Americans. We have shown that an African-derived rare variant in the TNFAIP3 gene (A125V) is associated with risk for IBD, yet is protective for SLE. However, the role of TNFAIP3 appears complex in SLE, as we also describe a common African-derived haplotype associated with risk for SLE, marked by the tagging SNP rs5029953. Thus, we can identify risk, neutral, and protective haplotypes in TNFAIP3 in African-Americans, and these haplotypes are defined by African-derived genetic elements. We tested the effects of nonsynonymous coding SNPs on the enzymatic activity of TNFAIP3 and found that the A125V mutation causes a decrease in the ability of TNFAIP3 to deubiquitinate and degrade the target protein TRAF2. Although our functional data suggest that this mutation causes a reduction in the activity of TNFAIP3, it is unclear whether this results in increased or decreased risk for developing inflammation. In fact, our genetic data argue that the effect of this polymorphism in inflammatory disease will be complex, as the same polymorphism is associated with risk of one condition and susceptibility to another.
We found that the minor allele of the African-American–specific tagging SNP rs5029953 marks the SLE-risk haplotype in 139 African-American samples (n = 485), but in none of the European ancestry samples (n = 324). Our study covered a range of ~10 kb centered on the TNFAIP3 gene. A closer examination of the linkage of rs5029953 with nearby SNPs in the International HapMap Project database’s YRI population suggests that this polymorphism lies within a linkage block of ~7 kb (Supplemental Fig. 2). There are nine other SNPs in LD with rs5029953 (r² > 0.50), including SNPs that fall within two LD blocks upstream and one LD block downstream of rs5029953 covering ~68 kb of the genetic region in and around the TNFAIP3 gene. All nine SNPs in high linkage with rs5029953 are of African origin (frequency range in YRI between 9–19%), with none of the minor alleles found in Europeans (Supplemental Table III). Thus, the SLE-risk haplotype we identify is of African origin. The SLE-risk signal in African-Americans is centered on the TNFAIP3 gene in our study, similar to the recent SLE meta-analysis in Europeans (27). The functional element on this African-derived SLE-risk haplotype causing SLE susceptibility remains unclear, and the most strongly associated SNP (rs5029953) has no predicted function. However, it is possible that this intronic SNP affects mRNA splicing, stability, or the binding of small regulatory RNAs. Other candidate functional elements include TNFAIP3 coding variants located within the same LD block as rs5029953 (F127C, R706Q, P765, and A766P). Although we find that F127C is associated with SLE risk, it is not independently contributing to the risk associated with rs5029953. We also found no additive effect between rs2230926 and rs5029953. Because we find limited functional or genetic effects with this SNP, it is possible that the result of this coding change in vivo is negligible or is compensated for in another way. Based on our analysis of the structure of the TNFAIP3 DUB domain, further functional studies that focus on the interaction of TNFAIP3 with various target proteins are needed to elucidate the extent of the F127C amino acid substitution on the enzymatic function of the protein. Another coding region SNP (rs5029956) on the risk haplotype is a synonymous base pair substitution in the proline residue 765 (CCC->CCT) in the ninth exon of TNFAIP3. This is a potential functional element, as protein levels can be modulated by synonymous base pair changes in codons. A variation in protein expression of ~250-fold was found by engineering synonymous mutations in GFP expressed by Escherichia coli which resulted in differential mRNA stability and folding (39). The effect of the synonymous of each protein band was determined, and the densitometry of the TRAF2 and TNFAIP3 bands are plotted using arbitrary units. One of three experiments is shown. B. The ability of TNFAIP3 single mutants to degrade the protein target TRAF2 was tested. Increasing amounts of either V5-tagged WT or A125V/F127C TNFAIP3 (1, 2.5, or 3.5 μg), or V5-tagged C103A TNFAIP3 (3.5 μg) were cotransfected with HA-tagged TRAF2 (0.5 μg) into HEK293 cells. Whole cell lysates were transferred to PVDF membrane and immunoblotted (IB) for HA, V5, or actin. The A125V/F127C mutant fails to degrade TRAF2 to the same extent as WT TNFAIP3, and is comparable to the known catalytic mutant C103A. Equivalent protein loading was confirmed by immunoblotting for actin. The relative intensity of the protein bands was determined, and the densitometry of the TRAF2 and TNFAIP3 bands are plotted in the lower half of the panel using arbitrary units. On the bar graph for F127C, the highest dose of F127C TNFAIP3 (5 μg) was not determined (ND). One of three experiments is shown.
P765 SNP on the expression of TNFAIP3 in vivo remains to be studied. Finally, the nonsynonymous coding SNPs rs3734553 (R706Q) and rs5029957 (A766P) are found within the SLE-associated LD block; however, the existence of these SNPs has not been fully verified, and these may be extremely rare variants. In addition, we found limited functional effects of these mutations. Further fine-mapping and sequencing along this ancestral risk haplotype, along with in vitro functional assays, will help to identify the precise functional element or elements that determine the association between the TNFAIP3 gene and increased SLE risk in African-Americans.

We demonstrated that the A125V human polymorphism in TNFAIP3 has a strong in vitro effect on protein function and also shows significant and opposing genetic effects in two distinct autoimmune diseases (OR 3 in IBD versus OR 0.33 in SLE). There are precedents for genetic variants that are associated with risk in one disease, but with protection in another. The nonsynonymous variant R620W in the PTPN22 gene is associated with risk for RA, T1D, SLE, and autoimmune thyroiditis (40).

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However, this same polymorphism is protective in CD and Behcet's disease (41, 42). The H131R variant in the Fc γ receptor 2A (FCGR2A) gene is associated with risk for SLE, T1D, Guillain-Barre syndrome, and multiple sclerosis; however, it is protective in ulcerative colitis in the Japanese population (12, 43–45). Thus, there are several examples of coding variants that have opposing disease associations in autoimmunity.

A central question that arises from this study is how a variant that decreases the function of TNFAIP3 is associated with risk for IBD yet protection from SLE. This may involve Th1/2 skewing of T cells, such that lead to SLE are increased in individuals with less functional TNFAIP3. Conversely, weaker inflammatory stimuli that might lead to SIBD (an intermediate Th1/2 response) are made sufficient (more cytokine milieus that are produced during the course of each disease, thus influencing the Th1/2 skewing of T lymphocytes. SLE is generally thought of as a Th2-type disease; whereas, IBD is more Th1-mediated. Loss of TNFAIP3 function results in more inflammation, which might alter the Th skewing of immune responses. Thus, inflammatory stimuli insufficient to initiate IBD (an intermediate Th1/2 response) are made sufficient (more toward a Th1 response) by reduced anti-inflammatory activity of TNFAIP3. Conversely, weaker inflammatory stimuli that might lead to SLE are increased in individuals with less functional TNFAIP3, leading to more inflammation and less susceptibility to SLE. This may involve Th1/2 skewing of T cells, such that a Th2 response that might lead to SLE is skewed toward a Th1 profile, reducing the risk for SLE. Our genetic findings are consistent with the observation that very few people are diagnosed with both SLE and IBD (46). Studies correlating TNFAIP3 SNPs with cytokine profiles in African-Americans with SLE versus IBD are needed to provide a basis for this hypothetical model.

The pathogenesis of autoimmune inflammatory diseases is complex, and likely involves both genetic and environmental factors. The identification of potential genetic associations has helped define possible mechanisms of disease initiation and progression. Genetic variation in the TNFAIP3 gene has been associated with several types of autoimmune disease in patients of European ancestry, and in this study we show novel genetic elements on African-derived chromosomes also modulate autoimmunity. Reconciliation of the differences between European- and African-American genetic studies of TNFAIP3 around functional elements will be a critical step in understanding disease biology. Our finding that the A125V polymorphism confers functional differences is an important advance in this effort. A better understanding of how polymorphisms in TNFAIP3 affect risk and protection in autoimmunity will provide valuable mechanistic insights into the pathogenesis and treatment of autoimmune disease.

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Disclosures
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