Alterations in the apoptosis of immune cells have been associated with autoimmunity. Here, we have identified a homozygous missense mutation in the gene encoding the base excision repair enzyme Nei endonuclease VIII-like 3 (NEIL3) that abolished enzymatic activity in 3 siblings from a consanguineous family. The NEIL3 mutation was associated with fatal recurrent infections, severe autoimmunity, hypogammaglobulinemia, and impaired B cell function in these individuals. The same homozygous NEIL3 mutation was also identified in an asymptomatic individual who exhibited elevated levels of serum autoantibodies and defective peripheral B cell tolerance, but normal B cell function. Further analysis of the patients revealed an absence of LPS-responsive beige-like anchor (LRBA) protein expression, a known cause of immunodeficiency. We next examined the contribution of NEIL3 to the maintenance of self-tolerance in Neil3−/− mice. Although Neil3−/− mice displayed normal B cell function, they exhibited elevated serum levels of autoantibodies and developed nephritis following treatment with poly(I:C) to mimic microbial stimulation. In Neil3−/− mice, splenic T and B cells as well as germinal center B cells from Peyer’s patches showed marked increases in apoptosis and cell death, indicating the potential release of self-antigens that favor autoimmunity. These findings demonstrate that deficiency in NEIL3 is associated with increased lymphocyte apoptosis, autoantibodies, and predisposition to autoimmunity.
Deficiency of base excision repair enzyme NEIL3 drives increased predisposition to autoimmunity

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Alterations in the apoptosis of immune cells have been associated with autoimmunity. Here, we have identified a homozygous missense mutation in the gene encoding the base excision repair enzyme Nei endonuclease VIII-like 3 (NEIL3) that abolished enzymatic activity in 3 siblings from a consanguineous family. The NEIL3 mutation was associated with fatal recurrent infections, severe autoimmunity, hypogammaglobulinemia, and impaired B cell function in these individuals. The same homozygous NEIL3 mutation was also identified in an asymptomatic individual who exhibited elevated levels of serum autoantibodies and defective peripheral B cell tolerance, but normal B cell function. Further analysis of the patients revealed an absence of LPS-responsive beige-like anchor (LRBA) protein expression, a known cause of immunodeficiency. We next examined the contribution of NEIL3 to the maintenance of self-tolerance in Neil3−/− mice. Although Neil3−/− mice displayed normal B cell function, they exhibited elevated serum levels of autoantibodies and developed nephritis following treatment with poly(I:C) to mimic microbial stimulation. In Neil3−/− mice, splenic T and B cells as well as germinal center B cells from Peyer’s patches showed marked increases in apoptosis and cell death, indicating the potential release of self-antigens that favor autoimmunity. These findings demonstrate that deficiency in NEIL3 is associated with increased lymphocyte apoptosis, autoantibodies, and predisposition to autoimmunity.

Introduction

The clearance of apoptotic cells is initiated by the recognition of their modified surface molecules and subsequent engulfment by phagocytes (1). An imbalance between the rates of cell death and the clearance of cellular debris leads to the release of normally concealed self-antigens. Immune recognition of these self-antigens promotes the development of autoimmunity (2). Defective clearance of DNA released from apoptotic cells can trigger autoimmunity, as evidenced by the identification of a homozygous loss-of-function variant in DNASeII3, encoding the deoxyribo- nuclease I-like 3, as a monogenic cause of systemic lupus erythematosus (SLE) (3). Alterations in apoptosis of immune cells have been associated with autoimmunity (4, 5). Autoimmunity has also been linked to defects in the central and/or peripheral tolerance checkpoints involved in the elimination of autoreactive T and B cells. Central T cell tolerance is maintained by deletion of autoreactive T cell clones in the thymus mediated by the transcription factor autoimmune regulator (AIRE) (6). Central B cell tolerance is established in the bone marrow by silencing autoreactive B cells using various tolerance mechanisms (7–10), and relies on signaling through the B cell receptor (BCR) and TLRs (11, 12). The activity of residual self-reactive T and B cells in the periphery is, in part, controlled by Tregs that express the transcription factor forkhead box P3 (FOXP3) (13, 14). A number of primary immunodeficiency diseases are associated with autoimmunity due to defects in central or peripheral tolerance. In particular, LPS-responsive beige-like anchor (LRBA) deficiency is associated with decreased Treg number and function (15). However, the impact of defective LRBA production on B cell tolerance checkpoints has not yet been determined.

Endogenous DNA damages occur at the rate of about 30,000 per human cell per day and are primarily repaired by the base excision repair (BER) pathway (16–19). This pathway is initiated by DNA glycosylases, which search the DNA for damage, then cleave...
The diagnosis of inflammatory bowel disease (IBD) was made based on a history of chronic diarrhea and on intestinal biopsy. B The diagnosis of IBD was made based on a history of diarrhea and family history. AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenia.

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**Table 1. Clinical features**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>7 years</td>
<td>7 years, 8 months</td>
<td>1 year</td>
</tr>
<tr>
<td>Failure to thrive</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Infections</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Sinopulmonary infections</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sepsis</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Opportunistic infections</td>
<td>Aspergillus pneumonia</td>
<td>Aspergillus pneumonia</td>
<td>Adenovirus pneumonia</td>
</tr>
<tr>
<td>Autoimmunity</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>AIHA (Coombs positive)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ITP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IBD</td>
<td>+*</td>
<td>+*</td>
<td>+*</td>
</tr>
<tr>
<td>Death</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Age of death</td>
<td>15 years</td>
<td>18 years</td>
<td>8 years</td>
</tr>
<tr>
<td>Cause of death</td>
<td>Lung hemorrhage</td>
<td>Sepsis</td>
<td>Sepsis</td>
</tr>
</tbody>
</table>

\*The diagnosis of inflammatory bowel disease (IBD) was made based on a history of chronic diarrhea and on intestinal biopsy. \*The diagnosis of IBD was made based on a history of diarrhea and family history. AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenia.

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**Results**

Clinical presentation and immunologic findings. The index patient (patient 1) and her 2 younger male siblings (patients 2 and 3), the products of a first-cousin marriage in a family from Kuwait, presented in childhood with recurrent respiratory tract infections associated with bronchiectasis, hemolytic anemia, recurrent bleeding episodes, chronic diarrhea, and failure to thrive (Table 1). Patients 1 and 2 developed *Aspergillus* pneumonia, and patient 2 had episodes of *Candida* esophagitis and streptococcal bactemia. All 3 patients had anemia, with a positive direct Coombs test demonstrable in patients 1 and 2, and all 3 had autoimmune thrombocytopenia with demonstrable anti-platelet antibodies in patient 3 (Table 2). Patient 3 also had anti-endomysial antibodies and anti-gliadin antibodies. Endoscopy revealed gastroduodenitis in patient 1 and small intestinal inflammation with crypt abscesses and lymphocytic infiltration in patient 2. No endoscopy was performed in patient 3. Patient 1 died at age 15 years of lung hemorrhage. Patients 2 and 3 died at 18 and 8 years, respectively, with a clinical picture of sepsis.

Patient 1 was found at the age of 9 years to have low IgG, low IgA, and normal IgM serum levels (Table 2). Patient 2 had normal Ig serum levels at presentation at age 7 years and 8 months, but repeated testing at the age of 11 years showed low IgG, low IgA, and normal IgM (Table 2); therefore, both patients were started on i.v. Ig replacement. Patient 3 was started on i.v. Ig at the age of 14 months despite normal serum IgG, IgA, and IgM levels (Table 2) because of recurrent respiratory tract infections and his family history. Lymphocyte phenotyping was performed on patient 2 at age 14 years, and on patient 3 at ages 18 months (Table 2) and 5 years, 8 months (data not shown). The absolute lymphocyte numbers and numbers of CD3\^+, CD4\^+, and CD8\^+ T cells were normal to slightly elevated, and the number of NK cells were decreased in patient 2 and normal in patient 3 (Table 2). The percentage of memory CD4\^+ cells was elevated in patient 2 and normal in patient 3. CD19\^+ B cells were normal for patients 2 and 3; however, most of the B cells were naive (Table 2). Because patients 1 and 2 had died by the time the study was initiated, lymphocyte proliferation and Ig secretion in vitro were examined only
Elevated levels of serum autoantibodies, defective peripheral B cell tolerance, and impaired Treg function. We determined the spectrum of autoantibodies in available sera from patients 2 and 3 as well as 2 control subjects using an array of 76 autoantigens. The patients had significantly increased levels of IgM antibodies to 24 autoantigens and IgG antibodies to 28 autoantigens compared with healthy controls (Figure 1, A and B). These autoantigens included cytoplasmic, structural, and nuclear proteins. There was no increase in the levels of autoantibodies in sera from the patients’ parents and unaffected siblings (data not shown).

To test the function of the central B cell tolerance checkpoint, antibodies expressed by FACS-sorted single CD19+CD20+CD21+IgM+CD27+ new emigrant/transitional B cells were cloned and their reactivity against dsDNA, insulin, and LPS was examined and found comparable in patient 3 and normal controls (Figure 2, A and B). In contrast, the reactivity of antibodies expressed by FACS-sorted single CD19+CD20+CD21+IgM+CD27+ mature naive B cells against HEp-2 cells was substantially higher (38%) in patient 3 than in healthy controls (19% averaged from 12 healthy controls) (Figure 2, C and D). These results indicate normal central B cell tolerance, but defective peripheral B cell tolerance in patient 3.

Because peripheral B cell tolerance is under the control of Tregs, we examined the presence and function of Tregs from patient 3. The proportion of CD4+CD25hiCD127loFOXP3+ Tregs among CD4+ cells was normal in patient 3 compared with controls (data not shown). We examined Treg function by testing the ability of CD4+CD25hiCD127lo T cells to suppress the proliferation of CD4+CD25lo T effector (Teff) cells sorted from a third-party healthy subject and stimulated with beads coated with anti-CD3, -CD28, and -CD2 mAbs. Tregs from the patient had a significantly decreased ability to suppress the proliferation of Teff cells compared with Tregs from controls (Figure 2, E and F), indicating that the patient’s Tregs were defective. Similar results were obtained when allogeneic Teff cells were used; hence the results were pooled. The patient’s Teff cells were suppressed by control Tregs comparably to the suppression of control Teff cells (data not shown). Thus, Tregs were qualitatively defective in patient 3.

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### Table 2. Laboratory investigations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>9 years</td>
<td>11 years</td>
<td>14 months</td>
</tr>
<tr>
<td>Platelets (cells/μl ×10^10)</td>
<td>86 (227–350)</td>
<td>74 (185–399)</td>
<td>47 (147–423)</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>7.4 (11.5–15.5)</td>
<td>7.8 (11.5–15.5)</td>
<td>10.2 (10.5–3.5)</td>
</tr>
<tr>
<td>Autoantibodies</td>
<td>Direct Coombs positive</td>
<td>Direct Coombs positive</td>
<td>Anti-platelet antibodies Anti-endomyosal antibodies Anti-gliadin antibodies</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IgG (mg/dl) at age</th>
<th>9 years</th>
<th>11 years</th>
<th>14 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>122 (633–1280)</td>
<td>352 (608–1572)</td>
<td>472 (294–1069)</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>11 (33–202)</td>
<td>&lt;45 (45–236)</td>
<td>46 (16–84)</td>
</tr>
<tr>
<td>IgM</td>
<td>54 (49–207)</td>
<td>196 (52–242)</td>
<td>135 (41–149)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Immunophenotype at age</th>
<th>8 years</th>
<th>14 years</th>
<th>18 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lymphocytes/ml</td>
<td>2,740 (1,900–3,700)</td>
<td>4,790 (3,600–8,900)</td>
<td>5,805 (3,600–8,900)</td>
</tr>
<tr>
<td>% CD3+CD4+ cells/ml</td>
<td>ND</td>
<td>2,753 (1,000–2,200)</td>
<td>3,358 (2,100–6,200)</td>
</tr>
<tr>
<td>% CD3+CD8+ cells/ml</td>
<td>ND</td>
<td>1,197 (530–1,300)</td>
<td>3,004 (1,300–3,400)</td>
</tr>
<tr>
<td>% CD4+CD45RA+</td>
<td>ND</td>
<td>7 (36–66)</td>
<td>59.8 (63–91)</td>
</tr>
<tr>
<td>% CD4+CD45RO+</td>
<td>ND</td>
<td>89 (18–38)</td>
<td>40 (12–12)</td>
</tr>
<tr>
<td>CD19+ cells/ml</td>
<td>ND</td>
<td>783 (720–2,600)</td>
<td>1629 (600–1,900)</td>
</tr>
<tr>
<td>% CD27</td>
<td>ND</td>
<td>979 (76.5–94.7)</td>
<td>96.6 (82.1–95.2)</td>
</tr>
<tr>
<td>% CD27+IgG</td>
<td>ND</td>
<td>1.7 (3.0–0.7)</td>
<td>1.7 (2.5–8.7)</td>
</tr>
<tr>
<td>% CD27+IgD</td>
<td>ND</td>
<td>0.10 (14–11.9)</td>
<td>0.4 (0.3–9.0)</td>
</tr>
<tr>
<td>CD16-/CD56+ cells/ml</td>
<td>ND</td>
<td>14 (70–480)</td>
<td>281 (180–920)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>B cell proliferation (cpm)</th>
<th>5 years, 8 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD40+IL-4</td>
<td>5,654 ± 1,076 (5,528 ± 510) NS</td>
</tr>
<tr>
<td>Anti-CD40+IL-21</td>
<td>7,058 ± 960.5 (14,350 ± 359.8) P &lt; 0.05</td>
</tr>
<tr>
<td>Background</td>
<td>1275 ± 31.50 (186.3 ± 37.25) NS</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>In vitro Ig secretion (ng/ml)</th>
<th>5 years, 8 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE</td>
<td>3.49 ± 1.89 (21.82 ± 3.56) P &lt; 0.05</td>
</tr>
<tr>
<td>IgG</td>
<td>7,973 ± 1,890 (35,470 ± 3,955) P &lt; 0.05</td>
</tr>
</tbody>
</table>

*Prior to Ig replacement therapy. B cell proliferation and Ig secretion were determined in 5 controls and in patient 3 on 2 occasions. Values shown represent mean ± SEM. Values for the controls are shown in parentheses. The age-matched normal ranges shown in parentheses are from refs. 78–82. ND, not determined.
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We identified in the second ROH 2 adjacent coding variants in NEIL3, which codes for nei endonuclease VIII-like 3 enzyme (36–38). In addition, we found variants in the coding sequences of 3 other genes outside the 2 ROH: KCNA10 (codes for potassium channel, voltage-gated shaker related subfamily A, member 10), ST7L (codes for suppression of tumorigenicity 7-like protein), and ACTR1B (codes for actin-related protein 1 homolog B). The

the NCBI’s Gene Expression Omnibus (GEO GSE85428) (46). Because there are 141 genes located in the 2 ROH, we performed whole genome sequencing (WGS) of gDNA from patients 2 and 3 and their mother and focused our analysis on coding/splice non-synonymous variants that were homozygous in the patients and heterozygous in their mother. The WGS data have been deposited in the NCBI’s Sequence Read Archive (SRA SRP082684).

Figure 1. Serum autoantibodies in patients 2 and 3. (A and B) Heat map display of IgM (A) and IgG (B) autoantibody reactivity against self-proteins (Pt.) and controls (Ctrl.). A value of 1 is equal to the mean of the controls. P values were obtained by 2-tailed Student’s t test. *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 2. Defective peripheral B cell tolerance checkpoint and impaired Treg function in patient 3. (A) Reactivity of recombinant antibodies expressed by single CD19^+CD10^+CD21^+IgM^+CD27^- new emigrant/transitional B cells from a representative healthy age-matched control and patient 3 against dsDNA, insulin, and LPS. Clones were considered polyreactive when they recognized all 3 antigens. The frequencies of polyreactive B cells are summarized in the pie charts, with the number of antibody-secreting B cells tested shown in the center. (B) Percentage of polyreactive new emigrant/transitional B cell clones from patient 3 and 12 controls. (C) Reactivity of recombinant antibodies expressed by single CD19^+CD10^-CD21^+IgM^+CD27^- mature naive B cell clones from a representative control and patient 3 against lysates of HEp-2 cells tested by ELISA. The frequencies of HEp-2- and non–HEp-2–reactive B cells are summarized in the pie charts, with the number of antibody-secreting B cells tested shown in the center. (D) Percentage of polyreactive new emigrant/transitional B cell clones from patient 3 and 12 controls. (E) Suppression by CD4^+CD25^+CD127^- Tregs from control (left panel) and patient 3 (right panel) of the proliferation of CellTrace Violet-labeled CD4^+CD25^- Teff cells sorted from a third-party healthy subject stimulated with anti-CD3/CD28/CD2 beads at a 1:1 Treg/Teff ratio. (F) Quantification of the suppressive function of Tregs sorted from controls and patient 3 on Teff cells. Columns and bars in F represent mean ± SEM of 3 controls and patient 3. Dotted lines in A and C show the results from ED38. Horizontal lines show the OD_{405} cutoff for positive reactivity.
mutations in KCNA10, ST7L, and ACTRIB were not pathogenic, since they were found in the homozygous state in one or more of the healthy siblings (data not shown). The mutation in NEIL3 was homozygous in both patients and heterozygous in the parents and all 4 healthy siblings (Figure 3, A and B). The 2 adjacent coding variants in NEIL3 are in a single codon in exon 3 (chromosome 4 genomic position 178,256,958-178,256,959; c.395_396AC>TG) and would lead to a change of an aspartic acid (D) residue to valine (V) at position 132 in the protein (p.D132V; Figure 3C).

The D132V mutation affects NEIL3 expression as determined by immunoblot analysis of EBV-transformed B cell lines from patient 3 (Figure 3E). The crystal structure of the unliganded mouse ortholog of human NEIL3 (mNEIL3) showed that D133 in mNEIL3, the equivalent to the mutated D132 in the patients' NEIL3, is located in the DNA-binding pocket of the enzyme (38), suggesting that the D132 residue may be important for NEIL3 activity. To test this hypothesis, full-length WT and D132V NEIL3 were expressed in HeLa cells and immunoprecipitated, and their glycosylase activity was determined by immunoprecipitation-coupled glycosylase activity assay (47). Western blot analysis showed no difference in the expression levels of D132V NEIL3 compared with WT NEIL3 (Figure 3F), confirming that the D132V mutation does not affect the stability of the protein. However, the D132V mutant had severely decreased glycosylase activity compared with WT NEIL3 on single-stranded DNA substrates containing the S enantiomer of SP ([S]-SP) or Gh modification (Figure 3G). An empty vector control and a reaction blank with DNA substrate but no immunoprecipitation product were used as negative control. Recombinant mouse NEIL3 was used as positive control. Data in E–G are representative of 3 independent experiments.

The D132V mutation in NEIL3 did not affect protein expression as determined by immunoblot analysis of EBV-transformed B cell lines from patient 3 (Figure 3E). The crystal structure of the unliganded mouse ortholog of human NEIL3 (mNEIL3) showed that D133 in mNEIL3, the equivalent to the mutated D132 in the patients’ NEIL3, is located in the DNA-binding pocket of the enzyme (38), suggesting that the D132 residue may be important for NEIL3 activity. To test this hypothesis, full-length WT and D132V NEIL3 were expressed in HeLa cells and immunoprecipitated, and their glycosylase activity was determined by immunoprecipitation-coupled glycosylase activity assay (47). Western blot analysis showed no difference in the expression levels of D132V NEIL3 compared with WT NEIL3 (Figure 3F), confirming that the D132V mutation does not affect the stability of the protein. However, the D132V mutant had severely decreased glycosylase activity compared with WT NEIL3 on single-stranded DNA substrates containing the S enantiomer of SP ([S]-SP) or Gh modification (Figure 3G). An empty vector control and a reaction blank with DNA substrate but no immunoprecipitation product were used as negative controls and showed no product formation. Mouse
NEIL3, which has high activity on both substrates, was used as a positive control and showed maximal product formation. Taken together, these data show that the human D132V NEIL3 mutant has severely impaired glycosylase activity.

Presence of autoantibodies in an asymptomatic individual homozygous for the D132V mutation in NEIL3. A search of Boston Children’s Hospital’s genomic database of 400 individuals of Middle Eastern ancestry identified an asymptomatic adult female who was homozygous for the same NEIL3 mutation present in our patients (Figure 4A). Genomic DNA from this individual was sequenced in the course of working up her son for severe combined immune deficiency. Immunologic analysis of this asymptomatic subject revealed normal immune function with normal percentage of memory B cells and normal levels of serum IgG and IgM (data not shown). B cells from the NEIL3D132V/D132V subject proliferated normally in response to stimulation with anti-CD40+IL-4 and anti-CD40+IL-21 (Figure 4B) and secreted normal amounts of IgE and IgG in vitro (Figure 4C). However, the levels of several IgM and IgG autoantibodies were increased more than 2.5-fold and up to approximately 296-fold in her serum compared with the mean of 2 simultaneously studied healthy controls who had no mutation in NEIL3 (Figure 4D). These findings suggest that the homozygous D132V mutation in NEIL3 predisposes to the development of autoantibodies, but not to immunodeficiency.

Lack of LRBA protein expression in patient 3. The absence of immunodeficiency in an adult homozygous for the D132V mutation in NEIL3 prompted us to reexamine the ROH and the WGS data in patients 2 and 3 for intronic mutations associated with immunodeficiency. LRBA, which was not known to cause human disease at the start of this study, resides within the ROH shared by patients 2 and 3. Mutations in LRBA are now known to result in recurrent sinopulmonary infections and autoimmune disease, including enteropathy and cytopenias (15, 48–54). Reanalysis of the WGS data from patients 2 and 3 identified no exonic mutations; however, a 47.8-kb duplication spanning exons 49–53 of LRBA was identified in both patients. This duplication was asso-
associated with the presence of a transposable Alu element in intron 53 of the LRBA gene that was flanked by sequences from both introns 48 and 53 (Figure 5A). Reverse transcription polymerase chain reaction (RT-PCR) amplification of RNA extracted from an EBV-B cell line derived from patient 3 revealed that exons 1–28 of LRBA gave a product of size and intensity similar to that obtained from normal EBV-B cells. In contrast, RT-PCR amplification of exons 23–58 gave no detectable product in the patient, but a readily detectable product in the control (Figure 5B). These findings indicated that the promoter region of LRBA is functional, but that there was a structural variation between exons 23 and 58. RT-PCR and Sanger sequencing of cDNA from patient 3 demonstrated the duplication of exons 49 to 53, resulting in a frameshift at the junction of exon 53 and the duplicated exon 49, leading to a premature stop codon 4 aa after the start of the duplicated exon 49 (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI85647DS1). Immunoblot analysis using an antibody directed against an epitope N-terminal to the duplication (aa 907-1038) revealed absence of LRBA protein expression in EBV-B cell lysates from patient 3 (Figure 5C), confirming the deleterious effect of this structural variation. These results suggest that the immunodeficiency of the patients resulted from the absence of LRBA.

Defective peripheral B cell tolerance in single NEIL3 deficiency and single LRBA deficiency. Patient 3, who harbored deleterious mutations in both NEIL3 and LRBA, had intact central B cell tolerance, but defective peripheral B cell tolerance. To determine the effect of single NEIL3 deficiency and single LRBA deficiency on B cell tolerance, we examined the frequency of autoreactive B cell clones in the asymptomatic subject with the homozygous D132V NEIL3 mutation and in a patient homozygous for the R2629fs mutation in LRBA (NM_006726, exon54, c.7885delA, p.R2629fs) who presented with classical features for the R2629fs mutation in LRBA (NM_006726, exon54, c.7885delA, p.R2629fs) who presented with classical features for the R2629fs mutation in LRBA (NM_006726, exon54, c.7885delA, p.R2629fs) who presented with classical features for the R2629fs mutation in LRBA (NM_006726, exon54, c.7885delA, p.R2629fs). In both cases, we found an increased frequency of autoreactive B cell clones compared to healthy controls. However, unlike the patient with NEIL3 deficiency, the patient with LRBA deficiency had no detectable autoreactive B cell clones.

Neil3–/– mice had increased levels of IgM and IgG autoantibodies to several other autoantigens that trended toward statistical significance. Treg numbers and in vitro suppressive function were normal in Neil3–/– mice (Supplemental Figure 2E and Supplemental Figure 4A). However, Neil3–/– mice had significantly increased serum levels of IgM and IgG antibodies to 48 and 6 out of 83 autoantigens tested, respectively (Figure 7, A and B). These autoantigens encompassed cytoplasmic, structural, and nuclear proteins. In addition, Neil3–/– mice had increased levels of IgM and IgG autoantibodies to several other autoantigens that trended toward statistical significance. Treg numbers and in vitro suppressive function were normal in Neil3–/– mice (Supplemental Figure 2E and Supplemental Figure 3C), indicating that NEIL3 deficiency does not result in loss of peripheral tolerance in mice.

Neil3–/– mice remained healthy and developed no evidence of spontaneous autoimmune disease over a 9-month observation period, as determined by the maintenance of normal numbers.

Figure 5. Loss of LRBA protein expression in patient 3 as a result of the duplication of exons 49 to 53 of LRBA. (A) Schematic representation of exons 48–54 of LRBA in the control and patient 3 showing the presence of an Alu element and the duplication of exons 49–53 of LRBA from patient 3. (B) RT-PCR of LRBA cDNA from EBV-transformed B cells from patient 3 and a normal control shows normal amplification of exons 1–28, but absent amplification of exons 23–58 in patient 3, as compared with the control. (C) Immunoblot of LRBA in lysates of EBV-transformed B cells from patient 3 and a control showing absent LRBA protein expression in patient 3. Data are representative of 3 independent experiments.

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of red blood cells and platelets, absence of autoantibodies on the surface of these cells as determined by flow cytometry, and normal kidney histology (Supplemental Figure 4, B–D). Treatment of susceptible strains of mice with TLR ligands, which mimics the exposure to infectious pathogens in human patients, can provoke or accelerate the development of autoimmune kidney damage including glomerulonephritis (55). Neil3–/– mice on a C57BL/6 background and WT congenic controls were treated weekly over a period of 90 days with the TLR3/RIG-1/MDA5 ligand poly(I:C). Histologic examination of kidney sections stained with periodic acid–Schiff (PAS) stain revealed glomerulonephritis in Neil3–/– mice, but not WT controls, as evidenced by enlargement of the glomeruli, expansion of glomerular cells, and periglomerular infiltrates (Figure 8A). Moreover, interstitial peritubular infiltrates were present in the kidneys of Neil3–/– mice, but not WT controls (Figure 8A). The histologic scores for both glomerulonephritis and interstitial peritubular infiltrates were significantly higher in kidneys from Neil3–/– mice compared with WT controls (Figure 8B). Sera from Neil3–/– mice that received poly(I:C) had significantly increased levels of IgM antibodies to 86 out of 95 autoantigens tested (90%) and of IgG antibodies to 50 out of 95 autoantigens (53%) compared with poly(I:C)-treated WT controls (Figure 8C). This represents a broader autoantigen reactivity than what was observed in Neil3–/– mice that did not receive poly(I:C) (53% for IgM autoantibodies and 7% for IgG autoantibodies). These findings indicate that chronic TLR3/RIG-1/MDA5 stimulation exacerbates autoantibody production in Neil3–/– mice and predisposes these mice to develop nephritis.

Neil3 deficiency is associated with increased lymphocyte apoptosis and cell death. DNA modifications caused by oxidative stress that are not resolved by NEIL3 may result in cell death due to accumulated DNA damage. Increased lymphocyte apoptosis and cell death are associated with increased production of autoantibodies (4, 5). We examined apoptosis and cell death in splenic T and B cells. The percentage of annexin V7AAD+ apoptotic/dead cells among freshly isolated T and B cells was minimally increased in Neil3–/– mice compared with WT controls (Figure 9, A and B). Importantly, the percentages of annexin V7AAD+ apoptotic/dead T and B cells after culture in complete medium, with anti-CD3 ± anti-CD28 for 48 hours in the case of T cells and with LPS ± IL-4 for 24 hours in the case of B cells, were substantially and significantly higher in Neil3–/– mice compared with WT controls (Figure 9, A and B). These data demonstrate that lack of Neil3 impairs T and B cell survival.

The GC compartment is a highly proliferative in vivo environment where B cells are exposed to oxidative stress while diversifying their antibody repertoire (16, 56). GC B cells express high levels of Neil3 (for more information go to the Immunological Genome Project; www.imgen.org). We determined the rate of apoptosis of GC B cells freshly isolated from the PP of the intestine of Neil3–/– mice and WT controls. There was a significantly higher percentage of apoptotic annexin V+ cells among B220+Fas’GL7+ GC B cells from Neil3–/– mice compared with GC B cells from WT controls (Figure 9C). There was no significant difference in the percentages of annexin V+ cells among B220+Fas GL7+ non-GC B cells, and B220+ non-B cells between the 2 strains (Figure 9, D and E). These data indicate that Neil3 expression is important for the survival of the highly proliferating GC B cells in vivo, consistent with its high level of expression in this B cell population.

Autoantibodies have been associated with alterations in somatic hypermutation (SHM), which is mediated by activation-induced cytidine deaminase (AID, encoded by Aicda) expressed in GC B cells (56, 57). Moreover, knockout mice carrying a hypomorphic mutation in the BER DNA polymerase β (Pol β) have a dramatically increased frequency of SHM and develop autoimmune and lupus-like disease (58). As altered AID activity can result in apoptosis of GC B cells, we determined whether loss of Neil3 affects Aicda expression and the frequency of SHM. The level of Aicda mRNA was not significantly different in FACS-sorted B220+Fas’GL7+ GC B cells from PP of Neil3–/– mice compared with WT controls (Figure 9F). To determine whether Neil3 deficiency alters SHM, gDNA was extracted from GC B cells isolated from the intestinal PP of Neil3–/– mice and WT controls (n = 3 per group), the V[γ]558L framework 3 and J[γ]4 3′ flanking regions were amplified from the IgH locus, cloned in the TOPO-TA cloning vector, and 200 clones from each mouse were Sanger sequenced. A 551-bp fragment from each clone was analyzed for the presence of mutations and compared with the reference sequence of the C57BL/6J strain, as described previously (59). There was no significant difference in the frequency of total mutations in GC B cells between Neil3–/– mice and WT controls (1.92 ± 0.70 per 1000 bp in WT mice versus 12.58 ± 3.45 in Neil3–/– mice). In addition, the frequency of mutations per nucleotide was not different between Neil3–/– mice and WT controls (Supplemental Table 1). There was a modest increase in C to G transversions in Neil3–/– mice compared with controls (P = 0.04, Supplemental Table 2).

Discussion

We demonstrate that loss of function of the BER enzyme NEIL3 is associated with the development of autoantibodies in humans and mice and promotes the development of kidney inflammation in mice chronically exposed to the TLR3/RIG-1/MDA5 ligand poly(I:C) to mimic microbial stimulation.

We studied 3 offspring of a consanguineous family who suffered from severe infections, autoimmune disease, chronic diarrhea, and early death. Autoimmune manifestations included Coombs positive hemolytic anemia and autoimmune thrombocytopenia that was likely the cause of the fatal pulmonary hemorrhage in patient 1. Moreover, chronic diarrhea that was associated with intestinal inflammation was possibly autoimmune in origin. Hypogammaglobulinemia was demonstrated in 2 of the siblings. Phenotypic analysis performed on the patients revealed normal numbers of T, B, and NK cells and decreased memory B cells. Detailed immunologic studies in patient 3 revealed impaired Ig production by B cells in response to CD40 ligation in the presence of IL-4 and IL-21 in vitro. Sera available on the 2 younger siblings showed significantly elevated levels of IgM and IgG autoantibodies against a wide range of self-antigens (Figure 1). Studies on single B cells isolated from the youngest sibling revealed intact central B cell tolerance, but breakdown in peripheral B cell tolerance. Consistent with this finding, Treg function, but not number, was markedly impaired in the patient.
WGS of 2 of the patients and their mother identified a missense mutation in \textit{NEIL3}. The mutation substituted a conserved negatively charged aspartic acid residue with a neutral valine residue (D132V). The mutation did not affect the stability of the protein, but abolished its base damage excision repair enzymatic activity, demonstrating that it is a loss-of-function mutation. Two lines of evidence strongly indicated that the homozygous D132V mutation in \textit{NEIL3} results in the development of autoantibodies. First, as was seen in the patients, an unrelated individual homozygous for the same mutation in \textit{NEIL3} had elevated levels of serum autoantibodies. Second, mice that lacked \textit{Neil3} had elevated levels of a wide range of autoantibodies to cytoplasmic, structural, and nuclear proteins, including antibodies to glomerular basement membrane.

In contrast with the patients, the unrelated asymptomatic individual who was homozygous for the D132V mutation in \textit{NEIL3} had no recurrent infections or autoimmune disease, and her serum Ig levels, percentage of memory B cells, and B cell function in vitro were all normal. The decreased Ig production in the patient with double-\textit{NEIL3}/LRBA deficiency, but not in the subject with single-\textit{NEIL3} deficiency, could be a consequence of the low percentage of memory B cells in this patient, a finding shared with single-\textit{NEIL3} deficiency, patient 3, and 12 controls. The decreased Ig production in the patient with double-\textit{NEIL3}/LRBA deficiency; however, Tregs from this patient expressed CTLA-4 normally. Treg function in LRBA-deficient patients is associated with decreased CTLA-4 expression (15). This likely contributes to autoimmunity in these patients, as administration of CTLA-4-Ig dampens their autoimmunity (50). We were not able to examine the function of Tregs in the patient with the single \textit{NEIL3} deficiency; however, Tregs from an LRBA-deficient patient against dsDNA, insulin, and LPS tested by ELISA. Dotted lines show the results from a positive control designated ED38. Horizontal lines show the OD\textsubscript{405} cutoff for positive reactivity. The frequencies of reactive clones from patient 3 represented in Figure 2 was added to B and D for comparison with the subject with \textit{NEIL3} deficiency and the patient with LRBA deficiency. Each symbol represents 1 individual. The horizontal bar represents the mean of the controls. (E) Intracellular CTLA-4 staining in Tregs from 2 controls and the \textit{NEIL3}-deficient subject. The numbers in the plots represent mean fluorescence intensity (MFI).

\textit{NEIL3} and LRBA play discrete roles in preventing the accumulation of autoreactive mature naive B cells in the blood. Impaired Treg function in LRBA-deficient patients is associated with increased antibody-secreting B cell clones tested shown in the center. Clones are considered polyreactive when they recognize all 3 antigens. (B) Percentage of polyreactive new emigrant/transitional B cell clones from the \textit{NEIL3}-deficient subject (\textit{NEIL3}-def), the LRBA-deficient patient, patient 3, and 12 controls. (C) Reactivity of recombinant antibodies expressed by single mature naive B cell clones from a control, the \textit{NEIL3}-deficient subject, and the LRBA-deficient patient against lyses of HEp-2 cells tested by ELISA. Dotted lines show the results from the positive control ED38. Horizontal lines show the OD\textsubscript{405} cutoff for positive reactivity. The frequencies of HEp-2- and non-HEp-2-reactive B cells are summarized in the pie charts, with the number of antibody-secreting B cell clones tested shown in the center. (D) Percentage of HEp-2-reactive mature naive B cell clones from the \textit{NEIL3}-deficient subject, the LRBA-deficient patient, patient 3, and 12 controls. The percentage of reactive clones from patient 3 represented in Figure 2 was added to B and D for comparison with the subject with \textit{NEIL3} deficiency and the patient with LRBA deficiency. Each symbol represents 1 individual. The horizontal bar represents the mean of the controls. (E) Intracellular CTLA-4 staining in Tregs from 2 controls and the \textit{NEIL3}-deficient subject. The numbers in the plots represent mean fluorescence intensity (MFI).
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Figure 7. Elevated levels of autoantibodies in the sera of Neil3−/− mice. (A and B) Heat map display of IgM (A) and IgG (B) autoantibody reactivity against self-proteins in the sera of Neil3−/− mice compared with WT controls. A value of 1 is equal to the mean of the WT. Thirteen Neil3−/− and 9 WT mice were used in this assay. P values were obtained by 2-tailed Student’s t test. *P < 0.05; **P < 0.01.

immgen.org), while Ogg1 is ubiquitously expressed (26, 65). Furthermore, NEIL3 has a broad substrate specificity (36), including removal of DNA base damages from quadruplex DNA, unique DNA structures that have been implicated in DNA replication,
transcription, and telomere functions (66–68), while OGG1 activity is limited almost exclusively to removing 8-oxoguanine (19, 26). Differences in expression pattern and/or substrate specificity between NEIL3 and OGG1 might account for the role of NEIL3 in protecting against the development of autoantibodies.

To our knowledge, this is the first study implicating a DNA glycosylase in the development of autoimmune. The carrier rate of the NEIL3 D132V mutation was found to be approximately 2% in healthy Middle Eastern subjects. Thus, this mutation may confer a risk factor for autoimmune disease in Middle Eastern populations.

Methods

Flow cytometry. Anti-human antibodies with isotype–matched controls were used for staining CD19 (HIB19), CD27 (O323), and IgD (IA6-2) (all from BioLegend). Anti-mouse antibodies were used for staining B220 (RA3-6B2), CD3 (I45-2C11), CD4 (GK1.5), CD8 (53-6.7), CD25 (PC 61.5), and FOXP3 (FJK.16s) (all from eBioscience). Samples were collected with an LSRFortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.).

In vitro human B cell function. PBMCs were cultured with anti-CD40 (5 μg/ml; 626; Santa Cruz Biotechnology Inc.) with or without rIL-4 (5ng/ml; R&D Systems) or rIL-21 (20ng/ml; R&D Systems). Proliferation was assayed 4 days later by measuring 3H-thymidine incorporation into gDNA added for the last 16 hours of culture. The production of IgE and IgG was measured after 14 days by ELISA (eBioscience).

Autoantibody-producing B cells. Autoantibody production by sort- ed single CD19+CD10IgM+CD21+CD27+ new emigrant/transitional and CD19+CD10-IgM+CD21-CD27+ mature naive B cells was determined as previously described (69). A highly polyreactive antibody (ED38) (69, 70) was used as a positive control.

Treg suppression assays. CD4+ T cells were isolated by negative selection (Miltenyi Biotec). For isolation of CD4+CD25− T eff cells, CD25+ cells were depleted with anti-CD25 beads. Tregs were sorted for CD4+CD25+CD127− human cells, and CD4+CD25−CD39+ mouse cells. Teff cells were labeled with CellTrace Violet (Life Technologies) and stimulated with anti-CD3/CD28/CD2-2 coated beads (Treg Suppression Inspector, human; Miltenyi Biotec) or with mouse antigen-presenting cells and 1 μg/ml anti-CD3. Autologous or allogeneic Tregs were added at different ratios, and cell divisions were evaluated using flow cytometry.

Autoantibody assays. Human and mouse IgG and IgM autoantibodies were measured on an autoantigen proteomic array (University of Texas Southwestern Medical Center, Dallas, Texas, USA) as previously described (71). Levels of autoantibodies against self-antigens were visualized as heat maps. Values from control samples were averaged for each antigen, and the averages of controls plus 2 SDs were calculated to generate relative autoantibody reactivity. Data were visualized by Multiple Experiment Viewer (MeV) 4.8 (version 10.2) (72). Student’s t test was used to compare the differences between groups, using GraphPad PRISM (GraphPad Software).

Genetic analysis. Homozygosity mapping was performed with the Affymetrix genome-wide human SNP array 6.0 and analyzed with Birdsuite version 1.4, and PLINK version 1.07, as previously described (73). WGS was performed through Complete Genomics Inc. and analyzed with Mol-BioLib (74). The frequencies of the identified variants were determined in the Exome Variant database (Exome Variant Server, National Heart, Lung, and Blood Institute [NHLBI] GO Exome Sequencing Project; http://evs.gs.washington.edu/EVS/), the Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org), and the 1000 Genomes database (75).

Custom TaqMan primers (Applied Biosystems) were used to determine the frequency of the mutation identified in NEIL3 in the Kuwaiti population using Applied Biosystems 7900HT Real-Time PCR. Sanger sequencing was used to determine the mutation in the Lebanese populations.

Immunoblotting. Lysates from EBV-transformed B cells were immunoblotted with rabbit polyclonal antibodies against NEIL3 (AssayBioTech; C15635), LRBA (Sigma-Aldrich; HPA023597), and actin (Sigma-Aldrich; A1978).

Plasmids. Human NEIL3 cDNA with a C-terminal HA tag was cloned into the pEGFP-N3 plasmid (Invitrogen). QuikChange site-directed mutagenesis (Stratagene) was used to create plasmids with the D132V mutation. Primer sequences are in Supplemental Table 3.

Immunoprecipitation-coupled glycosylase activity assay. HeLa cells were transfected with WT or D132V pNEIL3-HA using Lipofectamine 2000 (Life Technologies) and lysed in NP-40 lysis buffer (Invitrogen) 24 hours later. Then 2 μg of anti-HA antibody (Abcam ab9110) were prebound to 50 μl Dynabeads protein G (Life Technologies) and incubated with lysates for 2 hours at 4°C with rotation. Washed beads were suspended in NEIL3 reaction buffer (40 mM HEPES pH 7.0, 50 mM NaCl, 0.01% NP-40, 1 mM EDTA, and 1mM DTT), and 5 μl beads were denatured and subjected to immunoblotting using anti-HA mAb. Glycosylase activity was measured as previously described (38).

Mouse T and B cell proliferation, cytokine and Ig secretion. Splenic T and B cells were purified using the Pan T Cell Isolation Kit II and CD43-bound microbeads, respectively (Miltenyi Biotec). T cells were stimulated for 72 hours with 1 μg/ml immobilized anti-CD3 (I45-2C11; eBioscience) with or without 1 μg/ml anti-CD28 (57.51; eBioscience) or 20 ng/ml IL-2 (R&D Systems). B cells were stimulated for 72 hours with 50 ng/ml IL-4 (R&D systems), 100 ng/ml anti-CD40 (1C10; eBioscience), and 10 μg/ml LPS (Invivogen) alone or in combination. Proliferation was determined as above. IL-2, IFN-γ, IgE, and IgG1 were measured by ELISA (BioLegend).

Antibody production. Mice were anesthetized with 2,2,2-trimethoxymethanol (Sigma-Aldrich), immunized i.p. on day 0 (10 μg/mouse), and boost ed on day 14 (2.5 μg/mouse) with 2,4,6-trinitrophenyl hapten conjugated to protein keyhole limpet hemocyanin (TNPα-KLH; Biosearch Technologies Inc.) in ALUM (Thermo Scientific). Mice were sacrificed at day 21 for T-D antibody production or were immunized with 25 μg/mouse of TNPα-Ficoll and sacrificed at day 14 for T-I antibody production. IgM and IgG anti-TNP and serum IgM, IgG, and IgA were detected by ELISA (76).

Treatment of mice with poly(I:C) and kidney histology. Mice were injected weekly with 50 μg/mouse of poly(I:C) (Invivogen) in 100 μl PBS for 90 days. Kidneys were then obtained, fixed in 10% buffered formalin, and embedded in paraffin. Multiple 4-μm sections were stained with PAS and visualized on a Nikon Eclipse E800 microscope (Nikon). Images were captured with Nikon DS F1i camera and NIS-Elements AR3.2 software (Nikon). Kidney damage was scored as previously described (77).

Apoptosis of GC B cells. PC were isolated and GC B cells were stained with antibodies against B220 (RA3-6B2), Fas (1SA7), GL7 (GL7), annexin V, and 7AAD (all from eBioscience).

Amplification, cloning, and sequence analysis of the J558VH-FR3-JH4 3’ intron and exon segments. Viable (7AAD−) GC (B220+...
Figure 8. Poly(I:C)-induced glomerulonephritis in the kidneys and elevated levels of autoantibodies in the sera of Neil3−/− mice. (A) Representative photomicrographs of kidney sections stained with PAS from Neil3−/− mice and WT controls treated with poly(I:C). Kidney sections from Neil3−/− mice show glomerulonephritis with periglomerular infiltrates (upper right panel, arrows) and interstitial infiltrate (lower right panel, arrow). Original magnification, ×40 (upper panels); ×20 (lower panels). Data are representative of 10 mice per group. (B) Histologic scores of glomerulonephritis and interstitial infiltrates, scored according to ref. 77. Columns and bars represent mean ± SEM of 10 mice per group. P values were obtained by 2-tailed Student’s t test. (C) Heat map display of IgM and IgG autoantibody reactivity against self-proteins in the sera of poly(I:C)-treated Neil3−/− mice and WT controls (n = 5 mice per group). A value of 1 is equal to the mean of the poly(I:C)-treated WT controls. P values were obtained by 2-tailed Student’s t test. *P < 0.05; **P < 0.01.
CD95+GL7+ B cells were FACS sorted and gDNA extracted. The VHJ58L framework3-JH4 3’ flanking region was PCR amplified as previously described (59). PCR product (600 bp) was purified (QIAGen Quick Gel Extraction Kit; QIAagen), dA tails were added with Taq polymerase (Thermo Scientific), cloned in TOPO-TA vectors (Invitrogen), and used to transform Escherichia coli DH5α competent cells. TA-plasmids were extracted from 200 clones using the Gene Jet Plasmid Miniprep Kit (Thermo Scientific) and Sanger sequenced; then a 551-bp fragment was analyzed for the presence of mutations (59).

RNA extraction and RT/PCR of LRBA. RNA was extracted from EBV-transformed B cells using TRIzol (Life Technologies) and used.
to amplify LRBA cDNA exons using the SuperScript III One-Step RT-PCR System with Platinum Taq (Life Technologies). Primers used are listed in Supplemental Table 4.

Statistics. Two-tailed Student’s t test and 2-way ANOVA were used to compare the differences between groups, using GraphPad PRISM (GraphPad Software). A P value of less than 0.05 was considered significant.

Study approval. Blood samples were obtained after securing informed consent approved by the Institutional Review Board of Boston Children’s Hospital. Deidentified DNA samples from 384 healthy Kuwaiti and 100 healthy Lebanese donors were used as controls. Neil3−/− mice have been described (40). Mouse studies were performed in accordance with Boston Children’s Hospital policies and procedures.

Author contributions

MJM, JZ, JC, HJ, EJ, HM, TKO, KS, MK, JK, EC, AM, PBK, SG, BGO, EM, and MM contributed to research design, conducted the experiments, and acquired and analyzed the data. DT, KT, and YN provided reagents. WAH, LDN, GCT, JPM, EAI, and HA contributed patient samples and advice. MJM, JC, SSW, and RSG contributed to writing of the manuscript.

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