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Autosomal recessive mutations in proteasome subunit β8 (PSMB8), which encodes the inducible proteasome subunit β5i, cause the immune-dysregulatory disease chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE), which is classified as a proteasome-associated autoinflammatory syndrome (PRAAS). Here, we identified 8 mutations in 4 proteasome genes, PSMA3 (encodes α7), PSMB4 (encodes β7), PSMB9 (encodes β1i), and proteasome maturation protein (POMP), that have not been previously associated with disease and 1 mutation in PSMB8 that has not been previously reported. One patient was compound heterozygous for PSMB4 mutations, 6 patients from 4 families were heterozygous for a missense mutation in 1 inducible proteasome subunit and a mutation in a constitutive proteasome subunit, and 1 patient was heterozygous for a POMP mutation, thus establishing a digenic and autosomal dominant inheritance pattern of PRAAS. Function evaluation revealed that these mutations variably affect transcription, protein expression, protein folding, proteasome assembly, and, ultimately, proteasome activity. Moreover, defects in proteasome formation and function were recapitulated by siRNA-mediated knockdown of the respective subunits in primary fibroblasts from healthy individuals. Patient-isolated hematopoietic and nonhematopoietic cells exhibited a strong IFN gene-expression signature, irrespective of genotype. Additionally, chemical […]

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Autosomal recessive mutations in proteasome subunit β 8 (PSMB8), which encodes the inducible proteasome subunit β5i, cause the immune-dysregulatory disease chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE), which is classified as a proteasome-associated autoinflammatory syndrome (PRAAS). Here, we identified 8 mutations in 4 proteasome genes, PSMA3 (encodes α7), PSMB4 (encodes β7), PSMB9 (encodes β1i), and proteasome maturation protein (POMP), that have not been previously associated with disease and 1 mutation in PSMB8 that has not been previously reported. One patient was compound heterozygous for PSMB4 mutations, 6 patients from 4 families were heterozygous for a missense mutation in 1 inducible proteasome subunit and a mutation in a constitutive proteasome subunit, and 1 patient was heterozygous for a POMP mutation, thus establishing a digenic and autosomal dominant inheritance pattern of PRAAS. Function evaluation revealed that these mutations variably affect transcription, protein expression, protein folding, proteasome assembly, and, ultimately, proteasome activity. Moreover, defects in proteasome formation and function were recapitulated by siRNA-mediated knockdown of the respective subunits in primary fibroblasts from healthy individuals. Patient-isolated hematopoietic and nonhematopoietic cells exhibited a strong IFN gene-expression signature, irrespective of genotype. Additionally, chemical proteasome inhibition or progressive depletion of proteasome subunit gene transcription with siRNA induced transcription of type I IFN genes in healthy control cells. Our results provide further insight into CANDLE genetics and link global proteasome dysfunction to increased type I IFN production.

Introduction

Monogenic autoinflammatory diseases are immune-dysregulatory conditions that often present in the perinatal period with sterile episodes of fever and excessive organ-specific inflammation (1); genetic defects in innate immune pathways can cause intracellular stress, leading to cytokine dysregulation (2). Autosomal recessive homozygous or compound heterozygous loss-of-function mutations in proteasome subunit β 8 (PSMB8), which encodes the inducible proteasome component β5i, cause a syndrome that has historically been referred to as joint contractures, muscle atrophy, microcytic anemia, and panniculitis-induced childhood-onset lipodystrophy (JMP) syndrome, Nakajono-Nishimura syndrome (NNS), or chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE). These conditions form 1 disease spectrum of proteasome-associated autoinflammatory syndrome (PRAAS) (3–6). Characteristic clinical features

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include early presentation with fever, nodular skin rashes, myositis, panniculitis-induced lipodystrophy, and basal ganglion calcifications. In contrast to what occurs in many currently known autoinflammatory diseases, patients with CANDLE/PRAAS do not respond to IL-1 inhibition. The expression of a strong IFN-response gene signature suggests a possible association of proteasome dysfunction and IFN dysregulation, but the type of IFN driving the signature has not been well defined (6).

The ubiquitin proteasome system (UPS) degrades intracellular proteins derived from self or foreign structures and has a major role in the removal of misfolded proteins (7). The 20S core of the proteasome comprises 2 outer α-rings and 2 inner β-rings in an \( \alpha_2\beta_2\alpha_2\beta_2\) configuration. It contains 3 proteolytically active sites, 1 located in the \( \beta_1\) subunit conferring caspase-like activity, 1 in the \( \beta_2\) subunit conferring trypsin-like activity, and 1 in the \( \beta_5\) subunit conferring chymotrypsin-like activity. The 20S-core complex can be capped with 19S regulatory units, which recognize ubiquitin-protein conjugates, or with the PA28 regulator (8), forming 26S proteasomes or hybrid proteasomes, respectively (9).

Thus far, CANDLE/PRAAS-causing mutations have only been reported in PSMB8, which encodes an alternative inducible catalytic proteasome subunit designated as \( \beta_i\) (also known as LMP7) (4–6, 10, 11). There are 2 other inducible active sites, \( \beta_i1\) (also known as LMP2 and encoded by PSMB9) and \( \beta_i2\) (also known as Mecl1 and encoded by PSMB10), which can be incorporated into nascent proteasome complexes to form immunoproteasomes and thus increase proteolytic capacity. In most tissues, immunoproteasome formation is induced by proinflammatory cytokines, but it is constitutively expressed in hematopoietic cells (12, 13).

Helper proteins govern the assembly and maturation of either standard or immunoproteasomes; one such protein, proteasome maturation protein (POMP), is essential for proteasome formation and cell viability and preferentially supports incorporation of the inducible \( \beta_i\) subunit. Most proteasomal \( \beta\) subunits, except for \( \beta_3\) and \( \beta_4\), are synthesized as proforms that are matured through autocatalytic cleavage during the assembly process, which liberates the active-site threonines (14, 15). Most proteasomal \( \beta\) subunits, except for \( \beta_3\) and \( \beta_4\), are synthesized as proforms that are matured through autocatalytic cleavage during the assembly process, which liberates the active-site threonines.

As \( \beta_i\)-deficient mice do not exhibit a spontaneous inflammatory or metabolic phenotype, the presence of inflammatory disease manifestations in patients with PSMB8 mutations is surprising and raises questions about disease-causing mechanisms. Mouse models link immunoproteasome dysfunction to oxidative stress, accumulation of toxic ubiquitin-rich aggregates, and cytokine dysregulation, suggesting a role for immunoproteasomes in tissue preservation during inflammatory processes (16, 17).

Here, we demonstrate that CANDLE/PRAAS can be caused by either monogenic or digenic inheritance of hypomorphic or loss-of-function mutations in immunoproteasome and in constitutive proteasome subunits. Our data suggest that global proteasome dysfunction irrespective of genotype and the specific proteolytic subunit affected is linked to the upregulation of type I but not type II IFN production. Thus, our data suggest that proteasome dysfunction can lead to chronic type I IFN induction, establishing CANDLE as an IFN-mediated autoinflammatory disease.

Results

Identification of novel mutations in constitutive and inducible proteasome genes suggests digenic inheritance. We studied 8 patients (patients 1–8) with the clinical phenotype of CANDLE (Figure 1, A–D, Table 1, and Supplemental Figure 1, A–D; supplemental material available online with this article; doi:10.1172/JCI81260DS1) who had no known PSMB8 mutation (patients 1, 4, 5, and 8) or were heterozygous for only 1 disease-associated mutation in the PSMB8 gene: p.T75M (patients 2 and 3) or p.K105Q (patients 6 and 7). Clinical and demographic features are presented in Supplemental Table 1. We hypothesized that additional proteasome genes may cause CANDLE and screened 14 proteasome candidate genes encoding the proteasome subunits and the proteasome assembly gene, POMP, by standard sequencing (patients 1, 2, 3, 4, 5, 6, 7, and 8). Three patients (patients 1, 2, 8) and the unaffected parents of patient 2 were evaluated by whole-exome sequencing (WES) to facilitate screening and to rule out additional shared variants that may contribute to disease.

Patient 1 was compound heterozygous for 2 PSMB4 mutations encoding the constitutive \( \beta_3\) subunit, a rare 5’ UTR point mutation, c.-9G>A (rs200946642), and a novel 9 bp in-frame deletion (c.634-642del/p.D212V214del). Both unaffected parents and 1 of 2 unaffected siblings were carriers for one of the respective mutations (Figure 1, E and F, Table 1, and Supplemental Table 2).

Two unrelated patients, patient 2 and patient 3, with a known, paternally inherited PSMB8 mutation (p.T75M) each had a different novel mutation in PSMA3, encoding the constitutive \( a_7\) subunit. Patient 2 had a de novo heterozygous 3-bp in-frame deletion in PSMA3 (c.696_698delAAC/p.R233del), and patient 3 inherited a splice-site mutation in PSMA3 (c.404+2T>G/p.H111fs*10) from his unaffected mother (Figure 1, E and F, Table 1, and Supplemental Table 2).

Two affected Jamaican siblings, patients 4 and 5, inherited a novel monolicelial PSMB4 (β7-encoding) variant (c.44insG/p.P166fs*45) from their father and a rare missense substitution affecting a highly conserved aa residue, c.494G>A/p.G165D (rs69395789), in the inducible subunit PSMB9 (βi1 encoding) from their mother (Figure 1, E and F, Table 1, and Supplemental Table 2).

Two affected Irish siblings, patients 6 and 7, inherited a novel monolicelial nonsense mutation in PSMB4, c.666C>A/p.Y222X, from their father and a novel heterozygous missense mutation in PSMB8, c.313A>C/p.K105Q, from their mother (Figure 1, E and F, Table 1, and Supplemental Table 2).

An adopted patient of Palestinian descent (previously described in ref. 18) had a novel heterozygous frameshift mutation in POMP (c.344_345insTTTGGA/p.E115DSfs*20) (Supplemental Figure 2A, Table 1, and Supplemental Table 2).

For the 8 novel or rare CANDLE-associated mutations in 1 inducible and 3 constitutive proteasome subunit genes and in POMP, the allele frequencies and pathogenicity predictions for the novel mutations are summarized in Supplemental Tables 2 and 3 and in Supplemental Results.

In silico modeling and gene-expression studies suggest effects of mutations on protein-folding proteolytic activity and protein expression levels. All mutations were predicted to be pathogenic based on their conservation in vertebrates and on structural modeling by using the x-ray structures of bovine (19) and mouse 20S proteasomes (ref. 20 and Figure 1, G–J).
Figure 1. Clinical findings and CANDLE/PRAAS-associated mutations in 4 proteasome-encoding genes and in silico modeling. (A) Marked facial edema during flare. (B) Lipoatrophy later in life. (C) CANDLE rash during acute flare. (D) Abdominal protrusion due to intraabdominal fat deposition. (E) Pedigrees and identified genotypes of patients and their direct relatives. Underline in red indicates maternal, in blue, paternal, and in green, de novo inheritance of mutant allele. (F) Schematic organization of PSMB4, PSMA3, PSMB9, and PSMB8 genes (exon-intron structure, black rectangles represent coding sequences, white rectangles represent UTRs) with positions of the identified mutations. (G) Species conservation of mutated aa (yellow). Hs, Homo sapiens; Pt, Pan troglodytes (chimpanzee); Mm, Mus musculus (mouse); Oc, Oryctolagus cuniculus (rabbit); Bt, Bos taurus (cattle); Clp, Canis lupus familiaris (dog); Xi, Xenopus laevis (frog). Dr, Danio rerio (zebrafish). Alignment was performed with ClustalW. (H) PSMB8 and PSMB9 mutations were modeled based on the x-ray structure of the mouse immunoproteasome (PDB entry code: 3UNH) (46), and the mutations in PSMA3 and PSMB4 were based on the bovine 20S proteasome (PDB entry code: 1I1R) (19). Mutated subunits α7 (orange), β7 (cyan), and β1i (purple) are located at the opposite side of the 20S particle compared with β1i (red). (I) Top view of a ring. Subunit α7 (orange) with mutant residue R233 (balls) highlighted. (J) Detailed perspectives of ribbon models of mutant proteins. Mutated residues are depicted in yellow with relevant interaction aa side chains shown with stick models. Novel mutations are highlighted in yellow rectangles. Catalytic active sites in β1i and β1i are marked with asterisks. H, heterozygous, NM, nonmutant.
The 3-aa deletion in β7, p.D212-V214 (family 1), was located at the N terminus of an α-helix forming an intramolecular hydrogen-bonding network that stabilized its C-terminal extension. The p.P165fs*45 (family 4) and the p.Y222X (family 5) mutations caused the loss of the C-terminal extension of β7 (Figure 1, G–J), which is essential for proteasome assembly (21).

The p.R233del deletion in PSMA3 (family 2) distorted a surface of an α-helix-stabilizing motif of the α7 subunit and likely affected the subunit folding (Figure 1, H and J) and attachment of regulatory complexes (Figure 1I).

The β1i variant, p.G165D (family 4), was located in a loop interconnecting 2 α-helices (Figure 1J) that define the position of a β1i/caspase-like activity conferred by threonine (T1). The PSMB8 mutation p.K105Q (family 5) affected αα positions that directly regulate to β5i/chymotrypsin-like activity (20).

We next assessed allele-specific transcription of various mutant transcripts in peripheral blood mononuclear cells (PBMCs) (patients 1–5). In family 1, the maternally inherited mutant PSMB4 allele (c.-9G>A) was expressed at lower levels than the WT allele, both in patient 1 and in his unaffected mother (Figure 2A). The unaffected father who carried the 3-aa deletion in the same gene may have had increased expression of PSMB4 transcripts, indicating transcriptional compensation.

The expression of the c.696_698delAAG mutant allele in PSMA3 (patient 2) was similar to WT. The increased expression level of 60% for both mutant and WT allele when compared with his unaffected mother (patient 2-M), who was mutation negative in proteasome-encoding genes, may suggest compensatory induction of WT and mutant transcripts (Figure 2B).

The PSMA3 c.404+2T>C splice site mutation (family 3) led to an unstable transcript (Figure 2C) due to the skipping of exon 5 (Figure 2E). The mutant transcript was only amplified with junction-specific primers spanning exons 4 and 6 in patient 3 and his mother (Figure 2F) and not in the father (patient 3-F) and was likely not expressed. Patient 3-F, who was a carrier for the PSMB8 mutation, had normal expression of the PSMA3 transcript (Figure 2C).

The early PSMB4 frameshift mutation, c.44insG, in patients 4 and 5 suggests nonexpression of the mutant allele in the 2 affected children and their unaffected father. The increased PSMB4 mRNA expression in the father carrying the mutation may suggest compensatory upregulation (Figure 2D).

Proteasome subunit mutations affect proteasome assembly and maturation in vitro. To assess the impact of each of the novel mutations on proteasome assembly, we ectopically expressed 6 mutations, excluding those resulting in nonexpressed transcripts and the PSMB9 mutation, as V5 epitope–tagged versions from viral promoters in HEK293 cells. We assayed the mutation by immunoblotting for the V5 tag in SDS and native PAGE analyses (for schematic representation of tagged subunits, see Figure 3A, and for a summary of the effects by mutation, see Supplemental Table 4).

The V5-tagged p.D212_V214del mutant of β7 (present in family 1) revealed poor maturation of the β7 subunit (Figure 3B) and poor incorporation into 20S or 26S proteasomes. However, the mutated protein was detected in proteasome assembly intermediates (Figure 3C). The overall β7 protein expression of the c.-9G>A mutant protein was lower than that of WT (Figure 3, B and D), with therefore less incorporation into proteasome complexes compared with WT (Figure 3C). The accumulation of β5i-, β6-, and POMP-containing proteasome precursor complexes in patient 1 and his father’s lymphoblastoid cell lines (LCLs) and fibroblasts confirmed the maturation defect (Supplemental Figure 3, A–C) and suggests that propeptide cleavage of the β5i subunit is impaired in mutant β7 subunits (Supplemental Figure 3B). The lower expressed but incorporated protein encoded by the c.-9G>A mutant gene (patient 1 and his mother) likely compensated for the failure of the paternal 3-aa-deleted β7 subunit to be incorporated into proteasomes and to become matured (Figure 3B and Supplemental Figure 3, A–C). The increase in free PA28 complexes indicates that both β7 mutations destabilized the interaction of 20S complexes with the PA28 regulator.

Table 1. CANDLE patients, ancestry, and sequence/aa alterations in various proteasome genes (PSMA3, PSMB4, PSMB8, PSMB9, POMP)

<table>
<thead>
<tr>
<th>Patients</th>
<th>Ancestry</th>
<th>Gene 1 maternal allele</th>
<th>Nucleotide alteration/aa alteration</th>
<th>Gene 2 paternal allele</th>
<th>Nucleotide alteration/aa alteration</th>
<th>Genetic model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>US resident, ME</td>
<td>PSMB4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5′ UTR: c.-9G&gt;A</td>
<td>PSMB4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.634-642del, p.D212_V214del</td>
<td>Compound heterozygous, monogenic</td>
</tr>
<tr>
<td>Patient 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>US resident, ME</td>
<td>PSMA3&lt;sup&gt;a&lt;/sup&gt; (de novo)</td>
<td>c.696_698delAAG, p.R233del</td>
<td>PSMB8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.224C&gt;T, p.I775M and WT</td>
<td>Double heterozygous, digenic</td>
</tr>
<tr>
<td>Patient 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Spanish</td>
<td>PSMA3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.404+2T&gt;C, p.H111Ffs*10</td>
<td>PSMB8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.224C&gt;T, p.I775M and WT</td>
<td>Double heterozygous, digenic</td>
</tr>
<tr>
<td>Patient 4</td>
<td>Jamaican, sister of patient 5</td>
<td>PSMB9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.494G&gt;A, p.G165D and WT</td>
<td>PSMB4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.44, 45insG, p.P165fs*45 and WT</td>
<td>Double heterozygous, digenic</td>
</tr>
<tr>
<td>Patient 5</td>
<td>Jamaican, brother of patient 4</td>
<td>PSMB9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.494G&gt;A, p.G165D and WT</td>
<td>PSMB4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.44, 45insG, p.P165fs*45 and WT</td>
<td>Double heterozygous, digenic</td>
</tr>
<tr>
<td>Patient 6</td>
<td>Irish, brother of patient 7</td>
<td>PSMB8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.313A&gt;C, p.K105Q and WT</td>
<td>PSMB4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.666C&gt;A, p.Y222X and WT</td>
<td>Double heterozygous, digenic</td>
</tr>
<tr>
<td>Patient 7</td>
<td>Irish, brother of patient 6</td>
<td>PSMB8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.313A&gt;C, p.K105Q and WT</td>
<td>PSMB4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>c.666C&gt;A, p.Y222X and WT</td>
<td>Double heterozygous, digenic</td>
</tr>
<tr>
<td>Patient 8</td>
<td>Palestinian</td>
<td>POMP</td>
<td>c.344_345insTTTGA/p.E115Dfs*20 and WT</td>
<td>None identified</td>
<td>Autosomal dominant, monogenic</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Clinical case description previously published; the heterozygous PSMB8 mutations in patients 2 and 3 have been reported (6). <sup>b</sup>Mutation in a gene encoding a constitutive proteasome component (PSMB4 or PSMA3). <sup>c</sup>Mutation in a gene encoding an inducible proteasome component (PSMB8 or PSMB9). ME, mixed European descent. NA, not applicable, as no other mutations in genes encoding either constitutive or inducible subunits were found.
The nonsense mutation p.Y222X (patients 6 and 7, family 5) in β7 was expressed, but failed to incorporate into the 20S or 26S complexes, thus confirming the importance of the C-terminal β7 tail for proteasome assembly (Figure 3, B and C).

Protein from the V5-tagged α7 mutant p.R233del (patient 2) was not detectable in the complexes (Figure 3B), although normal transcription levels from the plasmid CMV promoter were seen (Figure 3D), suggesting that the mutant α7 was not incorporated into the 20S and the mature 26S complexes (Figure 3C). Similar to the in vitro data, in fibroblasts and LCLs from patient 2, the α7 mutation resulted in slightly reduced expression of the affected subunit (Supplemental Figure 3, B and C) and in overall reduced proteasome content. In addition, this α7 subunit mutation affected the binding to the PA28 regulator complex indicated by the increase in free PA28 regulator (Supplemental Figure 3A).

The novel (K105Q) and 5 previously reported β5i mutant subunits showed variable incorporation and/or maturation defects in transfected HeLa cells (Figure 3, B and C). The T75M and the G201V mutations led to decreased proteasome assembly (4, 5). The K105Q mutation caused maturation defects due to inability to completely trim the β5i propeptide (presence of intermediate band in Figure 3B), the p.M117V and p.A92T mutations displayed normal maturation and assembly (Figure 3, B and C) and only affected proteolytic activity (see below), and the truncation mutation C135X led to nonexpression of the protein, explaining its absence in all proteasome complexes (Figure 3, B and C).

We were unable to express the mutant variants of PSMB9 and POMP in HeLa cells. The truncated protein resulting from the POMP mutation (Supplemental Figure 2A) is likely unstable. POMP depletion by siRNA caused assembly defects, resulting in precursor accumulation and reduced proteasome formation with final reduced overall proteasome activity (Supplemental Figure 2, B and C).

The proteasome subunit mutations reduce proteolytic function in hematopoietic cells. To evaluate the effect of the different mutations on proteasome function, we assessed the activity and proteasome content in patients’ hematopoietic (whole blood and EBV-transformed B cells) cells (Figure 4A and Supplemental Tables 4 and 5). As previously shown and corresponding to the β5i-active site of the proteasome, PBMCs from patients with the PSMB8 mutations, control patient C1 (T75M/T75M), and control patient C3 (T75M/A92T) had selectively impaired chymotryptic-like activity, while the patient C2, who was homozygous for the C135X-PSMB8
nonsense mutation, had reduction in all 3 proteasome activities. Patients with digenic inheritance (patients 2, 4, 5) and with the PSMB4 mutations (patient 1) had more variable proteolytic defects. PBMCs from patient 1, with 2 PSMB4 mutations, and patient 2, with 1 PSMB8 mutation and 1 PSMA3 mutation, had impairment in all 3 measured proteolytic activities (Figure 4A). Proteasome activity measured in EBV-transformed B cells from patients 1 and 2 showed a similar impairment pattern (Supplemental Figure 4, A and B).

PBMCs from patients 4 and 5 had severely reduced caspase-like activity consistent with the PSMB9 mutation, because WT PSMB9 confers caspase-like activity. Overall, the proteasome activities were not as severely impaired, as was seen in patients with PSMB8 mutations with chymotryptic activity impaired over 70%.

IFN stimulation increases immunoproteasome content and thus proteolytic activity (12); we therefore wanted to determine whether proteasome activity is increased in patients with other autoinflammatory diseases. We studied PBMCs from patients with the IL-1–mediated disease neonatal-onset multisystem inflammatory disease (NOMID), who had no IFN signature and a normal IFN score, and from patients with undifferentiated autoinflammatory diseases (UIDs), who are genetically uncharacterized, but who had showed increased IFN signatures on gene-expression studies (not shown) and elevated IFN scores, suggesting chronic IFN stimulation.

Ubiquitin aggregation and proteasome function in affected skin.

To assess proteasome assembly and function in cells of an affected tissue other than blood, we examined skin biopsies from patients 1 and 2 and primary keratinocytes from patients 1, 2, 4, and 5. As previously observed in PSMB8-CANDLE patients (4, 5) ubiquitin staining of lesional skin biopsies from patient 1 (PSMB4 mutation) and patient 2 (PSMB8/PSMA3 mutations) showed a significant increase in ubiquitin-positive keratinocytes in comparison with healthy controls and psoriasis, a control inflammatory disorder.
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**Figure 4. Proteasomal activity of PBMCs.** (A) Proteasome activity of PBMCs from CANDLE/PRAAS patients (turquoise, Pt.1; orange, Pt.2; purple, Pt.4 and Pt.5; red, CANDLE patients with PSMB8 mutations), patient family members or heterozygous carriers (gray), healthy controls (HC, black), patients with an undifferentiated interferonopathy disease who are negative for proteasome gene mutations (UID, green), and active pretreatment NOMID patients (blue) was analyzed and expressed as percentage relative to the average of healthy controls. The dashed lines indicate 100% activity. Whole blood RNA samples from the same date for patient and patient family members were analyzed by qRT-PCR for IFN-regulated gene expression and an IFN score was calculated. For patient 2, we used control samples from nonrelated heterozygous carriers for the PSMB8 T75M (HZMa, HZMb, and HZMc) for comparison. Measurements for patient 4, patient 5, and their parents were performed at only 1 time point before treatment. Patients with PSMB8 mutations, C1 (T75M/ T75M), C2 (C135X/C135X), and C3 (T75M/A92T), were assessed as CANDLE controls. Two-sample, 2-tailed t tests were performed, and P values were stated. Error bars indicate technical triplicates. (B) Patient proteasome activity was normalized to the UID mean for each of the proteasome activities. Paired t test was performed. (C) PBMCs from 3 healthy donors were stimulated with the indicated cytokines or none for 6, 12, or 24 hours. The cell numbers were counted and a proteasome activity assay was done. Fold changes of activity against no-treatment control was calculated. The dashed lines on the graph indicate the activity of no-treatment controls. The numbers on top of the data points are IFN scores. Data represent mean ± SEM from n = 3 samples. Two-sample, 2-tailed t tests were performed. *P < 0.05.

We asked whether patients’ keratinocytes accumulated ubiquitin-rich inclusions, which were in fact increased in primary keratinocytes, as analyzed by immunoblots (patients 1, 2, 4 and 5) (Figure 5B).

Next, we assessed proteasome formation and estimated the amount of proteasome content in keratinocytes. Consistent with the experiments in transfected HeLa cells and in patients’ hematopoietic cells and fibroblasts (Figure 3, Figure 4A, and Supplemen
tal Figures 3 and 4), proteasome assembly in keratinocytes was impaired with accumulation of proteasome precursor complexes in patient 1 and his father (patient 1-F) as well as in patients 4 and 5 (Figure 5C). Patient 2 displayed a general decrease in the total proteasome content in fibroblasts and keratinocytes. Proteasome impairment in these patients (Figure 5, D and E) was due to altered proteasome assembly and failed subunit incorporation.

Recapitulation of additive effect of proteasome defects on proteolytic function and type I IFN induction in siRNA knockdown experiments in primary fibroblasts. To further confirm the digenic effects of the proteasome subunit mutations on proteasome formation and function, we used healthy fibroblasts and silenced mRNAs for PSMA3, PSMB4, PSMB8, PsMB9 by siRNAs in single knockdown experiments or we knocked down combinations thereof that were occurring in patients, PSMA3/PSMB8, PSMB4/PSMB8, or PSMB4/PSMB9, respectively. As expected, knockdown of a single proteasome subunit mRNA caused a slight decrease in the chymotryptic- or caspase-like activity of the proteasome due to decreased total proteasome amounts in comparison with cells treated with off-target siRNA as controls (Figure 6, A–C). The depletion of the standard subunits α7 or β7 affected the system more (35%–40% decrease) than depletion of 1 of the 2 immunosubunits, β11 or β51 (10%–20%). However, additive depletion of 2 proteasome subunits by siRNA led to more severe assembly defects and decreases in proteolytic function, which is consistent with our concept of digenic inheritance causing additive proteasome defects. In healthy control fibroblasts treated with siRNAs targeting the expression of 2 different subunits, a stronger decrease of proteasome assembly (Figure 6D) and of their proteolytic activity (up to 70% decrease) was observed (Figure 6, A and B). Knockdown controls for the respective mRNAs by quantitative reverse-transcriptase PCR (qRT-PCR) showed an approximately 60% to 40% efficiency, respectively.

Moreover, the additive depletion of 2 proteasome subunits in the combinations found in CANDLE/PRAAS patients led to the induction of type I IFN (shown for IFNA21 encoding IFNα21 and IFNB1 encoding IFNβ, but not type II IFN (IFNG mRNA was not detectable in any of the samples) (Figure 6E). A single knockdown of a proteasome subunit mRNA even to about 50% of WT did not significantly induce transcription of IFNB1, MX1, IP10 (also known as CXCL-10), or IL1B genes. In contrast, additive targeting of 2 subunits resulted in specific induction of type I IFN genes exemplified by IFNB1 and IFNA21 as well as the IFN-inducible genes MX1 and IP10, whereas IL1B was not significantly induced (Figure 6E). This effect is not caused by cellular siRNA responses, because increasing amounts of off-target siRNA do not induce IFN gene transcription in the concentrations used in the double-knockdown siRNA experiments (Supplemental Figure 5A, B and D). These experiments mimic the additive effect of 2 different proteasome subunit mutations and reproduce the induction of type I IFN that we observed in the digenic CANDLE/PRAAS patients.

Proteasome dysfunction leads to type I IFN induction. A strong IFN gene expression signature was present in all patients irrespective of their mutations (Figure 7A). The secretion of IFN-stimulated chemokines (IP-10, MCP-1, MIG) and cytokines (IL-18) that are detected early in viral infections (22, 23) was significantly increased in CANDLE patients compared with relatives, controls, or NOMID patients (IP-10, P < 0.001; MCP-1, P < 0.01; MIG, P < 0.01; and IL-18, P < 0.01). In NOMID, IL-18 is increased due to constitutive inflammasome activation and not IFN signaling (Supplemental Figure 6A).

It remains unclear whether the IFN signature is induced by intrinsic or extrinsic factors in hematopoietic and nonhematopoietic cells in CANDLE patients. To confirm the observation in the siRNA knockdown experiments, we used 2 proteasome inhibitors, epoxomicin and bortezomib, to chemically induce global proteasome impairment in PBMCs and fibroblasts. Treatment of PBMCs and fibroblasts with either inhibitor elicited dose-dependent increases in IFNA and IFNB transcription as well as transcription of IFN-stimulated genes (IP10 or MX1), but did not affect transcription of cytokine genes (IL1, TNF, and IL6) (Figure 7, B and C, and Supplemental Figure 6, C and D), in agreement with the siRNA experiments (Figure 6). To determine the cellular origin of type I IFNs in PBMCs, FACS staining for IFN-α and IFN-γ confirmed that mainly type I IFN, but not type II IFN, is upregulated in patients’ NK and plasmacytoid dendritic cells (pDCs) (performed in patients 1 and 2) (Figure 7D). We also assessed IFNG transcription in different cell populations; while it was very low or undetectable in fibroblasts, IFNG-γ staining was increased in sorted NK cells from active CANDLE patients (Supplemental Figure 6B). Blockade of proteasome function in healthy control cells (NK cells, CD4+ and CD8+ T cells, and pDCs) showed consistently more IFN-α-positive...
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Production both in hematopoietic and nonhematopoietic cells, dysfunction is associated with increased predominantly IFN type in response to proteotoxic stress. Our data show that proteasome upregulate immunoproteasome content or function when required impaired proteolytic activity along with an inability to appropriately mutations in proteasome subunits cause assembly defects or altered T cell expansion and maintenance (12, 26).

As stimulation with both cytokines, IFN-α and IL-18, induces IFN-γ production in NK cells (24), the increase in IFN-γ production observed in patients’ NK cells is likely secondary, rather than directly caused by proteasome dysfunction.

In addition, we observed alterations in B cell subsets as well as a higher percentage of naïve T cells and immature neutrophils in CANDLE patients (Supplemental Figure 6, E–G). This is in agreement with previous work suggesting that immunoproteasome impairment in mice is linked to changed cytokine patterns (17, 25) and altered T cell expansion and maintenance (12, 26).

In summary, our studies suggest that monogenic and digenic mutations in proteasome subunits cause assembly defects or impaired proteolytic activity along with an inability to appropriately upregulate immunoproteasome content or function when required in response to proteotoxic stress. Our data show that proteasome dysfunction is associated with increased predominantly IFN type I production both in hematopoietic and nonhematopoietic cells, providing further clues to the pathomechanisms of CANDLE.

Discussion

The findings that the CANDLE/PRAAS disease spectrum is caused by recessively inherited loss-of-function mutations in the gene encoding the inducible proteasome subunit PSMB8 (3, 4, 5, 11, 27) suggested that CANDLE/PRAAS may be an “immunoproteasome disease.” Our genetic data showing that mutations in inducible and constitutive proteasome subunits can cause CANDLE/PRAAS illustrate that WES should supersede targeted sequencing of proteasome genes in patients with suspected CANDLE/PRAAS in whom only one or no definite mutation is identified in the PSMB8 gene. Our functional data further suggest that global proteasome dysfunction rather than specific immunoproteasome dysfunction leads to the disease.

While the absence of inducible proteasome subunits is tolerated in mice, the presence of constitutive proteasome subunits is essential (28). Consistent with these observations, a homozygous truncation mutation in PSMB8 causes complete β5i deficiency with an intermediate CANDLE phenotype (6); however, we have not found homozygous truncation mutations in the constitutive subunits. Either mutations in the constitutive α7 and β7 subunits are hypomorphic and result in the expression of mutant proteins with residual protein function, which may be critical for survival, as we see in patient 1, who is compound heterozygous for PSMB4 mutations, or the mutations that lead to nonexpression are only present as monoallelic variants (patients 4 and 5).

The finding of digenic mutations as a cause of CANDLE and the absence of clinical disease in heterozygous parents was initially puzzling; however, the calculated statistical probabilities of assembling WT versus mutant proteasome complexes could explain this discrepancy. Assuming mutant subunits are competent for assembly, a heterozygous carrier (parent or sibling) would assemble 25% of proteasomes with WT subunits, 50% with 1 mutant subunit, and 25% with 2 mutant subunits. In contrast, CANDLE patients with double-heterozygous mutation (digenic) inheritance would assemble only 6.25% proteasomes with no mutant subunits, 62.5% of proteasomes would have 1 or 2 mutant subunits, and 31.25% would have 3 or 4 mutant subunits (Supplemental Figure 7). The POMP mutation likely causes haploinsufficiency, which is supported by previous findings that an approximately 50% reduction of POMP levels is sufficient to cause impaired proteasome activity and cell death in vitro (14, 29). The additive effect of 2 different subunit mutations was mimicked by siRNA knockdown experiments of 2 proteasome subunits, which led to a more severe reduction in proteasome assembly and proteolytic function than knockdown of a single proteasome subunit, thus verifying the concept of digenic inheritance. Together, the genetic data suggest that impairment of the proteasome assembly and/or function caused by mutations in various proteasome components can lead to clinical disease. Other examples of digenic inheritance include conditions caused by double-heterozygous variants of structural proteins that are critical for organ function, including retinitis pigmentosa (30), the digenic inheritance of nonsyndromic deafness (31), Usher’s syndrome (32), Bartter syndrome (33), and Hirschsprung’s disease (34), and triallelic inheritance was found in patients with Bardet-Biedl syndrome (BBS) (35) and in isolated hypogonadotropic hypogonadism (36). In 2 recently described digenic conditions, the second gene acts as epigenetic or epistatic modifier, as observed in facioscapulohumeral muscular dystrophy (FSHD) (37) and in ataxia, dementia, and hypogonadotropism (38), respectively. The advent of next-generation sequencing will likely increase the power to identify new diseases with digenic/oligogenic inheritance.

β5i deficiency in mice causes no spontaneous phenotype; however, immunoproteasome formation is pivotal for the maintenance of proteostasis and the preservation of cell viability in tissues when exposed to inflammatory insults. In mouse inflammation models, cytokines, particularly IFNs, trigger the production of radicals, which induce oxidant-damaged proteins and the formation of ubiquitin-rich inclusions (39). The clearance of these inclusions in cells of the central nervous system and of the periphery is dependent on the induction of immunoproteasomes with...
We assessed proteasome function and the ability to upregulate proteasome function in our patients. In all CANDLE/PRAAS patients, regardless of their proteasome subunit mutations, the proteasome function is impaired because the mutation-induced subunit defects lead to lower expression and/or impaired proteasome assembly. The impairment in proteolytic function is not limited to chymotrypsin-like activity, as was previously seen their increased proteolytic capacity that is necessary to prevent IFN-induced cell death (9, 12, 16, 17, 40). The data in murine models suggest that, while WT cells can adapt to an increased demand for protein degradation through the induction of immunoproteasomes, in mutant cells, there seems to be a threshold at which cells start a survival program followed by apoptotic cell death when the capacity to clear inclusions becomes too low.

Figure 6. Additive depletion of proteasome subunits by siRNA simulated the digenic inheritance of proteasome mutations in patients. Primary human fibroblasts were depleted for PSMB8, PSMB4, PSMA3, or PSMB9 as well as combinations thereof: PSMB8 plus PSMB4; PSMB8 plus PSMA3; PSMB9 plus PSMB4 by siRNA. The following siRNA concentrations were used: off-target 1, off-target 2, PSMA3, PSMB8, and PSMB9 (10 nM); PSMB4 (15 nM). (A and D) Representative results from n = 3. (A) Native PAGE substrate overlays with lysates of siRNA-treated cells show reduction of proteasome activity. (B) Quantification of native PAGE substrate overlays. (C) Knockdown control for the respective genes with qRT-PCR shows approximately 60% to 40% efficiency, respectively. (D) Immunoblot stained for α6 shows decreased total amount of proteasomes. In cells treated with siRNAs targeting the expression of 2 different subunits, a stronger decrease of proteasomes and their activity was observed. (E) mRNA expression of type I IFN and IFN-regulated genes MX1 and IP10 was significantly upregulated in cells treated with 2 different proteasomal siRNAs as assayed by qRT-PCR. All data in bar graphs represent mean ± SEM. n = 3. Samples were normalized against off-target siRNA 1+2. Paired t tests were performed. *P < 0.05; **P < 0.01; ***P < 0.001.
in patients with mutations in PSMB8 (3), but affects trypsin- and caspase-like activity, depending on the respective proteasome subunits that are mutated. A limitation of the assay includes the use of whole-cell lysates, as the assessed proteolytic activities are not absolutely proteasome specific; however, the in-gel assays confirmed the localization of the proteolytic activities to the proteasome complexes. Although at least one of the proteasome activities in patients was consistently lower than that of healthy controls, the assay does not distinguish some heterozygous, clinically unaffected parents from a patient, as the impaired proteolytic activities of some unaffected carriers may be similar to those of an affected child. However, the patients’ proteasome activity may be maximally upregulated because of the chronic IFN stimulation, while the unaffected carriers who do not have evidence of chronic IFN upregulation do not upregulate proteasome function through the IFN mechanism. The fact that CANDLE patients have chronically high IFN stimulation, as assayed by the persistently high IFN response signature, indicates that we are comparing patient and unaffected carrier under different conditions. The proteolytic function measured in the patients may therefore not reflect the full extent of the impact of the proteasome defect. When we compared proteasome function in CANDLE patients with proteasome activity in patients with other IFN-mediated autoinflammatory syndromes who do not have proteasome subunit mutations, we found increased proteolytic activities in PBMCs from patients with non-CANDLE interferonopathies for all 3 proteolytic activities tested. The increase in proteasome activity was not seen in cells from untreated, clinically active, and symptomatic NOMID patients, whose disease is caused by chronic constitutive IL-1 production and stimulation (41). These findings are consistent with previously published data and our current data showing that type I and type II IFN, but not IL-1, induce proteasome subunit upregulation and proteasome function. The measurement of proteasome activity in patients with an interferonopathy may thus be a useful diagnostic tool in addition to genetic and molecular diagnostics to distinguish patients with a proteasome defect from patients with similar clinical phenotypes who do not have CANDLE/PRAAS.

The presence and persistence of the IFN-response gene signature in CANDLE (6) regardless of the causative proteasome subunit mutation raises the question of the molecular mechanism that links proteasome dysfunction to IFN production. Our functional data in PBMCs and fibroblasts show that type I IFN transcription is induced in a dose-dependent fashion upon global inhibition of proteasome function with chemical inhibitors and in siRNA knockdown experiments, suggesting that global proteasome dysfunction induces type I IFN transcription. The cellular origin of the type I IFN transcription/production is ubiquitous. Upregulation of type I IFN and not type II IFN was seen in all major sorted white blood cell populations and in primary fibroblasts from patients. The chronic induction of a type I IFN signature in CANDLE/PRAAS patients and not the heterozygous controls likely indicates that patients’ cells have reached a level of cumulative proteasome defects that caused increased cell stress and thus the transcription of type I IFNs. We observe an accumulation of ubiquitin-rich inclusions in patients’ keratinocytes, which leads to cell stress in murine models. Whether these inclusions directly trigger type I IFN gene transcription remains unknown. However, the inability to clear damaged proteins and the concomitant induction of type I IFNs may cause a vicious cycle of further induction of protein damage and impaired clearance and explain the severe inflammatory phenotype of CANDLE/PRAAS (42). This hypothesis is preliminarily supported by our initial observation of clinical improvement with treatment with JAK inhibitors that can block IFN signaling and thus the production of radicals (NCT01724580).

The IFN signature in CANDLE/PRAAS patients is similar to that seen in patients with Aicardi-Goutières syndrome (AGS) (43) and in patients with STING-associated vasculopathy with onset in infancy (SAVI) (35). In patients with AGS, the genetic defects cause loss of function in nucleases and enzymes associated with nucleic acid metabolism that are thought to lead to the accumulation of nucleotides in animal models and patient cells (44). The IFN signature in both AGS and SAVI is dependent on STING (35, 37). However, the signaling pathway inducing type I IFNs in CANDLE is not STING dependent, as proteasome inhibition in Sting−/− cells still leads to the induction of type I IFN transcription (Yin Liu, our unpublished data). Chemical proteasome inhibition is known to induce ER stress and the unfolded protein response. Along with the strong IFN signature in CANDLE patients (6), we found the induction of some genes that are typically upregulated in an ER stress response (Yin Liu, our unpublished data).

Although more work is needed to decipher the exact molecular mechanism of proteasome dysfunction and type I IFN transcription, our data suggest that the induction of type I IFN and the presence of the IFN signature in CANDLE indicate cell stress that likely drives disease pathogenesis. The understanding of the exact molecular pathways that link proteotoxic stress to type I IFN production will likely result in the identification of novel therapeutic targets not only for patients with CANDLE/PRAAS, but also for those with a growing spectrum of inflammatory diseases that are caused by the generation of intracellular stress that is coupled with IFN production.

**Methods**

**Patients.** The present study includes 8 patients with clinical disease manifestations consistent with CANDLE who were negative (n = 4) or heterozygous for PSMB8 mutations (n = 4) (Supplemental Table 1). We used 3 CANDLE patients (C1–C3) with known PSMB8 mutations, 7 NOMID patients, and 3 uncharacterized IFN-mediated autoinflammatory disease (UID) patients as disease controls, parents heterozygous for a PSMB8 mutation, and controls from the blood bank as healthy controls.

**Cell lines.** The primary keratinocyte, primary fibroblast, and EBV-immortalized B cell lines used in the paper were generated from skin biopsies or blood draws from patients, their parents, and healthy controls at the NIH after obtaining consent; standard protocols were used to generate the cell lines.

**In silico modeling of novel mutations.** Homology models were built based on x-ray structures of the mouse immunoproteasome (PDB entry code 3UNH) (20) for mutations in PSMA3, PSMB8, PSMB9 or from the bovine 20S proteasome (1IRU) for mutations in PSMB4 (19) using the SWISS-PDB viewer (45). The initial models were energetically minimized with GROMOS 43B1 force field (39) to avoid local distortions and visualized with PYMOL (The PyMOL Molecular Graphics System, Schrödinger LLC).
Proteasome activity assay. Frozen PBMCs were thawed in a 37°C water incubator and resuspended in 10% FCS RPMI 1640 at about 2 × 10^6/ml. Six hours later, cell numbers were counted and proteasome activities were measured with Proteasome-Glo cell-based assay kit (Promega). Chymotryptic-like, tryptic-like, and caspase-like activities were measured separately following the company’s recommended protocol. 4 × 10^4 cells per well were used. Average proteasome activity of the healthy pediatric and adult PBMCs was set as 100%, and proteasome activity of all other PBMC samples was calculated as percentage relative to the average of controls. EBV-B cells were cultured in RPMI 1640 supplemented with 10% FCS, 1× penicillin/streptomycin, and 1× MEM NEAA (Gibco; Thermo Fisher Scientific). Keratinocytes were cultivated in Keratinocyte-SFM supplemented with serum. β-actin (Santa Cruz Biotechnology Inc., sc-166553) was analyzed by qRT-PCR. Fold change was calculated for each condition relative to the score of 1. Two-sample t tests were performed. P values are stated. (D) Expression of IFNs from whole blood of 2 active CANDLE patients (patient 1 and patient 2) were analyzed by flow cytometry. (E) Expression of IFNs in PBMCs from a healthy donor treated with the proteasome inhibitor epoxomicin at indicated concentrations was analyzed by flow cytometry. (D and E) Representative results from n = 3 and n = 2, respectively.

IFN score calculation. The mRNA expression levels for IFI127, IFI44L1, IFI44, RSAD2, ISG15, and USP18 were quantified by qRT-PCR (TaqMan, Applied Biosystems) and normalized to 18S. Gene-expression levels were expressed as fold changes relative to a healthy control. IFN score for each gene was calculated as the difference between the average of ratios of healthy controls divided by the SD of the healthy controls (40). The sum of the scores for all the genes was used as the score for the sample.

Polyubiquitinated protein staining. Paraffin-embedded tissue sections from skin biopsies of a healthy individual, a patient with psoriasis, and two CANDLE patients were first deparaffinized, rehydrated, and then stained with anti-ubiquitin antibody (Santa Cruz Biotechnology Inc., sc-166553) at a 1:3,200 dilution, followed by incubation with HRP polymer-conjugated anti-rabbit system (Golden Bridge). Stained slides were viewed on a Leica DMR microscope equipped with a Leica DFC500 camera. Images were acquired with Leica Firecam software at ×25 magnification.

Methods for RNA sequencing, Luminex assay, Sanger sequencing and whole-exome sequencing, qRT-PCR, cloning and transfection of expression vectors, and FACS analysis are described in the Supplemental Methods. Consistent with the patients’ consent, we will make the WES and RNAseq data to researchers upon request.

Statistics. Descriptive statistics were used to summarize group differences between patients and healthy controls or disease controls. All analyses were performed using Stata, version 12 (StataCorp). In some experiments, paired t tests of the stimulated to baseline ratios or logs of ratios were compared (Figure 4C, Figure 6, B, C, and E, Figure 7B, Supplemental Figure 5, A and B, and Supplemental Figure 6, C and D). In other experiments, we used a 2-sample t test, either the standard or Welch’s version, to compare standardized values of cytokines between patients with similar mutations and either healthy controls or appropriate disease controls (undifferentiated interferonopathies without a proteasome defect) (Figure 4, A and B, Figure 7C, Supplemental Figure 4A, and Supplemental Figure 6B). We also used 2-sample t tests of serum concentrations or percentages of cell subsets to compare various groupings of patients, heterozygous parents, healthy controls, or NOMID patients (disease control) (Supplemental Figure 6, A and
E–G). In 1 experiment we compared standardized values for individual patients or parents separately with a population of healthy controls (Figure 5E) using mixed models. In Supplemental Figure 6A, the P values comparing CANDLE 3 distinct control groups are unadjusted. All statistical tests were 2 tailed. P < 0.05 was considered significant.

Study approval. The study was approved by the NIDDK/NIAMS Institutional Review Boards at the respective sites, and written informed consent was obtained from the subjects or their parents. A separate written informed consent document was provided for photographs appearing in the manuscript.

Author contributions
AB and YL conducted experiments, analyzed data, and contributed to the writing of the manuscript; AS, BM, EQ, ZQ, AB, AdJ, MP, WLT, EFR, IK, JJC, JB, VC, and YH conducted experiments and contributed to the interpretation of the data; FS, SM, MG, and PM analyzed and interpreted data; GSM, AR, SH, HK, HJL, AM, RDC, DB, AVC, LG, DC, DS, AT, AZ, KR, and PB conducted patient care and clinical data collection; RW conducted statistical analyses; CCRL and DLK conducted data interpretation; PWH conducted in silico modeling; IA designed and oversaw genetic analyses; EK designed the research study and contributed to the writing of the manuscript; RGM oversaw the project, patient care, and evaluation, designed the research study, and contributed to the writing of the manuscript. All authors reviewed and approved the final version of the manuscript.

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