Supplemental Data

Additive Loss-of-Function Mutations in Proteasome-Subunit-Genes induce Type-I-interferon production in CANDLE/PRAAS

Anja Brehm,[1] Yin Liu,[2]* Afzal Sheikh,[2,3*] Bernadette Marrero,[2] Ebun Omoyinmi,[4] Qing Zhou,[3] Gina Montealegre,[2] Angelique Biancotto,[5] Adam Reinhardt,[6] Adriana Almeida de Jesus,[2] Martin Pelletier,[7] Wanxia L. Tsai,[8] Elaine F. Remmers,[3] Lela Kardava[9], Suvimol Hill,[10] Hanna Kim,[2] Helen J. Lachmann,[11] Andre Megarbane,[12] Jae Jin Chae,[3] Jilian Brady,[3] Rhina D. Castillo,[13] Diane Brown,[13] Angel Vera Casano,[14] Gao Ling,[15] Dawn Chapelle,[2] Yan Huang,[2] Deborah Stone,[3] Yongqing Chen,[2]* Franziska Sotzny,[1] *Chyi-Chia Richard Lee,[16] Daniel L. Kastner,[3] Antonio Torrelo,[17] Abraham Zlotogorski,[18] Susan Moir,[9] Massimo Gadina,[8] Phil McCoy,[5] Robert Wesley,[19] Kristina Rother,[20] Peter W. Hildebrand,[21] Paul Brogan,[4] Elke Krüger,[1]* Ivona Aksentijevich,[3]* Raphaela Goldbach-Mansky[2]**

**These authors contributed equally to this work*

Contents

1. Supplemental Methods

Patients, disease-controls, and healthy controls. CANDLE patients with known *PSMB8* mutations were: C1 (homozygous for T75M), C2 (homozygous for C135X), and C3 (compound heterozygous for T75M and A92T). Patients with the IL-1 mediated disease Neonatal-onset Multisystem Inflammatory Disease (NOMID) had active disease before treatment with anakinra and three patients with a genetically undifferentiated disease had a strong interferon-gene-expression signature in blood, and were negative for mutations in proteasome components by whole exome sequencing.

Sanger Sequencing. We initially used a candidate gene approach to systematically screen 14 genes encoding the constitutive proteasome subunits, α1-α7 and, β1-β7, and the assembly protein POMP (112 exones) in addition to the three genes encoding the inducible immunoproteasome components, β1i, β2i, β5i, using standard BigDye Terminator version 3.1chemistry (Applied Biosystems) on an ABI 3130 Genetic Analyzer. Sequencing data were analyzed using Sequencher (Gene Codes). The allele frequencies of two *PSMB4* mutations (c.-9G>A and p.D212_V214del) and the *PSMA3* mutation (p.R233del) were evaluated in Caucasian control DNA samples using mass spectrometry (Sequenom Inc.). To facilitate screening and to rule out potential other disease causing variants we also performed whole exome sequencing in a subset of patients (WES).

Whole exome sequencing and confirmation of mutations. Whole exome sequencing was performed on patients 1, 2, 8 and the parents for pt.2. We either used commercial sources, Otogenics, (Norcross, GA) or the WES was performed by NISC (NIH, Rockville MD) using previously published procedures.

Luminex assay. Cytokine measurements in patients' sera or plasma were performed using a multiplex luminex assay kit from Bio-Rad (27 plex group-I, and 21 plex group-II). Median

fluorescence intensities were collected on a Bio-Plex-200 instrument (Bio-Rad), using Bio-plex Manager software version 6.2. Standard curves for each cytokine were generated using the premixed lyophilized standards provided in the kits. Cytokine concentrations in samples were determined from the appropriate standard curve using a 5 point-regression to transform mean fluorescence intensities into concentrations. Each sample was run in duplicate and the average was used to calculate concentrations.

qRT-PCR. Cell lysis and total RNA extraction was performed with TRIzol Reagent (Invitrogen) on indicated cells. cDNA was generated from 1µg of DNase treated total RNA using the Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Life Technologies SYBR green were utilized for qRT-PCR experiments and 20 µl reactions were run according to the manufacturer's instructions on ABI 7500 real time PCR system (Applied Biosystems Inc). qRT-PCR was performed using GAPDH or 18S RNA as endogenous controls.

Used qRT-PCR Primers for confirming allele expression in patient PBMCs in 5'-3' orientation:

Family1: PSMB4-UTR5-G_for GCTTTCATTTTTTCTGCTACCG,
PSMB4-UTR5-G.A_rev AACTTAACGCCGAGGACTGA,
PSMB4-UTR5-A_for GCTTTCATTTTTTCTGCTACCA;

Family2: PSMA3-232R_for CATGAAATTGTTCCAAAAGATATAAGA,
PSMA3-232R_rev CCAGGGCTAAATAGTTACATTGGA,
PSMA3-232Rdel_for CATGAAATTGTTCCAAAAGATATAGAA;

Family3: PSMA3-EX5_for ATTCCACTAAAACATCTTGCAGAC,
PSMA3-3'UTR_rev TTCCTTCAGAGATTCCTTAGCA,
PSMA3-ex4/6_for CTACAACATTCCACTAAAATTT;

Family4: PSMB4-for CAGGACAGTTTTACCGCATT,
PSMB4_rev CACCAAGCATGTCCACATAA.

For the siRNA experiments RNA was isolated from human fibroblasts with HighPure RNA Isolation Kit (Roche). cDNA synthesis was performed from 400 ng DNAse treated total RNA with Transcriptor High Fidelity cDNA Synthesis Kit (Roche). qRT-PCR experiments were run in 10 µl reactions on a Rotor Gene RG-3000 (Corbett Research) according to the manufacturer's instructions with TaqMan Gene expression assays (life technologies). As endogenous control HPRT1 was used.

The following primers form life technologies were used in different experiments:

HPRT1 (Hs02800695_m1);	PSMA3 (Hs00541061_m1);
PSMB4 (Hs01123843_g1);	PSMB8 (Hs00188149_m1);
PSMB9 (Hs00160610_m1);	IFNα5 (HS04186137_sH);
IFNα7 (Hs01652729_s1);	IFNα17 (Hs00819693_sH);
IFNα21 (Hs00353738_s1);	IFNβ (Hs01077958_s1);
IFNγ (Hs00174143_m1);	TNF (HS01113624_g1);
MX-1 (Hs00895608_m1);	IP10 (Hs00171042_m1);
IL-1β (Hs00174097_m1);	IL6 (HS00985639_m1);
OAS3 (HS00196324_m1);	18S (Hs99999901_s1).

RNA_sequencing. RNA was extracted from whole blood collected in Paxgene tubes from CANDLE patients and controls; mRNA sequencing libraries were prepared using *TruSeq* RNA sample preparation kit V2 (Illumina). RNA-Seq data were generated with an Illumina's HiSeq 2000 system. Raw sequencing data were processed with CASAVA 1.8.2 to generate fastq files. Reads of 50 bases were mapped to the human transcriptome and reference genome hg18 using TopHat 1.3.2.[5] Gene expression values as reads per kilobase per million reads (RPKMs) were calculated with Partek Genomics Suite 6.6, which was also used for the principal component analysis (PCA) and ANOVA analysis.

Cloning and transfection of expression vectors. Coding sequences of human *PSMA3*, *PSMB4* and *PSMB8* were cloned into pcDNA 3.1 V5/His (Invitrogen) and were used as templates to generate patient derived mutant variants via site directed mutagenesis. Expression vectors were cotransfected with pEGFP into HeLa cells with Fugene HD transfection reagent (Roche). Transfection efficiency (more than 90 %) was monitored by FACS analysis sorting for GFP signal.

FACS analysis. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation and re-suspended in 10% FCS RPMI 1640 medium. For B-cell subset analysis standard procedures were used.[6] For the epoxomicin experiments, PBMCs were treated with epoxomicin at indicated concentrations for 16 h and stained for surface marker and intracellular cytokines using standard procedures (see supplement).

2. Supplemental Results

Genetics. The following refers to data presented in **Table S2**. The two *PSMA3* mutations (Family 2 and 3) were absent in the Exome Aggregation Consortium (ExAC), which spans 61486 unrelated individuals and includes the 1000 Genomes Pilot project and NHLBI Exome Sequencing Project (ESP6500). The two variants were also absent in dbSNP, in our internal database of 278 exomes, or in any of 363 genotyped Caucasian controls.

Of the 4 *PSMB4* mutations, the c.-9G>A allele (rs200946642) (present in Family 1) was found in 110 individuals in ExAC (0.0009) including the 13 individuals that were found in ESP6500 (0.001). Four of 659 genotyped healthy Caucasian controls samples were also positive (0.006). The frame-shift mutation, c.44_45insG/p.P16Sfs*45 (Family 1), the three AA deletion (p.D212_V214del), (Family 4) and the nonsense mutation, c.666C>A/p.Y222X (Family 5) were absent in ExAC and dbSNP.

The p.G165D mutation in *PSMB9* (rs369359789) (Family 4), was present at a low frequency of 5/4656 in Africans and absent in 54,161 non-African samples, with an overall frequency of in ExAC of 0.000042.

The p.E115Dfs*20 mutation in *POMP* was absent in the Exome Aggregation Consortium (ExAC) databse, which spans 61486 unrelated individuals, and in 192 genotyped controls of Lebanese and Palestinian Arab ancestry. This mutation was also not found in 384 control DNA samples of Arab ancestry. Parental samples were not available (Table 1, Supplemental Figure 2, and Supplemental Table 2). We did not identify a second-disease causing mutation in this patient despite performing the WES analysis.

***In silico* modeling of the novel mutations.**

p.D212-V214*,* in Family 1:

This deletion shortens and/or destabilizes the α-helix and likely affects correct positioning of the C-terminal extension.

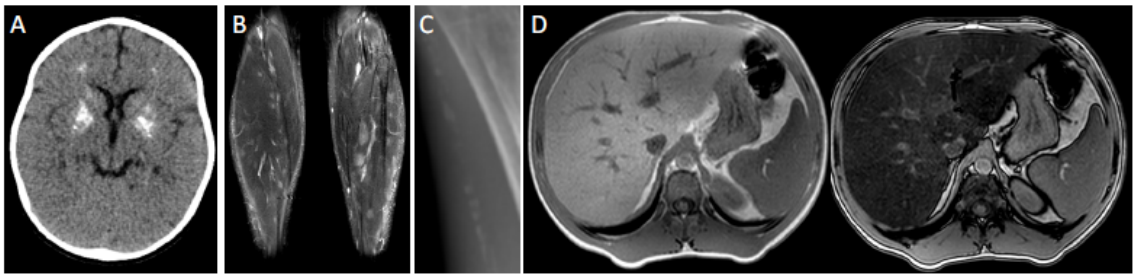
p.K105Q in Family 5:

The reported p.M117V and the p.A92T mutations in *PSMB8* change residues defining the conformation of the S1 substrate-specificity pocket, while the p.K105Q mutation directly affects an AA important for the active site. [1]

Expression of proteasome subunit mutations in HeLa cells. We were unable to express WT nor mutant PSMB9 in HeLa cells. However, the *PSMB9*-mutation causes severe proteasome dysfunction in patient cells (see **Figure 4** and **Figure 5B** below).

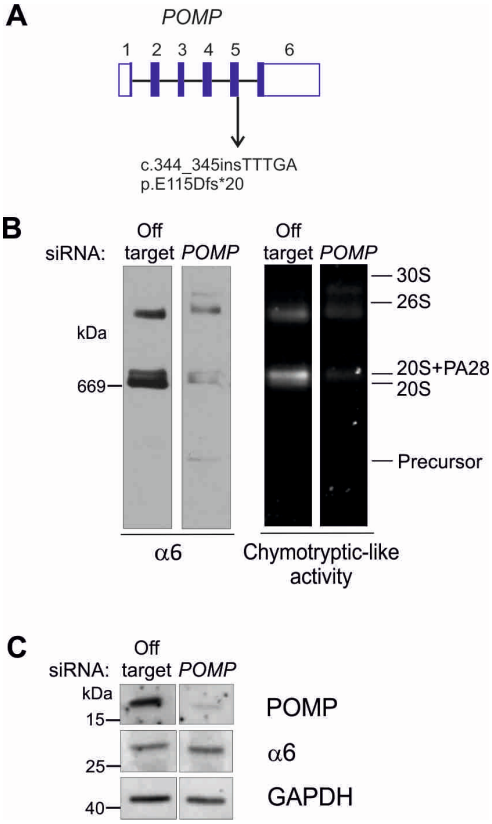
3. Supplemental Figures

Supplemental Figure 1



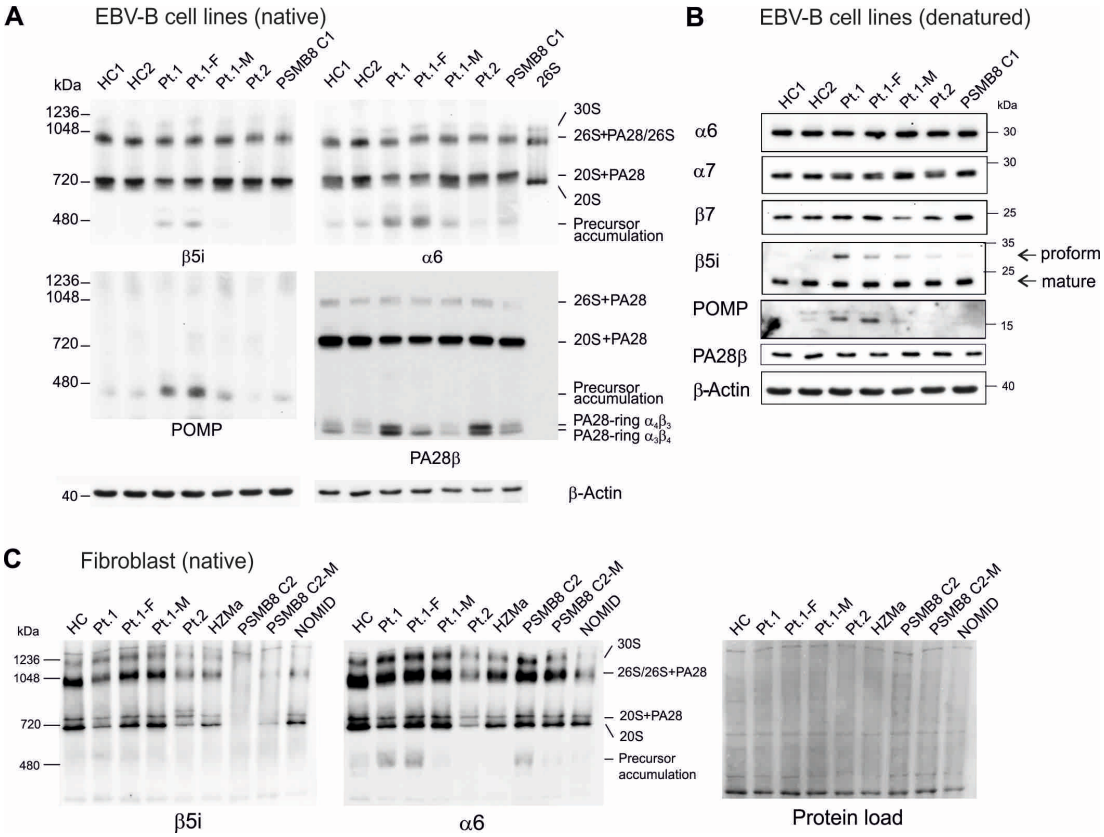
Supplemental Figure 1. Radiographic findings in CANDLE/PRAAS patients. (A) Brain CT image of basal ganglion calcifications. **(B)** Patchy inflammatory infiltrates on MRI STIR images of calf muscles. **(C)** Vascular calcifications in lower arm. **(D)** MRI in and out of phase image of liver dropping signal indicative of fatty liver.

Supplemental Figure 2



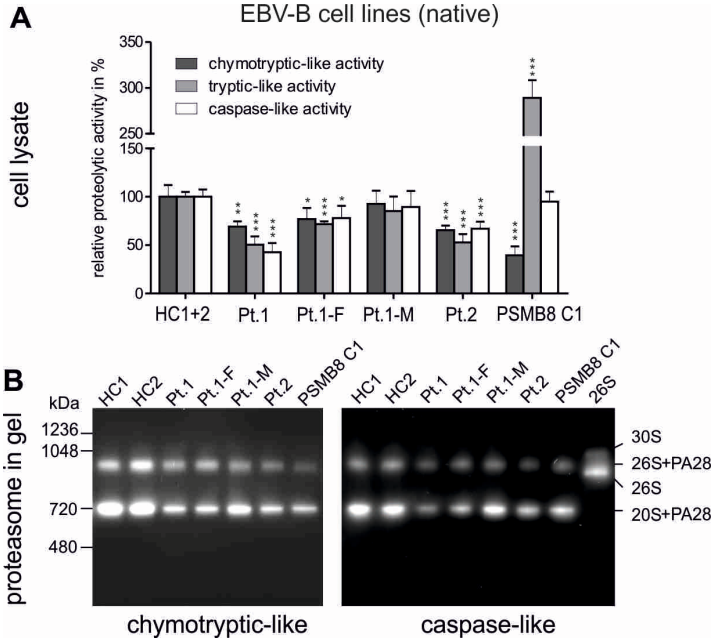
Supplemental Figure 2. CANDLE/PRAAS associated mutation in proteasomal maturation protein POMP. (A) Schematic organization of *POMP* gene (exon-intron structure, filled rectangles represent coding sequences, open rectangles represent UTRs) with position of the identified mutation leading to a truncation of the protein. **(B)** Knockdown of POMP expression via siRNA transfection of HeLa cells overnight was performed according to (Heink et al. 2005). Native cell lysates were separated on a native gel and reduced chymotryptic-like activity was measured in gel (overlay) compared to off target siRNA. Immunoblotting of the gel shows strong reduction of 20S proteasome amount after POMP siRNA treatment. **(C)** Cell lysates after siRNA treatment were separated on a SDS gel and immunoblotted for the indicated proteins. POMP expression was strongly reduced after POMP siRNA treatment. The lanes for off target siRNA and POMP siRNA were run on the same gel but were noncontiguous. Representative results from (n=3) independent experiments are shown.

Supplemental Figure 3



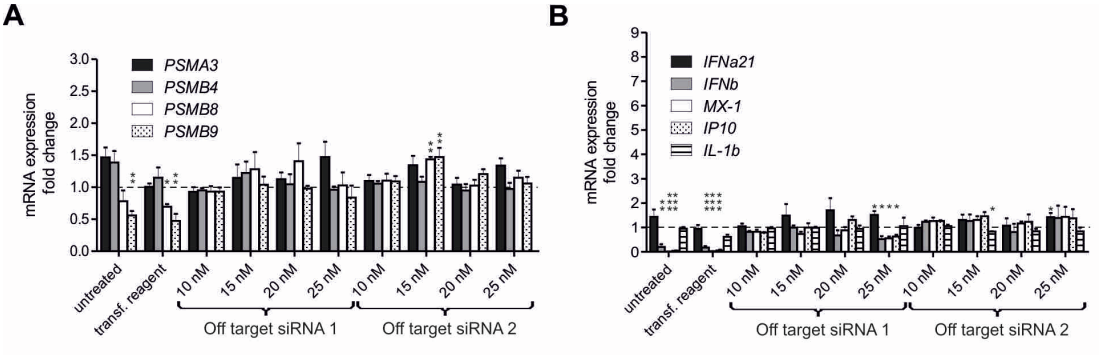
Supplemental Figure 3. Analyses of the proteasome assembly in patients EBV-B cells (Family 1 and 2) and fibroblasts (Family 1, 2 and 4). (A-C) EBV-B cells from depicted PRAAS patients, available relatives and adult healthy controls were lysed under native conditions. Representative results from (n=3) independent experiments are shown. **(A)** Lysates on native gels were blotted and analyzed with antibody to POMP, β5i, α6, PA28β and level of 26S (20S+19S) and 20S+11S/PA28 proteasomes as well as accumulation of precursors were assessed. A loading control with actin was performed with the same lysate on SDS gel. **(B)** Denatured SDS gel of lysate from EBV-B cells blotted with antibody to subunits α6, α7, β7, β5i as well as to POMP. Note the accumulation of the proform of β5i mainly in Pt.1 and his parents combined with accumulation of the assembly chaperone POMP. **(C)** Native PAGE of lysate from patients' fibroblasts blotted with antibody against α6 and β5i subunit. Protein load is determined after blotting with Amidoblack staining. Patient PSMB8 C2 (C135X +/+) shows an assembly defect with accumulation of proteasome precursors (α6 blot) and furthermore no β5i could be detected resembling a knock out situation for β5i. PSMB8 C2-M shows a reduced expression of β5i compared to NOMID and healthy control. Pts.1 and 2 shows an overall reduced formation of proteasome compared to heterozygous mutant for PSMB8 (T75M +/-) HZMa.

Supplemental Figure 4



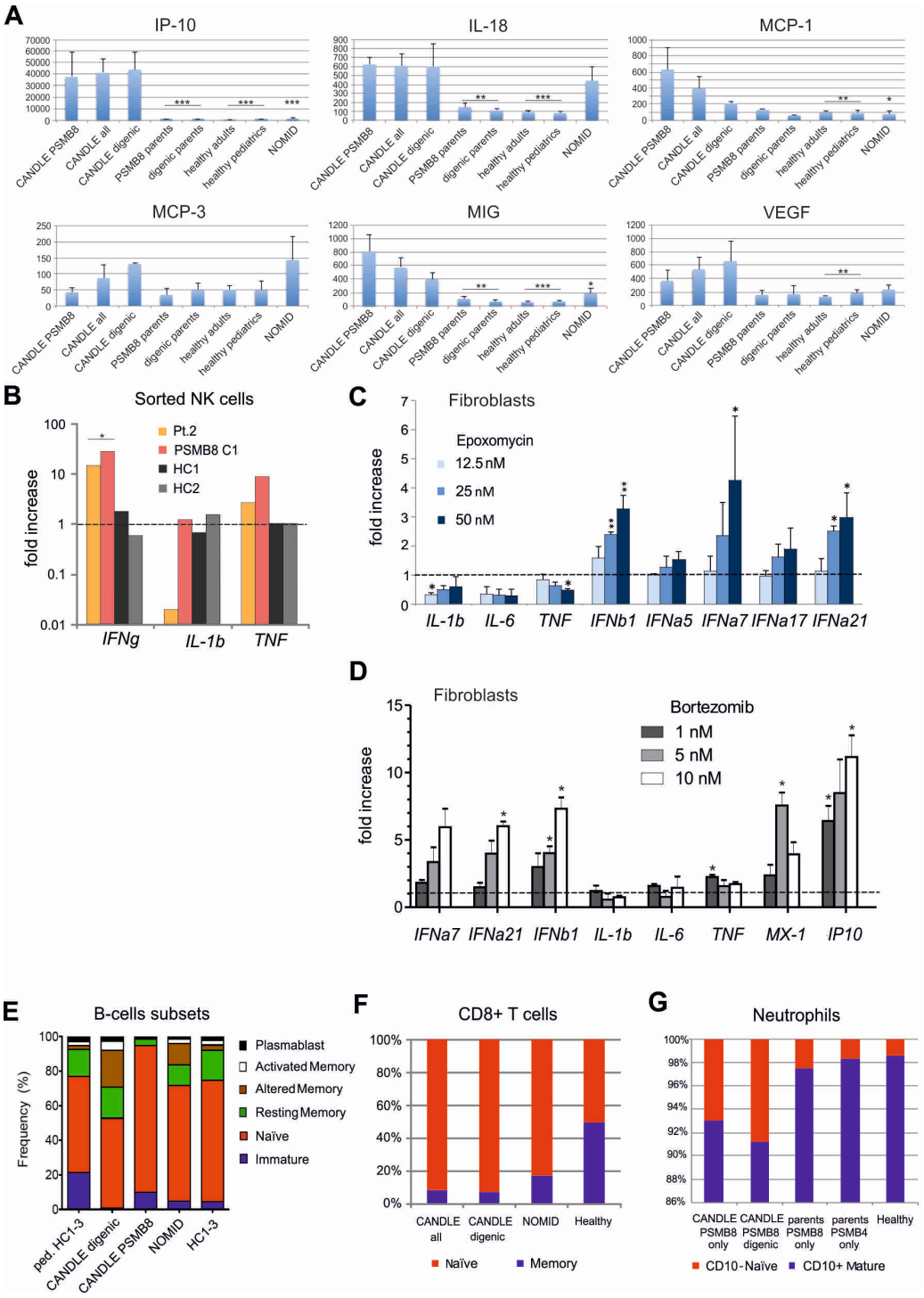
Supplemental Figure 4. Analyses of the proteasome in patients EBV-B cells and fibroblasts. (A-B) EBV-B cells from depicted PRAAS patients, available relatives and adult healthy controls were lysed under native conditions. **(A)** Plate reader activity assay from whole cell lysate directly after cell lysis. Graphic depiction of respective proteolytic activity as percentage of normal activity of two healthy controls (HC1+2) (mean+SEM; n=3 done in triplicates). Pts. 1+2 show a strong reduction in all 3 protease activities. 2-sample t-tests were performed compared to HC1+2 p-value < 0.05 *; p-value < 0.01 **; p-value < 0.001 ***. **(B)** Native PAGE substrate overlay for indicated proteolytic activities of proteasomes. The peptide-hydrolyzing activity for chymotryptic- and caspase-like activity is lower in patients and heterozygous controls than in healthy controls. Representative results from n=3.

Supplemental Figure 5



Supplemental Figure 5. Control siRNA experiments with increasing amounts of off target siRNAs. Primary human fibroblasts were incubated for 48 h with two different off target siRNAs with indicated concentrations. qRT-PCR analyses of samples were performed afterwards. **(A)** Compared to off target siRNA 1+2 (10nM) just minor induction or reduction of PSMA3, PSMB4, PSMB8 and PSMB9 can be detected in samples treated with higher concentration of siRNA. **(B)** Compared to off target siRNA 1+2 (10nM) just minor induction or reduction of IFNα21, IFNβ, MX-1, IP10 and IL-1β can be detected in samples treated with higher concentration of siRNA. All bar graphs represent the mean$\pm$SEM, n=3. Samples were normalized against off target siRNA 1+2 (10nM). Paired t-tests were performed, p-value < 0.05 * ; p-value < 0.01 ** ; p-value < 0.001 ***.

Supplemental Figure 6



Supplemental Figure 6. Functional Studies including serum cytokines, interferon production in NK cells and fibroblasts blocked with proteasome inhibitors and assessment of patient cell populations. (A) Cytokine Luminex assay on patients with *PSMB8* mutations (n=4), compared to digenic patients (n=4), healthy adults (n=25) and healthy pediatric controls (n=11, ages 3-4yrs (n=3), ages 7-8yrs (n=4), ages 11-15yrs (n=4)), as well as NOMID (n=8). Two sample t-tests were performed comparing all CANDLE patients to parents of patients (N=6); healthy controls; and NOMID patients. **(B)** RNA was extracted

from sorted NK cells from 2 patients with active disease and two healthy controls. Expression levels of IFNγ, TNF and IL-1 were analyzed by qRT-PCR. Results were normalized against 18S RNA. Fold changes were calculated for the cytokine transcription levels as indicated over the average of 2 healthy controls. A 2-sample t-test was performed in comparison to healthy control, n=2. **(C)** Fibroblasts from two healthy donors were treated with or without the proteasome inhibitor epoxomicin at indicated concentrations for 18 h. qRT-PCR was performed. Fold changes were calculated relative to untreated cells. Data are depicted as mean+SEM, n=2. **(D)** Fibroblasts from a healthy donor were treated with or without the proteasome inhibitor bortezomib at indicated concentrations for 17 h. qRT-PCR was performed. Fold changes were calculated relative to untreated cells. Data are depicted as mean+SD of triplicates, n=2. Mean fold-change values were compared to 1 using a one-sample t-tests of the logs of the values, which were close to normally distributed in PCR experiments. **(E)** B cell subsets were assessed and plotted as percent, on CANDLE *PSMB8* (n=3), digenic and PSMB4 compound heterozygous (n=3), NOMID (n=3) and healthy pediatric controls (n=3). **(F and G)** All CANDLE patients have a higher percentage of naïve CD8+ T cells and immature neutrophils (CD10-) compared to healthy age matched controls, $p<0.001$ for both comparisons. Separate 2- sample t-tests were performed for **(A-D)**; $p< 0.05$ *; $p< 0.01$ **; $p< 0.01$ ***.

Supplemental Figure 7

Heterozygous Mutation, Asymptomatic Carrier

Type	Combinations	Ratio / Percentage	
Wild type		1/4	25 %
One mutation		1/2	50 %
Two mutations		1/4	25 %

Double Heterozygous Mutations, Patient (digenic)

Wild type		1/16	6.25 %
One mutation		1/4	25 %
Two mutations		3/8	37.5 %
Three mutations		1/4	25 %
Four mutations		1/16	6.25 %

Supplemental Figure 7. Statistical probabilities for incorporating wildtype or mutant protein into the proteasome complex in patients with diallelelic mutations and healthy carriers. Patients carrying 2 monoallelic defects have a 6.25% chance of incorporating all 4 wildtype alleles into the proteasome. In comparison non-affected carriers of one mutation would have 25% of proteasomes with only wild type subunits incorporated.

4. Supplemental Tables

Supplemental Table 1. Demographics and clinical disease manifestations of CANDLE patients described

	FAMILY 1	FAMILY 2	FAMILY 3	FAMILY 4		FAMILY 5		
DEMOGRAPHICS	PATIENT 1[A]	PATIENT 2[A]	PATIENT 3[A]	PATIENT 4	PATIENT 5	PATIENT 6	PATIENT 7[A]	PATIENT 8[A]
Current Age years	8	6	5	3	2	22	16	10
Sex	Male	Male	Male	Male	Female	Male	Male	Male
Ethnicity/Origin	American/ Caucasian	American/ Caucasian	Spanish	Jamaican	Jamaican	Irish	Irish	Palestinian
Clinical Outcome	Alive, failure to thrive	Alive, failure to thrive	Alive	Alive, brother of Pt.5	Alive, sister of Pt.4	Alive, brother of Pt.7	Alive, brother of Pt.6	Alive
Age of clinical presentation	2 weeks	2 months	1 month	1 week	1 week	3 weeks	within 1 month	birth
Symptoms of initial presentation	Fever, rash, foot swelling, and periorbital erythema	Periorbital erythema and swelling	Fever and skin lesions	Fever, skin lesions, lipodystrophy and swallow difficulties	Fever, skin lesions and swallow difficulties	Fever, skin lesions and anemia	Fever, skin lesions and anemia	Fever, painful joints, subcutaneous nodules
Recurrent fevers and Elevated acute phase reactants (ESR, CRP)	+	+	+	+	+	+	+	+
Annular plaques/ violaceous eye lids	+	+	+	+ Hyperpigmented macules and scarring	+ Hyperpigmented macules and scarring	+	+	+
Myositis	+	+	+	+	-	+	+	-
Arthritis/arthralgia	+	+	+	+	-	+	+	+
Joint contractures	+	+		+	+	+	+	-
Lymphadenopathy	+	+	+	-	-	+	+	-
Aseptic meningitis	-	+	-	-	-	-	-	-
Hypergamma-globulinemia	IgG 770 nl ***IgM 321 hi*** ***IgA 223 hi***	IgG1240 nl IgM 203 nl ***IgA 373 hi***	Hypergamma-globulinemia	***IgG 152 lo*** IgM 39 nl IgA 25 nl	***IgG 190 lo*** IgM 84 nl IgA 26 nl	ND	ND	ND
Autoantibodies	ANA (+) now	ANCA 1:20 now	-	-	-	-	ANA (+)	ANA (+)

(intermittent)	negative	negative ANA (+) Anti-SmRNP (+) Anti-SSA (+)						
Anemia and other hematologic manifestations	Hypochromic microcytic anemia, thrombocytopenia, lymphopenia	Thrombocytopenia and lymphopenia	Hypochromic anemia	Anemia, thrombocytopenia, neutropenia, lymphopenia	Anemia, thrombocytopenia and lymphopenia	Hypochromic microcytic anemia and lymphopenia	Hypochromic microcytic anemia and lymphopenia	-
Lipodystrophy	Yes/ face upper and lower body	Yes/ face, upper and lower body	Yes	Yes / face, upper extremities and lower abdomen	Yes / face	+	+	+
Hepatospleno-megaly	+	+	-	-	-	+	+	-
Elevated LFTs	+	+	+	+	+	-	-	???
Lipid abnormalities	Hyper-triglyceridemia, low HDL	Hyper-triglyceridemia and low HDL	Low HDL	Hyper-cholesterolemia and high LDL	Hyper-cholesterolemia and high LDL	ND	Normal TG LDL/HDL ND	-
Intra-abdominal fat	+	+	+	ND	ND	-	+	?
Metabolic syndrome¶	Yes	Yes	ND	Yes	No	ND	ND	ND
Basal ganglia calcifications	-	-	-	+ other: Liver calcifications and peripheral calcinosis	ND	+	+	ND
Low weight and height	+	+	+	+	+	+	+	+
Conjunctivitis/epi-scleritis/ keratitis	-	-	+	+	+	-	-	-
Acanthosis nigricans	+	+	-	+	+	-	-	-
Pancreatic abnormalities	+	-		-	+	-	-	-
Lung manifestations	BOOP like	-	-	Interstitial infiltrate post pneumonia	-	-	-	-
Recurrent infections	Otitis and recurrent	Otitis and recurrent	+	Upper respiratory infections and	Sepsis from bowel perforation	Severe pneumonia with	Severe chronic gingivitis and	-

	sinusitis	sinusitis		urinary tract infections		multiorgan failure (on immunosuppres -sants)	periodontitis	
Epididymitis	-	+	-	-	NA	-	-	-
Steroid requirement (mg/kg/d)	High, 1.76	High, 0.48	NK	High, 1.23	High, 1.17	NK	NK	NK
MUTATIONS/ INHERITANCE	*PSMB4/PSMB4* Monogenic, CH[B]	*PSMA3/PSMB8* Digenic, DH[B]	*PSMA3/PSMB8* Digenic, DH	*PSMB4/PSMB9* Digenic, DH	*PSMB4/PSMB9* Digenic, DH	*PSMB4/PSMB8* Digenic, DH	*PSMB4/PSMB8* Digenic, DH	*POMP* Monogenic, AD[B]

[A] Clinically reported patients

NA denotes not applicable; ND denotes not done; NK denotes not known

[B] CH denotes compound heterozygous, DH denotes double heterozygous (an individual who has two different gene mutations at two separate genetic loci), and AD denotes autosomal dominant

Criteria for metabolic syndrome: Ford et al. Diabetes Care, 2005:28, 878-81. Three or more of the following: 1.Fasting glucose ≥ 110 mg/dL, 2.Waist circumference ≥ 90th percentile (sex-specific NHANES III, (The Third National Health and Nutrition Examination Survey)) 3.Triglycerides ≥ 110 mg/dL (age-specific NCEP (National Cholesterol Education Program)), 4.HDL-C < 40 mg/dL (all ages/sexes, NCEP), 5.Blood pressure ≥ 90th percentile (age-sex and height specific NHBPEP (The National High Blood Pressure Education Program))

Supplemental Table 2. Summary of novel CANDLE-associated mutations

Gene	Mutation type	Position	Nucleotide alteration	Protein alteration	Protein	Chromosome	NCBI Reference Transcript
PSMA3	Splice region mutation	Intron 5	c.404+2T>C	p.H111Ffs*10	α7	14q23.1	NM_002788.3
	Deletion, *(de novo)*	Exon 10	c.696_698delAAG	p.R233del			
PSMB4	Missense	5'UTR	c.-9G>A	NA	β7	1q21.3	NM_002796.2
	Frameshift, (insertion)	Exon 1	c.44_45insG	p.P16Sfs*45			
	In-frame deletion	Exon 5	c.634_642del	p.D212_V214del			
	Nonsense	Exon 5	c.666C>A	p.Y222X			
PSMB8 (LMP7)	Missense	Exon 3	c.313A>C	p.K105Q	β5i	6p21.32	NM_148919.3
PSMB9 (LMP2)	Missense	Exon 5	c.494G>A	p.G165D	β1i	6p21.32	NM_002800.4
POMP	Frameshift, (insertion)	Exon 5	c.344_345insTTTGA	p.E115Dfs*20	POMP	13q12.3	NM_015932

NA denotes not applicable
The protein nomenclature is according to Baumeister et al, Cell 92:367 (1998).

Supplemental Table 3. Allele frequencies and pathogenicity of CANDLE-associated mutations.

Gene	cDNA alteration	Protein alteration	rsID	ExAc allele frequency[A]	Allele frequency in genotyped controls	GERP score	Polyphen2	PROVEAN	SIFT	Mutation Taster
PSMB4	5'UTR:c.- 9G>A	/	rs200946642	0.0009189	0.006[B]	5.83	NA	NA	NA	Disease causing
	c.44_45insG	p.P16Sfs*45	NA	0	/	NA	NA	NA	NA	Disease causing
	c.634_642del	p.D212_V214del	NA	0	/	NA	NA	Deleterious	NA	Disease causing
	c.666C>A	p.Y222X	NA	0	/	NA	NA	NA	NA	Disease causing
PSMB8	c.224C>T	p.T75M	NA	0.00004242	/	5.91	Probably Damaging	Deleterious	Damaging	Disease causing
	c.274G>A	p.A92T	NA	0.000008463	/	5.91	Probably Damaging	Deleterious	Damaging	Disease causing
	c.313A>C	p.K105Q	NA	0	/	5.72	Probably Damaging	Deleterious	Damaging	Disease causing
	c.349A>G	p.M117V	NA	0	/	5.72	Probably Damaging	Deleterious	Damaging	Disease causing
	c.405C>A	p.C135X	rs146254972	0.00004069	/	3.38	NA	NA	NA	Disease causing
PSMB9	c.494G>A	p.G165D	rs369359789	0.0000425	/	4.85	Benign	Deleterious	Tolerated	Disease causing
PSMA3	c.404+2T>C	p.H111Ffs*10	NA	0	0[C]	NA	NA	NA	NA	Disease causing
	c.696_698delAAG	p.R223del	NA	0	0[C]	NA	NA	Deleterious	NA	Disease causing
POMP	c.344_345insTTTGA	p.E115Dfs*20	NA	0	0[D]	NA	NA	NA	NA	Disease causing

[A] ExAC: Exome Aggregation Consortium, which spans 61486 unrelated individuals and includes the 1000 Genomes Pilot project and NHLBI Exome Sequencing Project (ESP6500).
[B] Four of 659 genotyped healthy Caucasian controls samples were positive for PSMB4 5'UTR: c.- 9G>A mutation (0.006).
[C] The two variants of PSMA3 were absent in any of 363 genotyped Caucasian controls.
[D] The p.E115Dfs*20 mutation in POMP was absent in 192 genotyped controls of Lebanese and Palestinian Arab ancestry.

Supplemental Table 4. Summary of the analyses for each mutation studied.

Gene	Patient/ family	Gene	Allele-specific Expression	Protein expression in transfected HeLa cell	In silico Modeling	Assembly in trans-fected HeLa cells
PSMA3	*Pt.2*	*PSMA3*: c.696_698delAAG/ p.R233del (*de novo)*	Normal mRNA level [B]	No protein expressed in transfection assay	Likely affects folding and attachment of regulatory complex	No protein expression, no incorporation
	Pt.3 (Fam.3)	*PSMA3*: c.404+2T>C/ p.H111Ffs*10	Skipping of exon 5, unstable mRNA, truncated protein[A]	Not tested	Loss of C-terminal half, likely not expressed	Large truncation, ND
PSMB4	*Pt.1 (Fam.1)*	*PSMB4*: 5'UTR: c.-9G>A/ NA	Decreased mRNA level[B]	Decreased protein expression (consistent with decreased mRNA level)	Mutation in 5' un-translated region. If expressed should be wildtype protein or 3 AA extension on N-terminus	Lower expression, less incorporation into 20S and 26S proteasomes
	Pt.1 (Fam.1)	*PSMB4*: c.634_642del/ p.D212_V214del	Normal mRNA level	Well expressed	Likely destabilizes the C-terminal extension	Poor maturation and incorporation into 20S and 26S proteasomes
	Pts. 4, 5 (Fam.4)	*PSMB4*: c.44insG/ p.P16Sfs*45	Lower mRNA level[A]	Not tested	Early frame shift leads to very large truncation, likely not expressed	Large truncation, ND
	Pts.6, 7 (Fam.5)	*PSMB4*: c.666C>A/ p.Y222X	Normal mRNA level	Well expressed	Loss of C-terminal extension, predicted assembly defects	Poor maturation and incorporation into 20S and 26S proteasomes
PSMB8	*Pt.2, Pt.3 (Fam.2, Fam.3)*	*PSMB8*: c. 224C>T/ p.T75M	Normal mRNA level	Decreased protein expression	Altered active binding site structure (analyzed in previous publication)	Lower expression, less incorporation into 20S and 26S proteasomes
	Pts.6, 7 (Fam.4)	*PSMB8*: c. 313A>C/ p.K105Q	Normal mRNA level	Well expressed	Affects active site of chymotrypsin-like activity	Poor maturation but normal incorporation into 20S and 26S proteasomes
PSMB9	*Pts.4, 5 (Fam.5)*	*PSMB9*: c.494G>A/ p.G165D	Not tested	Not tested	Predicted to affect active site of caspase-like activity	ND

[A] Tested only in patients cells; [B] Also tested in patents cells; ND denotes not done

Supplemental Table 5. Effect of the mutations on proteasome activity and assembly assays in patients' cells.

Patient (Family)	Maternal Allele	Paternal Allele	PBMC proteasome assay	Assembly and function of EBV transformed B cells	Assembly and function of fibroblasts	Assembly and function of keratinocytes
Patient 1 (Family 1)	*PSMB4*: 5'UTR: c.9G>A/ NA	*PSMB4*: c.634_642del/ p.D212_V214del	Reduced chymotryptic (70%), tryptic (60%) and caspase (55%) activity	Reduced total proteasome content due to assembly defects and low expression of the maternal allele	Reduced total proteasome content due to assembly defects and low expression of the maternal allele	Reduced total proteasome content due to assembly defects and low expression of the maternal allele
Father (Family 1)	NA	*PSMB4*: c.634_642del/ p.D212_V214del	Nl. chymotryptic (90%) and tryptic (95%) but reduced caspase (50%) activity	Reduced proteasome content due to assembly defects, compensation by healthy allele	Reduced proteasome content due to assembly defects, compensation by healthy allele	Reduced proteasome content due to assembly defects, compensation by healthy allele
Mother (Family 1)	*PSMB4*: 5'UTR: c.-9G>A/ NA	NA	Nl. activities for chymotryptic (90%), tryptic (95%) and caspase (85%) activity	Maturation defect but normal proteasome content, likely compensated by healthy allele	Reduced expression of the subunit, almost completely compensated by healthy allele	ND
Patient 2 (Family 2)	*PSMA3*: c.696_698delAAG/ p.R233del (*de novo)*	*PSMB8*: c. 224C>T/ p.T75M	Reduced chymotryptic (80%), tryptic (78%) and caspase (80%) activity	Reduced proteasome content due to reduced expression of the mutated α7/PSMA3 subunit	Reduced proteasome content due to reduced expression of the mutated α7/PSMA3 subunit	Reduced proteasome content due to reduced expression of the mutated α7/PSMA3 subunit
Mother (Family 2)	NA	NA	Nl., but is wildtype	NA	NA	NA
Patient 3 (Family 3)	*PSMA3*: c.404+2T>C/ p.H111Ffs*10	*PSMB8*: c. 224C>T/ p.T75M	ND	ND	ND	ND

Patient (Family)	Maternal Allele	Paternal Allele	PBMC proteasome assay	Assembly and function of EBV transformed B cells	Assembly and function of fibroblasts	Assembly and function of keratinocytes
Mother (Family 3)	*PSMA3*: c.404+2T>C/ p.H111Ffs*10	NA	ND	ND	ND	ND
Father (Family 3)	NA	*PSMB8*: c. 224C>T/ p.T75M	ND	ND	ND	ND
Patient 4 (Family 4)	*PSMB9*: c.494G>A/ p.G165D	*PSMB4*: c.44insG/ p.P16Sfs*45	NI. chymotryptic and tryptic but reduced caspase 50% activity	ND	ND	Reduced proteasome content due to assembly defects from the paternal β7 subunit mutation, reduced caspase activity due to mutated maternal allele
Patients 5 (Family 4)	*PSMB9*: c.494G>A/ p.G165D	*PSMB4*: c.44insG/ p.P16Sfs*45	NI. chymotryptic and tryptic but reduced caspase 75% activity	ND	ND	Reduced proteasome content due to assembly defects from the paternal β7 subunit mutation, reduced caspase activity due to mutated maternal allele
Father (Family 4)	NA	*PSMB4*: c.44insG/ p.P16Sfs*45	NI. chymotryptic and tryptic but reduced caspase 65% activity	ND	ND	Assembly defects from the β7 subunit mutation, compensated by the healthy allele
Mother (Family 4)	*PSMB9*: c.494G>A/ p.G165D	NA	NI.	ND	ND	No detectable defects probably by compensation by the healthy allele

NA denotes not applicable; ND denotes not done; NI. denotes normal

5. Supplemental References

1. Griffin TA, Nandi D, Cruz M, et al. Immunoproteasome assembly: cooperative incorporation of interferon gamma (IFN-gamma)-inducible subunits. *J Exp Med.* 1998; 187(1): 97-104.
2. Unno M, Mizushima T, Morimoto Y, et al. The structure of the mammalian 20S proteasome at 2.75 A resolution. *Structure.* 2002; 10(5): 609-18.
3. Kopp J, Schwede T. The SWISS-MODEL Repository: new features and functionalities. *Nucleic Acids Res* 2006; 34(Database issue): D315-8.
4. Christen M, Hunenberger PH, Bakowies D, et al. The GROMOS software for biomolecular simulation: GROMOS05. *Journal of computational chemistry.* 2005; 26(16): 1719-51.
5. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics.* 2009; 25(9): 1105-11.
6. Moir S, Fauci AS. Insights into B cells and HIV-specific B-cell responses in HIV-infected individuals. *Immunol Rev.* 2013; 254(1): 207-24.