Early-onset Evans syndrome, immunodeficiency and premature immunosenescence associated with tripeptidyl-peptidase II deficiency

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Key Points

Deficiency of tripeptidyl-peptidase II is associated with Evans syndrome and viral infection susceptibility

TPP2 deficiency links premature immunosenescence of T and B cells with severe autoimmunity

Abstract

Autoimmune cytopenia is a frequent manifestation of primary immunodeficiencies. Two siblings presented with Evans syndrome, viral infections and progressive leukopenia. DNA available from one patient showed a homozygous frameshift mutation in tripeptidyl peptidase II (TPP2) abolishing protein expression. TPP2 is a serine exopeptidase involved in extralysosomal peptide degradation. Its deficiency in mice activates cell death programs and premature senescence. Similar to cells from naïve, uninfected TPP2 deficient mice, patient cells showed increased MHC I expression and most CD8+ T-cells had a senescent CCR7-CD127-CD28-CD57+ phenotype with poor proliferative responses and enhanced staurosporine-induced apoptosis. T-cells showed increased expression of the effector molecules perforin and IFN-γ with high expression of the transcription factor T-bet. Age-associated B-cells with a CD21- CD11c+ phenotype expressing T-bet were increased in humans and mice, combined with antinuclear antibodies. Moreover, markers of senescence were also present in human and murine TPP2 deficient fibroblasts. Telomere length was normal in patient fibroblasts and granulocytes and low normal in lymphocytes, compatible with activation of stress-induced rather than replicative senescence programs. TPP2 deficiency is the first primary immunodeficiency linking premature immunosenescence to severe autoimmunity. Determination of senescent lymphocytes should be part of the diagnostic evaluation of children with refractory multilineage cytopenias.
Introduction

Evans syndrome is defined by the simultaneous or sequential development of immune thrombocytopenic purpura and autoimmune hemolytic anemia. In about 50% of cases, it is associated with systemic autoimmune disease such as systemic lupus erythematosus, lymphoproliferative disease or with primary immunodeficiencies. In this latter group of diseases, the variety of predisposing genetic defects illustrates the multiple checkpoints that can be affected in the loss of immunological tolerance. However, despite the increased molecular understanding, the question whether a genetic predisposition contributes to the autoimmune cytopenia remains unresolved for most patients.

Immunosenescence is one pathomechanism that has been associated with autoimmunity. For T cells, age-associated skewing of the antigen-receptor repertoire related to decreased thymic output and homeostatic proliferation of potentially autoreactive clones as well as age-associated alterations in the antigen-receptor signaling network have been put forward as potential explanations. For B cells, a decline of B cell generation in bone marrow with age and shifts in naïve and antigen-experienced peripheral B cell subsets could be linked to autoimmunity. Premature immunosenescence can occur as a consequence of chronic immune stimulation, such as persistent viral infections. In addition, genetic factors favoring premature differentiation and/or persistence of senescent immune cells could be a predisposing factor for autoimmunity, even in the absence of persistent infections.

Tripeptidylpeptidase II (TPPII) is a molecule that has previously been linked to immunosenescence. TPPII is a cellular protease that operates mostly downstream of proteasomes in cytosolic proteolysis. It is important for cell proliferation and survival, in particular under conditions of cellular stress, and may contribute to an antiapoptotic phenotype. In mice, lack of TPPII activates cell death programs leading to proliferative apoptosis in T cells and premature senescence particularly of CD8+ T cells. In addition,
murine TPP2 deficiency also causes premature senescence in fibroblasts and degenerative alterations at the level of the entire organism. However, despite their immunological alterations, no autoimmunity or immunodeficiency phenotype has so far been described in TPPII deficient mice.

Here we report two siblings with early-onset Evans syndrome, variable lymphoproliferation and mild infection susceptibility, who had loss-of-function mutations in the gene encoding TPPII. Immunological studies in one of these patients were compared to those obtained in naïve, uninfected TPPII deficient mice in an attempt to differentiate primary consequences of TPPII deficiency from those of infections. Our results document that premature senescence in human TPPII deficiency also affects B cells in addition to CD8+ T cells and fibroblasts and is associated with autoimmunity and immunodeficiency.
Patients and Methods

Two siblings with early onset Evans’ syndrome and variable infection susceptibility

The index patient (P1), second child of consanguineous Palestinian parents, presented at the age of 21 months with Coombs positive autoimmune hemolytic anemia and immune thrombocytopenia, cervical and axillary lymphadenopathy and mild to moderate intermittent splenomegaly (Table S1). He was initially responsive to steroids and IVIG, but remained steroid-dependent and developed recurrent episodes of severe cytopenia despite treatment with cyclosporin, mycophenolate mofetil, several courses of rituximab and more than 6 months on sirolimus. While on immunosuppressive therapy, he developed disseminated and prolonged cutaneous chickenpox. He had repeated low-level CMV viremia without clinical sequelae, which could be controlled with ganciclovir and foscavir. At 10 years of age, he developed numerous flat hypopigmented 1-2 mm papular lesions on the face (Fig. S1). PCR was positive for HPV type 15. Otherwise, his infection history was unremarkable. Mild developmental delay with significant verbal and comprehension impairment was diagnosed and education for children with special need was recommended. Due to the refractory course of his Evans’ syndrome, he recently underwent haematopoetic stem cell transplantation (HSCT) from an HLA-identical healthy sibling donor following conditioning with fludarabine, targeted low levels of busulfan, thiotepa and ATG. In October 2014, six months after HSCT, the child remains well with 97% donor cell chimerism and no infectious, autoimmune or lymphoproliferative manifestations. He is off any immunosuppressive medication. Thus, at this early time point, the clinical features that led to the decision to transplant have resolved.

An older sibling (S1) had been followed in another hospital from 18 months of age when he presented immune thrombocytopenia followed by Coombs positive hemolytic anemia 5 months later. She had some lymphadenopathy but no splenomegaly. She was treated with IVIG and steroids, but became increasingly unresponsive to therapy that included
splenectomy at 33 months of age. She developed a left hemispheric ischemic event leading to right-sided hemiparesis at 30 months. At 37 months she died in the context of an acute hemolytic crisis. She had normal psychomotor development and no increased infection susceptibility. Limited laboratory information and no DNA were available from that sibling.

P1 intermittently had significantly increased IgG and IgM and increased IgG was also documented in S1. IgA and IgE were normal (Table S1). At age 11 years, P1 had seroconverted to CMV, EBV, VZV and had detectable tetanus antibodies. P1 and S1 were intermittently positive for antinuclear antibodies (scored 1-2 on a scale from 0-4). P1 had normal lymphocyte counts and subpopulations at 21 months. At 10 years he had mild leukopenia and lymphopenia with a reduction in the fraction of naïve CD4 T cells and low B cell counts (3 years after rituximab treatment). He had normal levels of serum vitamin B12 and sFasL, no elevation of CD3+TCRαβ+CD4-CD8- double negative T cells, a moderate elevation of γδT cells (18%) and a normal proportion of CD4+CD25+FOXP3+ regulatory T cells.

Five patients with FAS mutations (ALPS-FAS, age: 6-32 years) and nine patients with autoimmune cytopenia and lymphoproliferation in the context of the clinical diagnosis “CVID” (http://esid.org/Working-Parties/Registry/Diagnosis-criteria), aged 7-17 years were recruited as disease control groups for analysis of senescent T and B cells. Two of the “CVID” patients had biallelic LRBA mutations, two had activating mutations in PIK3CD and one a heterozygous CTLA4 mutation, while in the remaining 4 patients, a defined genetic diagnosis has not yet been established. Two siblings with autosomal-dominant TERC mutation and clinical dyskeratosis congenita (DKC) served as controls for telomere lengths determination.

Patient and parental consent were given for this study. The study was performed with the approvals of the ethical committees of Hadassah Medical Center and the Ministry of
Health and of Freiburg University Medical Center, and in accordance with the Declaration of Helsinki.

Mice

Mice rendered deficient in TPPII by gene targeting have been described previously\textsuperscript{15,16}. Mice were analyzed between 6-12 months of age. All mouse experiments were approved by the Regierungspräsidium Freiburg (G-06/34).

All experimental procedures are described in the supplementary material.
Results

A homozygous frameshift mutation in TPPII segregates with the disease phenotype

To elucidate the presumed genetic basis of the disease in the two siblings with early-onset Evans syndrome and variable susceptibility to viral infections, whole exome sequencing was performed using DNA extracted from whole blood of P1, a healthy sister and both parents. The patient exome was well covered with an average depth of X 326 (94.5% of the exons were covered > X10 and 92.8% were covered > X 20) Aligning the patient 196.94 million reads to the reference human genome revealed 261,516 variants. We removed variants, which were of low depth (<x8), deep intronic, heterozygous and those present in dbSNP132 or in the in-house dbSNP. Seven variants survived this filtering (Table S3) but only one was predicted pathogenic by MutationTaster17. This was a homozygous frameshift mutation, chr13: 10326878 c.432delG (p.Ala145Profs*25) in the gene encoding TPPII (Fig. 1A). The frameshift started at Ala145 generating a premature stop codon 25 residues later. The mutation segregated with the disease in the family and was absent from the 6503 healthy individuals whose exome analysis results were available through the Exome Variant Server, NHLBI Exome Sequencing Project, Seattle, Washington, USA (http://evs.gs.washington.edu;EVS-v.0.0.21) (accessed Feb. 18, 2014). No mutations were detected in the genes encoding PRKCD, PI3KD, STK4, CD95 or CD95L. A Western Blot using an anti-TPPII antibody recognizing amino acids 165-303 failed to detect protein (Fig 1B). In the course of the study 22 children with autoimmune cytopenia and moderate lymphoproliferation in the absence of FAS mutations were screened and no additional patients with mutations in TPPII were identified.

TPPII deficient mice and humans share features of immune dysregulation and premature fibroblast senescence

Since we only had access to blood from a single patient, we asked whether the main clinical features of the two siblings could also be observed in mice with an inactivated
TPPII gene\textsuperscript{15,16}. Lymphadenopathy and/or splenomegaly were inconsistently observed in about 50% of the mice used in this study (data not shown). Two of 16 mice developed antinuclear antibodies, 8 had anti-nucleolar or anti-cytoplasmatic antibodies (Fig.S3A). Hypergammaglobulinemia was not observed. Older mice had leukopenia (Fig. S3B), as reported previously. Additional characteristic previously reported features in these mice included increased MHC class I expression and premature senescence of fibroblasts\textsuperscript{15,16}. Similar to the murine findings\textsuperscript{16}, expression of MHC class I on T and B cells was higher in the patient compared to his HLA-identical healthy heterozygous sister (Fig.1C) and a group of 10 healthy adult controls. Pretreatment of control T cells with the TPPII inhibitor butabindide\textsuperscript{18,19} enhanced MHC I upregulation upon PHA stimulation (Fig. 1D). Finally, as described in murine cells, fibroblasts of P1 also showed increased staining with β-galactosidase indicating cellular senescence (Fig 1E).

**TPPII deficiency leads to premature T cell senescence**

Encouraged by these phenotypic similarities, we performed a parallel immunological analysis of patient and TPPII deficient murine lymphocytes to further understand the basis of this genetic disorder. Since TPPII deficient mice have increased CD44\textsuperscript{16} memory CD8+ T cells, we carefully analyzed T cell differentiation using established surface markers for human (CCR7 and CD45RA\textsuperscript{20}) and murine T cells (CD62L and CD44). More than 90% of CD8+ T cells from P1 had an effector memory or TEMRA phenotype (Fig. 2A,B). A similar effector skewing was observed in naïve TPPII deficient mice (Fig 2B). Advanced differentiation was also observed among patient and murine CD4+ T cells (Fig. 2A,B). Further analysis of patient CD8+ T cells revealed a predominant CD27-CD28-CD127-phenotype that was not present among CD4+ T cells (Fig. 2C). Terminal differentiation of the majority of CD8+ T cells was further reflected by expression of CD57 (Fig. 2C,D). Unexpectedly, these cells were largely negative for KLRG1 (Fig. 2C), which is usually expressed on CD57+CD8+ T cells. The proportion of CD57+CD8+ T cells in P1 was higher than in ALPS patients, while pediatric patients with autoimmune cytopenia and
lymphoproliferation fulfilling the clinical criteria for CVID, including patients with activated PI3K delta syndrome, LRBA and CTLA-4 deficiency showed variable results (Fig. 2D). There was also an increased fraction of CD127- CD8+ T cells in naïve TPPII deficient mice (Fig 2), indicating that this enhanced differentiation state was not only a consequence of the infection history of the patient. However, in contrast to the patient, most of these murine cells showed increased KLRG1 expression as usually observed for senescent T cells (Fig.2E).

While T-bet expression in CD57+CD8+ T cells of P1 was high as expected for terminally differentiated T cells, the expression of Eomesodermin (Eomes) was reduced and lower compared to CD57+CD8+ T cells from control donors (Fig. 3A). The advanced T cell differentiation was associated with an enhanced effector state. Thus, in contrast to controls, almost all CD8+ T cells and also a relevant fraction of CD4+ T cells expressed perforin (Fig. 3B). Furthermore, the fraction of cells responding to PMA/ionomycin stimulation with expression of IFN-γ was enhanced among both patient and murine TPPII deficient CD4+ and CD8+ T cells (Fig. 3C,D).

To assess whether the increased CD8 T cell differentiation was linked to the enhanced MHC I expression associated with TPPII deficiency, we compared EBV lines or PBMC from the patient and his heterozygous healthy HLA-identical sister in their ability to induce proliferation of allogeneic healthy donor responder T cells. However, no differences could be detected (Fig. S4). Analysis of the T cell repertoire revealed significant skewing among CD8+, but not among CD4+ T cells in the patient (Fig. S2A). In contrast, TPPII deficient mice had a normal polyclonal T cell repertoire, suggesting that the oligoclonal expansions in the patient were not a direct consequence of the genetic defect (Fig. S2B).

**TPPII deficiency reduces T cell proliferation and lymphocyte survival**

Previous murine experiments had indicated reduced proliferation associated with enhanced activation induced cell death in TPPII deficient T cells. The proliferative
response of patient cells to stimulation with anti-CD3/28 coated beads or with PHA was reduced, but present for CD4+ T cells, while no response could be induced in CD8+ T cells (Fig.4A). Moreover, proliferation of control T cells was diminished after pretreatment with butabindide (Fig. 4B). Due to the poor proliferative response, we could not generate enough activated human T cells to study restimulation-induced cell death. We therefore analyzed apoptosis induction in EBV lines from the patient, his heterozygous sister, a healthy donor and a patient with autoimmune lymphoproliferative syndrome cause by a germline FAS mutation (ALPS-FAS). While irradiation or stimulation with anti-Fas antibody or etoposide induced apoptosis in patient cells to a similar extent as in controls, patient EBV lines were more susceptible to staurosporine induced cell death (Fig. 4C). Pretreatment of control EBV lines with butabindide also enhanced the apoptosis response to staurosporine (Fig. 4D). In combination with previous reports in mice, these results suggest that TPPII deficiency confers a lymphocyte proliferation and survival defect.

TPPII deficiency leads to premature senescence of B cells

Analysis of the B cell compartment was performed 3 years after rituximab treatment. There was a mild relative reduction of IgD+CD27+ marginal-zone like (5.3%) and IgD-CD27+ class-switched memory B cells (2.7%) and a moderate increase in the percentage of CD38+ IgM+ transitional B cells (18%). Most prominent was an elevated fraction of CD21low cells, that in patients with CVID or autoimmune diseases have been characterized as a preactivated, functionally attenuated and autoreactive population21,22 (Fig. 5A). Recently, a related population of CD11c+ age-associated B (ABC) cells has been described that accumulates in aged humans and mice23,24 and has been associated with autoimmunity24,25. Indeed, most CD21low B cells of P1 expressed CD11c (Fig. 5B) and the transcription factor T-bet. This population was not observed in healthy adult donors or patients with ALPS, but in a proportion of pediatric patients with autoimmunity and lymphoproliferation fulfilling the clinical criteria for CVID. Of note, among the few disease control patients with a molecular diagnosis including activating PI3K delta
syndrome, LRBA or CLTA-4 deficiency, some had elevated ABCs and some elevated CD57+ CD8+ T cells without obvious correlation. Importantly, we also observed a significant increase in the fraction of CD11c+CD11b+ B cells in TPPII deficient mice, suggesting that the relative abundance of this B cell population resulted from the genetic defect and was not induced by the particular medical history of the patient (Fig. 5C).

**TPP2 deficiency does not affect telomere integrity**

To assess whether TPPII deficiency affects telomere integrity, we analyzed telomere length using flow-FISH technology in PBMC from P1, his healthy sister and two patients with dyskeratosis congenita (DKC) as a positive control. While lymphocyte telomere lengths of the sibling were at the 50th percentile of healthy children, patient lymphocytes showed a telomere length below the 25th percentile (Fig. 6A-C). This is much higher than what can be observed in DKC patients. Considering that patient lymphocytes contained 40% of CD57+CD8+ T cells, this is an expected result and does not indicate impaired telomere maintenance. In addition, both siblings had similar telomere lengths between the 25th and 50th percentile in granulocytes (Fig. 6D) and further analysis of fibroblasts revealed even longer telomere length (data not shown). These data suggest that TPPII deficiency is not associated with enhancement of replicative senescence.
Discussion

We detected biallelic TPPII mutations leading to absent protein expression in a child presenting with early-onset Evans syndrome. The following arguments suggest a causal relationship between this genetic defect and the clinical phenotype: (i) Features of cellular immunosenescence in patient T and B cells including phenotypic alterations, defects in apoptosis and proliferation and functional effector skewing were also observed in TPPII deficient mice. In addition to published reports, this was supported by parallel experimentation with human and murine cells in this study. (ii) The reduced proliferation, increased apoptosis and increased MHC class I expression of patient cells could be reproduced by treating control cells with the TPPII inhibitor butabindine. (iii) Similar to P1 and the possibly affected sibling, TPPII deficient mice show an increased incidence of autoantibodies. (iv) TPPII deficient mouse as well as patient fibroblasts show premature senescence. (v) A recent congress abstract described a similar phenotype of autoimmune cytopenia, infection susceptibility and developmental delay in two siblings with TPPII deficiency and premature fibroblast and CD8 T cell senescence.

Current understanding of the function of TPPII in vivo is mainly based on observations in TPPII deficient mice. Previous studies have documented an immunosenescence phenotype associated with enhanced cellular death programs. The mice showed age-related lymphopenia associated with enhanced apoptosis of immature thymocytes and accelerated thymic involution. In addition, proliferative apoptosis of peripheral T cells was increased. The T cell phenotype was skewed towards memory T cells and anti-CD3 stimulated T cells stained positive for acidic β-galactosidase further indicating premature senescence. An increased basal activity of NFκB, attenuated NFκB activation as well as increased p53 expression in TPPII deficient T cells further supported cellular senescence. Moreover, MHC class I expression was increased, as is observed in age-associated
inflammation\textsuperscript{27}. Clinically, TPPII deficient mice showed variable splenomegaly and/or lymphadenopathy associated with extramedullary hematopoiesis.

The knockout mice also indicated that TPPII is not only relevant in the hematopoietic system\textsuperscript{15,16}. Thus, premature senescence of fibroblasts was associated with aged appearance, reduced body weight and premature death of elderly mice\textsuperscript{15}. Despite the immunological alterations, there was only a minor phenotype after infection with lymphocytic choriomeningitis virus (LCMV). The mice showed a minimal reduction in the number of virus-specific T cells, but cytotoxicity was normal and virus elimination was unimpaired (\textsuperscript{16} and Peter Aichele: personal communication).

Based on these previous findings, it was highly surprising that human TPPII deficiency was observed in a patient with a life-threatening immunodeficiency. Nevertheless, the underlying cellular phenotype of our patient well reflected the immunosenescence observed in mice. In CD8+ T cells we observed a highly differentiated CCR7-CD45RA+CD27-CD28- and CD57+ phenotype\textsuperscript{28}. Such characteristics of senescent cells can also be induced by repeated proliferation induced by persistent viruses such as CMV\textsuperscript{29}. However, even though our patient had intermittent low-level CMV reactivation, the extent of accumulation of senescent CD8 T cells was very unusual for his age. Moreover, we confirmed this phenotype in the absence of infection in TPPII deficient mice, extending previous observations\textsuperscript{15}. The dominance of the transcription factor T-bet relative to the expression of Eomes could be related to the advanced T cell differentiation. T-bet drives T cell differentiation with enhanced effector activity, while Eomes expression is linked to memory cell formation with high proliferative capacity and self-renewal\textsuperscript{30}. Both patient and murine cells showed an excessive IFN-\gamma response and patient T cells highly expressed perforin. It is conceivable that this enhanced IFN-\gamma production contributes to the increased MHC class I expression, that was observed in the patient as in the TPPII deficient mice\textsuperscript{16} and can also be seen in aged individuals\textsuperscript{27}. Reduced proliferation is also in line with T cell senescence\textsuperscript{15}. 
Confirming murine observations, patient fibroblasts also showed features of premature senescence. Moreover, the mild developmental delay observed in our patient was also reported by Hambleton. TPPII deficiency thus appears to be a syndromic disease that is not restricted to the immune system. However, although the further course remains to be evaluated, at the age of 12 years, in our patient none of the potential extrahematopoietic manifestations was considered severe enough to represent a contraindication for HSCT.

It is a new finding that TPPII deficiency causes increased infection susceptibility. While a not further specified broad susceptibility to infections from early childhood was noted in the patients reported by Hambleton, our patient by the age of 12 years had a history of widespread cutaneous varicella infection, intermittent CMV viremia and cutaneous HPV infection. These infections may in part be explained by immunosuppressive therapy. However, extensive and recalcitrant HPV infection is unusual. In particular, beta-HPVs such as the genotype 15 detected in our patient are weakly virulent and unusual in healthy individuals, even under immunosuppressive therapy, while they are characteristic for epidermodysplasia verruciformis (EV, OMIM 226400), a rare genodermatosis. Infection by EV-specific beta-HPV genotypes has also been demonstrated in two autosomal recessive disorders affecting T-cell immunity; RHOH deficiency and MST1 deficiency. TPPII deficiency thus represents a further T cell deficiency predisposing to EV-specific HPVs.

It was even more unexpected, that TPPII deficiency is associated with autoantibody-mediated autoimmunity, manifesting as severe Evans syndrome leading to a transplant indication in P1 and early death in S1. Autoimmune cytopenia in two and autoimmune hepatitis in one patient was also reported by Hambleton. We could further document that most TPPII deficient mice developed antinucleolar, anticytoplasmic or antinuclear antibodies, which were also detected in P1 and S1. Aging is associated with increased autoantibody formation. A subset of B cells termed age-associated B cells (ABCs) accumulating in the elderly has recently been characterized in humans and mice by the expression of CD11b, CD11c and T-bet. Interestingly, these ABCs appear earlier in
autoimmune prone mice and are found in humans suffering from autoimmunity\textsuperscript{24}, including patients with common variable immunodeficiency\textsuperscript{21,22,25}. Their depletion in mice leads to a reduction in autoantibodies. We found ABCs in the 12 year-old patient as well as in TPPII deficient mice, illustrating premature immunosenescence also in the B cell compartment and providing a potential link to the observed autoantibody-mediated disease in TPPII deficiency.

What are the molecular mechanisms leading to immunosenescence in the absence of TPPII? In principle, two forms of cellular senescence can be differentiated. Whereas replicative senescence is associated with shortened telomeres, stress-induced senescence is associated with DNA damage, activated stress pathways or a dysbalance of proliferation-associated transcription factors\textsuperscript{35}. The observation of normal telomere length in patient fibroblasts and the expected shorter telomeres in predominantly senescent lymphocytes indicates that TPP2 is not required for general maintenance of telomere integrity such as in dyskeratosis congenita. In fact, previous observations support that TPP2 rather protects cells under conditions of cellular stress\textsuperscript{10}. As a peptidase, TPPII may have an impact on the intracellular concentration of short peptides modulating protein-protein interactions or a direct role in signaling by targeting natural substrates\textsuperscript{36}. One conceivable target is the Akt/mTOR pathway. Its activation has previously been linked to senescence associated β-galactosidase activity in human fibroblasts\textsuperscript{37} and to CD8 T cell senescence\textsuperscript{38}. In fact, recent observations in patients with gain-of-function mutations in the PI3K catalytic subunit or loss of the regulatory subunit p85 suggest that activation of this pathway may be associated with immunodeficiency and autoimmunity in human patients\textsuperscript{39-40}. We found slightly enhanced S6 phosphorylation in patient T cells (data not shown), but could not reproduce this in murine T cells. The lack of a clinical response to sirolimus in our patient may further suggest that this is not a major pathway linking TPPII deficiency to autoimmunity and immunodeficiency. Thus, the molecular link from TPPII deficiency to premature cellular senescence remains elusive.
In summary, in combination with the findings of Hambleton, our observations in a single patient and the corresponding gene-inactivated mice identify TPPII deficiency as a new syndromal primary immunodeficiency leading to an increased susceptibility to viruses and autoimmunity. It is the first primary immunodeficiency clearly linking premature senescence with autoimmunity. The combination of an accumulation of senescent cells, autoimmunity and immunodeficiency appear to be recurrent themes in pediatric patients with a number of recently described diseases including CTLA4 deficiency, activated PI3K delta syndrome, PI3K p85 deficiency or LRBA deficiency. Although not specific for a particular genetic condition, the determination of senescent T and B cells should be part of the diagnostic evaluation of any child with refractory multilineage cytopenias.
Conflict of Interest statement

There is no conflict of interest for any of the authors.

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Author contributions

PS – identified the patient, provided all clinical information, revised the paper; ARE – performed most human experiments, wrote the paper; RG, EF, SZ, HE, GN – performed mouse experiments; SRV – analyzed skin lesions; UF, SN – performed protein analysis; SF, MR, BK, MR – assisted with human lymphocyte analysis; OE, VMP, AB – performed genetic and bioinformatics analysis; FB, THB - analyzed telomere lengths; SE – coordinated research and wrote the paper
References


14. Hong X, Lei L, Glas R. Tumors acquire inhibitor of apoptosis protein (IAP)-
mediated apoptosis resistance through altered specificity of cytosolic proteolysis. 

immunosenescence-like phenotype in TPPII knockout mice. *Proc Natl Acad Sci


17. Schwarz JM, Rodelsperger C, Schuelke M, Seelow D. MutationTaster evaluates

18. Duensing S, Darr S, Cuevas R, et al. Tripeptidyl Peptidase II Is Required for c-
MYC-Induced Centriole Overduplication and a Novel Therapeutic Target in c-

19. Marcilla M, Villasevil EM, de Castro JA. Tripeptidyl peptidase II is dispensable for
the generation of both proteasome-dependent and proteasome-independent

T lymphocytes with distinct homing potentials and effector functions. *Nature.* 

in a dysfunctional memory B cell compartment in HIV-infected viremic

common variable immunodeficiency resemble tissue homing, innate-like B cells. 

uniquely responsive to innate stimuli accumulates in aged mice. *Blood.* 
2011;118(5):1294-1304.

accumulation of a novel CD11c(+) B-cell population is important for the

cells contain mostly autoreactive unresponsive clones. *Blood.* 

27. Assounga AG, Warner CM. Transcription of major histocompatibility complex class I (Kb) and transporter associated with antigen processing 1 and 2 genes is up-regulated with age. *Immunology*. 2004;113(3):378-383.


Figure legends

Figure 1: Human TPPII deficiency reproduces features of the murine knock-out phenotype

(A) Illustration of the single nucleotide deletion at position c.432delG of the TPPII coding sequence in the index patient (P1) and his asymptomatic sister (B) Western blot analysis of lysates from EBV lines of P1, the healthy heterozygous sister and a healthy donor (HD) incubated with anti-TPPII antibodies (upper panel) and anti-actin antibodies as a loading control (lower panel). (C) MHC class I expression on fresh T and B cells of P1 compared to his HLA identical healthy heterozygous sister or 10 adult controls (adult HD). (D) Upregulation of MHC class I expression on control CD4 or CD8 T cells after PHA stimulation in the absence (gray) or presence (black) of the TPPII inhibitor butabindide. (E) β-galactosidase activity of primary fibroblasts isolated from skin biopsies of the patient (P1) or a healthy donor (HD). Experiments C-E were performed three times with similar results.

Figure 2: Advanced differentiation of TPPII deficient T Cells

(A) PBMC of the patient (P1, age 11) and his asymptomatic heterozygous sister (age 7) were analyzed for expression of the differentiation markers CCR7 and CD45RA (gated on CD3+CD4+ and CD3+CD8+ T cells, respectively). (B) Left panel (human PBMC): Proportions of naïve (CCR7+CD45RA+, naïve), central memory (CCR7+CD45RA-, CM), effector memory (CCR7-CD45RA-, EM) and terminal differentiated T effector memory cells reexpressing CD45RA+ (CCR7-CD45RA+, TEMRA) among CD4+ or CD8+ T cells of the patient (P1) compared to adult healthy donors (HD, n=20). Right panel (mouse splenocytes): Proportions of naïve (naïve, CD44-CD62L+), central memory (CM, CD44+CD62L+), effector memory (EM, CD44+CD62L-) and double negative (DN, CD44-CD62L-) cells were determined among CD4+ or CD8+ T cells of 6-12 months old TPPII deficient (KO, n=16) compared to age-matched wildtype mice (WT, n=7). (C) Expression
of the indicated activation/differentiation markers on CD4 and CD8 T cells of the patient (P1) and his healthy heterozygous sister. (D) Percentage of CD57 expressing CD8 T cells in the patient (P1) compared to adult healthy controls, FAS mutant patients with autoimmune lymphoproliferative syndrome (ALPS; pediatric and adult) and pediatric patients with autoimmune cytopenia and lymphoproliferation in the context of “CVID”. Of these “CVID” patients two had LRBA deficiency (diamonds), two had activating PIK3CD mutations (squares) and one patient had a CTLA4 mutation (circle). (E) Expression of KLRG1 and CD127 on CD8 T cells of TPPII deficient (KO) and age matched wild-type mice (WT).

**Figure 3: Enhanced effector functions in TPPII deficient T cells**

(A) Expression of the transcription factors Eomesodermin (Eomes) and T-bet in CD8 T cells of the patient (P1) and a healthy adult control (HD). (B) Perforin expression in CD3+CD4+ and CD3+CD4- T cells of the patient (P1), his healthy heterozygous sister and an adult healthy control (HD). (C) Intracellular Interferon-γ and IL-17 expression in CD4+CD45RO+ T cells after stimulation with PMA/ionomycin. The percentage of IFNγ positive T cells in the patient was compared to a group of healthy adult controls (HD, n=24). (D) Intracellular IFN-γ of splenic CD3+CD4+ and CD3+CD8+ T cells from TPPII deficient (KO, n=4) and control mice (WT, n=4). The experiment was repeated twice with similar results.

**Figure 4: Defective proliferation and enhanced susceptibility to apoptosis**

(A) PBMC of the patient (P1) or his healthy heterozygous sister were left untreated or stimulated with PHA or anti-CD3/CD28 beads and CFSE dilution of CD4+ or CD8+ T cells was determined after 5 days incubation (unstimulated: gray, stimulated: black). The experiment was performed three times with similar results. (B) Control PBMC were labeled with CFSE and stimulated with PHA or anti-CD3/CD28 beads in the absence (gray line) or presence (black line) of the TPPII inhibitor butabindide. CFSE dilution of
CD4+ and CD8+ T cells was analyzed after 5 days. The experiment was repeated three times with similar results. (C) EBV lines of the patient (P1), his healthy heterozygous sister, a healthy control (HD) and a FAS-mutant ALPS patient were stimulated with increasing concentrations of anti-Fas antibody cross-linked with protein A, the topoisomerase inhibitor etoposide (eto), the protein kinase inhibitor staurosporine (sts), or were sublethally irradiated (30 Gy). Viable Annexin V/PI negative cells were determined by flow cytometry after 24h or on day 0, 2 and 4, respectively. Results are representative of 3 independent experiments with similar results. (D) Cells of an EBV line from a healthy donor were stimulated with increasing concentrations of staurosporine in the absence (filled squares) or presence (open squares) of the TPPII inhibitor butabindide and viable cells were determined after 24h. The experiment was repeated twice with similar results.

Figure 5: Abnormal B cell differentiation in TPPII deficiency

(A, B) Human CD19+ B cells were analyzed for expression of differentiation and senescence markers. The proportion of CD11c+CD21- age-associated B cells (ABC) was compared between the patient (P1), 8 adult controls (HD), 5 untreated ALPS-FAS patients (ALPS) and pediatric patients with autoimmune cytopenia and lymphoproliferation in the context of “CVID” (for explanation of symbols see Fig. 2). Patient B cells were analyzed for T-bet expression (C) The proportions of CD11b+ and CD11b/c+ double positive splenic CD19+ B cells were determined in 6-12 months old TPPII knock out (KO) and wildtype (WT) mice (n=4). The experiment was repeated twice with similar results.

Figure 6: Telomere lengths of TPPII deficient lymphocytes and granulocytes

(A) Exemplary FACS plot of the flow-FISH analysis: human lymphocytes, granulocytes and cow thymocytes were discriminated by forward scatter (FSC) and LDS 751 staining. (B) Telomere lengths were determined by subtracting telomere-Alexa-Fluor 488 intensity of stained from the unstained lymphocytes of the patient (P1) or the healthy carrier sister.
Cow thymocytes with known telomere length were used as an internal control and to calculate telomere lengths in kilobases. Absolute telomere lengths of lymphocytes (C) and granulocytes (D) of the patient, his sister and 2 patients with dyskeratosis congenita (DKC) are shown in the context of age-dependent percentiles (black lines: 99th, 50th and 1st percentile, dotted lines: 75th and 25th percentile).
Figure 1

A. Electropherograms showing the c.432delG (p.Ala145ProfsX25) mutation in HD, P1, and Sister (carrier).

B. Western blot analysis for TPPII and β-actin in HD, sister, and P1.

C. Flow cytometry analysis showing CD4+, CD8+, and CD19+ cell populations in P1 vs. HLA identical sister and P1 vs. Adult healthy donors.

D. Histograms showing the % of Max for CD4+ and CD8+ cells in Med and PHA conditions, with Grey: - and Black: +Butabindide.

E. Microscopic images of HD and P1 samples.
Figure 2

A

![Graph showing CD4+ and CD8+ cell percentages with CCR7 and CD45RA axes.]

B

![Graph showing CD4+ and CD8+ cell percentages with CD44 and CD62L axes.]

C

![Graph showing CD4+ and CD8+ cell percentages with CD27 and CD127 axes.]

D

![Graph showing % of CD57+ of CD8+ cells with CD27, CD127, and CD57 axes.]

E

![Graph showing % of CD57+ of CD8+ cells with KLRL1 and CD127 axes.]

Legend:
- TEMRA
- EM
- CM
- naive
- DN
- EM
- CM
- naive
- KO
- WT
- P1
- HD
- P1
- Adult ALPS CVID
- KO
- WT
- +
- +/-
- +/+
Figure 6

A

B

C

D

Lymphocytes

Granulocytes

Telomere length (kb)

Age (years)

Telomere length (kb)

Age (years)
Early-onset Evans syndrome, immunodeficiency and premature immunosenescence associated with tripeptidyl-peptidase II deficiency

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