Activation of cellular death programs associated with immunosenescence-like phenotype in TPPII knockout mice

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The giant cytosolic protease tripeptidyl peptidase II (TPPII) has been implicated in the regulation of proliferation and survival of malignant cells, particularly lymphoma cells. To address its functions in normal cellular and systemic physiology we have generated TPPII-deficient mice. TPPII deficiency activates cell type-specific death programs, including proliferative apoptosis in several T lineage subsets and premature cellular senescence in fibroblasts and CD8+ T cells. This coincides with up-regulation of p53 and dysregulation of NF-κB. Prominent degenerative alterations at the organ level were a decreased lifespan and symptoms characteristic of immunohematopoietic senescence. These symptoms include accelerated thymic involution, lymphopenia, impaired proliferative T cell responses, extramedullary hematopoiesis, and inflammation. Thus, TPPII is important for maintaining normal cellular and systemic physiology, which may be relevant for potential therapeutic applications of TPPII inhibitors.

We have generated TPPII-deficient mice and provide here genetic evidence that TPPII is important for proliferative survival of nonmalignant cells. Lack of TPPII activates cell death programs, including proliferative apoptosis in several T lineage subsets and premature senescence of CD8+ T cells and fibroblasts. In addition, lack of TPPII prematurely causes degenerative alterations at the level of the entire organism. These include abnormalities that coincide with symptoms characteristic of immunohematopoietic senescence and premature death of elderly knockout (KO) mice.

Results

Generation of TPPII-Deficient Mice. To generate TPPII-deficient mice, we used the Cre-loxP and FLP-FRT systems [supporting information (SI) Fig. S1A]. Crossing of floxed mice to CMV-Cre-transgenic mice deleted exons 11 and 12, the latter bearing the active-site serine of TPPII’s subtilisin-like catalytic triad (13). TPPII protein expression and proteolytic activity are completely abolished in cells from KO mice (Fig. S1 B and C). The overall cellular AAF-AMC-degrading activity is reduced by 80% in KO splenocytes or fibroblasts.

Young mice with a ubiquitous TPPII deletion are viable and grossly indistinguishable from WT littermates. Over 1 year of age KO mice show elevated mortality (Fig. S1D) associated with aged appearance, disheveled fur, large bald areas, reduced body mass, and loss of vigor.

Enhanced Apoptosis of Immature Thymocytes and Accelerated Thymic Involution in TPPII KO Mice. A tightly regulated balance among cell proliferation, survival, and cell death is fundamental to normal thymocyte development. In addition, the thymus is known to involution with age. The DN1, DN2, DN3, and DN4 stages of early CD4−CD8− double-negative (DN) thymocytes are followed by the CD4+CD8+ double-positive (DP) and the CD4+CD8− or CD4−CD8+ single-positive (SP) stages. Maturation involves two waves of proliferative expansion [in DN2 (in newborn mice in DN1/DN2) and DN3/DN4] and two waves of apoptosis (in DN4 and DP) (14, 15).

Whereas young TPPII KO mice had normal thymic cellularity, elderly (>1 year) mice showed a more pronounced thymic...
involution compared with WT littermates (Fig. L4). Thymic subpopulations showed a slight decrease in DP and slight increases in SP and DN cells of KO mice, respectively (Fig. 1B). Similar slight alterations in thymocyte subpopulations are found in very old WT mice and in strains with rapid thymic involution, in which increased apoptosis of DN thymocytes has been reported as well (16–18).

Cell cycle analyses (sub-G1 fraction) and annexin V labeling consistently revealed increased apoptosis of TPPII-deficient DN thymocytes directly ex vivo. This was seen for all DN subpopulations (Fig. S2) and animals of different ages (Fig. 1C and D and Fig. S2). Importantly, the KO/WT difference in DN apoptosis was much stronger in elderly than in young adult mice (Fig. 1D). The normal total thymocyte numbers in young KO mice are likely due to a compensation of the small apoptotic difference by slightly increased DN cell proliferation. In vivo BrdU incorporation into KO DN thymocytes was slightly increased (Fig. 1E), although the difference did not reach high statistical significance. This is consistent with a slightly enhanced proportion of blastoid cells in total KO thymocytes, both newborn and aged (Fig. S3). The faster proliferation may be associated with altered cell cycle kinetics, as indicated by a relative abbreviation of the S/G2/M phases in DN cells of KO mice (Fig. 1C and Fig. S3). The marked decrease in G2/M phase cells may reflect, in addition, enhanced mitotic apoptosis, consistent with a proposed role of TPPII in mitosis (9, 10).

Enhanced T Cell Receptor (TCR)-Mediated Apoptosis of Peripheral T Lymphocytes and Lymphopenia in TPPII KO Mice. Young KO adults already have reduced numbers of splenic T cells, for CD8+ cells more pronounced than for CD4+ cells (Fig. 2A). Peripheral T cells further decreased for CD8+ cells to ~50% of that of WT mice at around 1 year of age (Fig. 2A Upper). The proportions of memory CD8 T cells (CD44high) were increased in spleen and liver of most KO mice, indicating preferential decline in naive CD8+ cells (Fig. 2B).

Essentially all TPPII KO mice over 1 year of age displayed a strong lymphopenia. Absolute numbers of neutrophils in the blood were normal, with an increase in their percentage owing to the reduction of lymphocytes (Fig. 2C). The numbers of monocytes, platelets, and erythrocytes were normal as well (data not shown). The lymphopenia in blood was more pronounced than in lymphoid organs of KO mice.

The decrease in peripheral T lymphocytes could be caused by increased peripheral cell death. We therefore examined survival of splenic T cells upon TCR stimulation. When purified CD8+ and CD4+ cells were activated in anti-CD3-coated plates, cell recovery was reduced for CD8+ but not for CD4+ cells of KO mice (Fig. 3A). This was not caused by impaired T cell activation as judged by expression of the early activation marker CD69 on CD3+ cells (Fig. 3B). Carboxyfluorescein succinimidyl ester (CFSE) diming showed that the numbers of cell divisions were not significantly different between surviving KO and WT CD3+ cells (Fig. 3B). These data suggested that many KO CD8+ cells die and disintegrate upon proliferation. Increased apoptosis of activated KO CD8+ cells was confirmed by staining with annexin V (Fig. 3C). The serine protease inhibitor AAF-CMK inhibited proliferation and survival of anti-CD3-stimulated WT T lymphocytes (Fig. S4 Left), with the strongest inhibition at doses that maximally inhibit purified TPPII complexes in vitro (3).

Pointing at enhanced T cell apoptosis in vivo, freshly prepared KO splenic T cells contained approximately twice as many dead cells as WT controls (Fig. S4 Right), and CTL responses to lymphocytic choriomeningitis virus were reduced in KO mice (12). To assess T cell survival upon homeostatic proliferation in vivo, purified CFSE-labeled CD8+ and CD4+ T cells were inoculated into irradiated B6 recipient mice, and CFSE profiles as well as annexin exposure were determined several days later (Fig. 3D and E). Similar to anti-CD3-stimulated T cells subsets in vitro (Fig. 3A and B), fewer CD8+ cells, but similar numbers of CD4+ cells, were proliferating in the KO compared with the WT inoculum. Because the numbers of cell divisions and the proportions of undivided cells were similar between WT and KO T cells, the results suggest that proliferative survival of KO CD8+, but not CD4+, cells was impaired in vivo. The shift toward cells with more divisions among proliferating KO vs. WT CD8+ cells indicates a slightly faster proliferation, similar to thymocytes (see above).

Inflammation Associated with Extramedullary Granulopoiesis. Common signs of hematopoietic aging include increased proportions of blood myeloid cells, increased numbers of myeloid bone marrow (BM) progenitors, extramedullary hematopoiesis (19–

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Fig. 1. Increased DN thymocyte apoptosis and accelerated thymic atrophy in TPPII KO mice. (A) Total thymocyte numbers for young and elderly KO and WT mice (Left) and examples of thymi of 1-year-old littermates (Right). (B) Proportions of major thymocyte subsets determined by staining with anti-CD4 and anti-CD8. In A and B, n = 11 (for young) and n = 6 (for elderly) pairs of littermates, respectively. (C) Cell cycle distribution of DN thymocytes directly ex vivo. DNA staining was performed with propidium iodide. In elderly KO mice, an accurate assessment of S and G2/M phase cells could not be made. (D) Annexin V staining of DN thymocytes directly ex vivo. In C and D, n = 3–4 experiments with pooled thymocytes from two or three mice each. (E) In vivo BrdU incorporation into DN thymocytes 1 h after injection of BrdU into 3- to 5-month-old WT or KO mice (n = 3–4 mice per group).
22), and inflammation (23). All TPPII KO mice prematurely displayed some or all of these symptoms. Most pronounced were alterations typical of extramedullary hematopoiesis, including splenomegaly with enlargement of the red pulp (Fig. S5A) and elevated numbers of splenic granulocytes (Fig. S5 B and C). Whereas mature BM granulocytes were normal or moderately reduced (Fig. S5C), granulocyte progenitors in spleen and BM were increased in KO mice (Fig. S5D). Extramedullary hematopoiesis and increased myeloid BM progenitors may be attempts to compensate for inefficient hematopoiesis in the BM of TPPII-deficient mice.

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several days with the TPPII inhibitor AAF-CMK (50 μM) and the larger nuclei as visualized by DAPI staining. (C) Increased cell size of TPPII KO fibroblasts shown by forward scatter/ side scatter (FSC/SSC) analysis. (D) Binucleated KO fibroblasts observed by microscopy. (E) Higher percentage of cells with 4N DNA content. (F) Giant multinucleated KO fibroblasts. In all panels, representative experiments including different cell lines at low passage numbers (passage 4–10) are shown. Four different pairs of skin fibroblast lines (three from newborn mice and one from adult mice) were analyzed. (G) Premature senescence of CD8+ T cells. Although spontaneous and Fas-mediated apoptosis were similar between KO and WT fibroblasts (Fig. S7A), the sensitivity to TNF was enhanced in the former. Whereas WT fibroblasts are TNF-sensitive only when protein synthesis is inhibited, TPPII-deficient fibroblasts are inherently sensitive and the sensitivity is enhanced by cycloheximide (Fig. S7B). Dependency on blocked protein synthesis reflects that TNF-mediated apoptosis is normally counteracted by activation of NF-κB followed by the induction of antipapoptotic genes (31). Accordingly, NF-κB activation upon TNF addition was defective in TPPII-deficient fibroblasts, as seen by the greatly reduced nuclear accumulation of NF-κB p65 (Fig. 5A). In contrast, the antiproliferative tumor suppressor p53 was massively overexpressed (Fig. 5A). The increased TNF sensitivity of TPPII KO fibroblasts could be related to cellular senescence, in line with the up-regulation of p53 known to be an important senescence regulator (27, 28). p53 in KO cells upon TCR stimulation (Fig. 5B), but differences between WT and KO cells were not as pronounced as in fibroblasts. Whereas basal DNA-binding activity of NF-κB was increased in KO T cells, activation upon TCR signaling was attenuated (Fig. 5B). This is consistent with chronic inflammation (see above) and indicates that activation of NF-κB p65 is not generally defective but dysregulated in KO T cells upon TCR stimulation.

**Discussion**

We report here the generation and phenotypic characterization of mice lacking the giant protease TPPII and show that specific

26). Consistently, MHC class I and II cell-surface levels were elevated on freshly isolated TPPII KO splenocytes but rapidly decreased on in vitro cultured cells (12). Consistent with local inflammation are also histopathologically detected liver infiltrates of lymphocytes and mature granulocytes (data not shown) and increased NF-κB activity in freshly isolated splenocytes (see below).

**Premature Senescence of TPPII-Deficient Fibroblasts and CD8+ T Cells.**

Primary skin fibroblasts of newborn KO mice displayed reduced growth rates (Fig. 4A) and considerably increased cell size compared with WT controls (Fig. 4B, C, and F), associated with premature cellular senescence. Senescence is a state in which cells are irreversibly growth-arrested but remain alive and metabolically active for some time, secreting tissue-destructive enzymes and inflammatory cytokines. Premature senescence can be induced by various types of cellular stress, including DNA damage induced by oxygen radicals or aberrant mitogenic signaling, e.g., by activated oncogenes (26–28). At low passage numbers, 20–40% of TPPII KO but only 1–3% of WT fibroblasts showed signs of senescence, including an enlarged, flattened appearance of the cells and nuclei (Fig. 4B and F), a granular appearance, and accumulation of β-gal (Fig. 4B Top) (28). Similar to some reports on senescent phenotypes (29, 30), up to 10% of the KO fibroblasts (corresponding to 30–40% of the β-gal-positive cells) exhibited two nuclei (Fig. 4D). This indicates inability to complete mitosis, in line with the increased percentage of cells with 4N DNA content among TPPII-deficient fibroblasts (Fig. 4E). Giant multinucleated cells were also observed, albeit infrequently (Fig. 4F). A senescence-like phenotype was also seen upon treating primary WT fibroblasts for several days with the TPPII inhibitor AAF-CMK (50 μM) (Fig. S6). Interestingly, a considerable proportion of CD8+ T cells stained positive for acidic β-gal after a few days of stimulation on anti-CD3. Stimulated WT T cells did not stain positive (Fig. 4G).

**Deregulation of p53 and NF-κB in TPPII-Deficient Fibroblasts and T Cells.**

Although spontaneous and Fas-mediated apoptosis were similar between KO and WT fibroblasts (Fig. S7A), the sensitivity to TNF was enhanced in the former. Whereas WT fibroblasts are TNF-sensitive only when protein synthesis is inhibited, TPPII-deficient fibroblasts are inherently sensitive and the sensitivity is enhanced by cycloheximide (Fig. S7B). Dependency on blocked protein synthesis reflects that TNF-mediated apoptosis is normally counteracted by activation of NF-κB followed by the induction of antipapoptotic genes (31). Accordingly, NF-κB activation upon TNF addition was defective in TPPII-deficient fibroblasts, as seen by the greatly reduced nuclear accumulation of NF-κB p65 (Fig. 5A). In contrast, the antiproliferative tumor suppressor p53 was massively overexpressed (Fig. 5A). The increased TNF sensitivity of TPPII KO fibroblasts could be related to cellular senescence, in line with the up-regulation of p53 known to be an important senescence regulator (27, 28). p53 was induced in T cells after TCR stimulation (Fig. 5B), but differences between WT and KO cells were not as pronounced as in fibroblasts. Whereas basal DNA-binding activity of NF-κB was increased in KO T cells, activation upon TCR signaling was attenuated (Fig. 5B). This is consistent with chronic inflammation (see above) and indicates that activation of NF-κB p65 is not generally defective but dysregulated in KO T cells upon TCR stimulation.

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TPPII ablation is associated with cell-type-specific death programs such as aggravated T cell apoptosis and premature cellular senescence of fibroblasts and CD8^+ T cells. These defects likely contribute to the overall phenotype of the KO mice, which is dominated by the premature appearance of immunosenescence-like symptoms and higher age-related mortality. Apoptosis and senescence are both irreversible cell death programs, are important antitumor mechanisms, contribute to aging, and are affected by common signaling pathways (26–28). p53 is critical for control of both cell death programs. Under most circumstances, NF-κB has been shown to be antiapoptotic (31). TPPII deficiency is associated, as we show here, with alterations in p53 expression and NF-κB activation. The alterations in NF-κB activation, including decreased basal activity in fibroblasts, increased basal activity in other tissues (e.g., T cells), and attenuated activation by classical stimuli, have repeatedly been observed in association with cellular senescence and systemic aging (23, 32). However, we have so far no evidence for a direct molecular link between TPPII and p53 or NF-κB. Signal-induced degradation of the NF-κB inhibitor IκBα, which is mediated by proteasomes, was tested and was not found to be delayed in TPPII KO cells, either in TNF-treated fibroblasts or in anti-CD3-stimulated T cells (data not shown).

A common cause for the activation of apoptosis and senescence in TPPII-deficient cells may be mitotic errors. The reduction of TPPII-deficient thymocytes in the G2/M cell cycle phase together with increased apoptosis is consistent with problems in mitosis (Fig. S3). Similar to TPPII KO fibroblasts (see Fig. 4 D and F), incomplete mitoses with binucleated and multinucleated cells have been observed upon TPPII siRNA silencing in tumor cell lines (9, 10). Mitotic dysregulation with increased binucleation is also found in fibroblasts of old persons and progeria patients and seems to be causally related to systemic aging (33).

Similar findings have been reported for senescence induced by Ras or by lack of the stress kinase MKK7 (29, 34).

Several lines of evidence suggest a relationship among mitotic errors, apoptosis induced by p53, and TPPII. Mitotic errors often provoke p53 up-regulation thus activating the apoptotic machinery (35). In cell lines, TPPII overexpression conferred resistance to apoptosis induced by p53 (10). TCR stimulation in primary T cells causes p53 up-regulation (see Fig. S5B) as do the various stimuli causing cellular senescence (26–28). p53 is also up-regulated at the DN thymocyte stage (36). TPPII may thus be particularly important for survival of proliferating cells during conditions that lead to up-regulation of p53, and lack of TPPII expression may aggravate the antiproliferative responses to p53. The abnormalities of TPPII KO mice that coincide with symptoms of immunohematopoietic senescence in mice and humans are also found in part in mice lacking NF-κB pathway components (25) and mice with augmented p53 expression (37). These alterations include accelerated thymic atrophy associated with increased DN thymocyte apoptosis (16–18), T lymphopenia with particular reduction in naive CD8^+ cells as found in human aging (23, 25, 37, 38), and impaired mitogen-induced and homeostatic T cell proliferation due to increased T cell apoptosis (23, 25, 39). Furthermore, these symptoms include greater proportions of blood myeloid cells accompanied by more myeloid BM progenitors and compensatory extramedullary hematopoiesis as found in aging mice (19–22) and chronic inflammation associated with MHC up-regulation (23, 24).

Inflammation may be sustained by secretion of proinflammatory cytokines by senescent cells (26, 28) or by extramedullary granulopoiesis (25) and may accelerate lymphoid degeneration in TPPII-deficient mice. Similarly, many degenerative processes during normal systemic aging are associated with chronic inflammation (23).

Because previous studies have suggested that TPPII is up-regulated in many tumors and therefore likely important for tumor growth, TPPII has been considered as a target for tumor therapy. This study shows that TPPII is required for normal cellular and organismic physiology, although TPPII KO mice are viable. The value of putative TPPII inhibitory drugs in tumor therapies will thus depend on the nature and severity of the predictable systemic side effects.

Materials and Methods

Generation of TPPII KO Mice. Using genomic clones containing exons 11–15 of the TPPII gene, we constructed a targeting vector for homologous recombination in W4 embryonic stem cells and used it for homologous recombination in W4 embryonic stem cells to generate mutant mice (see SI Materials and Methods for a detailed description).

Fibroblast Lines, β-galactosidase Assays, and DAPI Staining. Fibroblast lines were generated from the skin of newborn mice. β-gal activity was detected with the senescence β-gal staining kit (Cell Signaling), and nuclear DNA was visualized by adding DAPI (3 μg/ml) after permeabilization with 0.1% Triton X-100.

Cell Proliferation. Cell proliferation was measured by counting viable cells after staining with trypan blue or flow cytometrically after labeling with CFSE (Fluka) at 1 μM concentration or after in vivo BrdU labeling (see SI Materials and Methods for details).

Cell Cycle Analysis. DNA content was monitored flow cytometrically by using propidium iodide or 7-aminoactinomycin D staining.

Mitogenic Stimulation of Splenic T Cells. Total or CD8^+ splenic T cells were isolated with the Pan T Cell Isolation or the CD8 Negative Selection Kit (Miltenyi Biotec). A total of 1 × 10^6 cells were cultured per well in 24-well plates coated with anti-CD3 mAb (145-ZC11; 1 μg/ml) in the presence of IL-2 (25 units/ml), eBioscience.

Apoptosis Assays. Apoptotic cells were determined flow cytometrically after staining with FITC-labeled annexin V (Miltenyi Biotec) (see SI Materials and Methods for details).
Multicolor Flow Cytometry. For the Abs used, see SI Materials and Methods. Analysis was carried out by using a CYTOMICS FC500 flow cytometer (Beckman Coulter).

NF-κB DNA-Binding Assay. Nuclear extracts from fibroblasts or splenic T cells were analyzed with an NF-κB Family Transcription Factor Assay Kit (Chemicon) (for details see SI Materials and Methods).

Western Blotting. Cells and isolated nuclei were lysed in RIPA buffer or nuclear extraction buffer, respectively, and proteins were quantified by using a BCA assay (Pierce). For the primary Abs used, see SI Materials and Methods. Proteins were detected by ECL (Amersham).

Methylcellulose Colony Assay. The colony assay of single-cell suspensions of BM or spleen was done with MethoCult M3534 (Stem Cell Technologies) with the Proteins were detected by ECL (Amersham).

Peripheral Blood Counts. Complete hematological profiles were obtained from tail vein blood of age-matched mice by using an automated complete blood cell counter.

Histology. Organs were fixed, paraffin-embedded, and then stained with hematoxylin/eosin.

Statistical Analysis. All data are presented as mean ± SD and analyzed by a two-tailed Student t test with unequal variance. P < 0.05 was considered significant.

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