

Effects of purine nucleoside phosphorylase deficiency on thymocyte development

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Background: Inherited or acquired defects in purine nucleoside phosphorylase (PNP) impair purine metabolism, as well as the survival and function of T lymphocytes. However, the effects of PNP deficiency on thymocyte development are not well known. **Objectives:** We sought to study thymocyte development in PNP-deficient (PNP-KO) mice.

Methods: Maturation, proliferation, and apoptosis were determined in thymocytes from PNP-KO mice and hematopoietic stem cells from these mice grown *ex vivo* into thymocyte-like cells.

Results: Reduced percentages of CD4⁺CD8⁺ double-positive (DP) thymocytes with normal percentages of CD4⁺CD8⁺ and CD4⁺CD8⁻ single-positive thymocytes were found in the thymi of PNP-KO mice. Similarly, reduced DP-like thymocytes grew *ex vivo* from hematopoietic stem cells of PNP-KO mice. Thymi of PNP-KO mice contained increased apoptotic DP thymocytes. Increased apoptosis of PNP-deficient DP thymocytes occurred after exposure to deoxyguanosine (dGuo), although not after Fas ligation, and could be prevented by restoring PNP activity within the cells. In DP thymocytes from PNP-KO mice, dGuo caused mitochondrial membrane potential dissipation and induced release of cytochrome c from the mitochondria followed by nuclear DNA fragmentation. Inhibition of the caspase pathway prevented dGuo-induced nuclear DNA fragmentation but not mitochondrial membrane potential dissipation, indicating that PNP deficiency induces apoptosis that is initiated in the mitochondria of DP thymocytes. 5-Bromo-2-deoxyuridine incorporation demonstrated that PNP deficiency does not interfere with DP or single-positive thymocyte proliferation.

Conclusions: PNP is important for the survival of DP thymocytes. Accumulation of dGuo in cases of PNP deficiency leads to mitochondria-initiated apoptosis of DP thymocytes, which can be prevented by restoring PNP activity in the cells. (J Allergy Clin Immunol 2011;■■■■:■■■-■■■.)

Key words: Purine nucleoside phosphorylase, thymocytes, apoptosis, proliferation, mice, immune deficiency

Purine nucleoside phosphorylase (PNP) is an intracellular enzyme responsible for the phosphorolysis of deoxyguanosine (dGuo), which can be further metabolized to uric acid or salvaged into the nucleotide pools (Fig 1, A). Inherited defects in PNP function cause accumulation of dGuo in the cells, which can be phosphorylated to deoxyguanosine monophosphate (dGMP), deoxyguanosine diphosphate, and deoxyguanosine triphosphate (dGTP) by the mitochondrial deoxyguanosine kinase (dGK) enzyme.¹ Excess dGTP is particularly toxic to lymphocytes, and therefore synthetic PNP inhibitors, such as forodesine, have been developed that affect lymphocyte survival and function.² Studying peripheral blood lymphocytes from patients with chronic lymphocytic leukemia treated with forodesine suggested that PNP has a role in preventing mitochondria-induced apoptosis.² Also, analysis of thymocytes lacking adenosine deaminase (ADA), another purine enzyme important for lymphocyte development and function, has indicated that abnormal purine homeostasis can cause increased mitochondria-induced apoptosis.³ Understanding the functions of PNP in thymocytes is particularly important for the management of PNP-deficient patients, as well as for the many patients with malignancy and immune dysregulation conditions who are treated with synthetic PNP inhibitors.⁴ However, the effects of PNP deficiency on thymocytes have not been thoroughly investigated.

Analysis of PNP-deficient thymocytes have been hampered by the limited availability of thymi from PNP-deficient patients and the confounding effects that prolonged infections, malnutrition, or immunomodulating agents might have on thymi obtained from autopsies. To better appreciate the functions of PNP, we previously knocked out the catalytic domain of PNP in mice and showed that PNP-deficient (PNP-KO) mice recapitulated many of the metabolic and immune abnormalities seen in PNP-deficient patients.^{5,6} The urine and sera of PNP-KO mice contained increased dGuo concentrations, whereas thymocytes had increased dGTP concentrations, particularly in the mitochondria. PNP-KO mice exhibited a profound decrease in the number and function of peripheral T lymphocytes, as well as marked perturbations in thymocytes. In addition, we showed that dGuo could induce apoptosis in thymocytes from normal (PNP-proficient) mice treated

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Abbreviations used

ADA:	Adenosine deaminase
BrDU:	5-Bromo-2-deoxyuridine
dGK:	Deoxyguanosine kinase
dGMP:	Deoxyguanosine monophosphate
dGTP:	Deoxyguanosine triphosphate
dGuo:	Deoxyguanosine
DN:	Double negative
DP:	Double positive
FACS:	Fluorescence-activated cell sorting
HSC:	Hematopoietic stem cell
MMP:	Mitochondrial membrane potential
OP9-DL1:	OP9 bone marrow stromal cells expressing the delta-like ligand 1
PI:	Propidium iodide
PNP:	Purine nucleoside phosphorylase
PNP-KO:	PNP deficient
SP:	Single positive
TAT-PNP:	Purine nucleoside phosphorylase fused to TAT protein transduction domain
TCR:	T-cell receptor

ex vivo with synthetic PNP inhibitor and that the apoptosis was initiated in the mitochondria.⁵ However, these studies were not performed in PNP-deficient thymocytes, and induction of apoptosis in these cells required excessive dGuo concentrations; therefore the relevance of our findings to thymocyte development in PNP deficiency was questioned.

Thymocyte development in mice is a well-defined process. CD4[−]CD8[−] double-negative (DN) cells reside in the thymus cortex and constitute 1% to 3% of the thymocytes in normal adult mice. As DN thymocytes transit through the cortex toward the medulla, they rearrange their V(D)J genes and eventually express a diverse T-cell receptor (TCR) repertoire. Many molecules influence maturation of DN cells, including Bim, which has been found to have an important role in physiological thymopoiesis. Indeed, in the absence of Bim, thymocyte maturation and proliferation are severely disrupted.⁷ TCR expression by thymocytes is followed by thymocyte proliferation and upregulation of CD4 and CD8 expression on thymocytes.⁸ Cells that express both CD4 and CD8, and therefore are considered double-positive (DP) cells, account for more than 80% of all thymocytes and are primarily detected at the corticomedullary junction of the thymus. Few DP thymocytes, which are positively selected and do not interact at high avidity with the thymic stroma, mature to become single-positive (SP) cells expressing either CD4⁺ or CD8⁺, which are found in the medulla. Most DP thymocytes fail selection and undergo apoptosis, which can be mediated by several mechanisms.⁹ The extrinsic apoptotic pathway often involves upregulation of Fas receptor expression and stimulation through the Fas receptor, followed by activation of the caspase pathway and cleavage of proapoptotic molecules. The intrinsic apoptotic pathway can be triggered by nuclear DNA damage,¹⁰ as well as induction of the proapoptotic molecule Bim, as recently shown in lymphocytes treated with the PNP inhibitor forodesine.² The intrinsic pathway might also be activated by downregulation of anti-apoptotic molecules, such as Bcl2, followed by disruption of mitochondrial membrane potential (MMP) and mitochondrial damage, as shown in thymocytes from ADA-deficient mice¹¹ and in cells treated with forodesine.² Additionally, we have shown

in normal thymocytes that accumulation of purine metabolites can bypass the extrinsic and nuclear apoptotic pathways, directly damaging the mitochondria and leading to dissipation of MMP, release of cytochrome c from the mitochondria to the cytosol, and cell death.⁵

Recently, OP9 bone marrow stromal cells expressing the delta-like ligand 1 (OP9-DL1 cells) were found to induce differentiation of hematopoietic stem cells (HSCs) into thymocyte-like cells *ex vivo*.¹² Avoidance of confounding factors that affect thymocytes *in vivo* and the synchronized production of large quantities of cells at similar maturation stages by the OP9-DL1 system enabled identification and analysis of several molecules and pathways important for thymocyte development.^{13–15}

To better understand the effects of PNP deficiency on thymocytes, we took advantage of the accessibility to thymic tissue from many PNP-KO mice, as well as the ability to generate *ex vivo* thymocyte-like cells from HSCs of PNP-KO mice. We show that accumulation of dGuo preferentially damages the mitochondria of DP thymocytes, resulting in cell apoptosis. Moreover, restoring PNP in PNP-deficient thymocytes can avert the dGuo-induced apoptosis.

METHODS**Mice**

Six-week-old PNP-KO (C57BL/6) mice and normal littermates maintained in a pathogen-free environment at the animal facility of the Hospital for Sick Children, Toronto, Canada, were used for all experiments. PNP deficiency was determined based on the results of Southern blot analysis and the absence of PNP enzyme activity in tail blood samples.⁵ The institution's Animal Care Committee approved all procedures in accordance to the Canadian Council of Animal Care Guidelines.

PNP activity and PNP fused to TAT protein transduction domain

PNP activity was measured and defined as previously described.^{5,16} The recombinant protein of the PNP fused to TAT protein transduction domain (TAT-PNP) was produced as previously described.¹⁶

Fluorescence-activated cell sorting analysis

Single-cell suspensions were stained with the appropriate antibodies or isotype-matched controls, as detailed in the **Methods** section in this article's Online Repository at www.jacionline.org.

DP and SP thymocyte isolation

Isolation of thymocytes was performed by means of magnetic depletion on MACS columns (Miltenyi Biotec, Auburn, Calif), as detailed in the **Methods** section in this article's Online Repository.

OP9-DL1 cultures

Lineage-depleted HSCs were isolated from freshly collected bone marrow by means of removal of cells expressing CD5, CD45R (B220), CD11b, GR1, 7-4, and Ter-119 with the Lineage Cell Depletion Kit (Miltenyi Biotec). Lineage-depleted HSCs (10⁵ cells/well) were added to near-confluent OP9-DL1 cells (kindly provided by J. C. Zúñiga-Pflücker, Toronto, Ontario, Canada). HSCs were maintained in α MEM (Invitrogen, Burlington, Ontario), 20% characterized heat-inactivated FBS (Thermo-Scientific, Nepean, Ontario), 5 ng/mL fms-like tyrosine kinase-3 ligand, and 1 ng/mL IL-7 (both from R&D Systems, Minneapolis, Minn), as previously described.¹² Nonadhesive cells were transferred every 3 to 4 days to

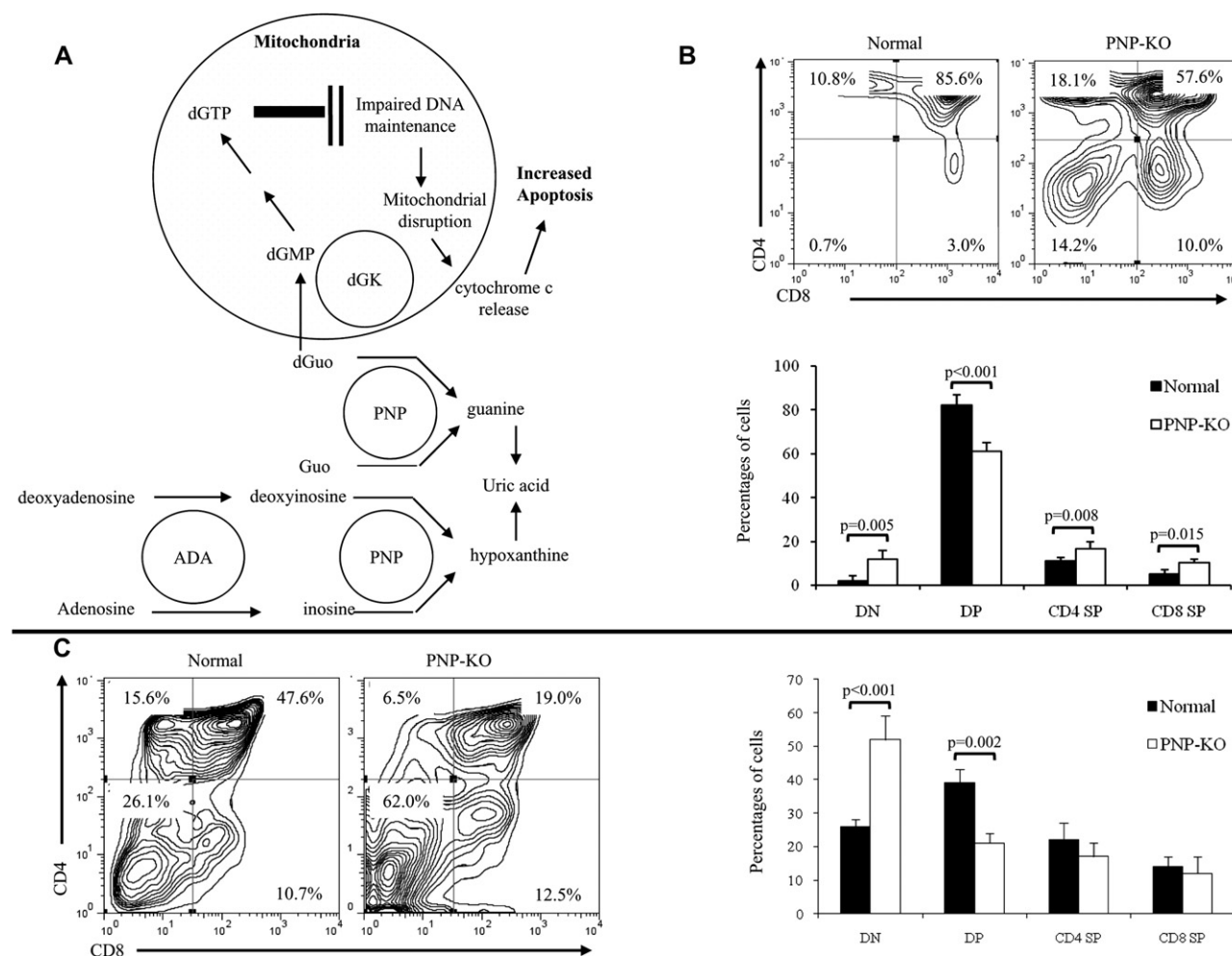


FIG 1. Effects of PNP deficiency on thymocytes. **A**, In the absence of PNP, dGuo can only be phosphorylated by the mitochondrial dGK to dGMP and subsequently to dGTP. Excess dGTP might impair mitochondrial DNA maintenance and disrupt the mitochondria, leading to cytochrome c release into the cytosol and apoptosis. **B**, Representative contour plots depicting the percentages of CD4-expressing thymocytes, CD8-expressing thymocytes, or both. Pooled data from 15 mice of each genotype are summarized in the bar graph (means $\pm$ SDs). **C**, Representative contour plots depicting the percentages of CD4-expressing thymocyte-like cells, CD8-expressing thymocyte-like cells, or both after 21 days of *ex vivo* differentiation on OP9-DL1 cells. Pooled data from 4 independent experiments are summarized in the bar graph (means $\pm$ SDs).

fresh OP9-DL1 cells. PNP activity was irreversibly inhibited in OP9-DL1 cells by means of treatment with 20 μ M forodesine (kindly provided by BioCryst Pharma, Durham, NC), followed by washes, before adding HSCs. dGuo (50 μ M/L; Sigma-Aldrich, Oakville, Ontario, Canada) was added to the cultures to simulate accumulation of dGuo in PNP-deficient patients and murine serum. Preliminary studies showed that neither forodesine nor dGuo affected the viability of OP9-DL cells or expression of delta-like ligand 1. Thymocyte-like cells were analyzed after removal of the large green fluorescent protein-expressing OP9-DL1 cells by using a 40- μ m filter, which was confirmed by the lack of fluorescence in unstained thymocyte-like cells.

Apoptosis

Thymocyte apoptosis was analyzed by means of fluorescence-activated cell sorting (FACS) with the Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, Calif), by means of dissipation of MMP with 25 nmol/L MitoTracker Red CMXRos (Invitrogen), or by means of nuclear DNA fragmentation with a fluorescein isothiocyanate-conjugated dUTP kit (Roche, Laval, Quebec, Canada). Apoptosis was further determined *in situ* by using

electron microscopy, as detailed in the [Methods](#) section in this article's Online Repository. Apoptosis was also evaluated in cultured thymocytes (10^7 cells/mL in 6-well plates) maintained in RPMI 1640 and 5% FBS at 37°C. Apoptosis was induced by 2' dGuo or 3.75 μ M/L dexamethasone (Sigma-Aldrich) or 5 μ g/mL anti-Fas Jo2 (BD Biosciences, Mississauga, Ontario, Canada). In some experiments zVADfmk (Santa Cruz Biotechnology, Santa Cruz, Calif) was added to the cultures 30 minutes before induction of apoptosis. In other experiments 10 U/mL TAT-PNP was added at the initiation of the culture. Bim expression was determined by means of Western blotting after lysis of 30×10^6 fresh thymocytes with rabbit anti-mouse antibodies to Bim (Cell Signaling Technology, Danvers, Mass) and peroxidase-conjugated anti-Rabbit antibody (Jackson laboratories, West Grove, Pa). Antibodies were detected by means of chemiluminescence (GE LifeScience, Baie d'Urfe, Quebec, Canada).

Cytochrome c release from the mitochondria

Cytochrome c was detected in the mitochondria and cytosol by means of Western Blotting, as detailed in the [Methods](#) section in this article's Online Repository and as previously described.¹⁷

Cell proliferation

Thymocyte proliferation was measured based on 5-bromo-2-deoxyuridine (BrdU) incorporation into thymocytes after 2 intraperitoneal injections of 1 mg of BrdU 4 hours apart. BrdU-incorporating cells were detected by means of FACS analysis with the FACS BrdU Flow Kit (BD Biosciences) and analyzed with allophycocyanin- or phycoerythrin-conjugated antibodies to CD8 and CD4, respectively, as described above. Proliferation of thymocyte-like cells grown on OP9-DL1 cells was measured 48 hours after adding BrdU (10 μ mol/L final concentration) to the cultures.

Statistical analysis

Values are expressed as means $\pm$ SDs. The statistical significance of the differences between the 2 groups was determined by using the 2-tailed Student *t* test, whereas the differences among 3 or more groups were determined by means of ANOVA with Tukey *post hoc* comparison adjustment for multiple comparisons. For data measured at multiple time points, repeated-measures ANOVA was used with multiple testing adjustments. A *P* value of less than .05 was considered statistically significant.

RESULTS

Effects of PNP deficiency on thymocytes

To investigate the effects of PNP deficiency on thymocytes, we used the PNP-KO murine model. Similar to PNP-deficient patients,¹⁸ the thymi of PNP-KO mice were small (0.062 ± 0.023 g compared with 0.085 ± 0.021 g in normal littermates, $P < .001$, $n = 50$ in each group). The thymi of 6- to 8-week-old PNP-KO mice also contained only $51.4 \pm 14.1 \times 10^6$ thymocytes compared with $169.9 \pm 32.6 \times 10^6$ thymocytes in normal control animals ($P < .001$, $n = 15$ in each group). Compared with normal littermates, the percentages of DP thymocytes in the thymi of PNP-KO mice were significantly reduced, whereas the percentages of DN and SP thymocytes were significantly increased (Fig 1, B). Similarly, the percentages of DP-like cells in cultures from PNP-KO mice were significantly lower compared with those seen in cultures from normal littermates (Fig 1, C).

PNP deficiency causes increased apoptosis of DP thymocytes

We previously hypothesized that PNP has an important role in survival of DP thymocytes.⁵ Quantitative assessment of apoptosis in thymocytes was performed by means of FACS analysis of Annexin V expression among propidium iodide (PI)-excluding cells (Fig 2, A). Annexin V was expressed by $3.7\% \pm 0.9\%$ of DP thymocytes from PNP-KO mice compared with only $1.5\% \pm 0.3\%$ of DP thymocytes from normal littermate mice ($P < .001$). In contrast, Annexin V was expressed by less than 1% of DN and SP cells from PNP-KO mice, which was not different than the expression seen in normal littermate mice (data not shown). Additionally, electron microscopy demonstrated that the corticomedullary junction of the thymus from PNP-KO mice contained cells at various stages of apoptosis (Fig 2, B). These included compaction of nuclear chromatin, margination of the nuclear chromatin to the periphery of the cell, and breakup of the chromatin into discrete fragments.

dGuo induces apoptosis of PNP-deficient DP thymocytes

PNP is required for phosphorolysis of dGuo, and the absence of PNP causes accumulation of dGuo and dGuo phosphorylation

products¹⁹; hence we suspected that accumulation of these purine metabolites in cases of PNP deficiency results in thymocytes apoptosis. We show here that the percentage of DP thymocytes from PNP-KO mice excluding PI and expressing Annexin V increased after incubation with dGuo (Fig 3, A). Moreover, a dose-dependent increase in the percentage of PI-negative, Annexin V-positive cells was observed after culture of PNP-KO DP thymocytes with 6.25 and 12.5 μ mol/L dGuo (Fig 3, B), concentrations similar to those found in the sera of PNP-deficient patients and mice.^{5,18} In contrast, dGuo did not significantly increase Annexin V expression by PI-negative SP thymocytes from PNP-KO mice or normal littermates analyzed separately or in combination (CD4⁺ and CD8⁺ cells). Additional confirmation of the effect of PNP and dGuo accumulation on DP thymocyte survival was provided by measuring MMP dissipation with the MitoTracker Red CMXRos, a fluorochrome that is lost from the mitochondria of apoptotic cells. As demonstrated in Fig 3, C, although dGuo treatment had practically no effect on MMP dissipation in DP thymocytes from normal mice, MMP dissipation increased significantly in DP cells from PNP-KO mice (from $6.7\% \pm 3.6\%$ to $20.1\% \pm 3.3\%$, $P = .004$). DP thymocytes from PNP-KO mice were also treated with the TAT-PNP recombinant protein, which enters PNP-deficient cells, restores phosphorolysis of dGuo to guanine, and prevents accumulation of dGTP.²⁰ Correcting PNP activity in PNP-KO DP thymocytes with TAT-PNP protected the cells from dGuo-induced Annexin V expression, confirming PNP's role in preventing apoptosis of DP thymocytes (Fig 3, D).

dGuo induces apoptosis in the mitochondria of PNP-deficient thymocytes

PNP is a cytoplasmic enzyme; however, phosphorylation of dGuo to dGMP and subsequently to dGTP is dependent on dGK, which is a mitochondrial enzyme. On the basis of experiments in normal (PNP-proficient) thymocytes treated with a PNP inhibitor, we had previously hypothesized that PNP deficiency induces apoptosis in the mitochondria of thymocytes. Here we confirm this hypothesis using thymocytes from PNP-KO mice. When the apoptotic signal is initiated within the mitochondria, MMP dissipation occurs before nuclear DNA fragmentation. Therefore we compared the temporal relationship of MMP dissipation and nuclear DNA fragmentation, as measured by means of FACS analysis with the MitoTracker Red CMXRos and terminal deoxynucleotidyl transferase-mediated UTP nick end labeling assays, respectively. As expected, 6 hours after dGuo-induced apoptosis in thymocytes from PNP-KO mice, less than 5% of cells showed MMP dissipation or fragmented nuclear DNA. In contrast, by 12 hours, a significantly higher percentage of cells had disrupted MMP, whereas less than 5% of the cells had fragmented DNA (Fig 4, A). By 18 hours, when apoptotic signals had already extended from the mitochondria to the cytoplasm, the percentage of cells with MMP dissipation was similar to the percentage of DNA-fragmented cells. Additionally, Western blotting demonstrated that parallel to dGuo-induced MMP dissipation in PNP-KO cells, cytochrome *c* expression was reduced in the mitochondria and increased in the cytosol, suggesting cytochrome *c* release from the mitochondria (Fig 4, B). The effect of dGuo on MMP dissipation and DNA fragmentation was similar, although not as pronounced as that induced by dexamethasone,

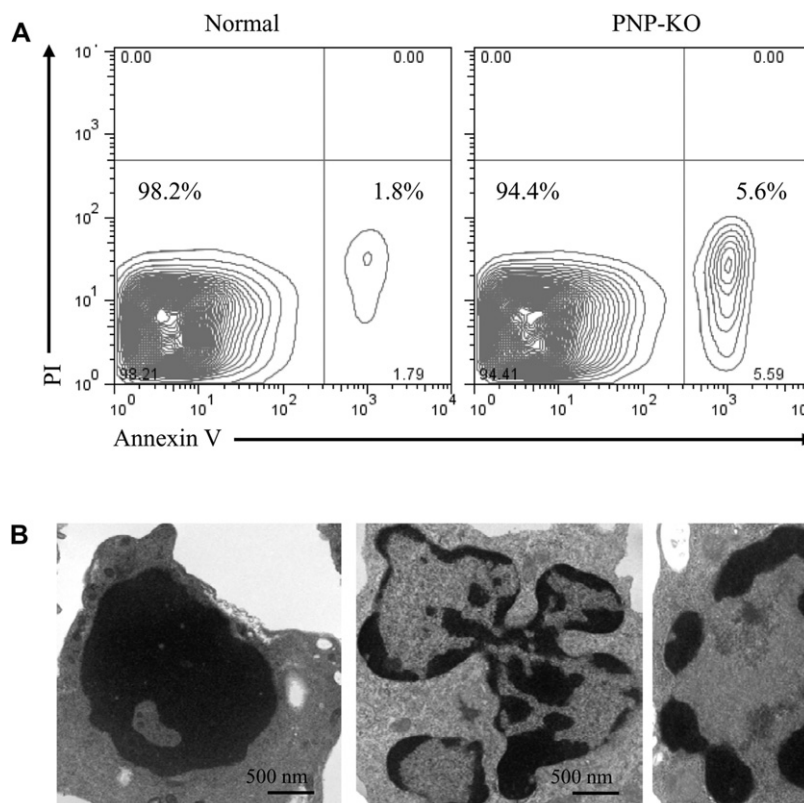


FIG 2. PNP deficiency causes increased apoptosis of DP thymocytes. **A**, Contour plots depicting the percentages of cells excluding PI and expressing Annexin V among DP thymocytes, as determined by means of FACS analysis. Results are representative of 10 mice per genotype in 4 independent experiments. **B**, Transmission electron microscopic view demonstrating cells at various apoptotic stages in the corticomedullary junction of the thymus of a PNP-KO mouse. These include compaction of nuclear chromatin into a sharply circumscribed uniform electron-dense structure (*left*), margination of the nuclear chromatin into the cell's periphery (*middle*), and chromatin breakups into discrete fragments (*right*). Results are representative of 3 mice from 2 independent experiments.

which was used as a positive control. Moreover, the pancaspase inhibitor zVADfmk, which was added to thymocytes from PNP-KO mice before dGuo-induced apoptosis, decreased nuclear DNA fragmentation from 72% to 24% without significantly reducing MMP dissipation (Fig 4, C), further indicating that PNP's primary role in these cells is to prevent mitochondria-initiated apoptosis. Other apoptotic pathways were not affected by PNP. FACS analysis showed that the percentage of cells expressing Fas receptor, which is often increased when the extrinsic apoptotic pathway is involved, were similar in thymocytes of PNP-KO mice and normal littermates (data not shown). Moreover, apoptosis after overnight Fas ligation among DP thymocytes from PNP-KO mice was not significantly different than that of normal littermates (Fig 4, D). Likewise, expression of the nuclear proapoptotic molecule Bim, as measured by means of Western blotting in DP thymocytes from PNP-KO mice and normal littermates, was similar (data not shown).

PNP deficiency does not affect proliferation or TCR diversity of thymocytes

Proliferation of peripheral T lymphocytes in response to various stimulations is impaired in cases of PNP deficiency.⁵ Therefore we studied PNP-deficient thymocyte proliferation

in vivo by BrdU "pulse" (Fig 5, A). The percentage of DP thymocytes incorporating BrdU 1 day after BrdU injection was similar among PNP-KO mice and normal littermates, suggesting that proliferation of DP thymocytes was not affected by PNP deficiency. Also, as expected after BrdU "pulse,"²¹ the percentage of DP cells incorporating BrdU in normal mice decreased at 5 days after BrdU injection, reflecting apoptosis of DP thymocytes and maturation of DP cells into SP thymocytes. The percentages of CD4⁺ and CD8⁺ SP cells among BrdU-incorporating thymocytes 5 days after BrdU injection were not significantly different between PNP-KO mice and normal littermates, suggesting that proliferation of SP thymocytes is also not dependent on PNP (Fig 5, B). In contrast, 5 days after BrdU injections, the percentage of DP thymocytes incorporating BrdU was significantly reduced in PNP-KO mice compared with that seen in normal littermates, further supporting our initial findings of accelerated apoptosis in PNP-deficient DP thymocytes. We also assessed proliferation of DP-like cells grown *ex vivo* from HSCs by using the OP9-DL1 culture system. Similar to the *in vivo* results, 2 days after pulsing the cultures with BrdU, 7.4% ± 3.2% of DP-like cells generated from HSCs of PNP-KO mice incorporated BrdU, which was not significantly different than the 5.0% ± 3.8% of BrdU-incorporating cells generated from HSCs of normal mice (Fig 5, C). Altered

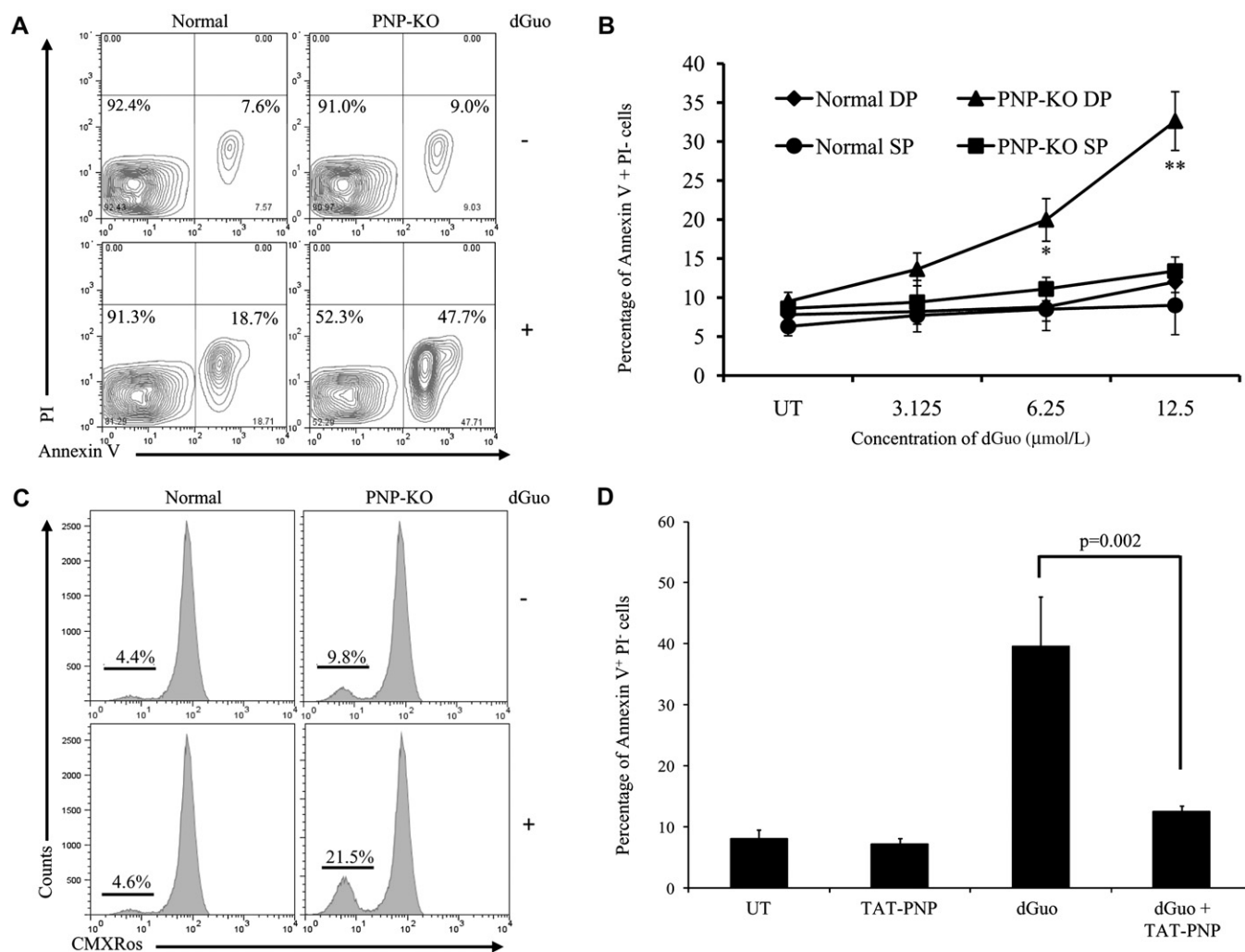


FIG 3. dGuo induces apoptosis of PNP-deficient DP thymocytes. **A**, Contour plots depicting the percentages of cells excluding PI and expressing Annexin V after overnight culture of DP thymocytes without (–) or with (+) 12.5 μmol/L dGuo. **B**, Bar graph depicting the percentages (means ± SDs) of PI-excluding and Annexin V–expressing DP, as well as CD4⁺ and CD8⁺ SP, thymocytes after overnight culture with increasing dGuo concentrations. UT, Without dGuo. * $P = .008$ and ** $P < .001$ between DP thymocytes from normal and PNP-KO mice. **C**, Histograms depicting the percentages of DP thymocytes not accumulating CMXRos (having disrupted MMP) after overnight culture with 12.5 μmol/L dGuo. **D**, Bar graph depicting the percentages (means ± SDs) of DP thymocytes from PNP-KO mice excluding PI and expressing Annexin V after overnight culture without (UT) or with 12.5 μmol/L dGuo. TAT-PNP (10 U/mL) was added to some of the cultures. All FACS analyses are representative of at least 4 independent experiments.

availability of purine bases might impair V(D)J recombination and limit TCRβ chain expression and diversity.²² Therefore we measured TCRβ expression by CD3⁺ thymocytes. FACS analysis demonstrated similar frequencies of various TCRVβ families among thymocytes from PNP-KO mice and normal littermates (Fig 5, D), suggesting that PNP deficiency does not restrict V(D)J rearrangement in thymocytes.

DISCUSSION

Studying the effect of PNP deficiency on thymocytes has been previously hindered by the limited availability of thymic samples. Here we overcame this obstacle by taking advantage of our access to thymocytes from PNP-KO mice and our ability to generate PNP-deficient thymocyte-like cells from the

bone marrow of these mice. A striking finding is the effect of PNP deficiency on DP thymocytes. DP thymocytes and DP-like cells in the thymus and in *ex vivo* cultures of PNP-KO mice, respectively, were markedly reduced. Maintaining a large enough DP population from which thymocytes expressing properly rearranged TCRs with appropriate affinity can be selected is critical for the generation of diverse functional T lymphocytes. Hence the decrease in thymocyte numbers at the DP stage has a significant biological effect on the development of lymphocytes.

The availability of large numbers of DP thymocytes from PNP-KO mice also enabled us to explore mechanisms directly disrupted by PNP deficiency. Absence of PNP *in vivo* and *ex vivo* caused increased apoptosis of DP thymocytes, which is similar to the apoptosis observed after PNP inhibition in

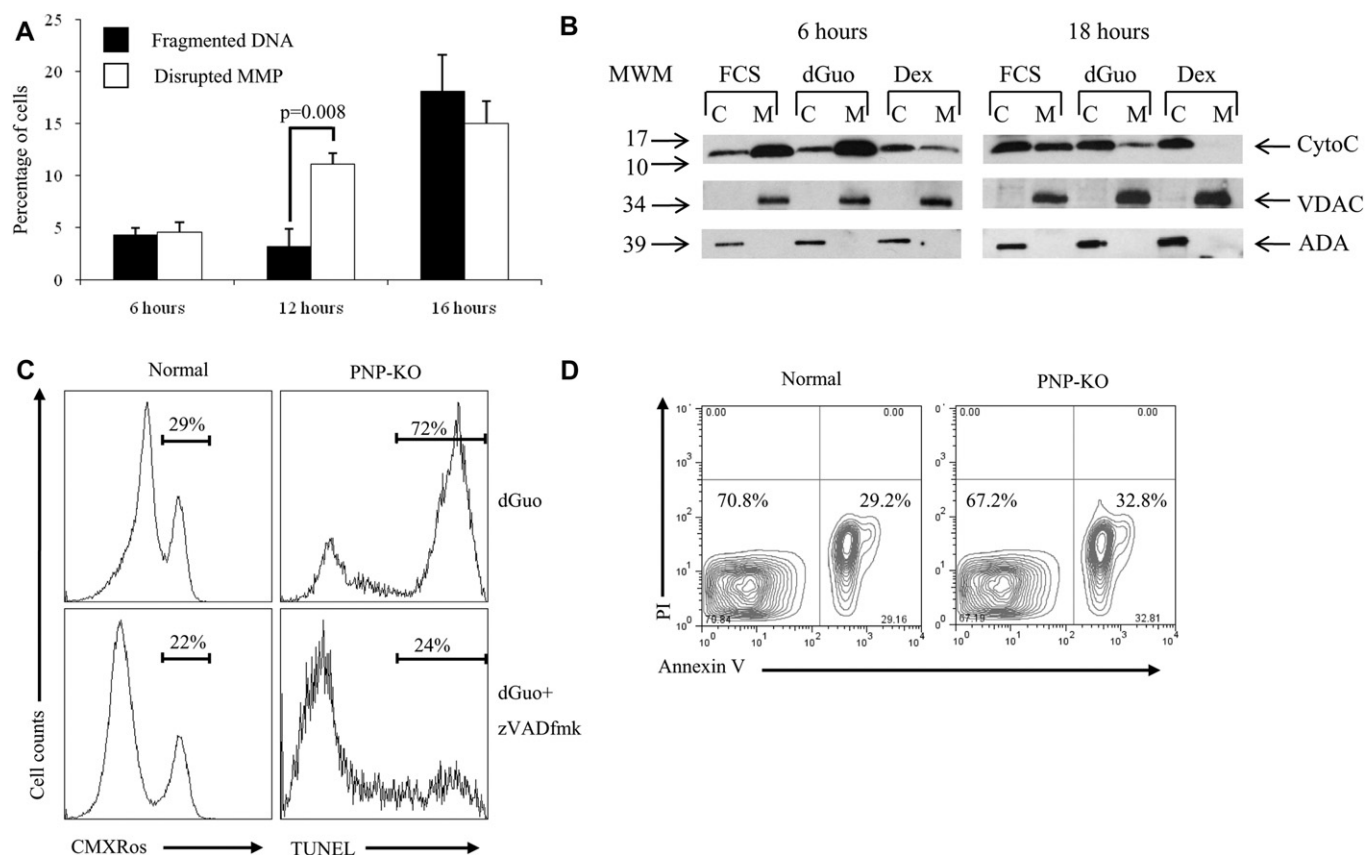


FIG 4. dGuo induces apoptosis in the mitochondria of PNP-deficient thymocytes. **A**, Bar graph depicting the percentages (means $\pm$ SDs) of DP thymocytes that have fragmented DNA (terminal deoxynucleotidyl transferase-mediated UTP nick end labeling positive) or disrupted MMP and hence not accumulating Mito-Tracker Red CMXRos after overnight treatment with 12.5 μ M dGuo, as determined by means of FACS analysis. Results represent pooled data from 4 independent experiments. **B**, Western blotting of cytochrome c (CytoC) in the mitochondria (M) and cytosol (C) of DP thymocytes from PNP-KO mice after treatment with 12.5 μ M dGuo or dexamethasone (Dex) for the indicated periods. Equal loading is determined with antibodies to the mitochondrial VDAC (Santa Cruz Biotechnology) and to the cytosolic ADA. Protein molecular weight markers (MWM) are provided. Results are representative of 3 independent experiments. **C**, Histograms depicting the percentages of DP thymocytes from PNP-KO mice with MMP loss (not accumulating CMXRos) and nuclear DNA fragmentation after 30 minutes of caspase inhibition with zVADfmk, followed by 24 hours of 12.5 μ M dGuo treatment. Results, as determined by means of FACS analysis, are representative of 4 independent experiments. **D**, Contour plots depicting the percentages of Annexin V-positive cells among PI-excluding DP thymocytes from normal littermates and PNP-KO mice after overnight Fas ligation, as determined by means of FACS analysis. Results are representative of 4 independent experiments.

activated peripheral lymphocytes of healthy subjects or lymphocytes from patients with lymphocytic leukemia.^{23,24} Depletion of PNP degradation products, such as guanosine triphosphate, was previously suggested as mediating the damage seen in patients with PNP deficiency²⁵; however, our results demonstrate that excess dGuo is the cause of thymocyte apoptosis. Moreover, the similar responses of thymocytes from PNP-KO and normal mice after Fas ligation suggest specific sensitivity of PNP-deficient DP thymocytes to dGuo. Our studies also suggest that in DP thymocytes PNP governs an apoptotic pathway, which is initiated in the mitochondria. Moreover, we clearly demonstrate that PNP deficiency causes MMP dissipation and release of cytochrome c from the mitochondria to the cytosol. The direct effect of dGuo on mitochondria that we observed is not surprising because dGK, which is the only enzyme that can metabolize the excess dGuo in cases of PNP deficiency, is

exclusively expressed in the mitochondria.^{26,27} Indeed, we and others have previously shown that in the absence of PNP, dGTP concentrations in the mitochondria of thymocytes are markedly increased.^{5,28} The involvement of the mitochondria in PNP-KO thymocytes is also similar to the mitochondria-mediated apoptosis reported in cells and tissues from patients with lymphocytic leukemia treated with dGuo and the PNP inhibitor forodesine.² Additionally, that lack of Fas pathway activation and the similar Fas-induced apoptosis in PNP-KO DP thymocytes further supports a Bcl2-dependent apoptotic pathway in cases of PNP deficiency, as already suggested by us⁵ and demonstrated previously in ADA-deficient thymocytes.⁹ Thus we conclude that in DP thymocytes PNP deficiency leads to accumulation of dGuo, which is then phosphorylated into dGMP and subsequently to dGTP in the mitochondria, resulting in damage to the mitochondria, MMP dissipation, release of

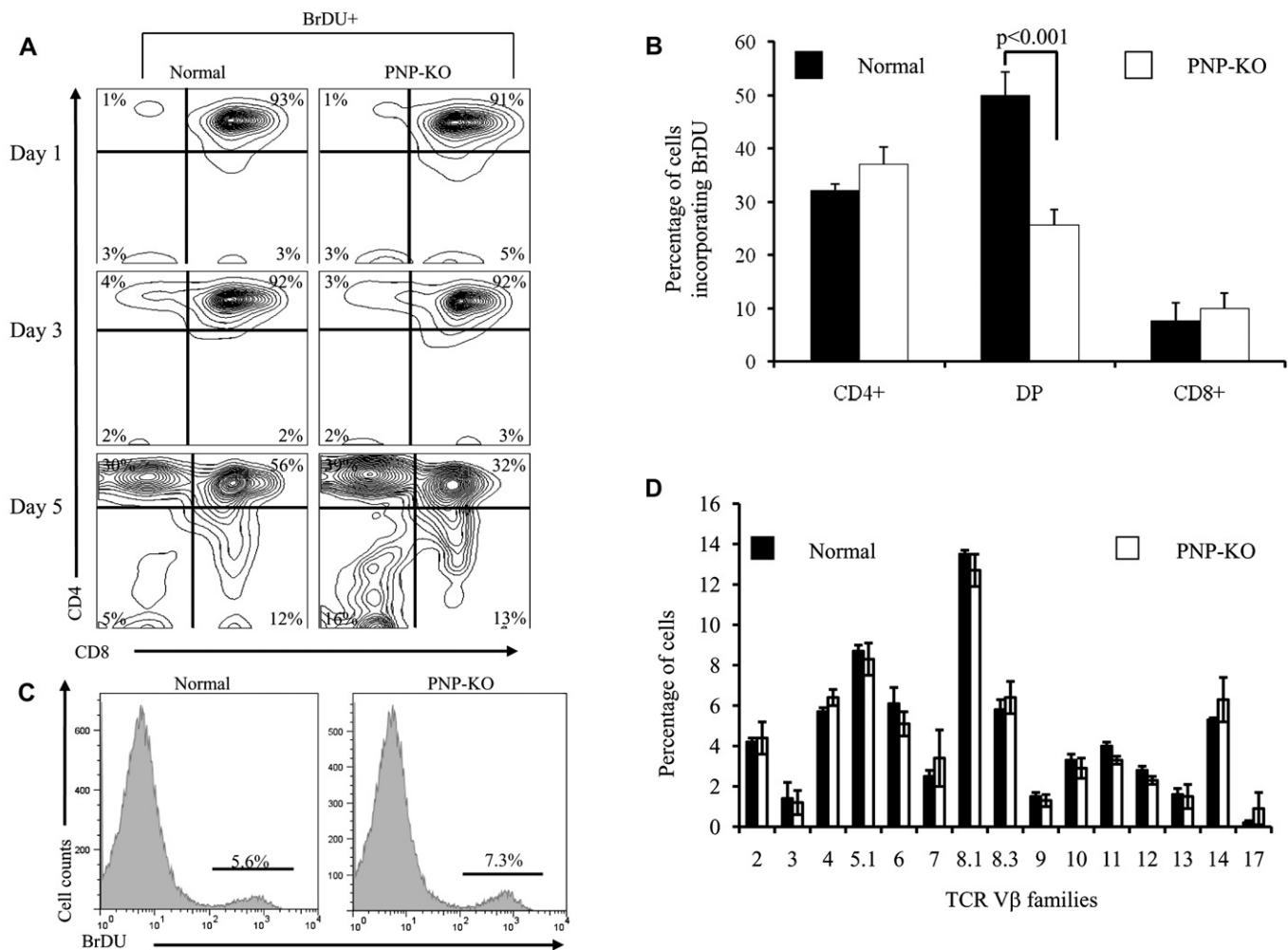


FIG 5. PNP deficiency does not affect the proliferation or TCR diversity of thymocytes. **A**, Contour plots depicting the percentages of CD4-expressing thymocytes, CD8-expressing thymocytes, or both among BrDU⁺ cells at the indicated days after BrDU injection. Results, as determined by means of FACS analysis, are representative of 3 independent experiments with 3 animals of each genotype per experiment. **B**, Bar graph depicting the percentages of DP, CD4⁺, and CD8⁺ SP thymocytes among BrDU⁺ cells, as determined by means of FACS analysis, 5 days after BrDU "pulse." Results (means $\pm$ SDs) are pooled data from 3 independent experiments with analysis of 3 animals of each genotype per experiment. **C**, Histograms depicting the percentages of thymocyte-like cells after 21 days of *ex vivo* differentiation on OP9-DL1 cells that are incorporating BrDU 2 days after BrDU pulse. Results are representative of 3 independent experiments with 3 animals of each genotype per experiment. **D**, Bar graph depicting the percentages (means $\pm$ SDs) of CD3⁺ thymocytes expressing different V β chain families, as determined by means of FACS analysis, from 5 independent experiments.

cytochrome c, activation of the caspase cascade, and apoptosis (Fig 1, A). Importantly, restoring PNP within PNP-deficient DP thymocytes prevented the accelerated apoptosis of these cells, suggesting that PNP supplementation might correct thymocyte defects in PNP-deficient patients. The latter is also supported by our observations that treating PNP-KO mice with TAT-PNP can prevent and reverse abnormalities of DP thymocytes.¹⁶

Contrary to the effects on DP thymocytes, the proliferation and apoptosis of SP thymocytes were not significantly affected by PNP deficiency. Similar uneven effects on thymocytes were previously reported in ADA-deficient mice, with increased apoptosis at the corticomedullary junction of the thymus, an area highly populated by DP thymocytes. Indeed, although DP

thymocyte numbers were markedly reduced in the thymi of ADA-deficient mice, the proportions of DN thymocytes, as well as mature CD4⁺ and CD8⁺ cells, were relatively normal.²⁹ The uneven effect of PNP deficiency on different thymocyte populations might be related to reduced expression of antiapoptotic proteins, such as Bcl-2, in DP thymocytes compared with Bcl-2 expression in DN or SP cells, which contributes to the increased susceptibility to apoptosis that normally occurs in DP cells.³⁰ Thus we hypothesize that the preferential death of DP cells in cases of PNP deficiency is caused by the effects of the increased "physiological" tendency of DP thymocytes to undergo selection and apoptosis amplified by the hostile microenvironment in the thymus corticomedullary area, where most thymocytes die and release excessive amounts of purine metabolites.

Similar to observations in PNP-deficient patients, thymopoiesis in PNP-KO mice is not as dramatically and severely affected as in most other immune deficiencies. At birth, T-cell production is normal or near normal in many PNP-deficient patients,^{1,18} as well as in PNP-KO mice (unpublished data). However, the gradual accumulation of toxic purine metabolites exacerbated by the release of purines from thymocytes undergoing excessive apoptosis results in progressive damage to the thymus. Indeed, a similar although more severe process has been observed in ADA-deficient patients and mice, as well as in ADA-deficient thymocytes expanded *ex vivo* in “fetal thymic organ cultures.”^{1,11,29} Thus early identification and treatment might better restore thymocyte development in these patients.

The adequate TCR β formation in thymocytes of PNP-KO mice also suggests that the SP thymocytes that do develop in the thymus are normal. Thus the significant abnormalities that we and others have observed in peripheral lymphocytes in PNP-deficient patients and mice^{31,32} are likely the result of an additional role or roles of PNP in peripheral lymphocytes, which were not studied here. Indeed, ADA deficiency was also shown to directly affect signaling in peripheral T lymphocytes³³ in addition to the role of ADA in thymocyte development that was described above.

We showed previously many similarities between human and murine PNP deficiency, including accumulation of purine metabolites and decreases in the number and function of peripheral T cells,^{5,6,16} which is not surprising given the conserved structure and function of PNP throughout evolution. Thus although the results presented here pertain to the effect of PNP deficiency on murine thymocytes, we suspect that human thymocytes are similarly affected. Therefore thymic function should be closely monitored in PNP-deficient patients, as well as in patients receiving synthetic PNP inhibitors and dGuo analogs.

In conclusion, using thymocytes from PNP-deficient mice and thymocyte-like cells generated from HSCs of these mice, we show, for the first time, that dGuo accumulation in cases of PNP deficiency induced mitochondria-mediated apoptosis in DP thymocytes.

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Key messages

- PNP deficiency preferentially causes apoptosis of DP thymocytes.
- Accumulation of dGuo in cases of PNP deficiency leads to mitochondria-initiated apoptosis of DP thymocytes.
- Restoring PNP in thymocytes prevents cell apoptosis.

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METHODS

FACS analysis

Dead cells were eliminated from analysis by means of forward and side scatter and PI exclusion. Two- and 3-color fluorescence analysis was performed with the FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo software (TreeStar, Inc, Ashland, Ore) software. Antibodies used included biotin-conjugated anti-mouse Gr-1, CD11b, CD11c, NK1.1, TER-119, CD19, B220, Mac-1, CD44, CD25, CD4, and CD8; phycoerythrin-conjugated anti-CD4, CD44, CD25 and Fas; fluorescein isothiocyanate-conjugated anti-CD8 and CD25; and allophycocyanin-conjugated anti-TCR β , all from BD Biosciences, eBiosciences (San Diego, Calif), and Miltenyi Biotec. Allophycocyanin-conjugated anti-biotin antibody was from Miltenyi Biotec. Cell-surface expression of 15 TCRV β families was determined by using the Mouse V β TCR Screening Panel (BD Biosciences).

DP and SP thymocyte isolation

For isolation of thymocytes, single-cell suspensions from single or pooled thymi were incubated with biotinylated antibodies to Gr-1, CD11b, CD11c, NK1.1, TER-119, CD19, B220, and Mac-1 (Depletion mix), followed by anti-biotin microbeads (Miltenyi Biotec) and magnetic depletion on MACS columns (Miltenyi Biotec). Enrichment of DP thymocytes was confirmed by means of FACS analysis. For isolation of DP thymocytes, selection was performed with the Depletion mix and biotinylated antibodies to CD44 and CD25, as described above, followed by selection with biotinylated antibodies to CD4 and CD8. Selection with the Depletion mix and antibodies to CD44 and CD25 was performed as described above,

together with antibodies to either CD4 or CD8, respectively, to isolate CD8⁺ or CD4⁺ SP thymocytes.

Electron microscopy

Ultrathin sections were cut from tissue fixed in 2.5% glutaraldehyde, postfixed in 1% OsO₄, dehydrated, and embedded in Embed 812-araldite (Electron Microscopy Sciences, Hatfield, Pa). Sections were stained in uranyl acetate and lead citrate and examined in a JEOL JEM 1011 transmission electron microscope (JEOL USA, Peabody, Mass). Images were recorded with a CCD camera (AMT Corp, Danvers, Mass).

Cytochrome c release from the mitochondria

Cytochrome c was detected by permeabilizing 30×10^6 cells with digitonin, 250 mmol/L sucrose, 70 mmol/L KCl, 137 mmol/L NaCl, 4.3 mmol/L Na₂HPO₄, 1.4 mmol/L KH₂PO₄ (pH 7.2), and a protease inhibitor cocktail (Sigma-Aldrich). After centrifugation, the supernatants containing the cytosol fractions were carefully removed. The pellets were further solubilized with 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid, 0.2% Triton X-100, 0.3% NP-40, and the protease inhibitor cocktail, followed by centrifugation at 15,000 rpm to obtain the supernatant containing mitochondria. The mitochondria and cytosol fractions were separated on 16% to 18% SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membranes. Cytochrome c immunoblotting was performed with murine anti-cytochrome c (BD Biosciences), followed by anti-mouse IgG (Jackson Laboratories). Equal loading was determined with the murine mAb to mitochondrial VDAC 1/2/3 (Santa Cruz Biotechnology) and the cytosolic ADA purine enzyme (Sigma-Aldrich), respectively.