THE EFFECTS OF PURINE NUCLEOSIDE PHOSPHORYLASE (PNP) DEFICIENCY ON THYMOCYTE DEVELOPMENT

by

Taniya Papinazath

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Abstract

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M.Sc Thesis, 2010

Institute of Medical Science, University of Toronto

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PNP is a crucial enzyme in purine metabolism, and its inherited defects result in severe T-lineage immune deficiency in humans. I hypothesized that PNP deficiency disrupts the development of late CD4+CD8− double negative (DN) thymocytes and induces mitochondrial-mediated apoptosis of CD4+CD8+ double positive (DP) thymocytes. By using PNP-deficient (PNP−/−) mice as well as an OP9-DL1 co-culture system simulating PNP-deficient conditions, I demonstrated that PNP deficiency interferes with the maturation of DN thymocytes at the transition from DN3 to DN4 stage. Although PNP deficiency does not affect the generation or proliferation of DP thymocytes, PNP−/− DP thymocytes were observed to undergo apoptosis at a higher rate. My results suggest that apoptosis is induced through a mitochondrial mediated pathway. Additionally, re-introduction of PNP into PNP+/− thymocytes protected the cells from the toxic effects of deoxyguanosine by preventing the formation of deoxyguanosine triphosphate, indicating that the toxic metabolite in PNP deficiency is deoxyguanosine.
Acknowledgements

First and foremost, I would like to thank my supervisor, Dr Eyal Grunebaum for his wonderful supervision, guidance and mentoring. In the past two years under his supervision, he has been encouraging and patient in both my scientific and artistic pursuits. Also, thank you for allowing me to explore without inhibition and learn from my mistakes, I am truly grateful for this experience.

I would like to extend my appreciation to the members of my program advisory committee Dr. Yigal Dror, Dr. Cynthia Guidos and Dr. Chetankumar Tailor. I thank you all for sitting through the numerous PAC meetings listening to me talk time and time again about how PNP deficiency causes T-cell immune deficiency, and for giving me valuable suggestions, without which the successful completion of this masters thesis would not have been possible.

A special thank goes out to the entire Grunebaum lab. Weixian Min for helping with all the animal handling, Yongmao Yu for giving timely input on flow data, Alka Arora for making me feel at home when I first joined the lab, and Alireza Mansouri for bringing an element of humour and joy into a sometimes frustrating scientific environment (and not to forget all the entertaining coffee breaks).

I would also like to acknowledge the members of the Dror lab; for being patient in answering all my western blot queries, for allowing me to borrow the cell separation magnets God knows how many times, and for hearing me out in times of despair. Thank you all for making me feel like an “honorary” member of your lab, inviting me for lab luncheons and other lab celebrations.

My thanks is also extended to members of the PMH flow facility, Casey and Francis for helping me get started in the world of flow cytometry.

My graduate life would be nothing without all the friends I made along the way. You are far too many to list but thank you for your camaraderie and friendship. A special shout out goes to Geneve Awong for clearing my OP9-DL1 co-culture queries.

Last but definitely not the least; I’d like to thank my loving family for all their support and encouragement, giving me the strength to overcome all obstacles faced during my graduate studies.
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<th>Description</th>
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<tbody>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>Ado</td>
<td>Adenosine</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>Alpha</td>
</tr>
<tr>
<td>( \alpha\text{-MEM} )</td>
<td>Alpha- minimum essential medium</td>
</tr>
<tr>
<td>( \beta )</td>
<td>Beta</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>dAdo</td>
<td>Deoxyadenosine</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCK</td>
<td>Deoxycytidine kinase</td>
</tr>
<tr>
<td>dGuo</td>
<td>Deoxyguanosine</td>
</tr>
<tr>
<td>dGMP</td>
<td>Deoxyguanosine monophosphate</td>
</tr>
<tr>
<td>dGDP</td>
<td>Deoxyguanosine diphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>dGK</td>
<td>Deoxyguanosine kinase</td>
</tr>
<tr>
<td>dIno</td>
<td>Deoxyinosine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative (CD4(^{-})CD8(^{-}))</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive (CD4(^{+})CD8(^{+}))</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Flurescein isothiocyanate</td>
</tr>
<tr>
<td>FTOC</td>
<td>Fetal thymic organ culture</td>
</tr>
<tr>
<td>Gua</td>
<td>Guanine</td>
</tr>
<tr>
<td>Guo</td>
<td>Guanosine</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HGPRT</td>
<td>Hypoxathine guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>Hx</td>
<td>Hypoxanthine</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Ino</td>
<td>Inosine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Lck</td>
<td>Lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>Lin⁻</td>
<td>Lineage negative</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic activated cell sorter</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>MNGIE</td>
<td>Mitochondrial neurogastrointestinal encephalomyopathy</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>OP9-DL1</td>
<td>OP9 Delta like-1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PNP</td>
<td>Purine nucleoside phosphorylase</td>
</tr>
<tr>
<td>PNP⁻/⁻</td>
<td>PNP-deficient</td>
</tr>
<tr>
<td>pTα</td>
<td>Pre-T-alpha</td>
</tr>
<tr>
<td>PTD</td>
<td>Protein transduction domain</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency syndrome</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive (either CD4⁺ or CD8⁻)</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T cell-acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>TAT</td>
<td>Transactivator of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>UT</td>
<td>Untreated</td>
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CHAPTER 1: INTRODUCTION
1.1. PNP Deficiency

Purine nucleoside phosphorylase (PNP) is a ubiquitous enzyme essential for the degradation of purine nucleosides; guanosine (Guo), deoxyguanosine (dGuo), inosine (Ino) and deoxyinosine (dIno) into uric acid, or their salvage into nucleic acids (Figure 1). An inherited deficiency in PNP causes severe T-cell immune dysfunction in patients, contributing to increased susceptibility to infections, failure to thrive and death in the first few years of life. Some patients manifest with autoimmunity and malignancy (Markert, 1991; Cohen et al., 2000). Few patients also exhibit neurological abnormalities. The fundamental role of PNP in the functioning of organisms makes it vital to research both the enzyme and the compounds affecting its activity.

History:

The significance of the purine salvage pathway in the maintenance and function of the immune system became evident by the discovery that genetic deficiency in the purine salvage enzyme adenosine deaminase (ADA) causes severe combined immunodeficiency syndrome (SCID) in infants. Further screening of immunodeficient patients led to the discovery that deficiency in the next enzyme in the purine salvage pathway, PNP, resulted in severe T-cell immune deficiency (Giblett et al., 1975), emphasizing the importance of the purine salvage pathway in lymphoid cells. PNP deficiency is inherited as an autosomal recessive disorder and is less frequent than ADA deficiency. It has been estimated that 4% of SCID are due to PNP deficiency (Markert, 1991) and to date more than 50 families have been reported worldwide with PNP deficiency. However, the wide spectrum of clinical
manifestations associated with this disorder suggests that many cases have not been documented.

Figure 1: *Schematic representation of the role of PNP in purine metabolism, salvage of purines from ribo- and deoxyribonucleosides* (adapted from Bzowska et al., 2000). PNP catalyzes the phosphorolysis of the products of the ADA reaction, Ino and dIno, to yield Hx and either R-1-P or dR-1-P. PNP also phosphorolyses Guo and dGuo to Gua and the respective ribose phosphates. The purine bases, Hx and Gua, may then be recycled by the action of hypoxanthine guanine phosphoribosyl transferase (HGPRT) to inosine monophosphate (IMP) and guanosine monophosphate (GMP), which can undergo further phosphorylation to guanosine triphosphate (GTP) which is salvaged back to the guanine nucleotide pool. Alternatively, the formed Gua and Hx can be acted upon by guanase (Guase) and xanthine oxidase (Xox) respectively to get converted to xanthine (Xan) and finally to uric acid and excreted in the urine.
Clinical Aspects:

Immunological abnormalities: The most characteristic immune abnormality in PNP-deficient patients is a profound T-cell defect which makes them susceptible to various pathogenic infections within the first two years of their life.

Viral infections including cytomegalovirus, parainfluenza type 3, herpesviruses, Epstein Barr virus have been documented. Persistent Varicella zoster infections which are difficult to treat have also been observed (Baguette et al., 2002). Pathogenic bacteria such as Haemophilus influenzae, Pseudomonas and Streptococcus pneumoniae have been documented, as well as certain opportunistic infections caused by Candida albicans and P. jiroveci.

On chest x-rays, the thymus is absent and the tonsils are either absent or reduced in size (Staal et al., 1980). Immunological evaluation has revealed T-lymphopenia and markedly decreased CD3+ T-cells (often less than 500 cells/ml or less than 15% of total lymphocytes), which further decreases with age. T-cell function is similarly reduced as assessed by response to various mitogens and by the mixed lymphocyte reaction (MLR).

PNP deficiency is associated with an increased risk of autoimmune disorders. The most commonly reported autoimmune occurrences are autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura and systemic lupus erythematosus. The abnormal immune surveillance in PNP-deficient patients also predisposes them to develop malignancies such as lymphoma and lymphosarcoma.

Non Immunological abnormalities: Patients present with mild hepatomegaly and splenomegaly. Many PNP-deficient patients also suffer from neurological dysfunction unrelated to the immunological abnormalities (Market, 1991). These neurological
imbalances include ataxia, developmental delay and spasticity, possibly reflecting the
importance of purine homeostasis in brain development, as demonstrated in other diseases
with abnormal purine metabolism such as Lesch-Nyhan syndrome (HGPRT deficiency) and
ADA deficiency (Pelled et al., 1999; Tezcan et al., 1995).

Another example of the role of purine metabolites in other tissues includes the effect
of PNP deficiency on bone marrow. PNP-deficient marrow has been found to be
hypersensitive to irradiation and many patients develop dysplastic morphology (Dror et al.,
2004).

**Biochemical findings:** Most PNP-deficient patients have undetectable levels of PNP
activity. In addition, the deficiency is strongly indicated by low serum and urinary levels of
uric acid, which is the final breakdown product in the PNP pathway. High serum levels of
PNP substrates; inosine (Ino) and guanosine (Guo) and high urinary levels of Ino,
deoxyinosine (dIno), Guo and deoxyguanosine (dGuo) are also often found (Hershfields and
Mitchell, 2001). The diagnosis of PNP deficiency is established when PNP activity is
undetectable and is confirmed by molecular analysis of the PNP gene.

**Human PNP structure, chemistry, genome and mutations:**

PNP has been isolated from a wide variety of both mammalian and bacterial species
(Pugmire and Ealick, 2002). PNP is a ubiquitous enzyme and has been purified from many
different tissues (Agarwal et al., 1975; Carson et al., 1977). In humans, the highest activity is
found in the kidney, peripheral lymphocytes, and granulocytes. The enzyme exists in the
trimeric form in which each subunit of the functionally active trimer has a molecular weight
of ~ 32 kDa (Zannis et al., 1978; Osbourne, 1980; Stoeckler et al., 1978; Goddard et al.,
1983). Mutant enzymes can be completely inactive or may exhibit residual activity. The
enzyme has been crystallized from erythrocytes, and studies of the active site have shown specificity for binding of guanine (Gua) and hypoxanthine (Hx) and their analogues but not adenine (Ealick et al., 1990).

The human PNP gene has been cloned, sequenced and mapped to chromosome 14q13 and spans 9kb of genomic DNA (Ricciuti and Ruddle, 1973; Williams et al., 1984). Over two dozen mutations have been identified; the bulk are missense mutations. The most common appeared to be the point mutation Arg234Pro (Markert et al, 1996), but many mutations have been detected since. Residual PNP activity has been observed to cause a milder phenotype (Broome et al., 1996; Markert et al., 1997) but the diversity of the mutations and the small number of patients makes it difficult for genotype-phenotype correlations.

**Purine Metabolism:**

Purine nucleotides are synthesized by the de novo or the salvage pathway. The de novo pathway creates phosphorlayted ring structures from simple precursors such as CO₂, glycine and glutamine. The salvage pathways reutilize purine and pyrimidine bases. Inosinic acid (IMP, inosine monophosphate) is the central product in both pathways in purine metabolism and is central to the interconversion of adenine and guanine nucleotides (Nyhan, 2005). These ubiquitous purine metabolic pathways are responsible for the proper balance between the production of dephosphorylated purines, detoxification by further degradation to uric acid, and salvage by metabolism back to the nucleotide level.
**Pathophysiology:**

PNP (EC 2.4.2.1) catalyses the reversible phosphorolysis of four purine nucleosides guanosine (Guo), deoxyguanosine (dGuo), inosine (Ino) and deoxyinosine (dIno) to yield guanine (Gua) and hypoxanthine (Hx) respectively, and ribose-1-phosphate (R-1-P) or 2’-deoxy ribose-1-phosphate (dR-1-P) (Figure 1). Specifically, PNP catalyzes the cleavage of the glycosidic bond of ribo and deoxyribonucleosides in the presence of ortho inorganic phosphate (Pi) in order to generate the purine base and R(dR)-1-P (Bzowska et al., 2000), schematized in the following reaction:

\[
(2’\text{-deoxy}) \text{nucleoside} + \text{Pi} \leftrightarrow \text{base} + (2’\text{-deoxy}) \text{ribose 1-phosphate}
\]

**Proposed mechanisms of the cellular immunodeficiency in PNP deficiency:**

The central feature of this disorder is profound impairment in cellular immunity characterized mainly by the ability of T-cells to accumulate dGTP. Several mechanisms have been proposed to explain the effects of PNP deficiency, but most researchers have focused on the accumulation of PNP substrates and their respective metabolic products (Hershfield and Mitchell, 2001). There has been extensive evidence pertaining to T-cells, showing that dGuo interferes with DNA synthesis via its phosphorylated product dGTP. dGTP is thought to cause feedback inhibition of ribonucleotide reductase (Carson et al., 1977; Mitchell et al., 1978; Chan, 1978; Ullmann et al., 1979; Kredich and Hershfield, 1989), which in turn causes depletion of intracellular dNTP pools resulting in inhibition of DNA synthesis (Reichard, 1978; Martin and Gelfand, 1981) (Figure 2). Arpaia et al further suggested a mitochondrial basis for immune deficiency (Arpaia et al., 2000). They postulated that, similar to the effects of dATP in ADA deficiency, the abnormalities observed in PNP deficiency are due to the selective accumulation of dGTP in the mitochondria of T-cells,
leading to abnormal mitochondrial DNA (mtDNA) synthesis which in turn impedes cell division resulting in apoptosis. A more detailed explanation of the specific effects of dGuo on thymocytes/T-cells will be discussed in the thymocyte section of this introduction.

Although T-cells are the most severely affected in PNP deficiency, abnormalities in other tissues have also been observed but not much attention has been paid on the specific effects of PNP deficiency on brain and other tissues. However, neurological abnormalities associated with PNP deficiency indicate a correlation with purine metabolism and normal brain development. Although increased levels of dGTP has been implicated in the T-cell dysfunction of this disorder, in contrast, the neurological symptoms associated with PNP deficiency has been postulated to be caused by a depletion of GTP, similar to the effects seen in HGPRT deficiency (Simmonds et al., 1987). Reduced GTP maybe because of the inability to produce Gua, or secondary to over-consumption of phosphoribosyl pyrophosphate, ultimately interfering with de novo purine synthesis (Barankiewicz et al., 1982). Decreased intracellular levels of GTP observed in PNP deficiency is unlikely to cause the immune dysfunction, as a similar decrease in intracellular levels of GTP associated with HGPRT deficiency has no effect on the immune system (Sidi et al., 1989). Therefore, the two major clinical consequences of PNP deficiency may reflect differing etiologies; neurological effects may result from deficiency of the PNP enzyme products (GTP) while the immunodeficiency might be caused by accumulation of the PNP enzyme substrates (dGuo, dGTP). The former hypothesis is yet to be elucidated.
Figure 2: *Metabolic Effects of PNP Deficiency* (adapted from Bzowska et al., 2000). PNP deficiency causes the accumulation of dGTP which subsequently inhibits ribonucleotide reductase and consequently hinders DNA synthesis. Abbreviations; dGK, deoxyguanosine kinase, NDP, nucleoside diphosphate.
PNP as a target enzyme for chemotherapeutic applications:

Selective T-cell immunodeficiency associated with PNP deficiency suggested that PNP can be targeted to selectively regulate T-cell immune response. The finding of the enzyme structure gave way to the discovery of potential PNP inhibitors for chemotherapeutic applications. The observation that in the absence of PNP there is an accumulation of dGuo in the plasma and an accumulation of dGTP in T-cells has been the impetus to develop a number of PNP inhibitors and PNP resistant dGuo analogues. Over the years, studies have shown the beneficial use of PNP inhibitors as a therapeutic agent for treating lymphoproliferative malignancies (Furman et al., 2007), certain autoimmune disorders such as rheumatoid arthritis, psoriasis (Morris and Omura, 2000) and to suppress graft vs host disease (Bantia et al., 2002). One such effective inhibitor of PNP is BCX-1777 (Forodesine/immucillin-H) (Miles et al., 1998; Bantia et al., 2001) (Figure 3), which was the inhibitor used over the course of this study.

![Structure of BCX-1777](image)

**Figure 3: Structure of BCX-1777** (Bantia et al., 2003). BCX-1777 is a potent inhibitor of human PNP and designed based on the transition state structure.

Previous studies have shown that the cytotoxicity of BCX-1777 in the presence of dGuo is specific to T-cells without affecting other non T-cell tumor lines (Kiscka et al., 2001). This
lead to a phase 1 clinical trial of BCX-1777 in patients with advanced T-cell malignancies (Balakrishnan et al., 2006). BCX-1777 in the presence of dGuo inhibited the proliferation of CEM-SS (a T-acute lymphoblastic leukemia (T-ALL)) cells accompanied by a 154 fold increase in endogenous dGTP. BCX-1777 also inhibited the proliferation of activated human T-cell lymphocytes induced with various agents such as IL-2, MLR and phytohemagglutinin (Bantia et al., 2003).

Individuals with genetic deficiency in PNP maintaining even 5% of the catalytic activity of the enzyme fail to accumulate dGuo and exhibit T-cell immune deficiency. Therefore it is essential to completely inhibit PNP (>95%) to achieve cytotoxicity of T-cells due to increase in dGuo (Morris et al., 1998). The necessity for near complete PNP inhibition makes BCX-1777 an ideal pharmacological inhibitor to study PNP deficiency as it binds ≈1000 times more tightly to PNP than previously studied inhibitors (Bantia et al., 2001).

**Treatment Options:**

PNP-deficient patients usually succumb to this disorder within the first two decades of life due to opportunistic infections, autoimmune hematological disturbances or malignancy. As of the time being, hematopoietic stem cell (HSC) transplant is considered the treatment of choice. Although it has been able to provide long term immune reconstitution in a few patients, the rarity of this disorder makes it imperative to have a definitive treatment option. Hence, research is still underway to develop a treatment modality to better manage this disorder.

Some of the previous attempts to alleviate symptoms associated with PNP deficiency include; erythrocyte transfusions as a means of PNP enzyme replacement. This therapeutic...
approach caused partial reconstitution of T-cell mediated immunity, however, it did not protect from devastating infections (Rich et al., 1980; Sakiyama et al., 1989). This gave way to a polyethylene glycol (PEG) bound enzyme replacement therapy.

After the relative success of PEG-ADA in the treatment of ADA deficiency (Markert et al., 1987; Hershfield et al., 1991), PEG-PNP injections were tested on PNP^{+/−} mice (Roifman –personal communication). Data suggesting that PEG-ADA provided only partial and temporary immune reconstitution in ADA-deficiency (Chan et al., 2005; Husain et al., 2007), probably because the enzyme was not being targeted into the cell, difficulties in producing PEG-PNP, and moreover the high cost of production, did not make this type of treatment a favorable one. This prompted us to develop a TAT-PNP treatment, an alternative enzyme replacement therapy that targeted PNP into the cells.

**TAT-PNP:** TAT-PNP treatment will be highlighted in more detail as this is the technique that was used to introduce PNP into the PNP^{+/−} thymocytes in this study. Protein transduction domains (PTD) are a group of peptides that can cross biological membranes in a receptor independent manner (Wadia et al., 2003). One such PTD is the HIV– transactivator of transcription (TAT) protein (Gump and Dowdy, 2007). Since only 11 amino acids of TAT is required for transduction, and its relatively easy production has made it the PTD of choice (Schwarze et al., 1999; Nagahara et al., 1998). Various *in vitro* studies have shown that PTD can transport cargo molecules into many different cell types (Dietz et al., 2004; Kwon et al., 2005). Based on this, recently, our lab generated a TAT-PNP fusion protein by replacing the ATG of the human PNP cDNA with the coding sequence for the 11 amino acids of the TAT-PTD (Toro et al., 2006). Repeated TAT-PNP injections were able to correct thymocyte development and T-lymphocyte function in PNP-deficient mice. PNP activity was detected
in all the organs tested and also increased survival of these mice (Toro and Grunebaum, 2006). Further, pre-clinical studies are currently being conducted to establish the benefit of PTD-PNP for PNP-deficient patients.

More recently our lab investigated the possibility of gene therapy using a lentivirus based vector that expressed human PNP. The preliminary studies carried out in PNP+/− mice have been promising (Liao et al., 2008). Currently our lab is optimizing conditions to ensure long term gene expression which can translate into better gene therapy for PNP-deficient patients.

**Study Model:**  

**PNP Knockout mice:**

There are no known naturally occurring models of PNP deficiency. This led to the development of two relevant mouse models recapitulating the human disorder. The first model by Snyder et al was developed by introducing three point mutations in the PNP gene locus by male germ cell mutagenesis (Snyder et al., 1997). In their mouse model, progressive loss of thymic cellularity due to reduction of CD4+CD8+DP thymocytes was observed, with corresponding reduction in peripheral T-lymphocyte numbers and function. Although these mice shared some similarities with the human phenotype, the residual levels of PNP activity, the lack of dGTP accumulation in thymocytes, the delayed onset of the immune deficiency and the normal life span of the mice were not representative of PNP-deficient patients (Snyder et al., 1997). This prompted another group led by Dr. Roifman, to develop a PNP deficient (PNP−/−) mouse model by replacing the catalytic domain of the murine PNP gene with a neomycin cassette, thereby creating mice with no PNP activity (Arpaia et al., 2000). These PNP−/− mice exhibited nearly all the metabolic, immune and non-
immune abnormalities typical of PNP-deficient patients. Some of the important similarities include; very early death (8-12 weeks), PNP activity in these mice was found to be <0.2% of normal, increased concentrations of PNP substrates were detectable in the urine, uric acid production diminished (Arpaia et al., 2000), and the mice failed to gain weight (Toro and Grunebaum, 2006). The mice developed a hypoplastic thymus characterized by reduced number of DP cells, markedly reduced number and function of T-cells and relatively preserved B-cell numbers (Arpaia et al., 2000). The mice displayed autoimmuity with splenomegaly and hepatomegaly. Mice also manifest with neurological abnormalities as tested by their inability to stay on a spinning rotorod, which is indicative of possible ataxia and abnormal motor coordination (Grunebaum unpublished). The above mentioned characteristics of the PNP−/− mice generated by Dr. Roifman’s group led us to choose this model, around which all the experiments for this study were designed.

1.2. Murine Thymocyte Development

Introduction

T-cells form an integral part of the adaptive immune system. T-lymphocytes are derived from hematopoietic precursors that arise in the fetal liver or adult bone marrow and migrate through the blood stream to the thymus. In the thymus, hematopoietic precursors go through well defined developmental steps which are required for formation of mature thymocytes characterized by a functional T-cell receptor (TCR). These mature T-lymphocytes are released to the periphery where they can subsequently act on invading antigen and fight infections when given the proper activation signals.
The thymic environment is essential for specifying the T-cell lineage fate for the hematopoietic precursors. Once the T-cell lineage fate is adopted the thymus further specifies whether thymocytes become either \( \alpha \beta \) or \( \gamma \delta \) TCR bearing cells and whether \( \alpha \beta \) cells will express either CD4 or CD8 co-receptors. During each stage of thymocyte differentiation, there are cellular controls that delegate whether the cells are in a position to proliferate and move forward in the developmental pathway. Thus, formation of T-cell repertoire involves stepwise fate determination in anatomically distinct thymic microenvironments (Nitta et al., 2008).

**T-Cell Receptor (TCR):**

All mature thymocytes and T-cells express a T-cell receptor (TCR) on their cell surface. The TCR is a complex of integral membrane proteins responsible for recognizing antigens bound to major histocompatibility complexes (MHC) when presented by antigen presenting cells such as dendritic cells, macrophages and B-cells (Mazza et al., 1998). The TCR is a heterodimer consisting of two polypeptide chains either \( \alpha \) and \( \beta \) or \( \gamma \) and \( \delta \). The TCR is also associated with a co-stimulatory CD3 complex which is composed of a \( \gamma \), \( \delta \), \( \epsilon \) and \( \zeta \) subunits (Frank et al., 1990). Once a specific peptide:MHC ligand binding occurs, the cytoplasmic region of the CD3 complex transduces signals to the nucleus which initiates a signaling cascade leading to the activation of the T-cell (Kane et al., 2000). The TCR together with the CD3 subchains forms the TCR complex.

Signalling from the TCR complex is enhanced by the presence of co-receptors CD4 or CD8 (Janeway, 1992). The co-receptor specifies antigen specificity as well as the functional lineage of the T-cell. The co-receptor CD4 binds to class II MHC molecules and receptor engagement leads to activation of the adaptive immune system. CD4\(^+\) T-cells are
termed “helper” T-cells as they have no direct cytotoxic or phagocytic activity and their main function is in aiding other immune cells. CD8 co-receptor binds to class I MHC and activation of CD8+ T-cells leads to the direct lysis of the target cell and hence are called “cytotoxic” T-cells.

**Thymic Organization:**

The thymus is a bilobed organ found in the upper portion of the chest just behind the sternum (Miller, 1961). The thymic lobes are separated by mesenchymal septae and are further divided into the outer cortex and the inner medulla regions each characterized by specific stromal cell types (Anderson and Jenkinson, 2001). The two regions are separated by the cortico-medullary junction (CMJ) and the cortex is surrounded by the subcapsular zone (SCZ) (Penit, 1988; Lind et al., 2001). At each stage of maturation, thymocytes occupy distinct regions in the thymus. Progenitors enter at the CMJ, migrate through the cortex to the outer SCZ during the early progenitor stages, and then move back toward the CMJ and into the medulla (Figure 4) (Lind et al., 2001; Petrie and Zúñiga-Pflücker, 2007). The thymus builds up a stock of T-cells before birth and early childhood and is largest at puberty, after which it starts to involute and get replaced by fat (Scollay et al., 1980).
Figure 4: Traffic of thymocytes through the thymus. Progenitor cells from the BM travels through the blood stream to the CMJ of the thymus. The earliest double negative (DN) population DN1 cells strongly rely on notch signaling from the thymic epithelium to assume a T-cell fate. DN1 cells differentiate into DN2 and relocate outwards to the subcapsular region of the thymic cortex where they differentiate into DN3 cells. The process of \( \beta \)-selection takes place here and these DN4 thymocytes proliferate as a result of pre-TCR signaling. The cortical epithelium is responsible for the process of positive selection and the positively selected DPs start relocating towards the medulla. SP cells enter the medulla for negative selection and ultimately mature T-cells leave the thymus for the periphery. The irregularly shaped cells represent the thymic epithelial cells. The dark blue cells represent thymic stromal cells expressing notch ligand. Abbreviations: HP, hematopoietic progenitor.
**Murine Thymocyte Development:**

Lin'Sca1^+CD117^+ BM derived progenitors seed the thymus, where they undergo differentiation which is broken down into distinct stages characterized by the presence of co-receptors CD4 and CD8 (Allman et al., 2003). Cells lacking both CD4 and CD8 have been identified as double negative (DN) thymocytes (Scollay et al., 1984; Scollay et al., 1988). The early DN subpopulation can be identified by the expression of CD117 (c-kit) and further classified based on the expression of adhesion molecules CD44, and the IL-2 receptor α, CD25 (Godfrey et al., 1993) which are expressed in a stage specific manner. The sequence of DN transition is as follows: DN1 (CD117^+CD44^+CD25^−), DN2 (CD117^+CD44^+CD25^+), DN3 (CD117^−CD44^−CD25^+), DN4 (CD117^−CD44^−CD25^−). Thymocyte differentiation is summarized in Figure 5.

DN1 cells move away from the site of entry into the deeper cortex as they differentiate into DN2 cells. Progression into the DN2 stage is marked by an upregulation of CD25 (Porrit et al., 2003). DN2 cells still retain some capacity to become natural killer (NK), dendritic (DC) cells and macrophages (Wu et al., 1996, Schmitt et al., 2004; Bell and Bhandoola, 2008). Migration to the outer cortex will lead to further differentiation of DN2 cells to the DN3 stage. The DN3 stage is characterized by a down regulation of CD44. During the transition from the DN2 to the DN3 stage, recombination of the variable (V), diversity (D) and joining (J) DNA elements of the antigen receptor genes is initiated at the β, γ and δ loci. This process is driven by the transient expression of the VDJ recombination activating gene (RAG)-1 and RAG-2 proteins, to give rise to unique TCRs (Fugmann et al., 2000). As this occurs the DN3 cells become irreversibly committed to the T-cell lineage (Ciofani et al., 2006). If the DN thymocytes successfully rearrange the γδ loci, they will...
follow along the γδ lineage. Alternatively, if they successfully rearrange their β-locus the
cells will adopt the αβ lineage. γδ T-cells reside in the epithelial layers of tissues such as
skin, intestinal epithelium, tongue and lung where they act as the first line of defense.
However, their development and selection processes are less well characterized than the αβ
lineage (Xiong and Raulet, 2007).

Further differentiation of the αβ lineage is triggered once the TCRβ chain assembles
with the invariant pre-Tα (pTα), and CD3 signaling chains to form the pre-TCR complex
(Saint Ruf et al., 1994; von Boehmer et al., 1997). αβ T-cells make up 95% of the T-cells in
the thymus of an adult mouse. Signaling through the pre-TCR complex leads to “β-
selection” (Michie and Zúñiga-Pflücker, 2002), the first checkpoint in thymocyte
differentiation ensuring that only thymocytes that have successfully rearranged their TCRβ
chain continue to differentiate into CD4⁺CD8⁺ DP stage. Therefore, activation through the
pre-TCR cements the αβ lineage commitment (Hayday et al., 1999).

Pre-TCR signaling and the resulting process of β-selection is a crucial checkpoint in
thymocyte differentiation. The expression of the pre-TCR complex initiates a number of
events including survival and proliferation (Falk et al., 2001; Vasseur et al., 2001; Kruisbeek
et al., 2000). Many DN3 cells do not productively rearrange their TCR loci and will die by
apoptosis in the thymus. Pre-TCR signaling also results in the down regulation of CD25
expression giving rise to DN4 cells. Upregulation of CD4 and CD8 expression allows the
DN4 cells to transit into the DP stage.

DP cells continue to rearrange the TCRα genes to express an α chain. Pairing of an
α chain with the β chain results in the formation of a mature functional αβ TCR (Petrie et al.,
1993). DP thymocytes expressing TCR αβ on their cell surface are localized in the cortex.
At this stage, the DP cells undergo further positive and negative selection that ensures that only self-MHC restricted and self-tolerant thymocytes will become a mature SP immunocompetent cell that can leave the thymus for the periphery (Sebzda et al., 1999).

During positive selection, only those DP thymocytes that receive TCR signals with ligand interaction of weak affinity will survive. The necessity for positive selection is to ensure that the T-cells reaching the periphery are only reactive to antigen when presented in context with self-MHC. During the positive selection process most of the cells are induced to undergo apoptosis and only 3-5% of DPs within the thymus survive this checkpoint and are selected for the next selection criteria, negative selection (Ashton-Rickardt et al., 1993; Surh and Sprent, 1994; Takahama et al., 1994). Positively selected thymocytes migrate from the cortex to the medulla where majority of the negative selection takes place (Witt et al., 2005).

Negative selection is a crucial step in eliminating self-reactive T-cells (Ohashi, 2003). This selection procedure ensures that the T-cells reaching the periphery are self-tolerant, without which undesirable autoimmunity would ensue. In contrast to positive selection, TCRs which bind MHC peptide complexes presented by a variety of cells like macrophages and dendritic cells, with high affinity, will be given an apoptotic signal and induced to die, thus preventing the exit of a self-reactive T-cell to the periphery (Hoffman et al., 1992; Sprent and Kishimoto, 2002). The divergence of the CD4+/CD8+ SP lineage is dependent on the specificity of the TCR to either class I MHC or class II MHC. CD4 is actively repressed in MHC-I restricted TCRs and CD8 in MHC-II restricted TCRs (Germain, 2002). Figure 5 summaries the development of murine thymocytes.
Figure 5: Schematic representation of murine thymocyte development (adapted from Kruisbeek et al., 2000). The CD4−CD8−DN subset is characterized by the expression of cell surface markers CD117, CD44 and CD25. The DN compartment is subdivided into distinct stages DN1-DN4. The first developmental checkpoint, β-selection, occurs at the DN3 stage. Only those cells that have successfully rearranged their β-locus by the process of VDJ recombination and express the pre-TCR will develop into the DN4 stage and become CD4+CD8+ DP cells. At the DP stage, TCR signals are activated resulting in either CD4+ or CD8+ SP lineage fate divergence. DPs expressing mature TCRs undergo two selective steps; positive selection and negative selection. CD4+ or CD8+ SP cells then move into circulation. The pre-TCR components are depicted in the box. Abbreviations; HSC, hematopoietic stem cell.
The process of thymocyte development requires a crosstalk between the lymphoid cells of the thymus and the thymic stroma which constitutes the non lymphoid portion (Petrie et al., 2001). The interplay of various thymic stromal cells creates microenvironments through which the cells traverse in an orderly manner. The stroma surrounding thymocytes at each stage of maturation provides signals which are crucial in directing the cell into distinct stages of development through direct lympho-stromal interactions, various thymopoeitic cytokines and other soluble growth factors important for T-cell repertoire selection and maturation (Petrie and Zúñiga-Pflücker, 2007). Furthermore, recent studies have shown that chemokines produced by thymic stromal cells are pivotal in guiding the traffic of developing T-cells in the thymus (Takahama, 2006). Consequently, thymocyte development is not a cell autonomous process but has several molecular players that enable the thymus to carry out its function (Di Santo and Rodewald, 1998).

**Notch Signaling:**

Many pathways have been identified in recent years to be important for determining the fate of T-lineage cells. Among these, Notch signaling has been extensively studied, as it is an evolutionarily conserved mechanism that is important in determining different cell fates during development (Artavanis Tsakonas et al., 1999). In mammals, the Notch transmembrane receptor family consists of four members (Notch 1–4) which interact with ligands of the Delta-like (DL) family (DL-1, DL-3, and DL-4) and Serrate-like ligands (Jagged-1 and Jagged-2) (Guidos, 2002).

The Notch pathway, particularly the Notch-1 plays a vital role in the commitment of progenitor cells to the T-lineage fate. Mice with a neonatally induced loss of Notch-1 function, or irradiated controls repopulated with Notch-1 deficient BM stem cells,
severely inhibited thymocyte development but not other hematopoietic lineages (Radtke et al., 1999). Accordingly, BM progenitors expressing constitutively active form of Notch-1 injected into mice favored the formation of DP T-cells and abolished B-cell development in the BM of these mice (Pui et al., 1999). Mouse thymocytes have been shown to have modulated expression of Notch-1 throughout thymocyte development (Hasserjian et al., 1996). Although multiple Notch ligands are expressed either in the thymus or on thymocytes themselves (Radtke et al., 2004), gain of function approaches have clearly indicated that DL ligands are capable of promoting T-cell development \textit{in vitro} and \textit{in vivo} (Visan et al., 2006). DL-1 and Jagged-1 in particular have been shown to promote T-cell development and is expressed in the thymic cortical and medullary epithelium (Lehar et al., 2005).

**OP9-DL1:**

In light of these findings, an osteopetrosis (OP) stromal cell line was stably transduced to express the Notch ligand DL1 to form the OP9-DL1 stromal cell line (Schmitt and Zúñiga-Pflücker, 2002). The lack of macrophage colony stimulating factor (M-CSF) production by OP9 cells prevented formation of macrophage cells and favored generation of lymphoid cells. Embryonic stem cells, fetal liver and bone marrow derived hematopoietic progenitors cultured on OP9-DL1 stromal cells were induced to commit to the T-cell lineage at the expense of B-cell lymphopoiesis (de Pooter and Zúñiga-Pflücker, 2007). This culture system is a valuable tool to study T-cell development \textit{in vitro} and was used in my studies.
**IL-7 and Flt3L:**

In addition to Notch signaling, lymphoid development is also regulated by a variety of cytokines. One such factor is IL-7, which is secreted by the thymic stroma. IL-7 is required mainly during early T-cell development in the thymus to protect DN2 and DN3 stages from apoptotic cell death (Kim et al., 1998). In IL-7 and IL-7 receptor (IL-7R) knockout mice, progression of thymocytes beyond DN2 stage is severely diminished (Peschon et al., 1994; von Freeden et al., 1995; Maki et al., 1996), indicating that IL-7 is essential for the expansion of the DN2 population to transition to the DN3 compartment. Indeed studies have indicated a role of IL-7 in thymocyte survival wherein IL-7 signalling was seen to upregulate the expression of the anti-apoptotic molecule, Bcl-2 (von Freeden et al., 1997; Akashi et al., 1997). Complimentary to these findings, overexpression of Bcl-2 was partially able to rescue the defects of IL-7R deficiency (Maraskovsky et al., 1997). More recently, Van De Wiele et al clarified the role of IL-7Rα in early thymocyte development using a transgenic background of ectopic IL-7Rα background. Their studies led them to conclude that the role of IL-7 signaling in early thymocyte development is between the time the thymocytes are fated to become T-cells and the β-selection stage instigated proliferative burst is initiated (Van de Wiele et al., 2007).

Another cytokine important for thymocyte development is FMS-like tyrosine kinase 3 ligand (Flt3L). Flt3 deficient mice have also shown a moderate decrease in the number of DN thymocytes (Mackerehtschian et al., 1995). Furthermore, Wang et al showed using the OP9-DL1 system; the function of Flt3L in thymopoiesis is primarily to support the survival and proliferation of progenitors (Wang et al., 2006). Interestingly, mice double deficient for
Flt3L and IL7R showed not only reduction in the DN1-DN4 and DP progenitors but also mature peripheral SP T-cells, demonstrating critical and complementary roles of Flt3 and IL-7 in early T-cell development (Sitnicka et al., 2007).

**Thymocyte abnormalities in PNP deficiency:**

Patients present with a small hypoplastic thymus and correspondingly reduced T-lymphocytes in peripheral blood. T-cell receptor excision circles (TRECs) which represent new thymic emigrants are also reduced in these patients. Concordant to the human observations, the number of thymocytes in PNP<sup>−/−</sup> mice is reduced compared to normal littermates (58±20 x10<sup>6</sup> cells/thymus vs 146±19 x10<sup>6</sup> cells/thymus, n=9 in each, my data). PNP<sup>−/−</sup> mice also exhibit increased numbers of DN thymocytes and decreased numbers of DP and SP thymocytes (Arpaia et al., 2000, my data).

**Effect of PNP deficiency on T-lymphocytes:**

PNP-deficient patients may exhibit normal T-lymphocyte numbers and function early on in life but deteriorates over a course of time as there is a buildup of toxic metabolites. The reduced response of T-cells from PNP-deficient patients to mitogens, and the increased susceptibility of these cells to ionizing radiation (Dror et al., 2004) are indicative that PNP deficiency, besides affecting thymocytes also directly affects peripheral T-cells. *In vivo* studies have also shown depressed T-cell function.

Similar to human observations, lymphocyte function in PNP-deficient mice is impaired as indicated by an inability to illicit an immune response in a mixed lymphocyte reaction (MLR) (Arpaia et al., 2000). In addition, recent findings from our lab showed increased thymidine incorporation into fresh T-lymphocytes of PNP<sup>−/−</sup> mice (Yu et al., 2009),
suggesting that T-lymphocyte abnormalities in PNP deficiency might result from exaggerated \textit{in vivo} activation of peripheral lymphocytes, possibly secondary to unrecognized infections or uncontrolled systemic inflammation. Alternatively, PNP deficiency might disrupt lymphocytes intracellular signaling, as studies have shown abnormal cytokine production by PNP-deficient lymphocytes (Arpaia et al., 2000; Dror et al., 2004; Toro et al., 2006).

\textbf{Selective dGuo toxicity to thymocytes and T-lymphocytes:}

Although there is accumulation of all 4 substrates (Guo, dGuo, Ino and dIno) in PNP deficiency, it has been suggested that dGuo plays a central role in mediating the effects of PNP deficiency. The possibility of dGuo being the potentially lymphotoxic metabolite in PNP deficiency was postulated back in 1977 by Cohen et al (Cohen et al., 1977). They found dGTP levels were ten-fold higher in PNP-deficient patients compared to normal patients (Cohen et al., 1977). It was demonstrated that immature human thymocytes were more sensitive to dGuo toxicity than peripheral T-lymphocytes (Cohen et al., 1980). The selective toxicity of dGuo to thymocytes may rest in the ability of these cells to trap dGTP due to high deoxyguanosine kinase and low nucleotidase activity (Carson et al., 1977; Gelfand et al., 1979).

Many different \textit{in vitro} models have been used to investigate the specific effects of dGuo on T-cell immune function. Some of the earlier studies have shown that continuously growing T-lymphoblastoid cell lines partially deficient for PNP are more sensitive to the cytotoxic effect of dGuo than B-lymphoblastoid cell lines (Mitchell et al., 1978). Moreover, these findings were also applicable to lymphoid cells under conditions simulating PNP deficiency wherein dGTP formation by thymocytes increased ten-fold (Fairbanks et al.,...
In fact, it has been postulated that the accumulation of dGTP and the preferential sensitivity of dGTP makes the T-lineage cells more prone to apoptosis in PNP deficiency. Evidence for this stems from the observation that dGuo has the ability to inhibit T-cell growth and in consequence, was associated with increase in intracellular dGTP (Gudas et al., 1978; Ullman et al., 1979), suggesting that toxicity is in fact due to the formation of dGTP and not due to the direct effects of dGuo. Furthermore, *in vitro* and *in vivo* studies carried out on lymphocytes from patients with T-cell malignancies in which PNP-deficient environment was simulated using chemical PNP inhibitors, demonstrated increased dGTP accumulation and reduced proliferation of malignant cells and normal human T-cells (Bantia et al., 2003; Conry et al., 1998; Gandhi et al., 2005).

**Thymocyte apoptosis associated with purine deficiencies:**

Several studies have proposed an apoptotic mechanism for the basis of immunodeficiency in ADA and PNP deficiency (Thompson et al., 2003; Arpaia et al., 2000). Considering that apoptosis is the fate of most thymocytes, it is not surprising that this may be the main cause for immune deficiency associated with these disorders.

- **Insights gained from ADA deficiency:**

ADA is another enzyme important for purine degradation and salvage. Importantly, similar to PNP deficiency, ADA-deficient patients suffer from severe T-cell immune deficiency that has been attributed to the accumulation of toxic purine metabolites. Although ADA deficiency is also associated with severe perturbations in other immune parameters, the close similarities between ADA and PNP deficiency suggest similar mechanisms.
Murine fetal thymic organ cultures (FTOC) have been used to investigate how ADA deficiency leads to a failure in thymocyte development (Thompson et al., 2000; Van De Wiele et al., 2002). FTOC is a technique where murine fetal thymic lobes at day 15 of gestation are cultured on cellulose ester filters sitting on gelfoam sponges at the air/media interface for periods of 3-5 days (Jenkinson et al., 1990). FTOCs are usually treated with dGuo to remove thymocytes and leave intact stroma so as to allow the seeded cells (either thymocytes or bone marrow progenitors) to repopulate the thymic niche. FTOCs performed on ADA-deficient fetuses or on normal fetuses treated with a potent ADA inhibitor revealed profound arrest of development at the DN3 stage (Thompson et al., 2000). This was associated with an increase in dATP levels suggesting that dATP is the toxic metabolite in these cultures. Moreover, the thymocytes could be rescued by using an inhibitor of adenosine kinase, an enzyme that phosphorylates dAdo to dAMP (Jenuth et al., 1996). This observed inhibition in differentiation was not due to a failure in β-selection as TCRβ and T-early α locus rearrangement occurred normally (Thompson et al., 2000). Rather, the cells in culture were found to be dying by apoptosis as the cultures could be rescued by inhibiting apoptotic pathways with the pan caspase inhibitor z-VADfmk (Thompson et al., 2000). This rescue was associated with an increase in the survival of cells at the β-selection checkpoint and normalization of dATP levels (Thompson et al., 2000). Similarly, the cultures were also rescued by Bcl-2 over-expression (Thompson et al., 2003). The large number of thymocytes that die coupled with the high ability of the thymus to phosphorylate dAdo and low capacity to dephosphorylate dATP explains the specificity of this organ to accumulate dATP and its high sensitivity to ADA deficiency.
Initial studies concentrated on the effect of ADA deficiency on development of DN thymocytes, recently there is evidence that ADA deficiency also affects DP thymocytes (Van De Wiele et al., 2006). The above results have been extrapolated in PNP deficiency research (discussed below) and have provided a basis for my study.

• **Insights gained from PNP deficiency:**

The specific T-lineage abnormalities characteristic of PNP deficiency have been attributed to the accumulation of dGuo and increased sensitivity to dGTP. Similar to ADA deficiency, studies using PNP\(^{-/-}\) mice demonstrated that thymocyte differentiation is severely affected at the DP stage, with possible increased apoptosis of DP thymocytes, although the exact stage of developmental abnormality had still not been elucidated (Arpaia et al., 2000; Snyder et al., 1997). However, Arpaia et al provided some insight on the mechanism causing this T-cell phenotype. They indeed pinpointed apoptosis as the cause, by demonstrating that Bcl-2 over expression protected against dGuo induced apoptosis in normal murine thymocytes treated with a PNP inhibitor (Arpaia et al., 2000). Further exacerbation of thymocyte damage may result from locally increased toxic purine metabolites released from dying thymocytes.

These findings led them to investigate the possibility that mitochondrial dGTP accumulation may predispose PNP\(^{-/-}\) T-cells to mtDNA damage.

The reasons for this speculation are as follows; firstly, human thymocytes were found to have increased deoxyguanosine kinase (dGK) activity and elevated levels of dGTP compared to peripheral T and B-lymphocytes (Refer Figure 2) (Cohen et al., 1980). dGK is an enzyme that phosphorylates dGuo to dGMP and finally to dGTP and is localized in the mitochondrial membrane (Wang et al., 1996; Johansson et al., 1996), as a consequence designating the site for dGTP formation and its subsequent accumulation. Thus Arpaia et al
postulated that the accumulation of dGTP in the mitochondria of PNP<sup>-/-</sup> T lymphocytes initiates apoptosis by interfering with the repair of mtDNA damage (Arpaia et al., 2000). Further support for this hypothesis came from the observation that mitochondria of PNP expressing thymocytes, incubated with dGuo in the presence of PNP inhibitor accumulated dGTP (Arpaia et al., 2000). Moreover, dissipation of mitochondrial membrane potential (MMP) in these cells was resistant to caspase inhibition, suggesting that the apoptotic signals originated within the mitochondria. Also, increased sensitivity of PNP<sup>-/-</sup> thymocytes and splenic T-cells to irradiation offers further evidence of DNA damage (Arpaia et al., 2000).

The importance of the balance in dNTP precursors for DNA replication and repair has been established for a long time now however, its relevance in human disease has gained momentum only in the past decade. Similar to the hypothesized mechanism of abnormal DNA synthesis and repair in PNP deficiency, studies carried out in thymidine phosphorylase and uridine phosphorylase double knockout mice showed nucleotide pool imbalance in the tissues of these mutant mice and partial mtDNA deletion in their brains (Lopez et al., 2009). Correspondingly, in dGK deficiency, the conversion of dGuo to dGTP does not occur and thus mtDNA reduces proportional to dGTP levels as dGTP is the largest dNTP pool in the mitochondria (Saada et al., 2008). This deficiency is seen to cause a hepatocerebral variant of mtDNA depletion syndromes (Ashley et al., 2007; Saada et al., 2008), further strengthening the importance of mitochondrial dNTP balance in various tissues.

Mechanisms of apoptosis involved in ADA deficiency may also explain apoptosis in PNP deficiency. The extracellular pathway via death receptor signaling was examined in ADA deficiency. T-cells treated with a potent ADA inhibitor and dAdo have demonstrated accumulation of dATP resulting in the inhibition of ribonucleotide reductase and imbalance
in the dNTP pool which leads to abrogation of DNA synthesis and repair (Ullman et al., 1979; Carson et al., 1977; Cohen A, 1986). Apoptotic signals via Fas was eliminated as a possible mechanism, nevertheless there is still some speculation regarding an alternative death receptor pathway (Figure 6, Pathway A) (Thompson et al., 2003). The extrinsic pathway was not studied in PNP deficiency. Another postulated mechanism is that accumulation of dATP in ADA deficiency triggers the release of mitochondrial cytochrome c initiating an apoptotic cascade (Figure 6, Pathway B) (Thompson et al., 2003). Similarly it has been postulated that mtDNA damage may be caused by the accumulation of dGTP in PNP deficiency leading to apoptosis via the release of cytochrome c from the mitochondrial membrane (Figure 6, Pathway B) (Arpaia et al., 2000). It is also possible that accumulated purine metabolites causes nuclear DNA damage which triggers an apoptotic signaling cascade (Figure 6, Pathway C). There is also some evidence for a p53-dependent apoptotic mechanism in ADA deficiency which is preventable by over-expression of Bcl-2 (Benveniste and Cohen, 1995). Of course it is still possible that pro-apoptotic Bcl-2 family members are involved, but through a p53-independent mechanism (Figure 6, Pathway D). Taken together, accumulation of dGTP in PNP deficiency may cause apoptosis by one or more of the above mentioned pathways.
Figure 6: A schematic representation of postulated apoptotic mechanisms caused by ADA and PNP deficiencies (adapted from Thompson et al., 2003). **Pathway A:** abnormal levels of purine metabolites cause induction of death receptor signaling (e.g. Fas). **Pathway B:** accumulated dGTP or dATP causes mitochondrial DNA damage leading to a loss in MMP causing the release of cytochrome c from the mitochondria. **Pathway C:** abnormal levels of purine metabolites lead to nuclear DNA damage finally disrupting the mitochondria, leading to activation of caspases. **Pathway D:** abnormal levels of purine metabolites causes induction of pro-apoptotic Bcl-2 family members, leading to the release of cytochrome c from the mitochondria.
CHAPTER 2: RATIONALE, HYPOTHESIS AND EXPERIMENTAL OBJECTIVES
Rationale, Hypothesis and Experimental Objectives

PNP deficiency in humans is associated with impaired thymic output; however the effect of PNP deficiency on thymocytes has been difficult to study due to the inability to obtain thymi from PNP-deficient patients. Arpaia et al and Snyder et al demonstrated increased numbers of DN thymocytes and reduced numbers of DP and SP thymocytes in PNP-deficient murine models, nevertheless, the thymus was not the prime interest of their studies (Arpaia et al., 2000; Snyder et al., 1997). Therefore little is known on the precise stage(s), cause(s) and mechanism by which PNP deficiency induces abnormality in thymocyte development, which was the focus of this study.

2.1. Rationale

The increased number of DN thymocytes and reduced number of DP thymocytes found in PNP-deficient mice could be due to a defect in one or more of the developmental stages described in Figure 7. However, two major reasons, outlined below, led us to speculate that the developmental delay is in the later DN stages, more specifically between the DN3 to DN4 stage transition. ADA deficiency, which as described earlier shares many metabolic and immunological features with PNP deficiency, has been shown to disrupt thymocyte development early within the DN3 stage. Thymocytes taken from 15-day old ADA-deficient mice and grown ex-vivo on FTOCs, revealed profound reduction in the number of CD44^-CD25^+ DN3 thymocytes (Thompson et al., 2000). Secondly, the transition between DN3 to DN4 is a major “check-point” in thymocyte maturation. Many immunodeficiency models (targeted deletions in Rag, pre-TCRα, TCRβ genes, lymphocyte specific tyrosine kinase-
Lck) associated with reduced thymic cellularity similar to PNP deficiency, affect the development of thymocytes between the DN3 to DN4 stage, which is the stage of β-selection (Godfrey et al., 1994; Levin et al., 1993; Fehling et al., 1995; Shinkai et al., 1992; Mombaerts et al., 1992). Therefore we hypothesized that a developmental delay between the DN3 to DN4 transition is the most plausible explanation for the reduced numbers of DP thymocytes associated with PNP deficiency.

Figure 7: Stages in murine thymocyte development. The red bar indicates the most likely stage at which thymocyte development is affected in PNP deficiency.

In addition to the increase in the number of DN thymocytes, there is a striking reduction in the number of DP cells in the thymus of PNP−/− mice, which could be caused by:

1. Decreased proliferation of early thymocytes, as previous studies have shown reduced proliferation of T-lymphocytes isolated from PNP-deficient patients and mice (Toro et al., 2006, Arpaia et al., 2000, Markert et al., 1991) as well as lymphocytes isolated from
patients treated in vitro or in vivo with chemical PNP inhibitors (Bantia et al., 2003; Conry et al., 1998; Gandhi et al., 2005; Kicska et al., 2001).

2. *Reduction in the percentage of cycling cells due to the effect of toxic purine metabolites.* Indeed, Thompson et al showed that the percentages of cycling cells are reduced in ADA-deficient cultures (Thompson et al., 2000). This finding is consistent with the reduced capacity of ADA deficient cultures to reach the CD44- CD25- DN4 stage that normally contains the highest percentage of rapidly cycling thymocytes in the DN populations (Tourigny et al., 1997).

3. *Increased apoptosis of DP thymocytes.* This is the most likely explanation as majority of normal DP thymocytes undergo apoptosis due to positive and negative selection (Surh and Sprent, 1994; Ohashi, 2003). In fact, Arpaia et al showed increased apoptosis in normal thymocytes that were treated with a PNP inhibitor and dGuo (Arpaia et al., 2000), and T-cell lines treated with BCX-1777 and dGuo also revealed significant apoptosis (Bantia et al., 2003). Furthermore, there is extensive apoptosis in the thymus of ADA-deficient mice (Apasov et al., 2001) and also in ADA-deficient thymocytes grown in FTOCs (Thompson et al., 2000; Van de Wiele et al., 2006).

Among the metabolic abnormalities found in human and murine PNP deficiency which may lead to increased apoptosis and reduced number of DP cells, we and others hypothesized that accumulation of dGuo, or a phosphorylation product of dGuo, such as dGTP is the most likely cause. Among the four substrates of the PNP enzyme (Figure 8), dGuo is the only substrate that has an alternate metabolic fate, as it can be phosphorylated by cytosolic dCK or mitochondrial dGK to form dGTP. Increased dGK activity has been observed in human thymocytes (Cohen et al., 1980) thus possibly explaining the preferential accumulation of
dGTP in thymocytes. Additionally, dGuo is also the only PNP substrate that has specifically been able to inhibit the growth of T-cells, which was associated with elevated dGTP levels in the cells (Gudas et al., 1978; Ullman et al., 1979). Furthermore, the inhibitory effect of dGuo was abolished by either blocking dGuo uptake into the cells or by inhibiting the enzymes dCK and dGK, thereby preventing the formation of dGTP (Ullman et al., 1979). These results are indicative that the formation of dGTP, secondary to the accumulation of dGuo, may be the actual cause for apoptosis.

The central role of dGTP (that is produced by dGK, an exclusive mitochondrial enzyme) in PNP-induced apoptosis suggests a mitochondrial-dependent apoptotic pathway (Figure 9). This is further supported by the observations that dGTP accumulated in the mitochondria of normal thymocytes treated with dGuo and a PNP inhibitor, that the apoptosis of these thymocytes was initiated in the mitochondria and that it could be inhibited by over-expression of Bcl-2, an anti-apoptotic factor localized primarily in the mitochondria and

Figure 8: *Schematic representation of the PNP pathway.* PNP substrates are depicted in the box.
important for maintaining mitochondrial membrane integrity (Arpaia et al., 2000). In
addition, similar involvement of the mitochondria was demonstrated also in ADA deficiency,
in which dATP accumulation induced a mitochondria-mediated apoptosis possibly by the
release of cytochrome c (Van de Wiele et al., 2002).

**Figure 9: Proposed mechanism of PNP deficiency.** The accumulated dGuo is
phosphorylated by mitochondrial dGK to dGTP which may interfere with mtDNA synthesis
and repair either directly or by inhibition of the mitochondrial ribonucleotide reductase
(mRR) eventually leading to apoptosis by the release of cytochrome C (cytC).
2.2. Hypotheses

Based on the rationale presented above, we hypothesized that PNP deficiency interferes with thymocyte development during the transition from the DN3 to the DN4 stage. In addition, we hypothesized that the reduced DP thymocytes was due to increased apoptosis induced by the accumulation of dGuo. We also hypothesized that the increased apoptosis of DP thymocytes in PNP deficiency is initiated in the mitochondria.

2.3. Specific Objectives

The overall objective of this work was to understand the effect of PNP deficiency on thymocyte development by using a PNP-deficient murine model.

The specific aims of this project were;

1) To analyse the effect of PNP deficiency on thymocyte development during the DN3 to DN4 stage transition.

2) To examine whether the reduced number of DP thymocytes is caused by increased apoptosis that is induced by the accumulation of dGuo.

3) To determine whether apoptosis of PNP-deficient DP thymocytes is initiated in the mitochondria.
CHAPTER 3: MATERIALS AND METHODS
Materials and Methods

3.1. Animals:

PNP-/- mice (C57/BL6 background) in which the catalytic domain of PNP was replaced by a neomycin cassette (Arpaia et al. 2000) and normal controls were maintained in a pathogen free environment at the Sick Kids Hospital Research Institute’s animal facility. Mice used in all the experiments were 6-8 weeks old. PNP deficiency was confirmed by the absence of PNP activity in the whole blood samples obtained from the tail of 18-20 day old mice. All procedures and experiments involving animals were approved by the Institutional Animal Care Committee of the Hospital for Sick Children, Toronto, Canada.

3.2. PNP enzyme activity:

PNP enzyme activity was determined by measuring conversion of inosine to hypoxanthine and has been previously described (Arpaia et al., 2000). Briefly, 14C-labeled inosine (Movarek Biochemicals, Brea, CA) was added to whole cell lysate and incubated at 37°C followed by a brief denaturation of the enzyme at 100°C for 1 min. The unconverted substrate and the product were separated by thin-layer chromatography (Fischer Scientific, Ottawa, ON) and measured using scintillation counting. One unit was defined as the amount of enzyme required to convert 1μmol of inosine to hypoxanthine in 1 min at 37°C.

3.3. Cell culture and media:

3.3.1. Thymocyte cultures were maintained for 8-24 hours in RPMI 1640 (Invitrogen, Burlington, ON) containing 5% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, Utah).
3.3.2. Jurkat cells were maintained in RPMI 1640 supplemented with 10% FBS.

3.3.3. OP9-DL1 cells were kindly provided by J.C. Zúñiga-Pflücker (University of Toronto, Toronto, Canada). OP9-DL1 and OP9-DL1 co-cultures were maintained in α-MEM (Invitrogen, Burlington, ON) supplemented with 20% FBS as previously described (Awong et al., 2008).

All culture media was supplemented with 1% penicillin/streptomycin (Invitrogen, Burlington, ON).

3.4. Antibodies and reagents:

3.4.1. Antibodies: Antibodies used in this study were biotin conjugated anti-mouse Gr-1, CD11c, CD3ε, NK1.1, TER-119, CD117, CD25, CD11b, B220, TCRβ, CD19, Mac-1, CD4, CD8, pycoerythrin (PE) conjugated anti-CD4 and anti-CD25, anti-CD71 and anti-Fas (eBioscience), fluorescein isothiocyanate (FITC) conjugated anti-CD8 and anti-CD25, and allophycocyanin (APC) conjugated anti-TCRβ (eBioscience). All biotin conjugated anti-mouse monoclonal antibodies were purchased from eBioscience (San Diego, CA) while APC conjugated anti-biotin antibody was from Miltenyi biotec (Auburn, CA). All other antibody-fluorochrome conjugates were purchased from BD Pharmingen (Mississauga, Ontario).

3.4.2. Pan caspase inhibitor: N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (z-VADfmk) was purchased from Santa Cruz Biotechnology (San Diego, CA).

3.4.3. MitoTracker® Red CMXRos:

Assessment of MMP was performed using MitoTracker® Red CMXRos (Molecular Probes, Invitrogen, Carlsbad, CA). This is a cell-permanent probe.
containing a mildly thiol-reactive chloromethyl moiety for labeling mitochondria. When it enters an actively respiring cell, it is oxidized and sequestered in the mitochondria, where it reacts with thiols on proteins and peptides to form an aldehyde-fixable conjugate.

3.4.4. **Apoptosis Inducing agents:**

- dGuo, dGMP and dGTP (Sigma Aldrich, Oakville, ON) were dissolved in 10mM NaOH.
- Dexamethasone (Dex) was purchased from Sigma Aldrich and dissolved in dimethyl sulphoxide (DMSO, Sigma Aldrich).

3.4.5. **PNP inhibitor:**

The PNP inhibitor BCX-1777 (Forodesine hydrochloride) was kindly provided by BioCryst (Durham, NC). 10mM solutions were prepared in DMSO.

3.5 **Flow cytometry:**

1-2x10^6 cells were washed and resuspended in staining buffer (PBS containing 1% FBS). Antibodies conjugated to PE, FITC and APC were added after careful titration. Cells were stained on ice for 30 minutes, and then washed with staining buffer. Live cells were identified by PI exclusion and by forward and side scatter profiles. All multicolour flow cytometric analysis was carried out on a dual laser FACScanLibur (Becton Dickinson (BD), Franklin Lakes, NJ), and data acquired was imported into either CellQuest (BD) or FlowJo (Tree Star, Ashland, OR) software for analysis.
3.6. **Bone marrow (BM) cell isolation:**

BM was isolated from the tibias and femurs of PNP−/− mice and age matched controls. The bones were crushed and passed through a 40μM nylon mesh filter to get a single cell suspension in α-MEM containing 1% FBS. Following erythrocyte lysis for 5 minutes with ammonium chloride (Stemcell Technologies, Vancouver, BC) and subsequent washes with 1x PBS (Invitrogen, Burlington, ON) supplemented with 1% FBS, the bone marrow cells were counted using a hemacytometer. 40x10^6 cells were taken for lineage depletion.

**Lineage depletion:**

Lineage (Lin) depletion was carried out using a mouse lineage cell depletion kit (Miltenyi Biotec), according to manufacturer’s protocol. Briefly, cells were labeled with biotin-conjugated monoclonal antibodies to CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4 and Ter-119, followed by anti-biotin antibodies conjugated to magnetic microbeads. The magnetically labeled cells were then passed through MS MACS column (Miltenyi Biotec) and separated in a magnetic field. The Lin-negative cells passed through the column while the positive cells were retained. Lin-negative cells were taken for co-culture experiments.

3.7. **OP9-DL1 co-cultures:**

OP9-DL1 co-cultures, maintained in α-MEM medium supplemented with 20% FBS, were carried out as previously described (Schmitt and Zúñiga-Pflücker, 2002). Briefly, 10^5 Lin-depleted BM cells or freshly isolated thymocytes were plated onto each well of a 6-well plate containing 90% confluent OP9-DL1 cells. IL-7 and Flt3L (R&D Systems, Minneapolis, MN) were added into the culture medium at concentrations of 1ng/ml and 5ng/ml respectively. Every 4-5 days the co-cultures were disrupted by pipette mediated
disaggregation and passed through a 40μM cell strainer (BD Falcon, Bedford, MA) to remove residual OP9-DL1 cells. Cells were pelleted and replated with fresh media and cytokines, onto fresh OP9-DL1 cultures. The BM co-culture was analyzed by flow cytometry every 7 days.

**OP9-DL1 cells** were maintained in α-MEM supplemented with 20% FBS. Culture plates containing confluent monolayers of OP9-DL1 cells were trypsinized using 0.25% Trypsin-EDTA (Invitrogen, Burlington, ON), pelleted down and resuspended into fresh medium. The cells were split into 4 parts into 10cm dishes (BD Falcon) for further maintenance and split every other day.

### 3.8. Thymocyte isolation:

PNP−/− mice and age matched controls were sacrificed by cervical dislocation. The skin surface was sterilized using 70% ethanol and removed to expose the rib cage. The rib cage was cut bilaterally through the costochondral junction exposing the thymus. The thymus, which is found to overlay the heart was removed using sterile surgical instruments. To prepare a single cell suspension of thymocytes, the thymus was crushed through a 40 μM nylon mesh filter placed in a petri dish containing α-MEM using the tip of a 3ml syringe (BD, Franklin Lakes, NJ). The single cell suspension was transferred into a 15 ml falcon tube and centrifuged. The residual erythrocytes in the resulting cell pellet were lysed by hypotonic lysis in ice-cold ammonium chloride for 5 minutes and subsequently washed with PBS containing 1% FBS. Live thymocytes were counted by trypan blue exclusion and the cells were taken for culture or analysis by flow cytometry.
3.9. **BrdU proliferation assay:**

In this method, 5-bromo-2-deoxyuridine (BrdU), which is an analog of the DNA precursor thymidine, is incorporated into newly synthesized DNA. The incorporated BrdU was stained with specific anti-BrdU fluorescent antibodies. The levels of BrdU incorporated by the cells were then visualized by flow cytometry.

**In vivo:**

PNP−/− and age matched controls received two intraperitoneal injections of BrdU (1 mg each, 4 hours apart). Thymocytes were isolated 1, 3 and 5 days later and stained for CD4 (PE), CD8 (APC) and anti-BrdU (FITC) as described in the previous section. BrdU incorporation into the thymocytes was detected with a BrdU flow kit (BD Biosciences, San Jose, CA) according to the Manufacturer’s instructions.

**In vitro:**

BrdU was added to the BM co-culture to obtain a final concentration of 10μM. The following day the cells were stained for CD4 (PE), CD8 (APC) and anti-BrdU (FITC) antibodies and analyzed by flow cytometry.

3.10. **Analysis of DN thymocytes:**

3.10.1. **Analysis of DN1-DN4 subsets:**

For analysis of DN thymocyte populations, single cell suspensions of thymus (1-2x10^6 cells/sample) were incubated on ice for 30 minutes with biotinylated antibodies specific for CD4, CD8ε, TER-119, B220, Mac-1, CD3ε, TCRβ, CD11b, CD11c, CD19 and Gr-1. Cells were then washed and stained with anti-biotin microbeads (Miltenyi Biotec) for 15 minutes at 4-8 °C. The prepared cell suspension was added to MS MACS columns and the unbound cells were collected in a tube. The tubes were
centrifuged to obtain a cell pellet which was then appropriately diluted for staining. The cells were stained with fluorochrome conjugated antibodies specific for CD25 (FITC) and CD44 (PE) and incubated on ice for 25 minutes. Cells were then washed and resuspended in staining buffer and analysed by flow cytometry.

3.10.2. Expansion of DN3 thymocytes ex-vivo:

Single cell suspensions of thymus from 6-8 week old mice were prepared and $10^7$ cells/sample were incubated on ice for 30 minutes with biotinylated antibodies specific for CD4, CD8ε, TER-119, B220, CD19, CD3ε, CD11b, and CD117. Cells were washed and subsequently incubated with anti-biotin microbeads and mature cells depleted using MS MACS columns in a magnetic field according to manufacturer’s instructions. The enriched fraction which is mainly CD25+ was plated onto OP9-DL1 cultures in which PNP was inhibited using 20 μM BCX-1777 and 50μM dGuo contained in a 24-well plate. The co-culture was supplemented with 5ng/ml of IL-7 and was analyzed three days later by double staining with either anti-CD4 (PE) and anti-CD8 (FITC) or anti-CD25 (FITC) and anti-CD71 (PE).

3.10.3. Analysis of intracellular TCRb expression on freshly isolated DN thymocytes:

Another method used to study the DN4 subpopulation involved isolating the desired DN subsets from fresh thymocytes as described above. Subsequently, the cells were stained with anti-CD25 (FITC) followed by fixation and permeabilization using BD cytofix/cytoperm kit (BD Biosciences). The fixed cells were stained for intracellular
TCRβ by staining with APC conjugated anti-TCRβ followed by analysis by flow cytometry.

3.11. **Apoptosis studies:**

3.11.1. **Annexin-V staining:**

Thymocytes (5x10^6 cells/ml) were cultured in RPMI media containing 5% FBS and treated with increasing concentrations of dGTP, dGMP and dGuo for 8-24 hrs. Apoptosis was detected by Annexin staining using the Annexin V-FITC Apoptosis Detection Kit from Biovision (Mountain View, CA), according to manufacturer’s protocol. In some experiments, thymocytes were stained with a combination of PE and APC conjugated antibodies before Annexin-V staining.

3.11.2. **Analysis of nuclear DNA fragmentation by TUNEL assay:**

Apoptotic nuclear DNA fragmentation was measured by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique using a FITC conjugated dUTP kit (Roche Applied Science, Laval, Quebec) according to manufacturer’s instructions. The frequency of apoptotic cells as detected by fragmented nuclear DNA was determined by flow cytometry.

3.11.3. **Inhibition of apoptosis using a pan-caspase inhibitor:**

Apoptosis was inhibited by pre-incubating thymocytes for 30 minutes with 50μM z-VADfmk followed by induction of apoptosis using either 12.5μM dGuo or 3.75μM Dex. The cells were cultured for 20-24 hrs, stained for Annexin-V, and analyzed by flow cytometry.
3.12. **Mitochondrial membrane potential (MMP):**

Thymocytes were incubated with 25nM MitoTracker® Red CMXRos in α-MEM containing 1% FBS at 37°C for 15-20 mins in the dark. The cells were washed and resuspended in staining buffer and analyzed by flow cytometry. Cell debris was electronically gated out based on the forward and side scatter. For experiments involving staining of cell surface antigens, MitoTracker® staining was carried out initially followed by staining for the cell surface markers.

3.13. **Re-introduction of PNP into PNP<sup>−/−</sup> cells:**

PNP<sup>−/−</sup> thymocytes were treated with 1μg/ml of TAT-PNP for 1 hour followed by induction of apoptosis using either 12.5μM dGuo or 125μM dGMP. Thymocytes were incubated for 20-24 hrs and analyzed by flow cytometry.

**TAT-PNP Production:** TAT-PNP was produced as previously described (Toro. A et al., 2006) with slight modifications. Briefly, PTD-PNP inserted into a pRSET vector with an N-terminal histidine tag was expressed in BL21(DE3) *E.coli*. PTD-PNP transformed bacteria were grown in Overnight Express™ Instant TB Medium (Novagen, Madison, WI), pelleted down and lysed using Bugbuster® Master Mix (Novagen). The lysate was applied to a TALON resin column (Clontech Laboratories Inc., Mountain View, CA) and washed with 15mM imidazole buffer (50mM sodium phosphate, 300mM NaCl). The proteins bound to the resin were eluted using 250mM imidazole buffer. The proteins were desalted using PD-10 columns (GE Healthcare, Piscataway, NJ) and concentrated using Amicon® ultra-15 centrifugal filter units (Millipore, Massachusetts). Protein concentration was determined by the BioRad assay with bovine serum albumin as a standard. Purity of the TAT-PNP preparation was determined by SDS-PAGE using a 10-12% tris glycine gel (Invitrogen) and
western blotting with rabbit anti-human PNP antibodies generated by our lab (Toro et al., 2006). The biological activity of the purified proteins was determined by assaying for PNP activity (as described earlier).

3.14. **Viable cell counting:**

Viable cells were counted using a hemacytometer (Hausser Scientific, Horsham Rd, PA). The cells were diluted accordingly and taken for trypan blue staining (Invitrogen). 10μl of this mixture was added to the wells of the hemacytometer and the number of cells overlying four 1mm² areas of the counting chamber was counted using a hand counter. Cell numbers were calculated using the formula;

\[
\text{Total cells counted in 4mm}^2 / 4 \times (\text{dilution factor}) \times 10^4 = \text{cells/ml}
\]

3.15. **Statistical analysis:**

Statistical analysis was carried out using a two-tailed Student’s t-test where applicable. The sample size (n) is indicated for each experiment. Differences were considered significant if the p value was less than 0.05. Statistical analyses were performed using the Microsoft Excel software.
CHAPTER 4: RESULTS
4.1. Abnormal lymphocyte subpopulation in thymus of PNP<sup>−/−</sup> mice.

We have consistently shown an increase in the percentage of CD<sup>4+</sup>CD<sup>8−</sup> DN thymocytes and decrease in the percentage of CD<sup>4−</sup>CD<sup>8+</sup> DP thymocytes in PNP<sup>−/−</sup> mice compared to normal littermates (Figure 10A). Furthermore, there was also an increase in the absolute number of DN cells with a significant decrease in the total numbers of DP thymocytes and CD<sup>4+</sup> and CD<sup>8+</sup> SP thymocytes in PNP<sup>−/−</sup> mice (Figure 10B- note the logarithmic scale).
Figure 10: Distribution of thymocyte populations in PNP<sup>−/−</sup> mice. Single cell suspensions of thymocytes from 6-week old PNP<sup>−/−</sup> mice and age matched controls were stained with anti-CD4 (PE) and anti-CD8 (FITC) antibody. (A) Percentage of cells in different subpopulations is indicated in the respective quadrants. The plot is representative of 9 independent experiments with one or two mice in each. (B) The graph displays the mean ± SD of the total number of cells in each thymocyte subpopulation isolated from PNP<sup>−/−</sup> mice (white bars) and PNP<sup>+/+</sup> controls (black bars) (n= 9 in each, ** p=0.0001, * p<0.001).
**Aim 1: To determine whether PNP deficiency interferes with the transition of thymocytes from the DN3 to the DN4 stage.**

4.2. **PNP deficiency disrupts thymocyte maturation at the DN3 to DN4 stage transition.**

ADA deficiency is another inherited defect in purine metabolism similar to PNP that causes abnormal thymocyte development. It was previously shown that ADA deficiency interferes with maturation of DN3 cells (Thompson et al., 2000). Therefore, we hypothesized that PNP deficiency also affects development of late DN cells.

To identify the effects of PNP deficiency on differentiation of DN thymocytes, and to tease out the stage at which development was being inhibited, several strategies were used;

1) To specifically study the DN thymocyte populations, DN cells were isolated by column purification and analyzed for the expression of CD25 and CD44. A representative example is provided in Figure 11A, demonstrating higher percentage of DN3 cells in thymi of PNP\(^{-/-}\) mice. Calculation of the absolute numbers of thymocyte subsets confirmed that DN3 cells were significantly increased in thymi from PNP\(^{-/-}\) mice (Figure 11B).
Figure 11: Characterization of maturation of DN thymocytes in PNP-deficient mice.

CD25 and CD44 expression were compared in freshly isolated thymocytes from PNP−/− mice and age matched controls. Single cell suspensions were stained with biotin-labeled antibodies against CD4, CD8, CD3, TER-119, NK1.1, CD19, B220, CD11b and Gr-1 followed by anti-biotin microbeads and separated using MS MACS columns. The resulting DN fraction was stained with anti-CD44 (PE) and anti-CD25 (FITC). (A) Representative data show expression of CD25 and CD44 in PNP−/− thymocytes and age matched controls. (B) Data show DN thymocyte subpopulation numbers in PNP−/− mice (white bars) and controls (black bars). Cells were characterized as CD44+CD25− (DN1), CD44+CD25+ (DN2), CD44−CD25+ (DN3) and CD44−CD25− (DN4). Data is representative of 5 independent experiments with one animal in each (*p =0.01, **p=0.004).
2) The strategy described above, in which thymocytes were collected at one point in time, allowed us to study thymocyte development at a steady state. To study development of thymocytes over time, I used a different strategy, which relied on expanding T-cells in vitro from normal BM derived stem cells using the OP9-DL1 co-culture technique. The pharmacological inhibitor of PNP (BCX-1777) was used to prevent the degradation of toxic purine metabolites by OP9-DL1 cells thereby simulating PNP-deficient conditions in vitro. dGuo was also added to the cultures to simulate the excess dGuo found in PNP−/− mice. dGuo or inhibitor added separately did not have any effect on the development of cells (data not shown). During the first week of culture, when most of the cells were still in the early DN stages (DN1 and DN2), there were no significant differences between cultures with normal or abnormal purine metabolism (Figure 12A). This finding suggested that early DN differentiation is not affected by PNP deficiency which was in concordance with our in vivo findings. In contrast, by 2-3 weeks of co-culture, when majority of the developing cells had matured into the later DN stages, a significant increase in the percentage of DN3 cells was evident in the PNP-deficient cultures (Figure 12B). Of note, CD4+ and CD8+ cells were not excluded from analysis, therefore our strategy can not definitely distinguish between abnormal DN3 to DN4 development or abnormal DN4 to DP development since the unstained fraction in the DN analysis constitutes DN4, DP and SP cells. Nevertheless, the significantly increased percentage of CD25+ DN3 cells and the markedly reduced percentage of DP cells in cultures with PNP-deficient conditions (Figure 12C), suggests that the confounding factors described above may have not been significant.
Figure 12: *Characterization of maturation of DN thymocytes under PNP-deficient conditions using OP9-DL1 co-culture*. BM cells were obtained and lineage depleted using MS MACS columns. Lin- cells were seeded onto near-confluent OP9-DL1 monolayers and were allowed to develop over a period of 3 weeks. Cells were harvested after (A) 7 days and (B) 21 days and analyzed for expression of CD25 (FITC) and CD44 (PE) (*p=0.0004, **p=0.0001). (C) Cells were analyzed for CD8 (FITC) and CD4 (PE) expression on day 21 of co-culture. Data represents the mean ± SD of 3 independent experiments. White bars indicate PNP+/+ BM development in PNP-deficient environment, black bars represent PNP+/+ BM development in PNP-proficient environment (dG+I, 50μM dGuo+ 20μM inhibitor).
3) In order to mimic *ex vivo* conditions in PNP+/+ mice more closely, a third strategy was used to assess the effects of abnormal purine metabolism in PNP deficiency by following the development of T-cells *in vitro* from Lin⁻ PNP+/+ BM (in contrast to PNP+/+ BM in strategy #2) cells using OP9-DL1 stromal cells (in contrast to the strategy 2 which tested normal BM). This strategy avoided the use of the chemical PNP inhibitor (BCX-1777) and PNP-deficient conditions were simulated using only excess dGuo (75μM vs 50μM in strategy 2). Similar to the *in vitro* findings in strategy 2, in this strategy, by the third week of co-culture, when most of the cells had transited to the later DN stages and the DP stage, PNP-deficient BM cultures had a higher percentage of cells in the DN3 stage compared to normal BM cultures (Figure 13A, 13C). Further, analysis of the DP and SP stages revealed a lower percentage of cells in the DP stage in the PNP-deficient BM cultures (Figure 13B), an effect that is markedly exacerbated in the presence of 75μM dGuo both in normal and PNP-deficient conditions. This suggests that PNP deficiency and the accumulation of dGuo interferes predominantly after reaching the DN3 stage. The caveats mentioned in strategy 2 also apply to this method.
Figure 13: Characterization of maturation of DN thymocytes under PNP-deficient conditions using excess dGuo. PNP$^{+/+}$ and PNP$^{+/+}$ Lin$^{-}$ BM was cultured on OP9-DL1 cells and analyzed for the expression of CD25/CD44 and CD4/CD8 after 18 days of co-culture. (A) Data is representative of CD25 (FITC) vs CD44 (PE) profiles. (B) Data is representative of CD4 (PE) vs CD8 (APC) profiles. Data is representative of two independent experiments. Percentage of cells in different subpopulations is indicated in the respective quadrants. (C) Graph is a representative of two independent experiments and depicts the percentage of DN subsets in PNP$^{+/+}$ BM (black bar) and PNP$^{-/-}$ BM (white bar) co-cultures after 18 days.
4) The fourth strategy employed to specifically delineate the DN3 and DN4 transition relied on measuring CD71 expression. The upregulation of CD71, the transferrin receptor, mediates iron uptake and is also critical for cell growth (Schneider et al, 1982; Trowbridge and Shackelford, 1986). Most DN3 thymocytes express low levels of CD71, whereas surface expression of this nutrient transporter is high in DN4 cells. Progression through β-selection is thus accompanied by upregulation of CD71 (Kelly et al., 2007). DN3 thymocytes isolated from PNP−/− and PNP+/+ mice were co-cultured for 3 days on OP9-DL1 cells in which PNP deficiency was simulated using BCX-1777 and dGuo as mentioned earlier. The limitation of this study is that the negative fraction obtained after column purification was enriched for DN3 and was not a homogeneous DN3 population as the technique employed does not allow for removal of DN4 cells. This may have been the reason why I was unable to detect differences in CD71 upregulation among DN4+ cells, and in the development of DP cells (Figure 14A and 14B). A more appropriate experiment would be to sort for DN3 cells by flow cytometry before plating onto OP9-DL1 cells, but one can argue that these cells are now labeled and can skew the experimental results. Further optimization is required to draw any conclusions from these experiments.

5) The DN4 subset is difficult to delineate due to the absence of a defined cell surface marker. Therefore, I employed another strategy which examined intracellular TCRβ expression. Early DN3 cells do not express TCRβ chain intracellularly, late DN3 cells start expressing TCRβ and DN4 cells will express intracellular TCRβ chain. The results indicate an increased percentage of early DN3 cells in PNP−/− mice with a significant reduction in the percentage of DN4 cells expressing TCRβ intracellularly compared to normal controls using
lineage depleted freshly isolated thymocytes (Figure 15A, 15B). This is indicative that the percentage of cells transiting from the DN3 to the DN4 stage is reduced in PNP⁻/⁻ mice compared to normal controls.

**A)**

![Image of flow cytometry plots](image1)

**B)**

![Image of flow cytometry plots](image2)

**Figure 14:** *Characterization of DN3 and DN4 subsets by upregulation of CD71 expression.* DN3 thymocytes were isolated from PNP⁻/⁻ mice and normal controls by depleting for CD4, CD8, CD3, CD11b, B220, CD19, TER-119, Gr-1 and CD117 positive cells using MS MACS columns, and the enriched DN3 fraction was co-cultured on OP9-DL1 cells for 3 days. The plot is representative of (A) CD71 (PE) vs CD25 (FITC) profiles and (B) CD4 (PE) vs CD8 (FITC) profiles after 3 days of co-culture for 5 independent experiments with one animal in each group.
Figure 15: Characterization of the DN3 and DN4 subsets by intracellular TCRβ staining.

DN3 and DN4 cells (characterized as negative for CD4, CD8, CD3, CD11b, B220, CD19, TER-119, Gr-1 and CD117) were isolated from PNP<sup>-/-</sup> mice and normal controls and stained for CD25 (FITC) and intracellular TCRβ (APC). (A) Data is representative of one of four experiments. Percentage of cells is indicated in each quadrant. (B) The graph depicts the mean ±SD of the percentage of DN3 and DN4 cells in PNP<sup>-/-</sup> mice (white) and PNP<sup>+/+</sup> mice (black). Data is representative of 4 independent experiments with one animal in each group (*p=0.0005).
CONCLUSION

The above data is indicative that abnormal PNP function and accumulation of dGuo affects thymocyte development within the DN3 stage or at the transition from DN3 to the DN4 and DP compartments. However, thus far I have not been able to identify the cause of abnormal DN development.
**Aim 2: To determine whether the reduced number of DP thymocytes in PNP deficiency is due to increased apoptosis caused by dGuo.**

4.3. **Increased apoptosis in freshly isolated PNP−/− DP thymocytes.**

As described earlier, in addition to the decreased percentage of DN thymocytes in PNP deficiency, there is also a marked reduction in the number and percentage of DP thymocytes. We hypothesized that the reduced DP could be caused by increased apoptosis. Therefore I initially measured apoptosis in freshly isolated thymocytes by flow cytometry. I found higher apoptosis (as measured by Annexin-V staining) in PNP−/− DP thymocytes than in PNP+/+ thymocytes (Figure 16A), as previously reported by Arpaia et al (Arpaia et al., 2000), which was statistically significant (p=0.02) although not dramatic (Figure 16B). This moderate increase in apoptosis can probably be attributed to the ability of resident macrophages to rapidly clear apoptotic thymocytes. No difference in apoptosis of DN and CD4+ or CD8+ SP populations was found between controls and PNP−/− mice.
Figure 16: *Increased apoptosis in freshly isolated DP thymocytes from PNP-deficient mice.* Freshly isolated thymocytes were analyzed by three colour flow cytometry for CD8 (APC), CD4 (PE) and Annexin-V (FITC). (A) Data is representative of Annexin positive cells among CD4⁺CD8⁺ DP thymocytes isolated from PNP⁺/⁺ (black) or PNP⁻/⁻ (white) mice. Percentage of apoptotic cells are indicated above the bars. (B) Data represents mean ± SD of apoptotic DP thymocytes (n=5 in each, *p=0.02).
4.4. **PNP Deficiency does not affect proliferation or the maturation of DP thymocytes.**

The most obvious consequence of PNP deficiency on thymocyte development is the reduction in the DP thymocytes. We reasoned that although increased apoptosis is the most likely cause for the DP phenotype observed in PNP deficiency, the reduction in DP thymocytes could also reflect a defect in expansion of DN precursors or reduced maturation of DP thymocytes. Therefore, I monitored the generation and subsequent maturation of PNP\(^{-/-}\) DP thymocytes 1 to 5 days after BrdU injection. One day after BrdU injection, the majority of PNP\(^{+/+}\) and PNP\(^{-/-}\) precursors that were labeled with BrdU were DP cells, in concordance with previous reports (Lucas et al., 1993; Matei et al., 2006) (Figures 17A, 17B). Similar proportions of DN and DP thymocytes from PNP\(^{-/-}\) and PNP\(^{+/+}\) mice incorporated BrdU one day after BrdU injection, demonstrating that PNP deficiency did not affect thymocyte proliferation or the initial generation of DP thymocytes. The percentage of BrdU labeled progeny becoming DP cells decreased dramatically over the next 5 days as some DP matured into SP thymocytes, while others died (Figures 17A, 17B, 17C). Importantly, the percentage of PNP\(^{-/-}\) precursors becoming SP thymocytes was similar to control after 5 days, indicating that maturation of DP into SP was not affected by PNP deficiency. Of note is the marked reduction in the percentage of BrdU\(^{+}\) DP thymocytes in PNP\(^{-/-}\) mice 5 days after BrdU injection (Figure 17C). This observation, together with the normal percentage of BrdU\(^{+}\) SP PNP\(^{-/-}\) thymocytes, indicates accelerated death of PNP\(^{-/-}\) DP cells which further supports our previous observations.

In conclusion, our data clearly demonstrates that the reduced numbers of DP thymocytes in PNP deficiency is due to a survival defect and not due to decreased proliferation of thymocyte precursors or defect in maturation of DP thymocytes.
A)

Day 1

Day 3

Day 5

B)

Day 1

Day 3

Day 5
Figure 17: BrdU labeling profiles for PNP^{−/−} mice show normal thymocyte proliferation but indicate a survival defect most strikingly obvious on day 5 after labeling. (A) Histograms represent BrdU labeling of total thymocytes from PNP^{−/−} mice and age matched controls at the indicated time points after BrdU injection. (B) The panels depict CD4 vs CD8 thymocyte profiles of PNP^{−/−} and PNP^{+/+} mice gated on BrdU^{+} cells at days 1, 3 and 5 after injection. Representative results of 3 independent experiments with one or two mice in each. (C) Graph represents mean ±SD of percentage of BrdU^{+} SP and DP thymocytes in PNP^{+/+} (black) and PNP^{−/−} (white) mice after 5 days of BrdU injection (n=3 in each,*p=0.001).
4.5. **dGuo causes increased thymocyte apoptosis.**

After determining that PNP deficiency induces thymocyte apoptosis, I was interested to understand the mechanism for this effect. It was thus hypothesized that the increased apoptosis of DP thymocytes is caused by accumulation of toxic PNP substrates, such as dGuo and its subsequent conversion to dGTP, and not by depletion of PNP products such as guanine (Gua) or GTP (Figure 18).

Figure 18: *Schematic representation of the role of PNP in purine degradation and salvage.* PNP catalyzes the phosphorolysis of dGuo to Gua. In the absence of PNP, dGuo accumulates and is phosphorylated by mitochondrial dGK eventually converting it into dGTP. dGTP inhibits mitochondrial ribonucleotide reductase causing mtDNA damage ultimately leading to apoptosis. PNP deficiency also prevents the formation of PNP products such as Gua, resulting in depletion of GTP.
I therefore determined the effects of dGuo on PNP-deficient thymocytes. PNP<sup>−/−</sup> thymocytes were cultured with increasing concentrations of dGuo for 24 hours and apoptosis was measured by flow cytometry after staining with Annexin-V. A dose-dependant increase in apoptosis was observed in PNP<sup>−/−</sup> thymocytes compared to PNP<sup>+/+</sup> thymocytes, most evident at 6.25 μM and 12.5 μM dGuo (Figure 19A), concentrations which are similar to those found in the serum of PNP-deficient patients and PNP<sup>−/−</sup> mice. Jurkat T-cells, another representative of PNP-proficient cells were resistant to dGuo induced apoptosis (Figure 19A), further confirming that dGuo causes apoptosis specifically in PNP<sup>−/−</sup> thymocytes.

**Direct effects of dGuo phosphorylation product dGMP on thymocyte apoptosis.**

We hypothesized that the dGuo phosphorylation to dGMP by mitochondrial dGK and subsequently to dGTP was the cause for thymocyte apoptosis (Figure 18). Therefore I tested the effects of increasing concentrations of dGMP on thymocyte apoptosis. dGMP showed a concentration-dependant loss of cell viability similar to dGuo (Figure 19B). As expected, dGTP, which is a positively charged molecule can not cross the cell membrane and had no effect on normal or PNP<sup>−/−</sup> thymocytes (data not shown).
Figure 19: Demonstration of apoptosis in thymocytes after incubation in vitro with different purine metabolites. Thymocytes were isolated from PNP<sup>−/−</sup> mice (dashed) and age matched controls (solid) and cultured for 24 hours in the presence of indicated concentrations of (A) dGuo (*p=0.014, **p=0.0018), (B) dGMP (*p=0.0002). Data represents the mean ± SD of 3 independent experiments with one or two mice in each experiment (UT, untreated).
**Re-introduction of PNP into the cells increases the survival of PNP<sup>−/−</sup> thymocytes.**

To further substantiate the hypothesis that dGuo and its phosphorylated products was the cause of thymocyte apoptosis in PNP deficiency, PNP activity was restored inside PNP<sup>−/−</sup> thymocytes using PNP fused to a protein transduction domain (TAT), an enzyme replacement therapy developed in our lab (Toro et al., 2006). TAT-PNP can restore phosphorolysis of dGuo to Gua thereby preventing accumulation of dGMP and dGTP (refer Figure 18). Addition of TAT-PNP protected PNP<sup>−/−</sup> thymocytes from dGuo induced apoptosis (Figure 20A). In contrast and as expected, TAT-PNP does not prevent apoptosis of cells treated with dGMP since the formation of dGMP is past the stage of PNP action (Figure 20B).
Figure 20: Introduction of PNP into PNP-deficient cultures increases the rate of thymocyte survival. Single cell thymocyte suspensions were prepared and cultured in the presence of (A) dGuo (12.5 μM) or (B) dGMP (125μM) for 24 hours after pre-treating with TAT-PNP (1μg/ml) for 1 hour. The data (A) represents mean ± SD for 5 independent experiments with one to two mice in each (*p=0.001). (B) Data is representative of two experiments (UT, untreated).
4.6. **dGuo preferentially causes the apoptosis of PNP⁺/⁻ DP thymocytes.**

In order to magnify the moderate increase in apoptosis observed in freshly isolated PNP⁺/⁻ DP thymocytes, an ex-vivo culture was set-up. Thymocytes were cultured in the presence of dGuo and apoptosis was measured in the various thymocyte subsets after 24 hours. A significant difference between PNP⁺/⁻ and PNP⁺/+ DP thymocytes was seen. As evident in Figure 21A and 21B, among PNP-proficient cells maintained 24 hours in culture with dGuo, only 21.58±4.4% of CD4⁺CD8⁺ DP thymocytes were apoptotic, compared to 42.74±8.7% of apoptosis among PNP⁺/⁻ thymocytes treated similarly (Figure 21C). There were no differences observed in the rate of apoptosis of CD4⁺ SP, CD8⁺ SP or CD4⁻CD8⁻ DN subpopulations in the dGuo treated groups (Figure 21B). These findings were in concordance with our *in vivo* results (refer Figure 16A and 16B).
A) Untreated thymocytes cultured for 24 hrs

- Untreated thymocytes (PNP+/+)
- Untreated thymocytes (PNP−/−)

DPs undergoing apoptosis

- PNP+/+: 8.4
- PNP−/−: 8.87

B) Thymocytes treated with 12.5μM dGuo cultured for 24 hrs

- Thymocytes treated with 12.5μM dGuo (PNP−/−)
- Thymocytes treated with 12.5μM dGuo (PNP+/+)

DPs undergoing apoptosis

- PNP+/+: 17.59%
- PNP−/−: 43.78%
Figure 21: **PNP−/− DP thymocytes undergo increased dGuo induced apoptosis.**

Thymocytes were incubated for 24 hours in the presence of 12.5 μM dGuo. Cells were analyzed by three colour flow cytometry after staining with anti-CD4 (PE), anti-CD8 (APC) and Annexin-V (FITC). (A) Histogram is representative of untreated PNP+/+ and PNP−/− DP thymocytes undergoing apoptosis after 24 hours. (B) Histogram is representative of PNP+/+ and PNP−/− DP thymocytes undergoing apoptosis after 24 hours of dGuo treatment. Numbers on the histograms represent the percentage of Annexin+ thymocytes in the DP subpopulation. The percentage of apoptotic cells in the different subpopulations are indicated beside the respective quadrants. (C) Graph represents the percentage of PNP+/+ (black) and PNP−/− (white) DP thymocytes undergoing apoptosis after 24 hrs of dGuo treatment. Data is the mean ±SD of 4 independent experiments containing one or two mice in each experiment (*p=0.005).
CONCLUSION

The above mentioned results are indicative that it is indeed increased apoptosis that causes the reduced numbers of DP thymocytes in PNP/−/− mice and not due to a proliferation defect. Furthermore, the results also show that dGuo is the PNP substrate that acts as a toxic metabolite possibly by its conversion to dGTP.
**Aim 3: To examine whether the increased apoptosis of DP thymocytes in PNP deficiency is initiated in the mitochondria.**

After demonstrating that PNP deficiency increases DP thymocyte apoptosis through elevated dGuo and likely through its phosphorylated products, I was interested to study the mechanism leading to apoptosis. As mentioned previously in the introduction chapter (Figure 6), there are two general apoptosis pathways; the intrinsic pathway which involves the release of cytochrome c from the mitochondria (mitochondrial pathway) and the extrinsic pathway or activation of death receptors such as Fas by ligand binding (death receptor pathway).

**4.7. Fas expression is not upregulated in PNP deficiency.**

Fas expression was analyzed in different thymocyte subpopulations by three colour flow cytometry. There was no difference in the expression of Fas in freshly isolated DP or DN thymocytes from PNP<sup>-/-</sup> or PNP<sup>+/+</sup> mice (Figure 22A). Fas expression in the different thymocyte subsets was similar to previous reports (Ogasawara et al., 1995). Moreover, even after 24 hours incubation of thymocytes with dGuo, which I have consistently shown to significantly increase thymocyte apoptosis in PNP deficiency, there was no difference in Fas expression (Figure 22B). These results suggest that upregulation of Fas may not be the mechanism by which PNP deficiency mediates apoptosis.
Figure 22: *Fas expression in subpopulations of PNP^{+/+} and PNP^{-/-} thymocytes.* Single cell suspension from the thymuses of 6-week old PNP^{-/-} mice and age matched controls were analyzed by three color flow cytometry for CD4 (APC), CD8 (FITC) and Fas (PE) expression. Histograms are representative of Fas expression on (A) freshly isolated and (B) 24 hour dGuo treated PNP^{-/-} DN (pink) and PNP^{+/+} DN (blue) thymocytes as well as PNP^{-/-} DP (green) and PNP^{+/+} DP (red) thymocytes. Figures are a representative of 3 independent experiments with one or two mice in each.
4.8. **Dissipation of mitochondrial membrane potential (MMP).**

The intrinsic pathway (Figure 6, Pathway B) involves the release of pro-apoptotic proteins that activate caspase enzymes from the mitochondria ultimately triggering apoptosis (Fulda et al., 2006; Mayer et al., 2003). If the apoptotic signals are generated intramitochondrially, the disruption of MMP occurs before nuclear DNA fragmentation. In order to determine whether PNP deficiency disrupts the mitochondrial membrane, the integrity of the mitochondrial membrane was analyzed by staining with MitoTracker® Red CMXRos.

Following 24 hours of dGuo treatment, a significantly higher percentage of PNP−/− thymocytes loses MMP (M2), as indicated by a decrease in fluorescence intensity (Figure 23A). Results demonstrate a significantly higher percentage of dGuo treated PNP−/− thymocytes losing MMP compared to the controls (Figure 23B).
Figure 23: Disruption of MMP. Thymocytes from PNP<sup>−/−</sup> mice and age matched controls were labeled with MitoTracker (a sensitive indicator of MMP) after 24 hours of dGuo treatment. (A) In the histograms, M1 indicates the percentage of cells maintaining MMP and M2 indicates the percentage of cells losing MMP. (B) Graph represents the mean ±SD of the percentage of PNP<sup>−/−</sup> (white bar) and PNP<sup>+/+</sup> (black bar) thymocytes losing MMP from 3 separate experiments containing one or two mice in each (*p=0.003).
4.9. **Changes in MMP occur before nuclear DNA fragmentation:**

When apoptotic signals are initiated within the mitochondria, the disruption of MMP occurs prior to nuclear DNA fragmentation (Ravagnan et al., 1999). Thus, I was interested to confirm that indeed change in MMP occurs prior to nuclear DNA fragmentation in PNP deficiency. Therefore, I measured MMP using a mitochondria specific dye, and DNA fragmentation by the TUNEL assay in dGuo treated PNP−/− thymocytes at several time points. The time points that I examined included:

6-8 hours treatment, which I hypothesized is prior to initiation of apoptosis,

12 hours treatment, which is when I hypothesized apoptosis is initiated,

16-24 hours of treatment, when apoptosis is fully developed in both the mitochondria and nucleus (summarized in Figure 24G).

As expected, 6-8 hours after treating PNP−/− thymocytes with dGuo, there was no increase in TUNEL+ cells or increase in the percentage of cells with abnormal MMP (Figure 24G). In marked contrast, at 12 hours, a significant higher percentage of cells had disrupted MMP (Figure 24A is a representative example), without a significant change in DNA fragmentation (Figure 24B), and the results of 4 separate experiments are summarized in Figure 24C. At 16-24 hours, the percentage of cells with changes in MMP (Figure 24D) was similar to the percentage of cells undergoing DNA fragmentation (Figure 24E), with the results of 4 separate experiments summarized in Figure 24F. In conclusion, these results suggest that apoptosis in PNP−/− thymocytes exposed to dGuo, is initiated in the mitochondria.
A) Mitotracker

B) TUNEL

C) 12 hour treatment

D) DNA fragmentation

E) Loss in MMP (M2)

UT dGuo

Percentage of cells

0 2 4 6 8 10 12 14

*
Figure 24: Effects of dGuo on MMP and DNA fragmentation after 12 hours and 16 hours of treatment. PNP−/− thymocytes were incubated in RPMI supplemented with 5% FBS in the presence of 12.5μM dGuo for the indicated time points. At the indicated time points the cells were stained with Mitotracker to investigate loss of MMP and nuclear DNA fragmentation was assayed by the TUNEL technique. (A) and (D) demonstrate changes in MMP at 12 h and 16 h respectively. Percentages of cells are indicated above the bars. (B) and (E) demonstrate nuclear DNA fragmentation measured by the TUNEL assay at 12 h and 16 h respectively. Percentages of apoptotic cells are indicated above the bars. (C) and (F) summarize DNA fragmentation (black) and changes in MMP (white) in untreated and 12.5μM dGuo treated samples at 12 h (*p=0.001) and 16 h respectively. The data represents mean ± SD for 4 independent experiments at each time point with one or two mice in each. (G) Graph summarizes the DNA fragmentation (black) and changes in MMP (white) profiles following 6 h, 12 h and 16 h dGuo treatment of PNP−/− thymocytes (*p=0.001) (UT, untreated).
4.10. **Caspase inhibition prevents dGuo induced DNA fragmentation but does not prevent apoptosis of the cells.**

To confirm that changes in MMP indeed occurred before DNA fragmentation I used an alternative strategy which determined the effect of caspase inhibition on MMP and DNA fragmentation. When the apoptotic signals are initiated within the mitochondria, the disruption of MMP does not involve the release and activation of caspases. Hence this event is considered to be a caspase independent process. In contrast, when apoptotic signals originate outside the mitochondria, then the subsequent disruption of MMP is a caspase dependant process (Ravagnan et al., 1999). Consequently, if apoptosis originated within the mitochondria then thymocyte apoptosis would not be affected by caspase inhibition. However, if apoptosis signals originated due to nuclear DNA damage then the apoptosis would be halted by caspase inhibition. Therefore I examined the effect of caspase inhibition (using the pan caspase inhibitor z-VADfmk) on dGuo induced apoptosis.

I first demonstrated that z-VADfmk prevented apoptosis (measured by the percentage of Annexin$^+$ cells) induced by dexamethasone (Dex) (Figure 25A), which is a well known caspase-dependent apoptosis inducing agent. In contrast, z-VADfmk was unable to protect thymocytes from dGuo induced apoptosis, as indicated by Annexin-V staining (Figure 25B). Interestingly, z-VADfmk did prevent dGuo induced DNA fragmentation, which is considered to be a late apoptotic event suggesting that in addition to the early mitochondria induced apoptosis, dGuo also has a late, DNA fragmentation effect (Figure 25C).
Figure 25: *Effects of pan-caspase inhibition on dGuo induced apoptosis and nuclear DNA fragmentation.* PNP− thymocytes maintained in RPMI 1640 medium with 5% FBS, were pre-treated for 30 minutes with 50μM z-VADfmk followed by the addition of (A) 3.75 μM Dex or (B) and (C) 12.5μM dGuo. 20-24 hours later, apoptosis was measured by Annexin-V staining (Figures A and B) and DNA fragmentation was measured by the TUNEL assay (Figure C). Results are representative of 4 independent experiments with one or two mice in each. The percentage of apoptotic cells are indicated above the bars.
CONCLUSION

In summary, these results demonstrate that a change in MMP occurs prior to nuclear DNA fragmentation. Also, the loss in MMP induced apoptosis is resistant to caspase inhibition suggesting it is a caspase independent mechanism. Taken together, these findings suggest that the accumulation of dGuo and its phosphorylated products in PNP⁻/⁻ thymocytes increased apoptosis in a mitochondrial mediated pathway.
CHAPTER 5: DISCUSSION
Discussion

PNP deficiency disrupts thymocyte development. However, the lack of availability of thymus tissue from affected patients has prevented detailed analysis of the precise effects of abnormal purine metabolism on thymocytes. PNP<sup>−/−</sup> mice displaying metabolic and immune phenotype similar to the human disease have helped understand some of the mechanisms associated with toxic purine metabolites, and hence were extensively used in this study. Although PNP is a ubiquitous enzyme, its preferential effect on thymocytes and T-cells is not surprising, as these cells, unlike many other cell types, are unique in that they undergo constant selection and proliferation during their lifetime. To maintain a constant homeostasis in these cells requires a balance in the nucleotide pools to compensate for the DNA damage and repair induced during the process of selection and subsequent proliferation. This may be the most likely explanation for the selective toxicity of thymocytes to the effects of PNP deficiency and thus, has been the area of focus for most researchers. However, preliminary data from our lab and previous observations by others suggests involvement of cells beyond the thymus. Neurological dysfunction has been reported in more than half of the patients (Markert, 1991). Similar to PNP-deficient patients PNP<sup>−/−</sup> mice manifest with neuroloigal abnormalities as tested by their inability to sustain on a spinning rotorod and increased apoptosis indicated by <i>in situ</i> TUNEL staining on purkinje cells from PNP<sup>−/−</sup> mice cerebellums (Grunebaum, unpublished). Also, PNP<sup>−/−</sup> skin fibroblasts from mice have been seen to proliferate at a slower rate compared to normal controls as tested by the thymidine uptake assay (Grunebaum, unpublished). Few patients also developed bone marrow abnormalities possibly due to hypersensitivity to irradiation (Dror et al., 2004). Reduced
B-cell numbers and abnormal humoral function have been reported amongst a few patients (Markert, 1987). These findings are indicative that PNP is essential in the maintainance and functioning of many cells in the body.

We and others have found significant increases in the number of DN thymocytes in the thymi of PNP<sup>−/−</sup> mice, which is particularly impressive when considering that the thymus of these mice is atrophic with marked reduction in other thymocyte populations. This is suggestive of abnormal maturation within the DN subset. Therefore our focus was initially targeted towards studying the development of DN thymocytes. The significant increase in the percentage and numbers of DN3 and reduction of DN4 and DP cells that I found in freshly isolated thymocytes and ex-vivo cultures simulating PNP deficiency, clearly demonstrate that maturation from the DN3 stage to the later stages are affected by PNP deficiency. The limitations of these experiments as mentioned earlier, is that by the third week of co-culture when most of the cells had developed into the later DN stages (DN3 and DN4) and to the DP and SP stage, CD4<sup>+</sup>/CD8<sup>+</sup> were not gated out to analyse the DN subsets. This makes interpretation of results difficult as the “DN4” subset is really a heterogeneous population consisting of DN4, DP and SP cells. However, the significant increase in the percentage of CD25<sup>+</sup> cells at this stage was evidence enough for us to conclude that the delay in thymocyte development in PNP deficiency begins at the DN3 stage. Another method to study DN generation and expansion would be to carry out a dynamic analysis by a short term BrdU pulse chase experiment. This would further show whether the early DN3s are being generated and proliferating at a proper rate. Moreover, investigating the exact stage of developmental delay, i.e. whether between DN3 and DN4 or between DN4 and DP stage, proved to be a challenge due to the absence of a distinct cell surface marker for the DN4
subset. The transition between DN3 to DN4 is a crucial check-point in thymocyte maturation, and it has been estimated that 4 out of 9 DN3 cells die at this stage as a result of failure to rearrange their TCRβ locus (Gärtner et al., 1999) and hence my results are not unexpected. I therefore also determined the percentage of PNP+/_- DN3 and DN4 thymocytes that express intracellular TCRβ chain. I found a decrease in the percentage of DN3 cells transiting to the DN4 stage by down regulating CD25 and upregulating intracellular TCRβ expression in PNP+/_- mice compared to controls, further indicating a developmental delay between the DN3 and DN4 stage. However, the similar percentages of late DN3 cells in PNP+/_ and PNP+/+ mice indicate that TCRβ rearrangement is taking place normally. My results are also in concordance to those reported in ADA deficiency, wherein ADA deficiency did not seem to compromise TCRβ chain rearrangement in developing thymocytes (Thompson et al., 2000). Collectively, these results suggest that rearrangement of the TCRβ locus and expression of the TCRβ chain is not affected by PNP deficiency. It will be beneficial to study the TCR-vβ repertoire in the periphery of PNP+/_- mice compared to controls by using a panel of antibodies to different TCR-vβ chains, which is a quantitative approach. A qualitative and more in-depth analysis of TCR-vβ repertoire is by a PCR technique such as spectra typing.

Another possibility for the decreased DN4 population could be related to sensitivity of these cells to factors released by dying thymocytes, such as toxic purine metabolites, particularly dGuo. Alternatively, the increased death of DN4 thymocytes could be due to abnormal signalling through the TCR complex, as has been seen in mice deficient in Lck (Levin et al., 1993), pTα deficient mice (Fehling et al., 1995), absence of ZAP-70 (Sugawara et al., 1998), RAG/- mice (Shinkai et al., 1992; Mombaerts et al., 1992) and knock down
models of pTα, CD3 ε, γ and ζ chains (Fehling et al., 1995; Haks et al., 1998; Dave et al., 1997; DeJarnette et al., 1998). This explanation, which needs to be examined by future experiments, is also supported by studies of peripheral PNP-deficient T-cells that showed reduced responses and decreased IL-2 secretion, again implicating impaired intracellular signalling (Arpaia et al., 2000; Dror et al., 2004; Toro et al., 2006).

In addition to the effect that PNP deficiency has on DN thymocytes described above, striking reductions in the numbers and percentages of DP thymocytes in PNP+/- mice were found. BrdU incorporation into the DNA of newly formed DP thymocytes in PNP+/- mice, as well as in DP cells developed ex-vivo, demonstrated that proliferation of PNP+/- DP cells was not impaired (refer Figures 16B, 16C). Moreover, the DP cells generated ex-vivo in PNP-deficient conditions was reduced, although there is no selection ex-vivo. Thus I concluded that proliferation and selection were not the causes for the decreased DP thymocytes. As an alternative explanation, I studied apoptosis in PNP+/- DP thymocytes. An indication for decreased cell survival was already provided by the rapid reduction in the percentage of BrdU labelled DP thymocytes, which was not accounted for by reduced proliferation of DN thymocytes or enhanced maturation into SP thymocytes. The increased apoptosis of PNP+- DP thymocytes was further confirmed by the significantly increased Annexin-V+ PI+ cells among PNP+/- DP thymocytes. The presence of Annexin-V binding is also an indication that the cells are truly undergoing apoptosis in contrast to other mechanisms of cell death such as necrosis. Although the value of apoptosis in DP cells increased (on average) only from 1.5% in normal control to approximately 3.5% among PNP+/- thymocytes, this increase was statistically significant, was observed consistently among PNP+/- mice and was similar to that reported previously (Arpaia et al., 2000). We suspect that the relatively low number of
apoptotic cells observed at a steady state among fresh thymocytes reflects both the ongoing gradual effect that toxic purine metabolites have throughout the 6 week period from birth to the time thymocytes were collected, and also the quick removal of apoptotic cells by the abundant scavenger macrophages found in the thymus of PNP<sup>−/−</sup> mice. This is in concordance to reports by Thompson et al., wherein a moderate increase in thymocyte apoptosis was not detectable by PI staining or <i>in situ</i> TUNEL staining in ADA-deficient cultures (Thompson et al., 2000).

Importantly, the increased apoptosis of PNP<sup>−/−</sup> DP thymocytes cultured ex-vivo indicated that the apoptosis was not caused by external events, such as surge of corticosteroids in PNP-deficient mice, unidentified infections, or enhanced signalling through the Fas-receptor. The latter hypothesis was also supported by demonstrating that PNP<sup>−/−</sup> DP thymocytes did not upregulate the surface expression of Fas receptor, further indicating that the extrinsic pathway mediated by Fas expression may not be the contributing mechanism for apoptosis. Moreover, death receptor signaling via Fas was eliminated as the apoptotic mechanism in ADA deficiency, as <i>lpr</i> mice (mice homozygous for lymphoproliferation spontaneous mutation (Fas<sup>lpr</sup>)) remained sensitive to ADA deficiency. However, the same group also showed that the use of a caspase-8 inhibitor prevented the apoptosis of ADA deficient thymocytes, indicating the possible role of extrinsic apoptotic mechanisms via other death receptor pathways such as tumor necrosis factor receptor (TNFR) family (Thompson et al., 2003), which was not tested in this study. It is also possible that p53 may be involved, since Benveniste and Cohen showed that p53<sup>−/−</sup> mice were resistant to apoptosis when induced with ADA deficient conditions using dCF and dAdo (Benveniste and Cohen, 1995). p53 can cause apoptosis by inducing transcription of pro-apoptotic Bcl-2 family members.
such as Bax, PUMA and Noxa (Miyashita and Reed, 1995, Yu et al., 2001, Oda et al., 2000). It was later shown by some preliminary studies that FTOCs from p53 knock-out mice were susceptible to the consequences of ADA inhibition (Thompson et al., 2003). Of course, it is still possible that a p53 independent mechanism may involve the pro-apoptotic Bcl-2 family members. None of the above mentioned apoptotic pathways were addressed in my study nevertheless, my preliminary experiments in attempt to study the extrinsic pathway, although not conclusive, together with findings from ADA deficiency studies, lead us to narrow the cause of apoptosis to an intrinsic mechanism.

We next asked whether the accelerated apoptosis of DP thymocytes in PNP deficiency were caused by increased dGuo and its phosphorylation products, dGMP and dGTP, or whether it was the depletion of PNP products (i.e. GTP). Both, increased dGuo and dGTP concentrations, as well as decreased GTP concentrations were observed in PNP-deficient thymocytes (Arpaia et al., 2000). Moreover, abnormal peripheral T-cell function have been associated with increased dGuo and dGTP (Kicska et al., 2001; Bantia et al., 2003) and reduced GTP. Here I demonstrated that apoptosis of PNP−/− thymocytes was induced by dGuo at concentrations equivalent to those recorded in the plasma of PNP-deficient patients (Hershfield and Mitchell, 2001) and mice (Arpaia et al., 2000). Moreover, apoptosis of thymocytes could be prevented by restoring PNP activity within the cells, which provided unequivocal evidence to the role of the abnormal purine homeostasis in induction of apoptosis. Apoptosis was also induced in PNP−/− thymocytes by dGMP, an intermediate metabolite between dGuo and dGTP, which further implicates the latter as the cause of apoptosis. Thus our study shows that accumulation of dGuo in PNP deficiency, followed by the conversion of dGuo to dGMP, and probably to dGTP, causes thymocyte apoptosis in
PNP−/− mice. We suspect that dGTP is the cause of apoptosis, as intracellular dGTP pools are normally kept under tight regulation (Bjursell et al., 1980; Reichard et al., 1985), and PNP inhibition using BCX-1777 in T-ALL, led to accumulation of dGuo in the plasma and dGTP in circulating leukemia cells that were induced to die rapidly (Bantia et al., 2003). The suspected role of dGTP in mediating the apoptosis of PNP-deficient thymocytes, also suggested that the mitochondria was the cellular compartment most affected by PNP deficiency, as dGTP formation is dependent on the function of dGK, which is a mitochondrial enzyme (Johansson et al., 1996). Indeed, I found that accumulation of dGuo led initially to the dissipation of mitochondrial membrane potential and that inhibition of caspases, prevented dGuo induced nuclear DNA damage, but not mitochondrial damage. In the experiments conducted to study changes in MMP, apoptosis induced by dGuo was not seen immediately but rather took 12 hours to observe the toxic effects, possibly recapitulating the gradual toxicity of purine metabolites to exert its effects on thymocytes in vivo. The mitochondrial abnormalities that I found in PNP−/− thymocytes were similar to previous observations in normal thymocytes treated with a PNP inhibitor (Arpaia et al., 2000), in ADA-deficient thymocytes (Thompson et al., 2000), and in inherited thymidine phosphorylase deficiency, which causes mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) disease (Nishino et al., 1999, Hiarano et al., 2006). Thus, our findings suggest particular sensitivity of mitochondria to abnormalities in purine homeostasis, which could be due to interference of purine metabolites with the down regulation of anti-apoptotic molecules such as Bcl-2, as introduction of a Bcl-2 transgene into PNP-deficient thymocytes increased their survival (Arpaia et al., 2000). Alternatively, the depletion of mtDNA due to particular sensitivity of mitochondria to abnormal purine
metabolism is also suggested by the mitochondrial abnormalities reported in ADA deficiency (Van De Wiele et al., 2002) and MNGIE (Lopez et al., 2009). Other observations also provide additional evidence that thymocyte damage in PNP deficiency may originate in the mitochondria. PNP-deficient cells were found to be sensitive to gamma irradiation but were still able to replicate their nuclear DNA in response to mitogen in the presence of IL-2 (Arpaia et al., 2000). Also, over-expression of dGK in the mitochondria was found to increase sensitivity to anti-cancer deoxyguanosine analogues in cancer cell lines (Zhu et al., 1998).

In conclusion, by this study, I demonstrated that PNP deficiency affects murine thymocyte development at two distinct stages, and my data also suggests the possibility of different mechanisms affecting the two stages. The first is at the maturation of thymocytes from the DN3 to DN4 stage. The precise mechanism leading to this defect is still not known and requires further investigation. The second thymocyte population affected in PNP-deficient mice is the DP population. PNP−/− DP thymocytes are undergoing apoptosis at an increased rate. The apoptosis is induced by accumulating dGuo, and probably by the secondary formation of dGTP, which interferes with mitochondrial integrity. Importantly, restoration of PNP activity within the cells increased survival, suggesting that enzyme or gene replacement therapy may help in correcting the thymocyte abnormalities in PNP-deficient patients.
Future Directions

1) Identify the cause of decreased DN4 thymocytes in PNP\(^{-/-}\) mice. Specifically, determine intracellular signalling in DN3 and DN4 thymocytes. Alternatively, measure cell cycling in PNP-deficient DN3 and DN4 thymocytes \textit{in vivo} and \textit{ex-vivo}.

2) Determine the contribution of GTP depletion in thymocyte abnormalities by GTP supplementation.

3) Distinguish between the effects of dGuo and those of dGTP by preventing phosphorylation of dGuo into dGTP, for example by cross-breeding PNP\(^{-/-}\) mice with dGK-deficient mice. If dGTP is the cause of apoptosis, then the double knockout will prevent the dGuo-induced thymocyte apoptosis.

4) Assess the importance of anti-apoptosis mechanisms in PNP\(^{-/-}\) thymocytes. Specifically, determine whether over-expression of Bcl-2 protects PNP\(^{-/-}\) deficient thymocytes from dGuo induced apoptosis, by cross breeding of PNP\(^{-/-}\) mice with Bcl-2 transgenic mice. Alternatively, measure pro-apoptotic molecules associated with the mitochondrial pathway, such as BAX and BAK.

5) Determine the sequence of events triggered by the mitochondrial damage that leads to cell death. Specifically, release of cytochrome c from the intermembrane space of the mitochondria to the cytoplasm, and activation of specific caspases, such as caspase 3 and caspase 9.

6) Improve the ability to follow development of PNP-deficient thymocyte \textit{ex-vivo} by
producing bone marrow stroma cells that do not express PNP, thereby more closely simulating *in vivo* conditions in PNP-deficient patients. This can be done by inhibiting PNP (using siRNA to PNP) in the OP9-DL1 cells.

7) Confirm that dGuo is indeed the metabolite responsible for thymocyte toxicity by preventing its uptake into thymocytes. This can be achieved by the use of a nucleoside transport inhibitor.

8) Explore the interactions (cross-talk) between thymocytes and the thymic epithelial cells, which are also affected by the abnormal purine metabolism and possibly contribute to the immune dysregulation observed in PNP-deficient patients and mice.
References


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