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Article

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Inhibition of T lymphocyte activation in mice heterozygous for loss of the *IMPDH II* gene

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Inosine 5′-monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme in the de novo synthesis of guanine nucleotides, which are also synthesized from guanine by a salvage reaction catalyzed by the X chromosome–linked enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT). Since inhibitors of IMPDH are in clinical use as immunosuppressive agents, we have examined the consequences of knocking out the IMPDH type II enzyme by gene targeting in a mouse model. Loss of both alleles of the gene encoding this enzyme results in very early embryonic lethality despite the presence of IMPDH type I and HPRT activities. Lymphocytes from *IMPDH II*$^{+/-}$ heterozygous mice are normal with respect to subpopulation distribution and respond normally to a variety of mitogenic stimuli. However, mice with an *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$ genotype demonstrate significantly decreased lymphocyte responsiveness to stimulation with anti-CD3 and anti-CD28 antibodies and show a 30% mean reduction in GTP levels in lymphocytes activated by these antibodies. Furthermore, the cytolytic activity of their T cells against allogeneic target cells is significantly impaired. These results demonstrate that a moderate decrease in the ability of murine lymphocytes to synthesize guanine nucleotides during stimulation results in significant impairment in T-cell activation and function.

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Introduction

Inosine 5′-monophosphate dehydrogenase (IMPDH) is a rate-limiting enzyme in the de novo pathway for synthesis of guanine nucleotides, which are essential for normal cell proliferation and function. IMPDH catalyzes the NAD-dependent conversion of inosine monophosphate to xanthine monophosphate, which is subsequently converted to guanosine monophosphate (GMP) by GMP synthase. The only alternative pathway for guanine nucleotide biosynthesis is through the salvage of guanine to GMP by hypoxanthine-guanine phosphoribosyltransferase (HPRT), an enzyme encoded by a gene on the X chromosome (Figure 1). The relative contributions of the de novo and salvage pathways to guanine nucleotide biosynthesis in different tissues and cell types have not been definitively determined, although this issue is clearly central to our understanding of both the pathophysiology of inherited disorders of enzymes in these pathways and the therapeutic effects of selected pharmacological inhibitors of these enzymes. IMPDH activity in human tissues is composed of the activities of two separate but very closely related IMPDH isoenzymes, termed type I (1) and type II (2), which are 84% identical at the amino acid level and possess indistinguishable catalytic activities. However, the regulation of expression of the two *IMPDH* genes differs dramatically (3, 4). The increased IMPDH activity observed in replicating or neoplastic cells is largely due to increased expression of the type II IMPDH mRNA, whereas expression of the type I gene is relatively unaffected by cell proliferation or transformation (5, 6). The expression of both genes is, however, significantly increased by mitogen activation of peripheral blood lymphocytes (7).

A survey of relative IMPDH mRNA levels in human tissues demonstrated significant variability in the pattern of distribution of the type I transcript, whereas expression of the type II transcript, while generally higher than that of type I, was far less variable (8). The observations that the expression of the *IMPDH* gene is tightly linked with both cellular proliferation and transformation (9, 10) have led to an interest in developing IMPDH inhibitors that deplete intracellular guanine nucleotide pools. It has been shown that administration of IMPDH inhibitors to cultured cells results in inhibition of DNA synthesis (11) and cell-cycle arrest at the G1-S boundary (12, 13). Inhibitors of IMPDH have also been shown to possess antineoplastic (14, 15), antiviral (16), antiparasitic (17), and immunosuppressive (18, 19) activities, and to induce

the differentiation of a variety of human tumor cell lines, including leukemic (20), breast cancer (21), and melanoma (22) cells.

In order to understand better the homeostatic mechanisms responsible for regulating intracellular guanine nucleotide synthesis through the de novo as opposed to the salvage pathway and to determine the relative biological roles of IMPDH type I and type II enzymes in the development and function of lymphocytes, we developed a specific gene-targeting construct to inactivate the murine *IMPDH* type II gene. Homozygous loss of the *IMPDH* type II gene results in early embryonic lethality. Although IMPDH II heterozygous or HPRT-deficient mice do not show any abnormal phenotype, T lymphocytes from mice with combined deficiencies of HPRT and IMPDH activities demonstrate both impaired proliferative responses to mitogen and decreased cytolytic function.

Methods

Construction of the IMPDH II targeting vector and embryonic stem cell selection. The pJNS2 vector (provided by Beverly Koller, Department of Medicine, University of North Carolina), containing *Neo* and *HSV-TK* genes under the regulation of the phosphoglycerate kinase (PGK) promoter, was used for making the knock-out construct. *IMPDH* type II gene fragments of 2.4 kb containing exons 1 through 5 and 4.6 kb containing exons 10 through 14 were inserted into NotI, XhoI, and XbaI sites 5′ and 3′ to the Neo gene, respectively (Figure 2). Mouse 129 strain embryonic stem (ES) cells were transfected using electroporation and selected in the presence of 200 μg/mL G418 and 0.5 μg/mL ganciclovir. Genomic DNA from selected ES clones was digested with BglII, separated on 0.8% agarose gels, transferred onto Zeta-Probe nylon membranes (Bio-Rad Laboratories Inc., Hercules, California, USA), and hybridized with an [α-^{32}P]-dCTP-labeled 0.6-kb DNA probe (3000 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) located 5′ to the first exon (Figure 2). Labeling was done using a Random Primer Labeling kit (Promega Corp., Madison, Wisconsin, USA). Two ES clones heterozygous for recombination at the *IMPDH II* locus were microinjected into C57BL/6 blastocysts to generate chimeric mice.

Breeding and genotyping of offspring. Male mice with 65–100% chimerism based on coat color were bred with C57BL/6 females (The Jackson Laboratory, Bar Harbor, Maine, USA). Genotyping was performed on 2- to 4-week-old F1 agouti offspring by Southern hybridization analysis of tail DNA using High Efficiency Hybridization System (Molecular Research Center Inc., Cincinnati, Ohio, USA).

PCR analysis of blastocysts. To obtain blastocysts, female mice were superovulated by injecting 5 U of pregnant mares' serum (Sigma Chemical Co., St. Louis, Missouri, USA) intraperitoneally followed 48 hours later by 5 U of human chorionic gonadotropin (Sigma Chemical Co.). Blastocysts were collected from 3.5-days-post-coitum (dpc) uteri, washed in M2 medium (Cell & Molecular Technologies Inc., Phillipsburg, New Jersey, USA) and PBS, and digested in 20 μL of PCR lysis buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.3], 0.01% gelatin, 0.45% NP-40, 0.45% Tween-20, and 100 μg/mL proteinase K) at 55°C overnight. One μL of sample was used in each PCR reaction containing 250 μM of the four deoxyribonucleoside triphosphates, 100 ng of each primer, and 0.5 units of *Taq* polymerase (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). Two sets of oligonucleotide primers were used in separate PCR reactions, since they gave rise to amplified fragments of similar size. The first set of primers, specific for the wild-type allele, includes a forward primer (E5F2) from exon 5 of the type II gene: 5′-GGAAGAGCATGACCGGTTCTTGGA-3′; and a reverse primer (E6R1) from exon 6: 5′-AGGGGCGACCACCAAATCTT-3′. The second set of primers, specific for the recombinant allele, includes a forward primer (PNT 3F) from the 3′ end of the Neo gene cassette: 5′-GAGATCAGCAGCCTCTGTTCCAC-3′; and a reverse primer (E10R1) from exon 10: 5′-GAGACCTTGTACACTGCTGTGGCT-3′.

Northern hybridization. Isolation of RNA and Northern blots were performed as described previously (4). IMPDH mRNAs were detected with 1.5-kb mouse IMPDH type I and type II full-length cDNA probes, and HPRT mRNA was detected with a 0.5-kb mouse HPRT cDNA probe extending from exon 3 to exon 9 obtained by RT-PCR.

In vitro lymphocyte activation. Mouse splenocytes were cultured in RPMI-1640 (Life Technologies Inc., Rockville, Maryland, USA) containing 10% heat-inactivated FBS (Sigma Chemical Co.), 55 μM β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. For T-cell stimulation, Con A (Pharmacia Biotech AB, Uppsala, Sweden) was used at 5 μg/mL; PMA and ionomycin (Calbiochem-Novabiochem Corp., San Diego, California, USA) were used at 10 ng/mL and 250 ng/mL, respectively; and 1 μg/mL of anti-CD3 (500-A2; Caltag Laboratories Inc., Burlingame, California, USA) and 2.5 μg/mL of anti-CD28 (37.51.1; Caltag Laboratories Inc.) in 100 μL volume were used to coat each of 96 wells at 37°C for 4–6 hours. For B-cell stimulation, LPS (Sigma Chemical Co.) was used at 20 μg/mL.

Lymphocyte proliferation assays. Splenocytes were plated in a 96-well plate at a density of 10^5 cells per well for T-cell mitogens, and 5×10^4 cells per well for LPS stimulation; 1 μCi of [methyl-^{3}H]-thymidine (80 Ci/mmol; Amersham Pharmacia Biotech) was added to each well during the final 6 hours of culture. Cells were harvested at the indicated time onto glass fiber filters (Packard Instrument Co., Meriden, Connecticut, USA) and counted in Microscint 20 scintillation fluid (Packard Instrument Co.) by a Top Count (Packard Instrument Co.). For testing drug sensitivity, cells were stimulated with either Con A or antibodies in the absence or presence of various concentrations of mycophenolic acid (MPA) and harvested after 72 hours.

^{51}Cr-release assay. To set up mixed lymphocyte reaction (MLR), 5×10^6 cells/mL of irradiated (30 Gy) DBA/2 (H-2^d) mouse splenocytes used as stimulators were mixed with 2×10^6 cells/mL of responder splenocytes (H-2^b) for 5 days. To prepare target cells for ^{51}Cr-release assays, 10^7 P815 cells (H-2^d; American Type Culture Collection, Rockville, Maryland, USA) were labeled with 150 μCi [^{51}Cr]-sodium chromate (250–500 mCi/mg Cr; Amersham Pharmacia Biotech) by incubation at 37°C for 45 minutes. To test specific killing activity of cytotoxic T cells generated from MLR, 100 μL of ^{51}Cr-labeled P815 cells (1×10^4) were mixed with 100 μL of effector cells at indicated effector:target (E:T) ratios and incubated at 37°C for 4 hours. The wells containing target cells with medium alone were used as spontaneous release controls, and wells containing target cells plus 5% Triton X-100 were used as maximum release controls. Results are expressed as a percentage of specific lysis as calculated by the following formula: (release with effector cells – spontaneous release) / (maximum release – spontaneous release).

Measurement of nucleotides. To extract intracellular nucleotides, cell pellets were extracted with cold 5% perchloric acid and incubated on ice for 10 minutes followed by a 10-minute centrifugation. Supernatants were then adjusted to pH 6–7 using 3M KOH/3M $KHCO_3$ solution. Nucleotide pools were quantitated by HPLC, using a Whatman Inc. (Clifton, New Jersey, USA) Partisil-10 SAX anion exchange column and a linear gradient of 0.5 M KH_2PO_4 pH 4.5 versus 7 mM KH_2PO_4 pH 3.8 monitored at 254 nm. Total intracellular nucleotide levels were calculated by comparing with peaks generated by calibrated amounts of pure ATP and GTP.

Results

Generation of IMPDH type II–deficient mice. To inactivate the *IMPDH* type II gene in mouse ES cells, we constructed an *IMPDH* type II gene targeting construct in which a 1.7-kb genomic XbaI fragment encompassing exon 6 through exon 9 is replaced with a PGK-Neo cassette (Figure 2a). A targeted recombination event with this vector would introduce a BglII site at the 3′ end of the *Neo* gene, giving rise to a distinct 6-kb BglII fragment in the recombinant as opposed to the 11-kb BglII genomic fragment as detected by a probe 5′ to exon 1 (Figure 2, a and b). ES cells were electroporated with the linearized vector and selected in the presence of G418 and ganciclovir. Cells containing the correctly targeted allele were identified by Southern blot hybridization and constituted 10–15% of the clones analyzed. Two ES clones, each containing a single targeted allele, were microinjected into C57BL/6 (B6) blastocysts to generate chimeric mice.

Chimeric male mice were then mated with B6 females with successful germ line transmission. The ES cell line (E14TG2a) that we used for the initial transfection is a variant of the E14 line and is deficient in X chromosome–linked HPRT activity (23). Since F1 female mice generated from this line will be HPRT$^{+/-}$, we backcrossed the IMPDH II heterozygous F1 male (IMPDH II$^{+/-}$, HPRT$^{+/o}$) with B6 wild-type females to generate offspring containing wild-type HPRT. To generate mice homozygous-deficient in IMPDH II, IMPDH II$^{+/-}$ mice were interbred. The genotypes of offspring were determined by Southern blot analysis of tail DNA. Of 159 offspring examined, 57 were wild-type (36%) and 102 were IMPDH II$^{+/-}$ (64%). No homozygous-deficient mice (IMPDH II$^{-/-}$) were found, indicating that loss of both alleles of the *IMPDH* type II gene results in embryonic lethality. Since HPRT is an enzyme that uses guanine in a phosphoribosylation reaction of the salvage pathway to generate guanine nucleotides (Figure 1), we supplemented drinking water with 2 mM guanosine to determine whether the salvage of guanine through the HPRT pathway could compensate for the loss of IMPDH type II activity. No *IMPDH II*$^{-/-}$ mice were found in 28 pups analyzed. *IMPDH*$^{+/-}$ mice were phenotypically and developmentally normal and were fertile.

To determine the stage of development at which the homozygous-deficient embryos were lost, blastocysts were isolated at 3.5 dpc from superovulated *IMPDH II*$^{+/-}$ females that had been bred with *IMPDH II*$^{+/-}$ males. PCR was performed using two sets of oligonucleotide primers as described in Methods. Of 122 blastocysts analyzed, 36 contained only wild-type alleles, 78 were heterozygous, and 8 were homozygous for presence of the recombinant alleles only (Figure 3). Since the percentage of blastocysts that are homozygous-deficient (6.5%) is much less than the expected 25%, this result indicates that the majority of the *IMPDH II*$^{-/-}$ mice did not develop to the blastocyst stage. Embryos from heterozygous matings were also analyzed at early gestation times using embryos from wild-type B6 matings as normal controls. At least ten sections were made for each uterus, and early embryonic development was determined by the finding of an ectodermal and endodermal egg cylinder within the decidual swelling by light microscopy (data not shown). The percentage of empty decidua was only slightly higher in the *IMPDH II*$^{+/-}$ matings compared with that in the B6 matings at age 7.5 dpc. These results indicate that few, if any, of the *IMPDH II*$^{-/-}$ blastocysts implanted to induce a decidual response, further supporting very early embryonic lethality.

In order to determine whether loss of guanine salvage by HPRT would enhance any phenotypic alterations of heterozygous *IMPDH II*–deficient animals, *IMPDH II* heterozygous, *HPRT* wild-type F1 males (*IMPDH II*$^{+/-}$, *HPRT*$^{+/o}$) were bred with *IMPDH II*$^{+/-}$, *HPRT*$^{+/-}$ F1 females to generate male offspring with four different genotypes: (a) wild-type for both *IMPDH II* and *HPRT* (*IMPDH II*$^{+/+}$, *HPRT*$^{+/o}$); (b) *IMPDH II* wild-type, *HPRT*-deficient (*IMPDH II*$^{+/+}$, *HPRT*$^{-/o}$); (c) *IMPDH II*$^{+/-}$, *HPRT*$^{+/o}$; and (d) *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$. Mice containing each of these genotypes had no evident growth or behavioral abnormalities and were fertile. Subsequent studies were conducted on these mice between 5 and 10 weeks of age.

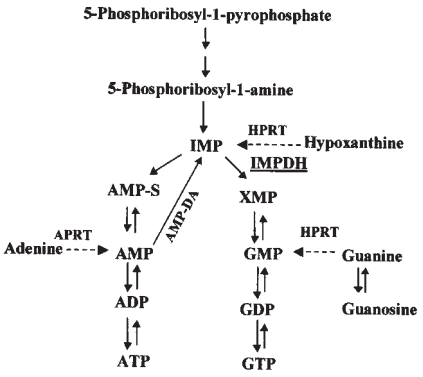


Figure 1
Schema illustrating the pathways of de novo and salvage purine nucleotide biosynthesis. Solid lines represent the de novo purine biosynthetic pathway, and dashed lines indicate salvage pathways. APRT, adenine phosphoribosyltransferase; AMP-DA, AMP-deaminase.

Effect of loss of IMPDH II or HPRT activity on IMPDH type I expression. In order to determine whether the IMPDH type I enzyme was increased in expression in *IMPDH* type II$^{+/-}$ and/or *HPRT*-deficient mice and whether the expression of the IMPDH and the HPRT were coordinately regulated, we measured the mRNA levels of IMPDH type I, IMPDH type II, and HPRT in tissues from mice with the four different genotypes. As expected, the mRNA levels for IMPDH types I and II, as well as HPRT, were markedly increased in splenic lymphocytes after mitogen stimulation (Figure 4a). The level of IMPDH type II mRNA in stimulated splenocytes from the IMPDH II$^{+/-}$ mice was approximately 70% of the wild-type level as calculated by densitometry after normalization to the 28S rRNA level. The level of IMPDH type I mRNA was similar in all groups, and HPRT deficiency did not result in an upregulation of IMPDH I or II mRNAs. The relative levels of RNAs from a number of tissues from wild-type and IMPDH II$^{+/-}$, HPRT$^{-/o}$ mice were also compared (Figure 4b). There was approximately a 30% reduction in type II mRNA levels in lung, liver, heart, and brain in IMPDH$^{+/-}$ mice as compared with wild-type after normalization to the 28S rRNA level. Of particular interest is the lack of detectable IMPDH I mRNA in mouse livers, suggesting that the type II enzyme represents the major enzyme for de novo synthesis of guanine nucleotides in this organ. Although expression of type I mRNA is not altered in most tissues from *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$ mice, there is a small increase (30%) in type I mRNA levels in the brains of these mutant mice as compared with the wild-type animals.

IMPDH types I and II proteins varied in rough proportion to the levels of these corresponding mRNAs. There was no alteration in expression of the type I protein in *IMPDH II*$^{+/-}$, *HPRT*-deficient mice, with the exception of a slightly increased amount of type I protein in the brain (data not shown).

IMPDH enzymatic activity is increased more than sixfold in wild-type splenic lymphocytes upon Con A stimulation. This increment is reduced by approximately 40% in *IMPDH II*$^{+/-}$ mice regardless of HPRT status (data not shown). This result suggests that expression of IMPDH type I in combination with a single allele of IMPDH type II does not compensate during lymphocyte activation either for the loss of the second allele of the type II gene or for the loss of HPRT activity.

T- and B-cell development and distribution. To examine the development of the T and B cells in mutant mice, flow cytometric analysis of thymocytes, bone marrow cells, and splenocytes was performed. There were no significant differences in the size of the thymus or spleen or in the number of cells from these organs. There were no significant differences among the four mice genotypes in T-cell development in the thymus, in B-cell development in the bone marrow, or in T- and B-cell distribution in the spleen (data not shown).

Effect of IMPDH and HPRT mutations on lymphocyte activation and drug sensitivity. Splenocytes from the four groups of mice were cultured in the presence of a variety of T- or B-cell activators, and cell proliferation was measured at 24, 48, and 72 hours after stimulation by measuring ^{3}H-thymidine incorporation into DNA. As shown in Figure 5, there were no significant differences in response to Con A stimulation of T cells (Fig-

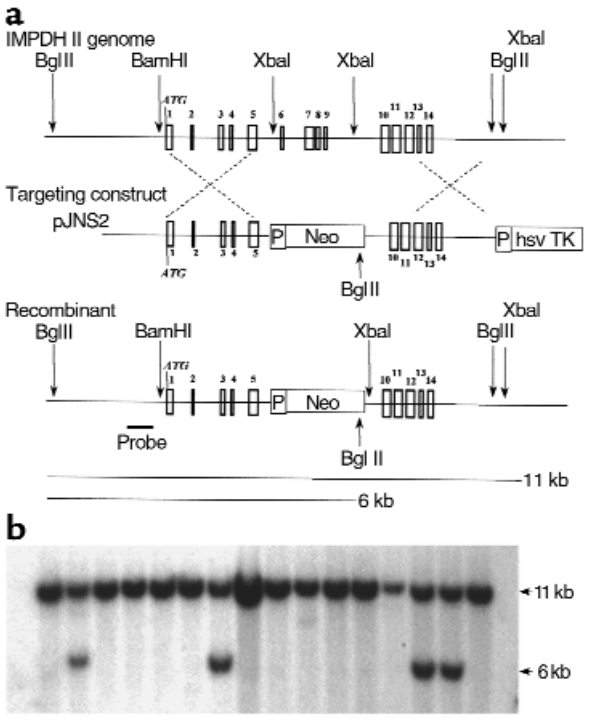


Figure 2
Targeted disruption of the IMPDH type II gene. (**a**) Restriction map of the wild-type IMPDH type II locus, the knock-out construct, and the expected recombinant allele. Exons are indicated by boxes. The two BglII fragments expected from the wild-type (11 kb) and recombinant (6 kb) alleles are shown, and the probe used for Southern hybridization is indicated. P indicates the PGK promoter. (**b**) Southern blot showing 11-kb and 6-kb bands in four heterozygous ES clones.

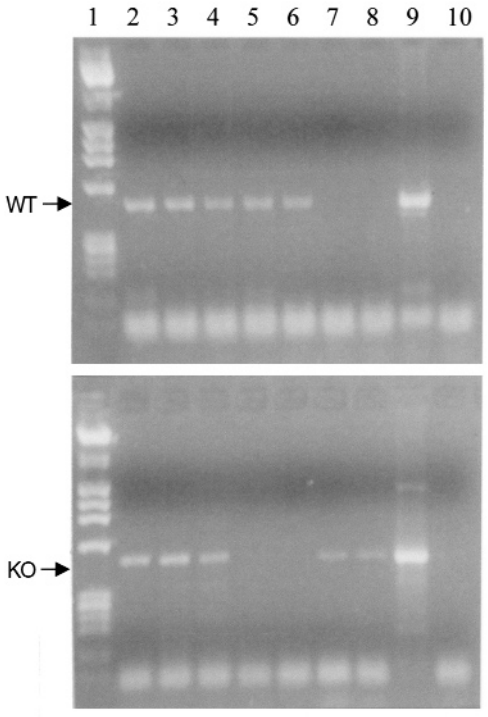


Figure 3
PCR genotyping of mouse blastocysts. Blastocysts were isolated from *IMPDH II*$^{+/-}$ breeding females at 3.5 dpc. PCR was performed using two sets of oligonucleotide primers. Lane 1 contains molecular weight markers of lambda HindIII plus PhiX174 Hae III. Lanes 2–4 are examples of heterozygotes, since they have both wild-type (WT) and knock-out (KO) bands; lanes 5 and 6 are examples of wild-type; and lanes 7 and 8 are examples of homozygotes. Lane 9 contains tail DNA from an IMPDH II heterozygous animal, and lane 10 represents a PCR reaction in the absence of DNA.

ure 5a) or LPS stimulation of B cells (Figure 5b). Proliferation in response to PMA plus ionomycin, however, was moderately reduced at 72 hours in lymphocytes from *IMPDH*$^{+/-}$ animals (Figure 5c). Very significant inhibition was seen with the use of anti-CD3 and -CD28 antibodies as costimulators, with a 50–75% reduction in proliferation in *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$ mice as compared with wild-type (Figure 5d). These results suggest that although guanine nucleotide synthesis is important for cell proliferation in general, T cells are more sensitive than B cells to these effects. Proliferation in response to T-cell receptor activation in conjunction with CD28 costimulation appears to be most sensitive to the effects of guanine nucleotide depletion.

To test whether T cells from *IMPDH*$^{+/-}$, *HPRT*$^{-/o}$ mice would be more sensitive to IMPDH inhibitor, T cells were stimulated by either anti-CD3 plus anti-CD28 antibodies (Figure 6a) or Con A (Figure 6b) in the absence or presence of various doses of MPA, a specific IMPDH inhibitor. Proliferation of splenocytes from mutant mice is significantly more sensitive to the inhibitor as compared with wild-type cells under both stimulation conditions.

To determine whether mutant mice produce normal levels of immunoglobulins, wild-type and mutant mice were injected with NP_{20}-chicken γ globulin to elicit a T cell–dependent B-cell response. Serum IgM and IgG level was measured before, 7 days after, and 19 days after immunization. No significant differences were found between wild-type and mutant mice (data not shown).

Cytotoxic T-cell function was assayed 5 days after an MLR as described in Methods. The cytolytic activity of the lymphocytes was determined by a standard ^{51}Cr-release assay. There was a consistent, statistically significant, 20–30% decrease in killing activity in cells from *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$ mice compared with cells from wild-type mice (Figure 7). To further determine whether mutant mice have a reduction in the ability to expand CD8-positive T cells upon viral infection, wild-type and mutant mice were injected with lymphocytic choriomeningitis virus and total CD8 T cells were quantitated before and 9 days after infection using flow cytometry. There was no significant difference in CD8-cell expansion between these groups (11.43 to 31.68% [± 4.9%], wild-type; 10.61 to 28.49% [± 7.5%], mutant).

Effect of IMPDH and HPRT mutations on purine nucleotide pools. Intracellular nucleoside triphosphate levels of ATP and GTP were measured in both resting and mitogen-stimulated splenocytes, and values were compared between the wild-type and *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$ mice (Figure 8). GTP levels were approximately 30% lower in lymphocytes from mutant mice as compared with the wild-type mice after anti-CD3 plus anti-CD28 antibody stimulation ($P < 0.003$). There was a slight decrease in GTP levels in Con A–stimulated cells that did not achieve statistical significance. There was no significant difference in ATP levels between the wild-type and mutant mice.

Discussion

IMPDH enzymatic activity is greatly increased in cells that are proliferating or have undergone malignant transformation (5, 9, 10) and is reduced in cells

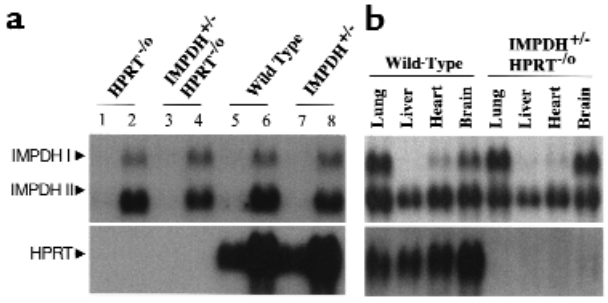


Figure 4
Northern blot analysis of IMPDH type I, type II, and HPRT mRNAs. (**a**) 15 μg of total RNA from resting (lanes 1, 3, 5, and 7) or Con A–activated (lanes 2, 4, 6, and 8) splenocytes from mice of four different genotypes was probed with IMPDH type I, type II, or HPRT cDNAs as indicated. (**b**) Northern hybridizations were performed on 20 μg of total RNA from various tissues of wild-type and *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$ mice.

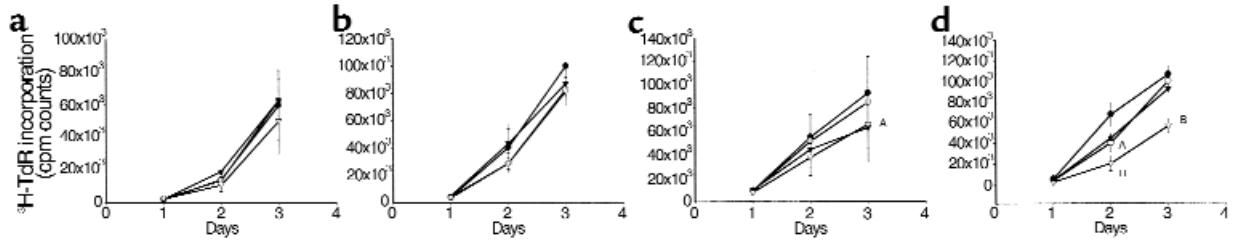


Figure 5
Effect of loss of IMPDH II and/or HPRT activities on mouse splenocyte proliferation. Mouse splenocytes were stimulated with Con A (**a**), LPS (**b**), PMA plus ionomycin (**c**), or anti-CD3 plus anti-CD28 (**d**) for 24, 48, and 72 hours. Proliferation of T or B cells from wild-type (closed circles), *HPRT*$^{-/o}$ (open circles), *IMPDH II*$^{+/-}$ (closed triangles), and *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$ (open triangles) mice was measured by ^{3}H-thymidine incorporation. n = 3 for *HPRT*$^{-/o}$ or *IMPDH II*$^{+/-}$ mice; n = 5 for *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$ or wild-type mice. Values are means ± SD. $^AP < 0.04$; $^BP < 0.003$.

induced to differentiate (20, 24). These observations constitute indirect evidence that the de novo pathway of guanine nucleotide biosynthesis is important in the overall regulation of cell growth and differentiation. Inhibitors of IMPDH activity are in clinical use as immunosuppressive drugs in the setting of organ transplantation and graft-versus-host disease (18, 19, 25). Despite this apparently selective clinical effect on T-lymphocyte responses in vivo, IMPDH inhibitors impair the activation of both T and B lymphocytes in vitro (26, 27) and, at somewhat higher concentration, also inhibit the proliferation of a number of cell lines (27, 28). Given the roughly equivalent inhibitory effects of IMPDH inhibitors on the activities of both type I and type II IMPDH isoenzymes, it has not been feasible to determine the relative roles of these isoenzymes in T-lymphocyte proliferative and functional responses. In order to determine the effects of loss of IMPDH type II expression on both cell growth and lymphocyte responsiveness in vivo, we have attempted to create a mouse model with selective deficiency of the *IMPDH* type II gene.

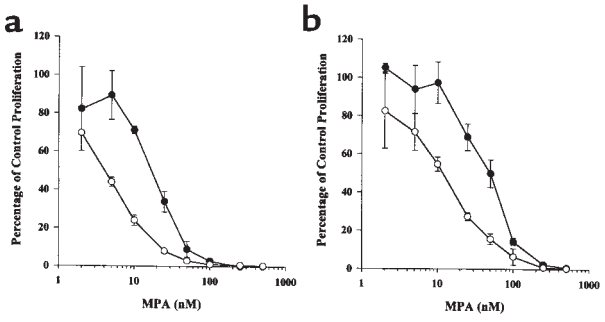
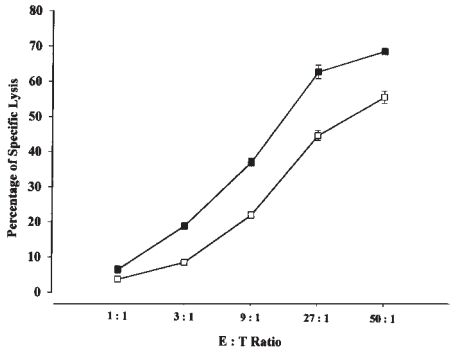


Figure 6
Effect of loss of IMPDH II and HPRT activity on drug sensitivity. Wild-type (closed circles) or mutant (open circles) splenocytes were stimulated with either antibodies (**a**) or Con A (**b**) in the absence or presence of increasing concentrations of MPA. Data represent one of three highly similar experiments performed in triplicate (± SD) and are plotted as the percentage of proliferation in the absence of drug.

We have demonstrated that homozygous loss of IMPDH type II gene expression results in very early embryonic lethality. Of the blastocysts analyzed, only 6% demonstrated targeting of both *IMPDH II* alleles, strongly suggesting that loss of the IMPDH type II enzyme does not permit the further development of fertilized ova. Previous studies have suggested a critical role for purines, especially for guanosine and hypoxanthine, in the maintenance of oocytes in meiotic arrest (29). Inhibitors of IMPDH such as MPA induced premature meiotic maturation and resulted in a significant loss of implantation capacity and viability after implantation (30). Our results also indicate that a small percentage of homozygous-deficient embryos may have implanted but underwent resorption shortly after, supporting an absolute requirement for IMPDH type II expression during this developmental stage and indicating that neither IMPDH type I activity nor HPRT activity with supplemental guanosine added to the maternal drinking water could substitute for IMPDH type II. Since a dramatic increase in embryonic growth rate is known to occur after implantation around 4.5 dpc (31), and since increases in the synthesis of guanine nucleotides are generally required to sustain increased cell proliferation, it seems highly likely that defective synthesis of guanine nucleotides is responsible for the embryonic lethality.

The requirement for guanine nucleotide biosynthesis for cell proliferation and the central role of IMPDH type II in this process are underscored by the upregulation of IMPDH II gene expression that occurs in the face of guanine nucleotide depletion (32). Our study indicates that, in the mouse model, neither IMPDH type I or HPRT expression can be increased in a similar compensatory fashion in the absence of IMPDH type II activity. We conclude from these data that the evolutionary conservation of two *IMPDH* genes encoding nearly identical proteins results from their differential regulation at the transcriptional level. The *IMPDH* type I gene is expressed in humans in a complex manner, with three different transcripts arising from three promoter regions 5′ to the gene (4), suggesting a requirement for tissue-specific and/or developmentally specific expression of this isoenzyme. Whether or not IMPDH type I is required for organ-specific develop-

Figure 7
Effect of mutations on the cytolytic activity of cytotoxic T lymphocytes. The function of cytotoxic T lymphocytes from wild-type (open squares) and *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$ (closed squares) mice was analyzed in a ^{51}Cr-release assay. A representative experiment performed in triplicate is shown. *P* values were calculated from four experiments performed in triplicate: $P < 0.05$ at E:T = 1:1; $P < 0.015$ at E:T = 3:1; $P < 0.012$ at E:T = 9:1, 27:1, and 50:1.



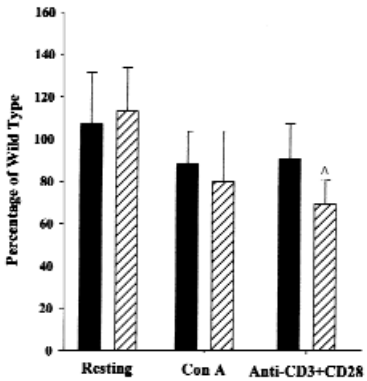
ment will have to be determined in studies on mice deficient in type I gene expression. IMPDH type II, on the other hand, is less restricted in its expression but is strongly upregulated at the transcriptional level by a promoter region that responds directly to growth stimuli (3, 33). The latter finding supports a more specific role in supporting cell proliferation.

A major reason for undertaking these studies was to determine whether loss of IMPDH type II protein would result in immunodeficiency analogous to that produced by inhibitors of the enzymes and, if so, by what mechanism. We have clearly shown that marked increases in the expression of both IMPDH isoenzymes in murine splenocytes result from T-cell mitogen stimulation. Loss of a single allele of IMPDH type II does not impair the mitogenic response, nor does it have any other demonstrable effects on the mouse phenotype. Given the prevailing hypothesis that deficiency of guanine nucleotides is causally related to the immunodeficiency resulting from IMPDH inhibition, we mated IMPDH heterozygous mice to HPRT-deficient mice to ask whether elimination of the salvage pathway would enhance the effects of loss of one *IMPDH II* allele. Although HPRT is frequently referred to as a "housekeeping" enzyme, it is clear from the present study that its expression also increases markedly with the activation of murine splenocytes.

Lymphocyte subsets from *IMPDH*$^{+/-}$, *HPRT*$^{-/o}$ mice were not significantly different from those in wild-type mice in the thymus, bone marrow, or spleen. However, the splenocytes from these animals demonstrated a very significant decrease in the proliferative response to anti-CD3 and anti-CD28 stimulation as compared with values from control mice and mice containing either mutation alone. In contrast, there was no significant difference in splenocyte responsiveness to LPS or Con A, and the response at 72 hours to PMA and ionomycin was only mildly reduced. Nucleotide pool data demonstrated a 25–30% reduction ($P < 0.003$), upon antibody stimulation, in the ability of *IMPDH*$^{+/-}$, *HPRT*$^{-/o}$ splenocytes to increase GTP levels, compared with the wild type, whereas the GTP levels in Con A–stimulated cells were reduced by only 10–15%. These data demonstrate that guanine nucleotide biosynthesis during lymphocyte activation via CD3 and CD28 costimulation is dependent on increased synthesis through both the de novo and salvage pathways. In addition, a relatively small reduction in the ability of lymphocytes to increase guanine nucleotide levels following activation via the T-cell receptor and CD28 significantly inhibits T-cell responsiveness. Explanations for these results include the possibilities (a) that lymphocyte response to mitogens such as Con A and LPS requires a lesser degree of GTP increase, and (b) that some component of the CD3/CD28 activation pathway is specifically dependent on a threshold level of GTP for signal transduction, as many studies have already demonstrated an important role for a number of G proteins and T-cell activation (34–36).

Finally, we have shown that the cytolytic T-cell function is reduced in *IMPDH*$^{+/-}$, *HPRT*$^{-/o}$ mice, although numerical expansion of CD8$^+$ T cells is similar in mutant and wild-type animals after LCMV infection. There are several pieces of evidence indicating that GTP is crucial in the signaling events in cytotoxic T lymphocytes that culminate in the exocytosis of cytoplas-

Figure 8
Alterations in ATP (closed bars) and GTP (hatched bars) levels in splenocytes of *IMPDH II*$^{+/-}$, *HPRT*$^{-/o}$ mice as a function of lymphocyte activation. Values were determined after 48 hours of stimulation with Con A or antibodies, are plotted as the percentage of values obtained from wild-type splenocytes under similar conditions, and represent the mean ± SD from duplicate determinations on five animals. $^{A}P < 0.0027$. Nucleotide levels (nmol/10^7 cells) for wild-type resting splenocytes were 2.105 (ATP) and 0.27 (GTP). Levels of GTP were increased by 3.4-fold ± 0.8-fold after Con A stimulation, and by 7.7-fold ± 2.6-fold following Ab stimulation.



mic granules containing perforin and the serine proteases granzyme A and B, causing apoptosis of target cells (37–39). The question of which signaling pathways are affected by impaired synthesis of guanine nucleotides will best be worked out in a genetic model of IMPDH deficiency.

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