Folate dietary insufficiency and folic acid supplementation similarly impair metabolism and compromise hematopoiesis

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ABSTRACT

While dietary folate deficiency is associated with increased risk for birth defects and other diseases, evidence suggests that supplementation with folic acid can contribute to predisposition to some diseases, including immune dysfunction and cancer. Herein, we show that diets supplemented with folic acid both below and above the recommended levels led to significantly altered metabolism in multiple tissues in mice. Surprisingly, both low and excessive dietary folate induced similar metabolic changes, which were particularly evident for nucleotide biosynthetic pathways in B-progenitor cells. Diet-induced metabolic changes in these cells partially phenocopied those observed in mice treated with anti-folate drugs, suggesting that both deficiency and excessive levels of dietary folic acid compromise folate-dependent biosynthetic pathways. Both folate deficiency and excessive dietary folate levels compromise hematopoiesis, resulting in defective cell cycle progression, persistent DNA damage, and impaired production of lymphocytes. These defects reduce the reconstitution potential in transplantation settings and increase radiation-induced mortality. We conclude that excessive folic acid supplementation can metabolically mimic dietary folate insufficiency, leading to similar functional impairment of hematopoiesis.

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Introduction

Given the genetic variability within the human population, divergent lifestyles, vastly variable diets, and inaccurate self-reporting, unambiguous links between diet and disease predisposition have been difficult to establish. Folate, a B vitamin, is an important factor for a number of metabolic pathways, including DNA methylation and the biosynthesis of nucleotides.[1] While dietary folate deficiency is a problem in much of the developing world, mandatory folate supplementation of grain products in the USA and Canada since the late 1990s has nearly eliminated dietary folate deficiency in these countries and reduced the rate of neural tube defects.[1,4]

Folate is important for the synthesis of purines and thymidylate, which are required for mitochondrial and cytosolic adenosine triphosphate (ATP), total nucleotide triphosphate (NTP), and deoxy-NTP (dNTP) production.[1] Folate also contributes to the one-carbon/methyl donor pathway, being critical for the production of S-adenosylmethionine (SAM), which is essential for the methylation of DNA, glutathione, and other macromolecules. Importantly, while natural folates in foods are primarily tetrahydrofolates (THF), folic acid (the synthetic oxidized form of folate) is the form primarily used for supplementation, due to its economical synthesis and good bioavailability.

High level folic acid intake is common in today's society, given both the supplementation of grains and the common consumption of additional vitamin supplements, energy drinks, and breakfast cereals with added folic acid.[2,3] Indeed, many breakfast cereals are fortified at 160-175% over reported levels,[4,5] and often consumed at well above the suggested serving sizes.[6] While the Recommended Dietary Allowance (RDA) for folate is 400 µg/day, folic acid supplementation above the recommended limit of 1000 µg/day is not uncommon for women of childbearing age.[7] Even higher daily doses, up to 5 mg, can be recommended for pregnant women with certain preconditions, such as obesity, diabetes, MTHFR status, or a history of pregnancies associated with neural tube defects.[8,9] Given that supplemented folate is primarily in the form of folic acid, which is not normally present *in vivo*, and that folic acid has been shown to inhibit at least one key metabolic enzyme,[10] it is imperative that we gain a full understanding as to how this supplementation impacts cellular metabolism.

Dietary folate levels have been linked to cancer risk in a puzzling way; dietary folate deficiency has been associated with increased risk of some cancers,[2] while excessive folic acid supplementation may also be associated with increased cancer risks.[2,3] For example, an inverse correlation between folate intake and the risk of colorectal adenocarcinomas have been supported by some studies carried out in both mice and humans.[11] In contrast, other trials indicate that folic acid supplementation (1 mg/day) after detection of polyps or in individuals with a history of colorectal adenoma is associated with increased progression to, or recurrence of, adenomas;[12-14] a connection further supported by mouse studies.[15] Moreover, a reversal in the downward trend of colorectal cancer incidence in the USA and Canada is evident, starting in 1996 and coinciding with the onset of folate supplementation in these countries.[3] Another clinical trial showed that supplementation with folic acid plus vitamin B12 increased cancer incidence and all-cause mortality in patients with ischemic heart disease.[16] Nonetheless, other studies have failed to observe such associations,[17] and differences in supplementation and the myriad of other genetic, dietary and lifestyle complications likely contribute to the lack of clear associations.

Both low and high levels of dietary folate have been shown to negatively impact immune function in humans. A study of postmenopausal women describes a bell-shaped curve for folate intake and natural killer (NK) cell cytotoxicity,[18] with reduced NK cell activity in both low and high intake groups. This study also noted an inverse association between unmetabolized folic acid in plasma and NK cell cytotoxicity, suggesting that free folic acid may negatively impact immune function. Maternal folate supplementation has been shown to associate with increased incidence of allergy-related respiratory impairment in children[18] and multi-generational respiratory defects in rats.[18] In rats, altering dietary folate levels reduces the percentages of circulating B cells and augments splenic lymphocyte responses to lipopolysaccharide (LPS; particularly in the context of folic acid supplementation).[19] Moreover, long-term and multigenerational exposure to folic acid supplementation can exacerbate neural tube defects associated with several different mutations in mice.[20] On the other hand, maternal folate supplementation is associated with a number of positive health outcomes (in humans and rodents), such as reductions of neural tube defects and congenital cardiac defects in children.[18] Taken together, these observations suggest that both insufficient and excessive dietary folate can impact multiple tissues in as of yet undefined ways, highlighting our lack of understanding of how alterations in dietary folate levels impact cellular homeostasis.

Given that both low and high dietary folate have been associated with various diseases, in the study herein we sought to determine how modulating dietary folate levels impact metabolic, developmental, and physiological processes in hematopoietic progenitor cells. Strikingly, we found that both insufficient and excessive dietary folate levels similarly compromised nucleotide metabolism, leading to functional defects in hematopoietic cells.

Methdos

Mice and folate supplementation

Mice were fed deficient (FD; 0.1 mg/ kg folic acid), control (CD; 2 mg/ kg folic acid), or supra-folate diets (SD; 10 mg/ kg folic acid). 2mg/kg folic acid complies with the recommendations of the American Institute for Nutrition for rodents.[21] The chow was purchased from Research Diets (AIN-76A, except that folic acid levels were varied), and was sterilized by irradiation. All chow was supplemented with the antibiotic succinylsulfathiazole to prevent folate production from gut bacteria.

Mass spectrometry analysis for organ metabolomics

Bone marrow (BM) B-cell progenitors were isolated by anti-B220 Magnetic Activated Cell Sorting (Miltenyi Biotec) and processed for ultra high performance liquid chromatography - mass spectrometry (UHPLC-MS) analysis as described in *Online Supplementary Methods.*

Untargeted quantitative 1H-nuclear magnetic resonance metabolomics analysis

Isolated B-cell progenitors from pooled animals were extracted and nuclear magnetic resonance (NMR) analyses performed on a Bruker 500 MHz spectrometer, as described in *Online Supplementary Methods.*

Bone marrow transplants

For competitive BM transplantation assays shown in Figure 6, whole donor BM from mice on various levels of dietary folate (green fluorescent protein (GFP)-) was mixed at a 3:1 ratio with competitor BM (GFP+) from GFP-expressing mice fed normal folate diets. For competitive BM transplantation assays shown in Figure 6, whole donor BM from mice on various levels of dietary folate was mixed at a 3:1 ratio with green fluorescent protein (GFP)-expressing competitor BM from mice fed normal folate diets.

Flow cytometric analysis and complete blood counts

For surface stains: Single-cell suspensions were plated in 96-well round-bottomed plates and washed in fluorescence-activated cell sorting (FACS) buffer [3% fetal bovine serum (FBS) + 1X phosphate buffered saline (PBS) + 2mM ethylenediamine tetraacetic acid (EDTA; v/v)]. After washing, cells were surface stained for 1 hour on ice in 50 µl of antibody solution, and analyzed by flow cytometry to identify the hematopoietic populations of interest. The antibodies used are listed in *Online Supplementary Methods.*
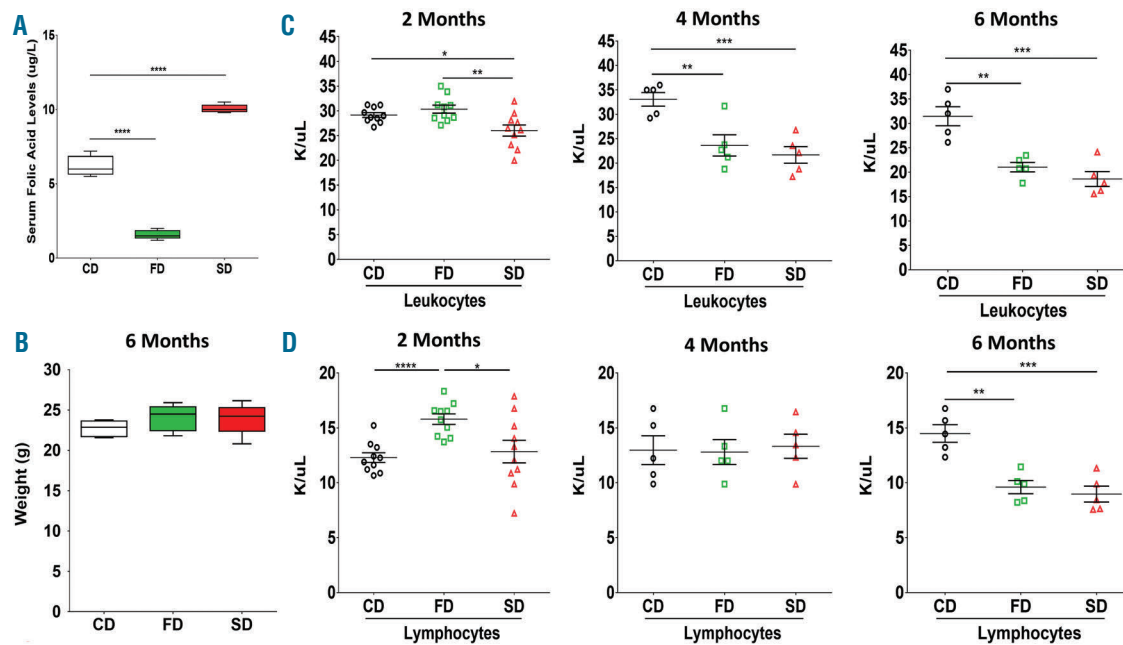
Figure 1. Mice on low and high dietary folate exhibit reduced peripheral leukocyte numbers. (A) BALB/c mice were fed control (CD), folate-deficient (FD) and supra-folate (SD) diets for 4 months and serum was collected and analyzed for the presence of folic acid using a folic acid microbiological test kit. Values represent mean ±SEM of (6 total samples). (B) The weights of BALB/c mice maintained on CD, FD and SD for six months are shown. Weights represent mean ±SEM of 2 independent sets of mice (6 total samples). (C and D) Complete blood counts were performed on peripheral blood collected from BALB/c mice kept on CD, FD and SD folate diets for 2, 4, and 6 months. Values represent mean ±SEM from 5-10 mice/diet at the various time points. All statistical analyses were performed using Student's t-test relative to CD for each experiment. Student's *t*-test was used in (A) and (B) comparing both FD and SD diets to CD. In (C) and (D) statistical analyses were performed using a one-way ANOVA test followed by Tukey's post-test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Complete blood counts

Peripheral blood was collected from the lateral tail vein in heparinized microfuge tubes at the indicated time points. Complete blood counts were performed on a Cell-Dyn 1700 (Abbott Laboratories, Abbott Park, IL, USA).

Statistics

Unpaired t-tests, the Cox proportional hazards, and one-way ANOVA were used to analyze experiments, with significance indicated by *P<0.05, **P<0.01, ***P<0.001, and **** P<0.0001. All errors shown represent biological replicates (different mice), not technical replicates. Statistical analyses were performed using GraphPad Prism (version 6.07; GraphPad Software). Survival curves were analyzed by the log-rank (Mantel–Cox) test. All results are expressed as mean ± SEM.

Results

Mice on low and high dietary folate exhibit reduced peripheral leukocyte numbers

In order to determine the effects of having too little or too much dietary folate, Balb/c mice were fed deficient (FD; 0.1 mg/kg), control (CD; 2 mg/kg), or supra (SD; 10 mg/kg) folate diets for 2 to 12 months. We analyzed how these diets affected circulating folate levels, weight, and the representation of peripheral blood cells. The folate-deficient (FD) diet resulted in a ~3-fold decrease in the amount of circulating folate while the supra-folate (SD) diet significantly increased serum folate levels by ~2-fold (Figure 1A). The observed folate levels for control and supra-folate diets in mice are within the range of variances observed in humans (5.9-24.6 µg/L depending on the extent of fortification and supplementation reported for participants from the USA between 2001 and 2004).[22] The observed folate levels for the FD diet in mice reflect what is considered folate-deficiency for humans (3 µg/L).[23]

Given the importance of folate in the production of nucleotides and ATP, complete blood cell counts were performed in order to determine if altering dietary folate levels impacted circulating blood cell populations. Leukocyte numbers were consistently reduced in mice on both FD and SD diets, which was apparent by 4-6 months on the altered diets (Figure 1C). By 6 months on these diets, reductions in peripheral lymphocyte numbers were also evident (Figure 1D). The representation of circulating neutrophils and thrombocytes was not significantly altered by modulating dietary folate levels for up to 6 months (*Online Supplementary Figure S1A-S1C*); however, hemoglobin and red blood cells were significantly reduced with FD diets in some experiments, but not others (*Online Supplementary Figure S1D*). Importantly, mice maintained on the FD, SD, and CD diets for up to a year were not distinguishable by observable features including differences in weight (Figure 1B and *Online Supplementary Figure S1E*), activity, or survival (*data not shown*). Taken together, these observations reveal that mice on the FD and SD diets maintained normal physical phenotypes, but exhibited reduced circulating levels of leukocytes and lymphocytes.

Control, folate-deficient and supra-folate diets alter cellular homeostasis in distinctive ways

Based on the observation that both FD and SD diets reproducibly reduced systemic lymphocyte numbers, we next determined how altering dietary folate levels affected
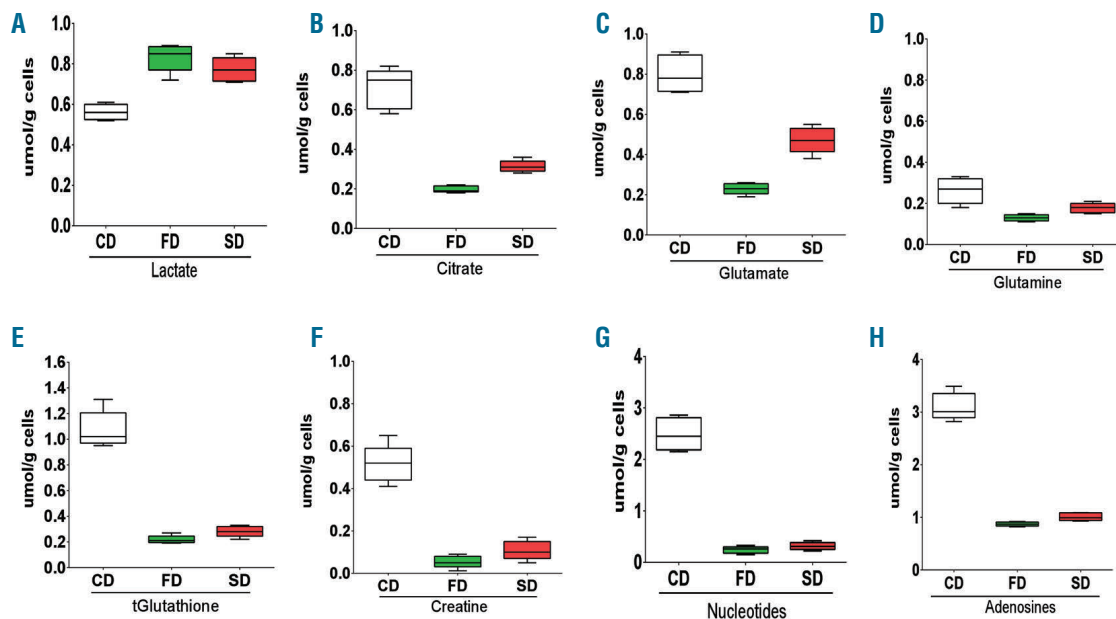
Figure 2. Both deficient and supra dietary folate levels alter metabolism in B-progenitor cells. (A-H) BALB/c mice were fed control (CD), folate-deficient (FD) and supra-folate (SD) diets for 4 months and B-progenitor cells were isolated using B220⁺ MACS selection. NMR analysis was performed on these cells in order to determine diet-induced metabolic changes. Values represent mean ±SEM from 2 independent experiments (5 pooled mice/group/experiment), which precluded statistical analysis due to the number of technical replicates (n=2).

metabolism in B-lymphocyte progenitor cells, given that this is a highly proliferative population in the BM. Altering dietary folate levels did not significantly change the representation of pro-B, pre-B, and immature B-progenitor cell populations in the BM (*Online Supplementary Figure S2*), although the ratios of pro-B to pre-B cells trended higher in mice on FD and SD diets. NMR metabolomic analysis of BM B220⁺ cells revealed that B-cell progenitors from mice on both the FD and SD diets exhibited significantly increased lactate levels (Figure 2A), with reductions in citrate, glutamate and glutamine levels, suggesting that unbalanced dietary folate levels alters central carbon metabolism (Figure 2B-D; *Online Supplementary Table S1*). Furthermore, levels of total glutathione (a key cellular reducing agent), which is derived from s-adenosyl methionine (SAM), glutamine and glutamate, were significantly reduced in B-progenitors isolated from mice on the FD and SD diets (Figure 2E). Additionally, both FD and SD diets significantly decreased creatine (which can be used to make ATP), total nucleotides, and total adenosine levels in B-cell progenitors (Figures 2F-H).

Due to the striking observation that both low and high levels of dietary folate similarly compromise metabolic activity in B-progenitor cells, we further explored metabolic perturbations resulting from altered folate intake using ultra-high pressure liquid chromatography combined with UHPLC-MS. This method employs a highly sensitive technique that allows for the robust and comprehensive detection of metabolites in a high-throughput manner (for full results see *Online Supplementary Table S2*). We additionally determined if alterations in dietary folate levels induce metabolic perturbations in the heart, liver, and intestinal tissues (*Online Supplementary Figure S3* and *Online Supplementary Table S2*). Unsupervised hierarchical clustering and partially supervised partial least squares-

discriminant analysis (PLS-DA) of metabolomics data revealed that both the FD and SD diets impacted metabolism in B-cell progenitors, the heart, liver, and intestines, with each altered diet eliciting distinct signatures (*Online Supplementary Figures S3B-S3F*). PC1 explains the highest percentage of the variance (20-30%) calculated on the basis of metabolic phenotypes of a given sample set. Notably, in all tested tissues PC1 could discriminate between the CD and the FD/SD diets, while the FD and SD diets either overlapped or were partially discriminated along PC2 (explaining < 15% of the variance). Results were even more striking when performing unsupervised hierarchical clustering for B-progenitor cells (Euclidean distance, furthest neighbor – *Online Supplementary Figure S3B*), which revealed co-clustering of FD and SD samples (t-test of square distances - *P*-value = 0.27) and separate clustering of CD samples (t-test of square distances - *P*-value$_{CD/FD}$ = 0.052 and *P*-value$_{CD/SD}$ = 0.016).

We performed a Metabolite Set Enrichment Analysis (MSEA) to identify metabolic pathways that were significantly enriched (False discovery rate (FDR) < 0.05) in the tissues from mice on both the FD and SD diets. Results revealed enrichment for purine metabolism and alanine, aspartate, and glutamate metabolism for B-progenitors (*Online Supplementary Figure S4*); protein biosynthesis for liver; purine metabolism, protein biosynthesis, gluconeogenesis, and the urea cycle for heart; and taurine and hypotaurine metabolism for intestines (*Online Supplementary Table S3*). These results further highlight that diets deficient in folate or having excess levels of folate alter cellular function in multiple organs, and that insufficient and excess dietary folate have profound and comparable impacts on metabolism.

Surprisingly, we did not observe significant changes within the one-carbon metabolism pathway leading to
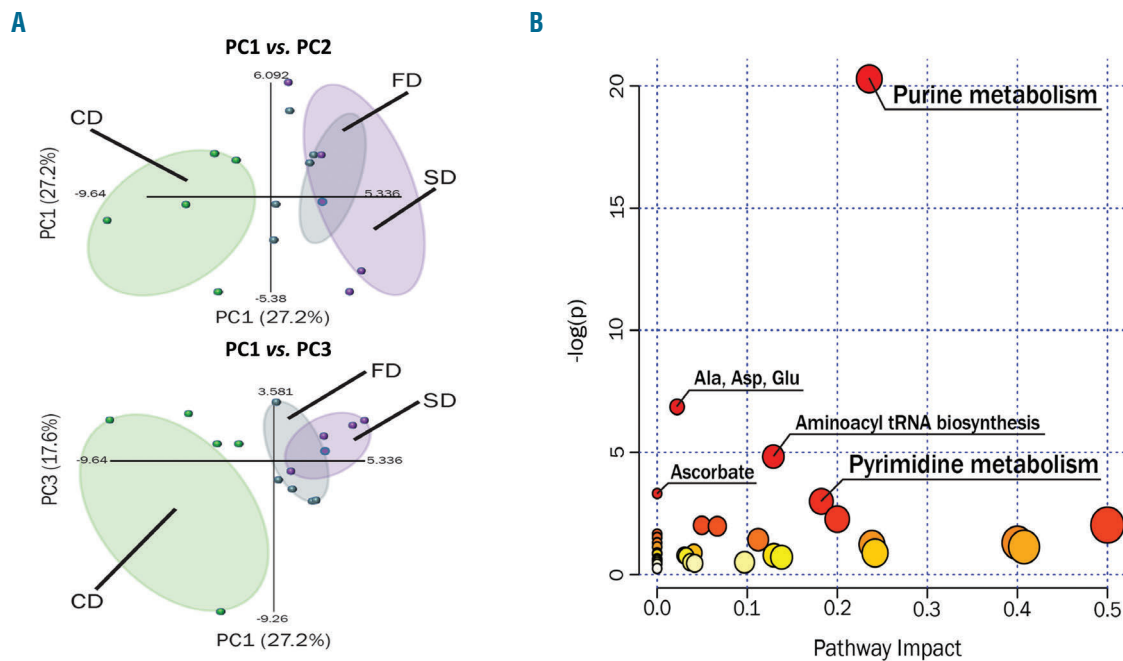
Figure 3. Both folate-deficient and supra-folate diets impact nucleotide metabolism in B-progenitor cells. BALB/c mice were fed control (CD), folate-deficient (FD) and supra-folate (SD) diets for 4 months (2 independent experiments with group sizes of 2 and 3 mice). Metabolites altered by changes in dietary folate levels were identified in B-progenitor cells (B220+ cells isolated by MACs selection) using UHPLC-MS (primary data are shown in *Online Supplementary Table S2* and B-progenitor cell profiles used for these experiment are shown in *Online Supplementary Figure S2*). (A) PLS-DA analysis was performed on the data from *Online Supplementary Table S2* to determine metabolites associated with nucleotide production in B-progenitor cells from animals on the three diets. (B) MSEA was performed to identify pathways that are affected by altered folic acid diets. Metabolic pathways are graphed based on scores from enrichment analysis (y-axis), which identifies enriched pathways based on the number of associated metabolites that are identified, and topology analysis (x-axis), which is a measure of a centrality for a particular pathway enriched in a given data set. The color of each circle is proportional to the *P*-value, with red representing relatively low *P*-value within the data set, and the size of each circle is proportional to the pathway impact value.

SAM, rather, there was a trend towards increased levels of SAM in B-progenitors from mice on the FD and SD diets (*Online Supplementary Table S2*). Methionine levels, along with most other amino acids, significantly increased in FD and SD B-progenitors (*Online Supplementary Figure S4A*). Given the significant impact of the FD and SD diets on nucleotide synthesis pathways, as shown using both NMR and MS approaches, we have focused on nucleotide metabolism and its impact on cell fitness.

Folate-deficient and supra-folate diets similarly reduce nucleotide metabolism in B-progenitor cells

Given the strikingly similar metabolic alterations induced by FD and SD diets on B-progenitor cells and observed reductions in lymphocyte numbers in peripheral blood, PLS-DA and hierarchical clustering were performed to identify similar pathways in these cells that were affected by altering dietary folate. Consistent with the enrichment for purine metabolism by metabolite set enrichment analysis (MSEA), PLS-DA analysis indicated a highly similar impact of both the FD and SD diets on nucleotide metabolism (Figure 3A). Pathway analysis of metabolites showing the highest loading weights along PC1 in PLS-DA analyses revealed that both the FD and SD diets (compared to CD) significantly and similarly impacted purine metabolism, pyrimidine metabolism and amino acid homeostasis (Figure 3B and *Online Supplementary Figure S3B*), most likely by altering the function of similar enzymes involved in these metabolic pathways (*Online Supplementary Figure S4C*). Nonetheless, certain metabo-

lites were differentially altered in B-cell progenitors from mice on the FD and SD diets. In particular, we observed increased levels of saturated and unsaturated fatty acid metabolites in FD B-progenitors, but decreased levels of many of the same metabolites in SD B-progenitors (*Online Supplementary Table S2*).

Various metabolites in the purine and pyrimidine nucleotide synthesis pathways were significantly perturbed by altering dietary folate. Reductions in the levels of inosine, adenylosuccinate, and guanine were evident in B-progenitors from mice on the FD and SD diets relative to those on the CD diet (Figure 4A,B). Moreover, we observed significant reductions in adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine monophosphate (GMP), guanosine diphosphate (GDP), and Guanosine-5'-triphosphate (GTP; Figure 4B) in B-progenitors from mice on the FD and SD diets. Large reductions in oxidative Pentose Phosphate Pathway intermediates, such as D-Glucono-1-5-lactone 6-phosphate and 6-phosphogluconate, coupled with increased non-oxidative phase intermediates such as sedoheptulose 1-phosphate and ribose 1-phosphate, were also observed in both FD and SD progenitors (*Online Supplementary Table S2*). Furthermore, we observed reductions in both oxidized and reduced glutathione (*Online Supplementary Figure S5*) and in the key second messenger, cyclic-AMP, perhaps as a consequence of reduced ATP availability, which could impact redox control and cell signaling. While levels of deoxyadenosine triphosphate (dATP) were modestly reduced in B-progenitors from SD
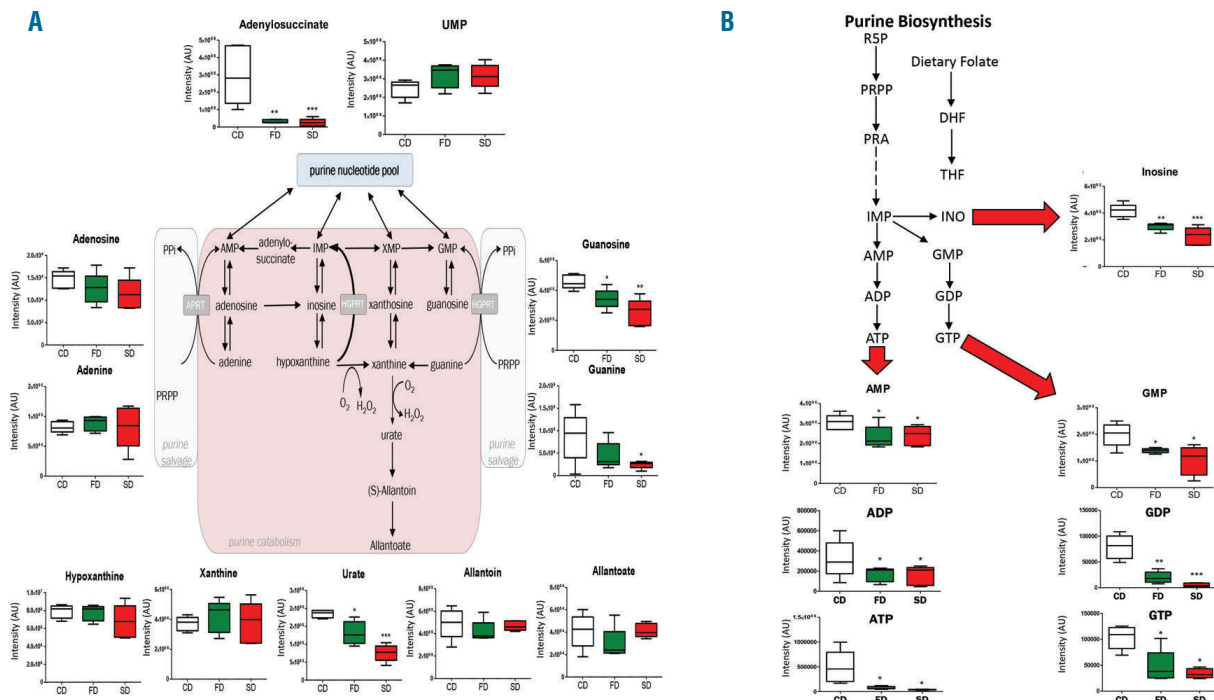
Figure 4. Folate-deficient and supra-folate diets compromise purine nucleotide metabolism. (A and B) BALB/c mice were fed control (CD), folate-deficient (FD), and supra-folate (SD) diets for 4 months as described in Figure 3, and specific metabolites in the purine nucleotide synthesis pathway that were altered by changes in dietary folate levels are graphed (data presented in *Online Supplementary Table S2*). Values represent mean ±SEM of 5 mice/diet from 2 independent experiments with group sizes of 2 and 3 mice. Each metabolite was analyzed using the Student's *t*-test with the CD values set as the control. *$P<0.05$; **$P<0.01$; ***$P<0.001$; UMP: uridine monophosphate; IMP; inosine monophosphate; AMP: adenosine monophosphate; ADP: adenosine diphosphate; ATP: adenosine triphosphate; GMP: guanosine monophosphate; GDP: guanosine diphosphate; GTP: guanosine triphosphate

mice, levels of deoxyguanosine triphosphate (dGTP) were reduced by 4.3- and 9.7-fold in FD and SD B-progenitors, respectively (*Online Supplementary Table S2*), which could contribute to impaired DNA synthesis. Defects were also observed for pyrimidine synthesis, with reductions in uridine monophosphate (UMP), uridine-5'-triphosphate (UTP), and deoxythymidine monophospate (dTMP) in FD and SD B-progenitors (*Online Supplementary Figure S6*).

Since both high and low folate intake result in a similar phenotype, we hypothesize that excess folate may exert negative inhibitory activity on rate-limiting enzymes of folate metabolism, particularly dihydrofolate reductase (DHFR). To test this hypothesis, we asked whether treatment of mice for 5 consecutive days with the DHFR inhibitor methotrexate (MTX), which is routinely used as a chemotherapeutic agent, would phenocopy purine metabolic defects observed in FD and SD mice in the B-progenitor cell compartment. Treatment of wild-type Balb/c mice (maintained on standard mouse chow with 2 mg/kg folic acid) with MTX resulted in patterns of impaired nucleotide metabolism in B-progenitor cells, which in some cases mirrored those observed for progenitors from mice on the FD and SD diets. Surprisingly, the impacts of MTX treatment on these metabolites were less than for the altered diets (Figure 5A and *Online Supplementary Figure S6*; for full results see *Online Supplementary Table S4*). For example, decreases in GMP and AMP observed in B-cell progenitors from mice on the FD and SD diets were recapitulated in B-cell progenitors isolated from MTX treated mice (Figure 5B).

The function of pro B-progenitor cells is impaired in mice fed folate deficient and supra-folate diets

We determined the effects of these diets on hematopoiesis in mice. The numbers of early hematopoietic stem and progenitor cells (HSPC) and myeloid progenitor cells in the tibias and femurs did not differ for mice on the CD, FD and SD diets (*Online Supplementary Figure S7*). Given that the FD and SD diets led to a reduction in the number of circulating lymphocytes and reduced metabolism in B-progenitor cells, we next determined if these diets altered DNA replication in B-progenitors using EdU (5-ethynly 2'-deoxyuridine) incorporation assays (Figure 6A). While the percentage of B-progenitor cells in S-phase did not change as a result of modulating dietary folate levels (Figure 6B), the FD and SD diets significantly reduced the rate of S-phase progression in pro-B-cells (particularly in the case of folate deficiency; Figure 6C) and compromised the efficiency of nucleotide incorporation (Figure 6D). Furthermore, we observed that FD and SD diets promoted persistent DNA damage in B-progenitor cells (Figure 6E).

Both low and high dietary folate impair hematopoietic reconstitution post-irradiation

Transgenic mice ubiquitously expressing GFP (GFP[TG]) were irradiated and transplanted with GFP[neg] whole bone marrow cells (BMC) isolated from mice fed CD, FD and SD diets, along with competitor GFP[TG] whole BM (at a 3:1 ratio). Recipient mice were maintained on a normal diet (2 mg/kg folate). While the contribution of donor CD BMC
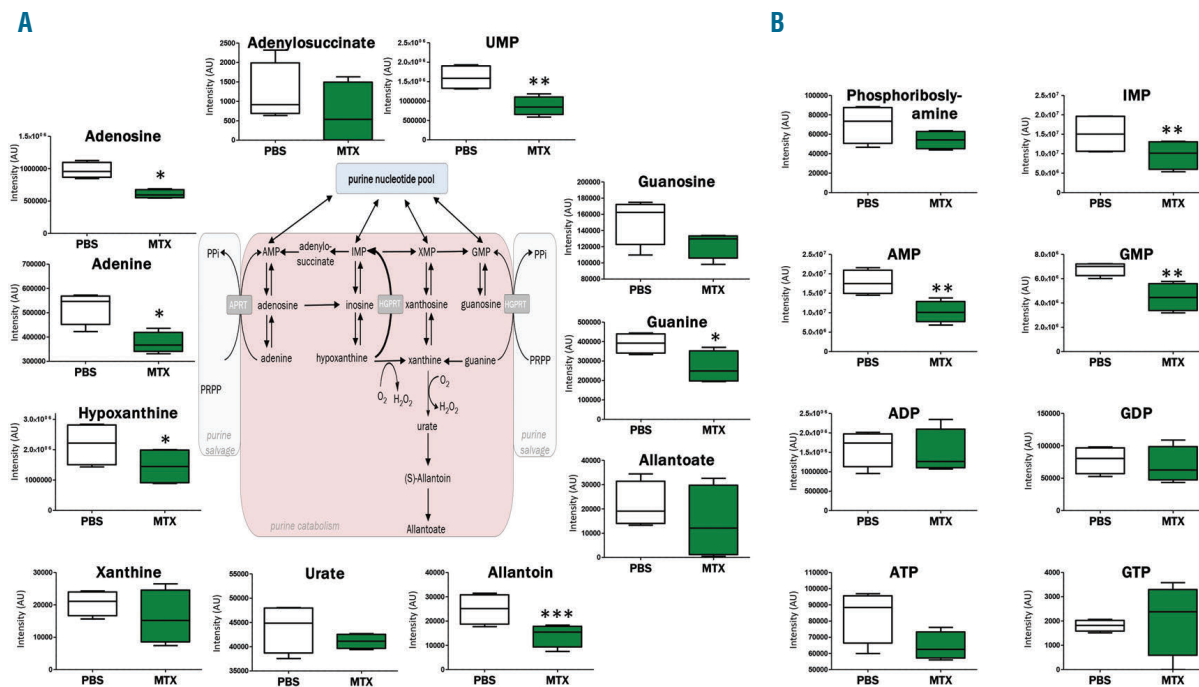
Figure 5. Methotrexate reduces purine synthesis in B-progenitor cells. BALB/c mice were treated for 5 consecutive days with vehicle (1X PBS) or methotrexate (MTX; 10 mg/kg) injected intraperitoneally. (A and B) On the day after the last injection, B-progenitor cells were isolated by MACs selections (B220⁺) and UHPLC-MS was used to determine the metabolic changes in the purine nucleotide synthesis pathway that resulted from treatment with MTX. Values represent mean ±SEM of 4 mice/treatment group. Student's t-test was used to compare metabolic changes induced in B-progenitors isolated from PBS and MTX treated mice. *$P<0.05$; **$P<0.01$; ***$P<0.001$. UMP: uridine monophosphate; IMP; inosine monophosphate; AMP: adenosine monophosphate; ADP: adenosine diphosphate; ATP: adenosine triphosphate; GMP: guanosine monophosphate; GDP: guanosine diphosphate; GTP: guanosine triphosphate.

continued to increase in recipient mice for over 6 months, we observed a significant and steady decline in hematopoietic contributions to peripheral blood cells from donor FD and SD BMC (Figure 7A). Upon sacrifice of recipient mice at 7 months post-transplant, clear reductions in contributions towards B-cell, myeloid, and multipotent progenitor populations from transplanted FD and SD BMC were evident (Figure 7B-D). While the decline in hematopoietic contribution from FD and SD donor BMC could result from altered methylation states, no significant total DNA methylation differences were observed in B-progenitor cells isolated from FD or SD mice (Figure 6F), consistent with the lack of effects on one-carbon metabolism. Since FD and SD diets did not significantly alter the number of long-term hematopoietic stem cells (*Online Supplementary Figure S7A-S7C*) or common myeloid progenitor cells (*Online Supplementary Figure S7D-S7F*), it is unlikely that the declining contributions of FD and SD BMC to hematopoiesis in recipient mice resulted from the transplantation of unequal numbers of HSCP. Therefore, transplanted FD and SD HSCP are likely compromised, leading to reduced hematopoietic reconstitution potential prior to or after seeding of the BM microenvironment of CD recipient mice.

Given these observations, we asked whether altered folate diets would impact survival post-irradiation for mice. Mice maintained on CD, FD and SD diets for 4 months were sublethally irradiated (5 Gy) and monitored for survival post-irradiation. Surprisingly, all mice fed low and high levels of dietary folate succumbed to irradiation-

induced complications within two weeks post-irradiation and required sacrifice, whereas only one CD mouse was removed from the study due to signs of morbidity (Figure 7E). The increased mortality for mice on the FD and SD diets post-irradiation likely, or at least in part, could result from reduced ability to restore hematopoiesis post-irradiation. Nonetheless, since the mechanism of death was not determined, impacts of altered dietary folate on other organs (i.e., the intestines) could contribute to enhanced radiation sensitivity.

Discussion

Data presented herein support a model whereby impairment of folate-dependent metabolism due to diets both with high and low folic acid dietary supplementation leads to hematopoietic defects (Figure 7F). As previously reviewed,[2,28,24] while folic acid supplementation has been shown to be beneficial in reducing neural tube defects in newborns, folic acid supplementation may also be associated with numerous health problems in humans. These include respiratory disorders, cancers, autism spectrum disorders, and multiple sclerosis (although cause and effect relationships were not established, and some studies fail to find such associations). A number of these associations have been tested and substantiated with rodent models. Dietary deficiency in folates is also linked to a number of disorders, including cancer and neural tube defects. However, our understanding of how both insufficient and
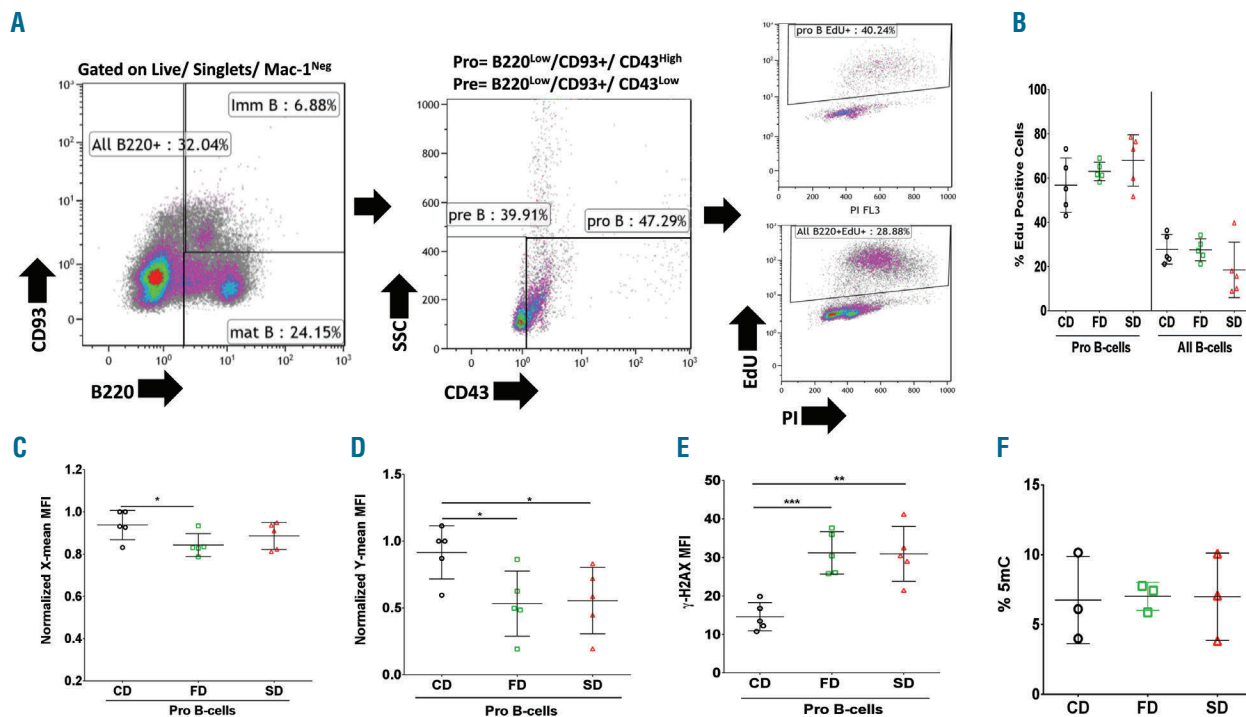
Figure 6. Alterations in dietary folate levels lead to functional impairments in B-progenitor cells. BALB/c mice were fed control (CD), folate-deficient (FD), and supra-folate (SD) diets for 5 months and *in vivo* EdU incorporation was determined in B-progenitor cell populations (A). The percentage of EdU positive B-progenitor cells (B) and the normalized X-mean and Y-mean mean fluorescence intensity (MFI) were calculated (C and D). The normalized X-mean and Y-mean MFI serve as proxies for progression through S-phase and the efficiency of nucleotide incorporation, respectively. The normalized X-mean MFI of EdU+ populations was calculated using the following formula: [(x-mean MFI of EdU+ cells) - (x-mean MFI of the G1 population)]. The normalized Y-mean MFI of EdU+ populations was calculated using the following formula: [(y-mean MFI of EdU+ cells) - (y-mean MFI of EdU- cells)]. (E) Bone marrow derived pro B-progenitor cells were identified using the gating strategy defined in (A) and intracellular staining was performed in order to determine phospho-γH2AX levels in this population using flow cytometric analysis. (F) For the DNA methylation assay, DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) from MACS-sorted B220+ cells (Miltenyi Biotec). Methylation levels were analyzed using MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Epigentek) per manufacturer's instructions. One representative experiment out of two is shown. Controls provided by the kit as well as 5-azacytidine-treated KG-1 cells as a hypomethylated DNA control were used (*data not shown*). Values in (A-E) represent mean ±SEM of 5 samples/ group. All statistical analyses were performed using a one-way ANOVA followed by a Tukey's post-test in order to compare the effects of all diets to each other.

excess folate could contribute to reductions in human health is limited.

Data presented herein demonstrate that both low and high levels of dietary folate compromise metabolism in multiple organs, with significant defects manifesting in the B-progenitor compartment. Although folate is required in multiple biosynthetic pathways (including for nucleotides, glutathione, SAM and amino acids), we primarily observed deficiencies in the nucleotide synthesis pathways for B-progenitors isolated from mice fed FD and SD diets, as demonstrated using both NMR and UHPLC-mass spectrometry. The impact of the FD and SD diets on reduced nucleotide synthesis in B-progenitor cells likely contributed to the S-phase proliferative defects observed using EdU analysis.

The observation that both low and high levels of dietary folate promote similar metabolic defects in hematopoietic cells is surprising. Nonetheless, based on mathematical modeling, Ulrich and colleagues have suggested that adverse effects of high folate diets might be explained by the ability of key enzymes, including in the purine and pyrimidine synthetic pathways, to be inhibited by products of folate metabolism.[2,25] For example, thymidylate synthase (TS) uses methylene-tetrahydrofolate (MTHF) to

make dTMP from deoxyuridine monophosphate (dUMP), producing dihydrofolate (DHF), which must be recycled by DHFR to regenerate tetrahydrofuran (THF). Importantly, DHF can inhibit TS activity.[2,25] Notably, supplemented folic acid requires reduction by DHFR (mostly in the liver) to DHF, but this reaction is more than 1000X slower relative to the reduction of DHF to THF, and DHFR exhibits variable activity in the livers of different people.[10] Accordingly, folic acid inhibits DHFR reduction of DHF.[10] A possible explanation for the metabolic defects observed in SD B-progenitors is that excessive folic acid may inhibit DHFR, thus impairing reduced folate dependent pathways. Indeed, conversion of folic acid to the reduced folates DHF and THF presents saturation kinetics (additional substrate cannot be processed effectively), and folic acid consumption above ~200 μg/day is estimated to overwhelm an individual's ability to convert folic acid to THFs, leading to unmetabolized folic acid in the circulation.[6,26] Notably, in a study of postmenopausal women from the USA, circulating folic acid was found in 78% of participants,[22] and both mandated fortification and further supplementation were shown to have increased circulating folic acid levels in the Framingham Offspring Cohort.[27] Thus, albeit speculative, the negative impact of high folate
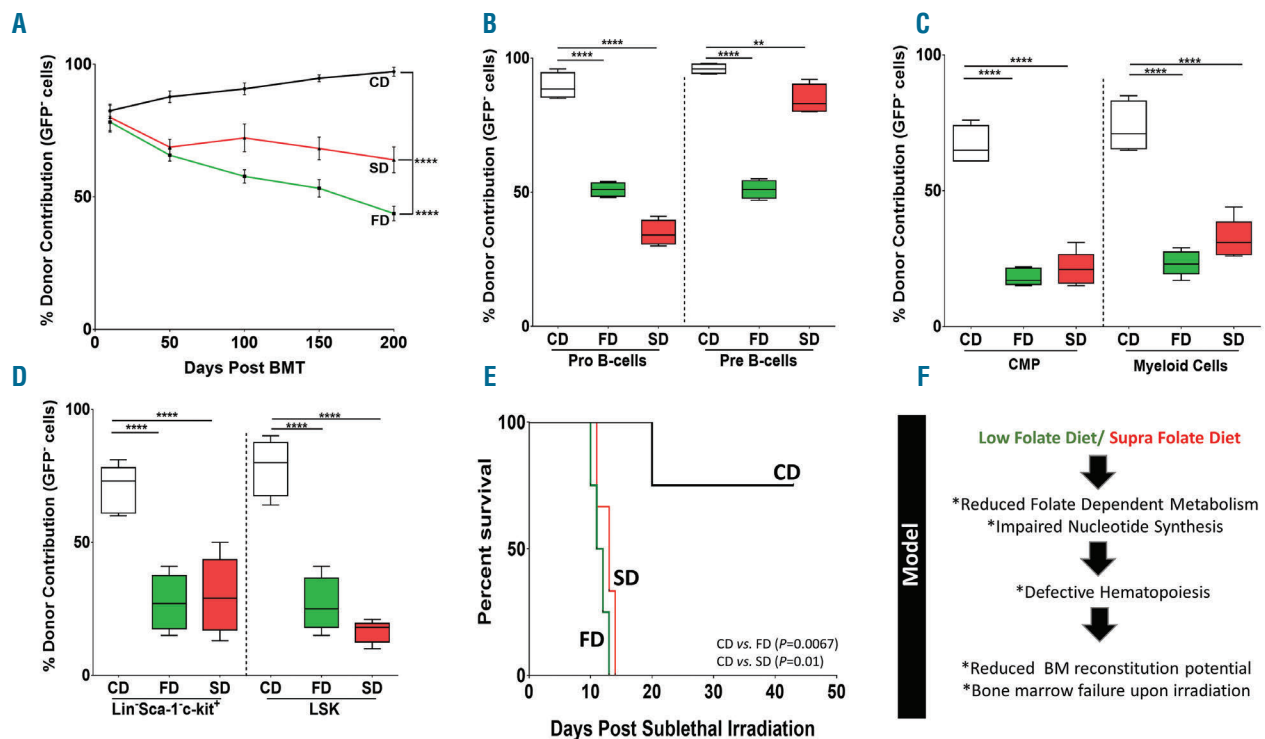
Figure 7. Alterations in dietary folate levels lead to reduced hematopoietic reconstitution potential and increased mortality following irradiation treatment. Two month old BALB/c GFP-expressing transgenic mice were lethally irradiated (10 Gy) and transplanted with GFP-negative whole BMC isolated from mice fed control (CD), folate-deficient (FD) and supra-folate (SD) diets for two months along with competitor BMC expressing GFP at a ratio of 3:1 (GFP^{Pos}: GFP^{Neg}). (A) The contribution of the donor BMC to hematopoiesis was assessed over 7 months by monitoring the percentage of GFP^{Neg} cells in the peripheral blood. The number of donor-derived B-progenitor cells (B), hematopoietic stem and progenitor cells (C), and myeloid progenitor cells (D) was determined by flow cytometric analysis. Values in (A-D) represent ±SEM of 4- 5 mice/ group. (E) BALB/c mice fed CD, FD, and SD diets for 4 months were sublethally irradiated (5 Gy) and survival monitored post-irradiation. The Kaplan-Meier curves reflect results pooled from 2 experiments with a total of 4 mice/ group. Statistical analyses in (A-D) were performed using a one-way ANOVA followed by a Tukey's post-test in order to compare the effects of all diets to each other, and in (E) the log-rank (Mantel-Cox) test was used to determine statistical significance. **$P<0.01$; ****$P<0.0001$. GFP: green fluorescent protein; BM: bone marrow; BMT: bone marrow transplant.

diets may not result from folates per se, but from supplementation with folic acid (as opposed to reduced folates);[28,29] however, further experimentation is required to test this hypothesis. Together with these previous studies, the results presented herein suggest that studies examining the impact of dietary folate on disease risk need to consider the source and type of folate.

We observed that both folate deficiency and supra-levels of dietary folate lead to reduced hematopoietic reconstitution potential in competitive bone marrow transplant (BMT) experiments and increased death post-irradiation, consistent with observed cell cycle defects in hematopoietic progenitors. Our BMT studies could indicate that the fitness defects apparent in hematopoietic progenitors are somatically heritable, in that they manifest in recipient mice that are maintained on normal diets. Still, given that hematopoietic progenitors in FD and SD mice appear to be deficient in their ability to reconstitute hematopoiesis post-irradiation, reduced competition in recipient mice could in part relate to an immediate failure in HSPC to reconstitute the host, independent of the impact of the altered diets on DNA methylation.

The paradoxical association of diets both low and high in folates with increased cancers has been ascribed to different effects of folate on cancer initiation and on the growth of preexisting tumors.[2,3] Folate insufficiency is thought to enhance cancer initiation by increasing the misincorporation of dUTP in DNA, leading to oncogenic mutations. For the latter, excessive folate is thought to fuel the growth of pre-existing tumors. However, we show that both insufficient and excessive folic acid are associated with impaired DNA synthesis. Our data suggest that common effects of insufficient or excessively supplemented folate on disease could have a common cause – impairments in folate-dependent metabolism. While it may seem counterintuitive that impairing pathways essential for cell proliferation (such as nucleotide synthesis) would increase the risk of cancer, these impairments would be expected to enhance selection for oncogenic events that are adaptive in this context.[30] In addition, increased levels of DNA damage could increase the frequency of potentially oncogenic events on which such altered selection could act.

Given our results that diets high in folic acid can, over time, have substantial negative impacts on tissue and progenitor cell metabolism and fitness, mirroring those of dietary folate deficiency, excessive folic acid consumption by many Americans could have unappreciated negative impacts on their health. There are clear indications that combined mandatory and voluntary folic acid supplementation are vastly exceeding the targeted level,[2,6,31,32] with the intake of folic acid for most Americans well above the

RDA. Indeed, 23% of the population of the USA (including 43% of children) were considered high in serum folates in the NHANES 1999-2000 study,[23] and the diets of many Americans are fortified at >1000 μg/day. With a substantial fraction of the North American population at risk for excessive folate intake, and much of the rest of the world potentially deficient in folate, the studies presented herein should stimulate parallel research in humans that could have significant public policy implications.

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