THE EFFECTS OF FOLIC ACID SUPPLEMENTATION ON NATURAL KILLER CELL ACTIVITY IN AN ANIMAL MODEL

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Nutritional Sciences
University of Toronto

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Abstract

An emerging body of evidence has linked high intake of folic acid, common in the Canadian population due to mandatory folic acid fortification and prevalent supplement use, to certain adverse health effects. High intake and blood levels of folic acid were associated with reduced natural killer cell cytotoxicity in a pilot study of post-menopausal women that has not yet been confirmed. As such, we investigated the effects of folic acid supplementation on natural killer cell activity in a mouse model. Folic acid supplementation, plasma folate, and plasma unmetabolized folic acid were associated with reduced NK cell degranulation in response to stimulation with malignant cells. Our data corroborate the previously observed inverse association between folic acid and natural killer cell cytotoxicity and suggests that reduced natural killer cell mediated tumour immunosurveillance may be an additional mechanism behind the purported tumour promoting effect of folic acid supplementation.
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This thesis is dedicated to my grandfather:

Franciszek Najuch [1924-2012]
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BDR</td>
<td>Basal dietary requirement</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine preceding guanine</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle to threshold</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DFEs</td>
<td>Dietary folate equivalents</td>
</tr>
<tr>
<td>DHF</td>
<td>Dihydrofolate</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>FA</td>
<td>Folic acid</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable</td>
</tr>
<tr>
<td>FPGS</td>
<td>Folypolyglutamate synthase</td>
</tr>
<tr>
<td>FR</td>
<td>Folate receptor</td>
</tr>
<tr>
<td>GCPII</td>
<td>Glutamate carboxypeptidase II</td>
</tr>
<tr>
<td>GGH</td>
<td>Gamma-glutamyl hydrolase</td>
</tr>
<tr>
<td>HFA</td>
<td>High folic acid</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer immunoglobulin-like receptor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MFD</td>
<td>Moderate folate deficiency</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylenetetrahydrofolate reductase</td>
</tr>
<tr>
<td>MTR</td>
<td>Methionine synthase/5-methyltetrahydrofolate-homocysteine methyltransferase</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NTDs</td>
<td>Neural tube defects</td>
</tr>
<tr>
<td>PABA</td>
<td>Para-aminobenzoic acid</td>
</tr>
<tr>
<td>PCFT</td>
<td>Proton-coupled folate transporter</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended daily allowance</td>
</tr>
<tr>
<td>RFC</td>
<td>Reduced folate carrier</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SHMT</td>
<td>Serine hydroxymethyltransferase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis inducing ligand</td>
</tr>
<tr>
<td>TS</td>
<td>Thymidylate synthase</td>
</tr>
<tr>
<td>UL</td>
<td>Tolerable upper intake level</td>
</tr>
<tr>
<td>UMFA</td>
<td>Unmetabolized folic acid</td>
</tr>
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**Chapter 1: Introduction**

Folate, a water-soluble B vitamin (B9) and its synthetic form, folic acid (FA) used in fortification and supplements are critical to biological one-carbon transfer reactions (1). As such, folate plays a crucial role in human health and is important to fundamental biological processes such as nucleotide biosynthesis and biological methylation reactions (1).

The combined effect of mandatory FA fortification policy and FA-containing supplement use by a large proportion of individuals has led to significantly increased intake and blood levels of folate and FA in the North American population (2, 3). Indeed, data from the Canadian Health Measures Survey conducted between 2007-2009 revealed that folate deficiency as defined by red blood cell (RBC) folate concentrations < 305 nmol/L is virtually non-existent in the Canadian population; whereas, 40% of Canadians exhibited RBC folate concentrations above the high cut-off of 1360 nmol/L (the 97th percentile of RBC folate concentrations from the National Health and Nutrition Examination Survey conducted in the U.S. between 1999-2004) (3).

Although the health benefits of FA supplementation in regard to neural tube defect (NTD) prevention are well established (4-6), possible health risks associated with high intake and blood levels of folate and FA have not been thoroughly examined. Indeed, several lines of evidence have linked high FA intake to adverse health outcomes including cognitive decline, masking of vitamin B12 deficiency, epigenetic changes, and the promotion of tumour progression depending on the time and dose of FA intervention (7, 8). Excessive FA appears to saturate the key enzyme (dihydrofolate reductase, DHFR) involved in FA biotransformation and unmetabolized FA (UMFA) enters the circulation (9). Persistent exposure to high levels of circulating UMFA may have unanticipated physiological consequences and as such some have
proposed that UMFA may be the driving factor behind the adverse health outcomes associated with excessive FA intake (10).

At present, the impact of folate status and in particular the effects of FA supplementation on immune function are largely unknown. However, a possible adverse effect of excessive FA intake on immune function was reported in a study of post-menopausal women, wherein high intake of folate and FA from dietary sources and supplementation was associated with reduced natural killer (NK) cell cytotoxicity, when compared to NK cell function in women consuming low dietary folate without supplementation (11). In addition, plasma UMFA detectability in all study participants and plasma UMFA concentrations in women aged ≥ 60 years were significantly and inversely associated with NK cell cytotoxic activity (11). NK cells are effectors of innate immunity that recognize and eliminate abnormal cells through cytotoxic mechanisms and cytokine production, and as such represent a primary line of defense against malignancies and microbial infection (12).

Thus, a possible reduction in NK cell cytotoxic function in response to high intake and/or circulating UMFA represents a significant health concern that warrants further investigation. Impaired NK cell function as a result of high FA intake may have particularly pronounced adverse health outcomes in elderly populations, in whom immune responses are already in the process of decline (13) and in whom high FA/folate intake and blood levels have been reported (2, 14). In addition, given the observation that FA supplementation may promote the progression of (pre)neoplastic foci (8), a reduction in NK cell cytotoxicity and tumour immunosurveillance as a result of high FA intake could provide an additional mechanistic explanation for this observed tumour-promoting effect. Finally, although widely cited, the reported inverse association between FA supplementation/UMFA and NK cell cytotoxicity remains unconfirmed
and unexplained from a mechanistic perspective, with very few studies providing conflicting evidence for this purported effect of FA on NK cell cytotoxicity.

Given this consideration, the main objective of this thesis was to examine the relationship between FA supplementation and NK cell function in an animal model, in order to confirm the purported effect of FA on NK cell cytotoxicity, and as such, to clarify the necessity for further studies examining this potentially harmful phenomenon. An additional objective of this thesis was to identify potential mechanisms by which FA supplementation may impair NK cell activity.
2.1 Folate and folic acid

The section “Folate and folic acid” (Section 2.1) is based, in part, on the publication (15) [by permission of the publisher: John Wiley and Sons Ltd]:

Warzyszynska, Joanna E and Kim, Young-In J (October 2014) Folate in Human Health and Disease. In: eLS. John Wiley & Sons, Ltd: Chichester. DOI:
10.1002/9780470015902.a0002268.pub2

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2.1.1 Overview

Folate, a water soluble B vitamin (vitamin B9), and its synthetic form folic acid (FA) used in fortification and supplements, are important to human health as they are critical to biological one-carbon transfer reactions involved in nucleotide biosynthesis and methylation reactions; as such, folate is important to processes such as cell division, DNA repair and maintenance, and regulation of gene expression through epigenetic mechanisms (1). Indeed, folate deficiency in humans is associated with a variety of poor health outcomes and diseases including megaloblastic anemia, hyperhomocysteinemia (which in turn is associated with cardiovascular disease), neuropsychiatric and cognitive disorders, congenital disorders, adverse pregnancy outcomes, and development of certain cancers (7, 8, 16).

The main source of folate for humans is the diet and naturally-occurring folates are present in many foods, including but not limited to green leafy vegetables, citrus fruits, organ meats and yeast (16). Mandatory FA fortification of white wheat flour and certain grain products was implemented in Canada and the U.S. in 1998 to ensure adequate folate status in women of child-bearing age, thereby reducing the rate of NTDs (17, 18). The combined effect of FA
fortification policy and prevalent supplement use has significantly increased intake and blood levels of folate and FA in the general North American population (2, 3, 19). Although FA fortification and supplementation are an undeniable public health success in regard to NTD prevention (4-6), concerns have been raised over potential adverse effects of FA supplementation and excessive FA intake, specifically in light of the current status of folate and FA in the North American population (7).

2.1.2 Chemical structure

Folate is a general term used to collectively describe a variety of folate vitamers with a characteristic chemical structure and similar nutritional properties (20). The parent chemical structure of folate consists of three moieties: an aromatic pteridine ring joined to para-aminobenzoic acid (PABA) through a methylene bridge, and glutamic acid joined to PABA via a γ-peptide bond (Figure 2.1) (1). The oxidation state of the pteridine ring varies between folate vitamers; while FA is fully oxidized, naturally occurring folates are typically reduced (1). In addition, in contrast to the monoglutamylated FA, naturally occurring folates contain a polyglutamate chain consisting of varying numbers of glutamate residues (maximum of 9) joined by γ-peptide bonds (1). One-carbon groups may be linked at the N-5 and N-10 positions of tetrahydrofolate (THF) (1).
Figure 2.1: Chemical structure of folic acid [A] and folate [B]. Folate consists of three moieties: an aromatic pteridine ring joined to para-aminobenzoic acid (PABA) through a methylene bridge, and glutamic acid joined to PABA via a γ-peptide bond. FA is fully oxidized and monoglutamylated. Naturally occurring folates are reduced and contain a polyglutamate chain consisting of up to 9 glutamate residues. One-carbon units can be linked to THF at the N⁵ and N¹⁰ positions: R = CH₃ (N⁵), CHO (N⁵ & N¹⁰), CH=NH (N⁵), CH₂ (N⁵ & N¹⁰) and CH= (N⁵ & N¹⁰). Adapted and reprinted by permission from the publisher (John Wiley and Sons Ltd): (8). Copyright © 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

2.1.3 Absorption, metabolism and biochemical functions

Folate is absorbed primarily in the proximal small intestine, where the enzyme glutamate carboxypeptidase II (GCPII), an exopeptidase found anchored to the intestinal apical brush border membrane, hydrolyses the polyglutamate chain of folate vitamers as folates containing more than three glutamate residues are unable to cross the cellular membrane (1, 8). Following hydrolysis, folate is transported across the intestinal apical cellular membrane mainly via the proton-coupled folate transporter (PCFT), although other transporters such as folate receptors (FR; where FR-α is the predominant isoform of FR in epithelial membranes) and the reduced folate carrier (RFC) are also expressed in the small intestine (Figure 2.2) (1, 8). The PCFT exhibits equal affinity for both reduced and oxidized forms of folate and mediates folate
transport at a low pH, consistent with the small intestine micro-environment (1, 21). Folate transport via the RFC is optimal at physiological pH and is ineffective at pH < 6.5 (1). In addition, the RFC exhibits markedly higher affinity for reduced folates relative to FA (1). The FRs (also known as folate binding proteins) exhibit high affinity for all forms of folate; however, their affinity is highest for FA (1).

Upon entering the cell, folate is converted to a polyglutamylated form by the enzyme folylpolyglutamate synthase (FPGS) as a mechanism of cellular retention, where efflux of polyglutamylated folates from the cell is an inefficient process (1, 8). In addition, polyglutamylated folates are better substrates for intracellular enzymes involved in folate metabolism (1). The enzyme γ-glutamyl hydrolase (GGH) removes terminal glutamate residues from polyglutamylated folates to facilitate their efflux from the cell (Figure 2.2) (1). Circulating folates are typically monoglutamylated, and are transported in the blood unbound, bound to albumin (which accounts for approximately 50% of protein-bound circulating folates) or bound to soluble folate receptors (mainly FR-γ) in low proportions (1, 22). Folates are transported into target tissues via FRs, the RFC, or the PCFT, where different tissues will express these receptors/transporters in varying proportions (1). Once within the cell, folates are retained by FPGS to mediate one-carbon transfer reactions in the target tissues (1).

FA is biotransformed in the liver and to a lesser degree in the small intestine by the action of dihydrofolate reductase (DHFR), which reduces FA first to dihydrofolate (DHF) and then further on to THF, which is metabolically active once methylated (23). 5'-methylTHF is the predominant form of folate found in the circulation (23). Upon saturation of the enzyme DHFR, unmetabolized FA (UMFA; i.e., FA in an unaltered state), enters into the circulation; intake
levels of > 200 µg FA have been reported to result in the appearance of UMFA in the plasma (24).

Folate is a critical component of one-carbon transfer reactions, which are involved in the methionine cycle, biological methylation reactions, and nucleotide biosynthesis (Figure 2.2) (1). The re-methylation of homocysteine to methionine is catalyzed by methionine synthase/5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) along with the co-factor cobalamin (vitamin B_{12}), and involves the transfer of a methyl group from 5-methylTHF to homocysteine, thereby generating methionine and tetrahydrofolate (THF) (1). Methionine can then be activated by ATP (adenosine triphosphate) and methionine adenosyltransferase (MAT), thereby producing S-adenosylmethionine (SAM), the major methyl group donor in DNA methylation and other biological methylation reactions (1). DNA methylation, in turn, is mediated by cytosine guanine dinucleotide (CpG) DNA methyltransferases, which use SAM as a methyl donor; DNMT3a and DNMT3b mediate de novo methylation of cytosines within CpG sequences, whereas DNMT1 is required for maintenance CpG methylation (25). DNA demethylation occurs either passively, via failure of maintenance mechanisms, or via active mechanisms that are not well characterized (25).

Serine hydroxymethyl transferase (SHMT) catalyzes the reversible conversion of THF and serine to 5,10-methyleneTHF and glycine (1). In a reaction catalyzed by the enzyme thymidylate synthase (TS), 5,10-methyleneTHF transfers a methyl group to deoxyuridine monophosphate (dUMP), generating deoxythymidine monophosphate (dTMP, thymidylate, a precursor of pyrimidylate biosynthesis) and dihydrofolate (DHF) (1). Folate also participates in purine synthesis, wherein formylated THF and 5,10-methyleneTHF can enter into purine synthesis (1).
DHF is reduced to THF via DHFR, and 5,10-methyleneTHF can be irreversibly converted to 5-methylTHF via methylenetetrahydrofolate reductase (MTHFR) (1).

Figure 2.2: Folate absorption, transport, and biochemical functions. Luminal folates are hydrolyzed by glutamate carboxypeptidase II (GCPII). Folate is transported across the intestinal apical cellular membrane mainly by the proton-coupled folate transporter (PCFT) but also via the reduced folate carrier (RFC) and the folate receptor (FR). Inside the cell, folate is converted to a polyglutamylated form by the enzyme folylpolyglutamate synthase (FPGS) and terminal glutamate residues are removed from polyglutamylated folates by the enzyme γ-glutamyl hydrolase (GGH). Folate is important to one-carbon transfer reactions involved in the methionine cycle, biological methylation reactions, and DNA synthesis. The re-methylation of homocysteine (Hcyst) to methionine (Met) is catalyzed by methionine synthase (MTR) and involves the transfer of a methyl group from 5-methyltetrahydrofolate (5-methylTHF) generating THF. Methionine can then be converted to S-adenosylmethionine (SAM), the primary methyl donor in most biological methylation reactions including methylation of DNA. DNA methylation is mediated by DNA methyltransferases (DNMT1,3a,3b) whereas DNA demethylation is putatively catalyzed by the methyl-CpG binding domain protein (MBD2). Serine hydroxymethyl transferase (SHMT) catalyzes the reversible conversion of THF and serine to 5,10-methyleneTHF and glycine. 5,10-methyleneTHF transfers a methyl group to deoxyuridine monophosphate (dUMP) generating deoxythymidine monophosphate and dihydrofolate (DHF). Formylated THF and 5,10-methyleneTHF can enter into purine synthesis. DHF is reduced to THF via dihydrofolate reductase (DHFR), and 5,10-methyleneTHF can be irreversibly converted to 5-methylTHF via methylenetetrahydrofolate reductase (MTHFR). Adapted and reprinted by permission from the publisher (John Wiley and Sons Ltd): (8). Copyright © 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
2.1.4 Dietary requirements

Except for de novo synthesis of folate by the intestinal microbiota, a proportion of which is absorbed across the colon and incorporated into host tissues (26-29), mammals are generally unable to synthesize folate (1, 8). Mammals have the ability to synthesize all of the components, but lack the enzyme required for coupling the pteridine ring to PABA (1, 8). Thus, the daily folate requirement must be obtained from dietary or supplemental sources (1). Folate is found naturally in sources such as green leafy vegetables, asparagus, broccoli, Brussels sprouts, citrus fruit, legumes, dry cereals, whole grain, yeast, lima beans, liver, and other organ meats (16). The bioavailability of naturally occurring folates depends on a variety of factors, including the food source and preparation methods; approximately 50-75% of folate content is lost through food processing and storage techniques (16). Naturally occurring folates are unstable and are very susceptible to oxidation under conditions of low pH (16). In contrast, FA is very stable and exhibits high bioavailability relative to naturally occurring folates. Folate intake is often described using dietary folate equivalents (DFEs), where 1 DFE is equal to 1 µg of naturally occurring dietary folate; 0.6 µg of FA in fortified foods or in a supplement taken with food; or 0.5 µg of FA ingested on an empty stomach (16). In North America, recommended daily allowance (RDA) of folate in adult men and women is 400 µg DFEs/day, and is increased to 600 µg DFEs/day during pregnancy and 500 µg DFEs/day during lactation (16). The tolerable upper intake level (UL) applies only to FA and is set at 1 mg of FA per day for adult men and women (16).

2.1.5 Measures of folate status

Folate status is typically assessed through measurement of serum and RBC folate concentrations. Whereas serum folate concentrations are reflective of short-term dietary intake
and supplemental use, RBC folate concentrations are considered to be more reflective of folate tissue stores (16). In humans, serum folate levels rise rapidly after folate consumption and peak at approximately 2 hours after intake (30). In addition, the half-life of monoglutamylated folates in the serum, as assessed by using stable isotope-labelled or radiolabelled folates, is only a few hours long (31). RBCs accrue folate only during maturation in the bone marrow, and therefore, RBC folate is reflective of bone marrow folate stores (16). Additionally, the 120-day turnover rate of RBCs confers resistance of folate levels to short-term variation in dietary folate intake and supplemental FA intake (16). RBC folate concentrations are thus used in diagnosis of clinical folate deficiency; where, according to current WHO standards, concentrations < 340 nmol/L are indicative of deficiency (32). Serum folate measurements < 10 nmol/L are also considered indicative of folate deficiency if observed repeatedly over a one month period (32). Raised plasma homocysteine concentrations (> 16 nmol/L) represent an additional accurate, albeit non-specific, biomarker of folate deficiency (16). Plasma homocysteine concentrations increase during folate deficiency due to diminished availability of 5-methylTHF in the remethylation of homocysteine to methionine (16). However, raised plasma homocysteine concentrations are not specific to depletion of folate stores, as inadequate vitamin B_{12} and B_{6} status and other factors (e.g., renal dysfunction, aging) can also contribute to increased levels (16). High cut-offs for RBC and serum folate concentrations are not well-defined, although levels of 1360 nmol/L [the 97th percentile of RBC folate concentrations in the National Health and Nutrition Examination Survey (NHANES) conducted in the U.S. in 1999-2004 (3)] and 45 nmol/L [an arbitrarily chosen cut-off based on the upper end of the assay calibration curve for serum folate quantification in NHANES 1991-1994 (phase II) and 1999-2000 (33)] respectively, have been used in scientific literature.
2.1.6 Impact of folic acid fortification and supplementation on folate status in the North American population

A compelling body of evidence regarding reduced risk of NTDs with periconceptional FA supplementation led to the implementation of mandatory FA fortification policies in many countries (4, 17, 18). At present, regulations for mandatory FA fortification of white wheat flour exist in 53 countries, including the United States, Canada, and Chile (4). Mandatory FA fortification has notably not been implemented in many western European countries (4), although voluntary fortification with FA may still take place (34).

Mandatory FA fortification was introduced in Canada and the United States in 1998 (17, 18). Canadian regulations mandate FA fortification of white wheat flour and cornmeal at a level of 150 µg/100 g and of enriched pasta at a level of 200 µg/100 g (17). In contrast, regulations in the U.S. mandate FA fortification of white wheat flour, cornmeal and enriched pasta at a level of 140 µg/100 g (17, 18).

In addition to consuming FA-fortified foods, ~30-40% of the North American population consume FA-containing supplements (19, 35-37); standard multivitamins typically contain 400 µg of FA (38). Use of dietary supplements is associated with the female gender, older age, higher socioeconomic status, and a healthy lifestyle (19, 35-37). Multivitamin supplement use is also common among cancer patients and survivors, with the highest prevalence reported for female breast cancer patients (57-62%). Supplement use among cancer patients and survivors is also associated with the female gender and higher socioeconomic status (39). Health Canada recommends that all pregnant women and women capable of becoming pregnant consume a daily supplement containing 400 µg of FA in addition to folate-rich foods (40). However, most prenatal multivitamin supplements in Canada contain ~1 mg of FA (41). Therefore, as a result of
fortification and widespread supplement use, intake and blood levels of folate and FA have significantly increased in the post-fortification era in North America (2, 3).

Data from the NHANES 1988-2010 in the United States revealed 2.5X and 1.5X increased geometric mean concentrations of serum and RBC folate, respectively, in the post-fortification era (1999-2010) relative to pre-fortification (1988-1994) concentrations (2). Data from the Canadian Health Measures Survey (CHMS) 2007-2009 revealed a virtual non-existence of folate deficiency (defined by Institute of Medicine 1998 standards as RBC folate concentrations < 305 nmol/L) in the Canadian population, whereas > 40% of the population had high RBC folate concentrations (defined as levels above the 97th percentile of RBC folate concentrations from NHANES 1999-2004 at 1360 nmol/L) (3).

The detection of circulating UMFA also appears to have increased in the North American population post fortification; in data from the Framingham Offspring Cohort, the prevalence of detectable UMFA was 74.7% post-fortification in comparison to 55.0% pre-fortification in supplement non-users and 80.7% post-fortification in comparison to 72.5% pre-fortification in supplement users (42). Data from NHANES 2001-2002 revealed detectable UMFA in 38% of the US population aged ≥ 60 years (14). A study examining post-menopausal women in the post-fortification era reported a 78% prevalence of detectable UMFA (11). In addition, the detection of UMFA is not limited to countries with mandatory FA fortification; UMFA was detected in the majority of samples in a study conducted in Ireland, where only voluntary FA fortification takes place (34).

2.1.7 Folate and folic acid in health and disease

Folate, as a critical factor in nucleotide biosynthesis and biological methylation reactions, plays an important role in DNA synthesis and repair (1) and in genomic stability and regulation
of gene expression (43). Consequently, folate status is vital to human health. Indeed, various deleterious health conditions, including megaloblastic anemia, adverse pregnancy outcomes, NTDs and other congenital disorders, neuropsychiatric disorders, cognitive decline, coronary heart disease, stroke and development of certain cancers have been associated with folate deficiency (7, 16). However, except for megaloblastic anemia and NTD risk, the nature and magnitude of the relationship between folate deficiency and these adverse health outcomes have not been clearly defined. Folate deficiency is caused by inadequate folate intake, increased folate demand or utilization, and impaired folate absorption and/or metabolism (16). In addition, chronic alcohol consumption, tobacco smoking, and various medications such as anti-folate, anti-epileptic, and anti-inflammatory drugs may cause or exacerbate folate deficiency (16, 44).

Although FA supplementation has been shown to be effective in treatment of megaloblastic anemia and in prevention of NTDs, where a 15-50% decrease in NTD incidence has been observed in North America following FA fortification (4-6), the effects of FA supplementation on other disease outcomes remain to be clearly determined. Conflicting results have been reported in regard to the effects of periconceptional FA supplementation on prevention of congenital defects other than NTDs, although the evidence generally supports a protective role for FA in prevention of congenital heart defects (45, 46). Despite some inconsistencies, data from randomized trials of FA supplementation with or without other B vitamins also generally support a beneficial effect of FA on amelioration of cognitive decline and symptoms of neuropsychiatric disorders (47, 48). In contrast, generally null effects have been reported for efficacy of FA and other B vitamins in secondary prevention of coronary heart disease outcomes in predisposed individuals with previous coronary events (49, 50). Although a
beneficial role of FA supplementation in stroke prevention is more strongly supported by the data than in coronary heart disease prevention, null findings have also been reported (49, 51, 52).

In addition, several lines of evidence have implicated high FA intake in adverse health outcomes, including the risk of masking vitamin B12 deficiency, particularly in the elderly; potential for development of tolerance or resistance to anti-folate drugs used in treatment of cancer, arthritis and inflammatory disorders; impaired cognitive function when combined with low vitamin B12 status; potential epigenetic modifications; and promotion of the progression of (pre)cancerous cells (7).

The influence of one-carbon nutrients such as folate on epigenetic modifications, particularly DNA methylation due to the role these nutrients play in the provision of SAM for use by methyltransferases, has been extensively studied, as these epigenetic changes could potentially alter susceptibility to certain chronic diseases (53). This is well illustrated by proof-of-concept experiments, such as those conducted in the agouti and AxinFused mouse models, where a maternal diet high in methyl donors, including FA, was shown to increase methylation in the promoters of the agouti and axin genes and to result in marked phenotypic differences in the offspring, such as coat colour and tail kinkedness, respectively (54-56). Furthermore, with respect to the agouti mouse model, methylation of the agouti promoter resulted in leaner offspring with lower tendency towards obesity, cancer and diabetes, relative to mouse counterparts with an active agouti promoter (54, 55). DNA methylation is involved in regulation of gene expression, maintenance of genome stability and integrity, chromatin modifications, and the development of mutations (53). Aberrancies in DNA methylation are mechanistically associated with the development of certain chronic diseases such as cancer (53). To date, the effect of folate status on DNA methylation has been observed to be tissue and gene specific, and
dependent on the timing, duration, and measure of folate intervention, as well as interactions
with other dietary/lifestyle factors, and variability in critical genes encoding enzymes involved in
the folate and one-carbon metabolic pathways (53). Moreover, results from FA intervention
studies conducted in animal models suggest that maternal FA supplementation can modulate
mammary and colorectal cancer risk in the offspring, in part via alterations in DNA methylation
(57, 58).

2.1.8 Dihydrofolate reductase and unmetabolized folic acid

The primary site of FA biotransformation is the liver, where FA is reduced to DHF and
subsequently THF by the enzyme DHFR (59, 60). A study by Bailey et al. revealed that human
DHFR exhibits poor efficiency and high individual to individual variability (5-fold variation) in
regard to FA reduction relative to rodent DHFR (9). The activity of human DHFR was shown to
be on average 56 times lower than that of rat DHFR, with FA as a substrate (9).

Saturation of DHFR can lead to the appearance of UMFA in the circulation (9, 24);
indeed, detectability of UMFA has increased in individuals post-fortification and is more
prevalent among supplement-users (42). Concerns over UMFA in the circulation have been
raised as the pharmacodynamic properties of UMFA are not known (10). Exposure of cells to
persistently high levels of UMFA could potentially lead to indirect disruption of, or preferential
shifts in, processes such as DNA synthesis and biological methylation reactions through
competition between FA and the appropriate folates for enzymes involved in provision of
substrates for these processes (7). Furthermore, FA can competitively inhibit the DHFR mediated
reduction of DHF to THF (9), a mechanism by which high levels of FA could lead to an
intracellular deficiency of active folate metabolites (20). In addition, DHF inhibits the activity of
several folate-dependent enzymes such as TS, MTHFR, and folate-dependent enzymes required for purine synthesis (7).

Excessive circulating FA could also dysregulate folate-associated processes by potentially modulating the gene and protein expression of folate-dependent enzymes and carriers/transporters involved in folate absorption and transport (7). For example, Ashokkumar et al. observed that the human intestinal cell line Caco2 and the renal cell line HK-2 cultured in a high concentration of FA (100 µmol/L) for 5 generations demonstrated reduced gene and protein expression of the RFC and reduced gene expression of the PCFT (61). In addition, FA uptake (at pH 5.5) was significantly and specifically reduced in both cell lines (61). Similarly, Dev et al. observed reduced FA uptake and reduced protein but not gene expression of the RFC and PCFT in the small intestine of Wistar rats receiving a 20 mg FA/kg diet (10X the basal dietary requirement) for a period of 10 days relative to rats receiving a control diet (62). However, these changes were not observed when supplementation occurred over a longer time period (60 days) (62). Collectively, these studies provide support for the hypothesis that UMFA may alter the expression of folate transporters and folate-dependent enzymes, thereby potentially influencing folate absorption and metabolism.

The impact of FA supplementation on DHFR expression in the liver and small intestine has not been well characterized. However, this may be of interest, as DHFR is an enzyme critical to maintenance of the folate pool and thus to biological functions such as DNA synthesis and cell division (60). This is well illustrated by the use of DHFR antagonists such as methotrexate in treatment of neoplasms (60). Expression of DHFR is maintained throughout the cell cycle; however, it is significantly upregulated at the G1/S stage boundary, as nucleotide requirements are drastically increased. DHFR has a TATA-less promoter and regulation of its transcription is
mediated by two key transcription factors: E2F1 and SP1 (60). Expression of DHFR may also be regulated post-transcriptionally via the microRNA, mir-24, as DHFR contains a mir-24 binding site in its 3’ UTR (60). Translational regulation of DHFR occurs via DHFR binding to its own cognate mRNA, which in turn inhibits the translation of its own mRNA. Binding of DHF by DHFR induces a conformational change within the enzyme that results in release of DHFR mRNA and consequent translational derepression (60). The same translational derepression is induced by binding of methotrexate, and as such, protein levels of DHFR are observed to increase with methotrexate treatment (60).

2.1.9 Folate and carcinogenesis

Epidemiological evidence suggests an inverse relationship between folate status and the risk of several human malignancies including cancer of the colorectum, oropharynx, oesophagus, stomach, pancreas, lungs, breast, cervix, uterus, and ovary, as well as neuroblastoma and leukemia (8, 38). Conversely, FA supplementation may reduce the risk of these cancers. This purported inverse association between folate status and cancer risk contrasts with the conceptual basis for anti-folate chemotherapies, where intracellular folate depletion resulting from disrupted folate metabolism decreases the provision of substrates for nucleotide biosynthesis, thereby preventing cell replication and cancer progression (8, 38). However, the nature, magnitude and consistency of the purported association between folate status and cancer risk have not unequivocally been demonstrated for all cancer types (8, 38). The relationship between folate deficiency and cancer risk has been best studied in colorectal cancer (CRC), where the majority of case-control and prospective epidemiologic studies collectively suggest a 20 to 40% increased risk of CRC or its precursor, adenoma, in those with the lowest folate intake compared to individuals with the highest folate intake (63-65). Epidemiologic evidence also suggests that
nutrient-gene interactions, such as those between folate and other one-carbon nutrients and genetic variations in the folate and one-carbon metabolic pathways, may modulate cancer risk (8). For example, the MTHFR C677T polymorphism has been reported to modify cancer risk in opposing directions depending on the cancer type and on folate status (66).

Animal studies have generally provided support for the purported inverse association between folate status and cancer risk observed in epidemiologic studies. However, these animal studies have collectively demonstrated a dual modulatory role of folate in cancer development and progression that is dependent on both the stage of cell transformation at the time of folate intervention and the dose of FA supplementation (Figure 2.3) (8). In chemically-induced or genetically predisposed rodent models of CRC, folate deficiency enhanced colorectal carcinogenesis in the normal colorectum, whereas FA supplementation at modest levels (4-10X the basal dietary requirement) inhibited colorectal carcinogenesis, if provided before neoplastic transformation was initiated in the colorectum (67). However, in the normal colorectum, supraphysiological levels of FA supplementation (> 20-fold the basal dietary requirement) potentiated colorectal carcinogenesis (8). In established (pre)neoplastic colorectal cells, folate deficiency inhibited the progression of tumours, whereas FA supplementation promoted the progression of (pre)neoplastic foci (8, 68).

Potential mechanisms behind the dual modulatory effects of folate status on carcinogenesis are related to the biochemical roles of folate in one-carbon transfer reactions: nucleotide biosynthesis and DNA methylation (Figure 2.3) (8). In normal tissues, folate deficiency can promote neoplastic transformation due to the lack of a sufficient nucleotide pool, leading to aberrant DNA synthesis, repair and consequently integrity (8). FA supplementation may prevent or correct this procarcinogenic milieu. In contrast, folate deficiency in
(pre)neoplastic foci confers a protective effect as the availability of DNA building blocks needed for the rapid proliferation of transformed cell is restricted, whereas FA supplementation promotes progression by providing these substrates (8). Aberrant DNA methylation resulting from variation in folate status can also contribute to carcinogenesis via global DNA hypomethylation, which is associated with reduced genomic stability, via hypermethylation of the promoter regions of tumour suppressor and cancer-related genes with consequent gene silencing, and via the inherent hyper-mutability of methylated cytosine (8, 43).

Figure 2.3: Dual modulatory role of folate in colorectal carcinogenesis. Evidence from animal models suggests that folate has dual modulatory effects on cancer development and progression depending on the dose and the stage of cell transformation at the time of folate intervention. In normal tissues, folate deficiency increases the risk whereas FA supplementation at modest levels decreases the risk of neoplastic transformation. However, in the presence of (pre)neoplastic lesions, folate deficiency inhibits whereas FA supplementation promotes progression of tumours. Possible mechanisms for these observed effects are listed. (↑: Increased, ↓: Decreased). Adapted and reprinted by permission from the publisher (John Wiley and Sons Ltd): (15). Copyright © 2001 John Wiley & Sons, Ltd. All rights reserved.

Several small human clinical trials have reported that FA supplementation can improve several functional biomarkers of folate metabolism and colorectal carcinogenesis (8). However, larger human clinical trials examining the effect of FA supplementation on the occurrence or recurrence of adenoma or CRC as the primary or secondary outcome have reported conflicting
results (8). Three randomized double-blind placebo-controlled trials examining the recurrence of adenoma over a period of 1-8 years with FA supplementation at doses of 0.5 or 1 mg/day reported either a null effect (69) or an insignificant trend towards lower risk of adenoma recurrence with FA supplementation (70, 71). The trial conducted by Wu et al. did, however, report a significant decreased risk of adenoma recurrence with FA supplementation among participants with low plasma folate at baseline (71). Additionally, a fourth clinical trial conducted to examine the effect of FA supplementation at 5 mg/day for 3 years on the recurrence of adenoma reported a 64% lower risk of multiplicity of recurrent adenomas associated with FA supplementation ($p = 0.023$) (72).

In contrast to the aforementioned trials, the Aspirin/Folate Polyp Prevention Study (AFPPS), a large multi-centre randomized double-blind placebo-controlled trial examining the recurrence of adenoma with 1 mg of FA supplementation per day, reported a significant 67% increased risk of advanced lesions and a significant 2.3-fold increased risk of multiple adenomas in participants receiving FA relative to placebo at a follow up period of 6 years (73). Furthermore, the risk of prostate cancer was significantly increased with FA supplementation in male participants of this study at approximately 10 years of follow-up (74). However, in supplement non-users, a non-significant inverse association was observed between baseline plasma folate concentrations and prostate cancer risk (74). Results from the AFPPS trial therefore provide human evidence that supports the observations made in animal studies regarding the potential tumour promoting effect of FA supplementation. Considering the dual modulatory role of folate in carcinogenesis based on the stage of cell transformation at the time of FA supplementation, it is possible that the observations made in the AFPPS trial might have resulted from the tumour promoting effect of FA supplementation on pre-existing undiagnosed
(pre)neoplastic lesions in the colorectum and prostate in these highly predisposed individuals (68).

In alignment with the purported cancer promoting effect of FA supplementation, ecological studies conducted in Canada, the U.S., and Chile have reported a significant increase in CRC rates concurrently with implementation of mandatory FA fortification programs in these countries, suggesting that FA supplementation might have played a role in this trend (75, 76). In contrast, two large population-based cohort studies conducted in the post-fortification era have reported a significant inverse association between total (77) or total and dietary folate intake and CRC risk (78).

Recent meta-analyses examining the effects of FA supplementation on overall cancer or specific CRC risk as a primary or secondary outcome in various study populations have also reported conflicting results. Two meta-analyses of clinical trials examining the effect of FA supplementation on recurrence of adenoma reported a null association (79, 80). Two meta-analyses of clinical trials investigating the effect of FA supplementation alone or in conjunction with other B vitamins on the secondary prevention of cardiovascular disease reported either a null effect on cancer incidence (50), or an increased risk of cancer incidence and mortality (81). Finally, two most recent meta-analyses encompassing trials of FA supplementation in both the general population, and individuals with history of adenoma or cardiovascular disease, reported either a null (82) or an increased (83) cancer risk with FA supplementation. The role of folate in cancer development and progression remains highly controversial at present and the effect of FA fortification and supplementation on cancer progression needs to be clearly elucidated in further studies.
2.2 Natural killer cells

2.2.1 Overview

Natural killer (NK) cells are effectors of the innate immune system important to defence against virally-infected and transformed cells (12). These cells were first identified based on the “natural” cytotoxicity (i.e., not requiring prior activation) or “killing” activity they exhibited against tumour cells in vitro (12). The major functions of NK cells include cytotoxic function against abnormal cells, such as virally-infected, or malignant or allogeneic cells, the production of pro-inflammatory cytokines (IFN-γ, TNF-α) that shape further immune responses, and the regulation of inflammatory responses through cell-cell interactions and elimination of activated immune cells (12).

NK cells are considered to be “innate” lymphocytes, as they express germ-line encoded receptors that mediate recognition of and distinguish between healthy and allogeneic (“non-self”) or abnormal cells such as virally-infected cells or cells expressing markers of cellular stress; in contrast to lymphocytes of the adaptive immune system such as T and B-lymphocytes which express receptors that undergo somatic recombination (84). NK cell receptors can be inhibitory, recognizing ligands generally expressed on healthy cells, such as major histocompatibility complex class I (MHC class I) molecules, or activating, recognizing ligands associated with cellular stress (84). MHC class I molecules are often down-regulated in abnormal cells, reducing inhibition of NK cell cytotoxic activity. Down-regulation of MHC class I molecules is a mechanism of immune evasion observed in viral infection and carcinogenesis, NK cells are however capable of recognizing and targeting these cells in a process termed “missing-self” recognition (12). In contrast, “induced-self” recognition describes the process by which activating NK cell receptors recognize molecules that are upregulated in abnormal cells (85).
integration of opposing inhibitory and activating signals determines the functional outcome of NK cells (84). NK cell cytotoxic activity is mediated primarily via the release of cytotoxic granules containing perforin and granzymes, and via expression of cell surface molecules such as Fas ligand (FasL) (84, 86). Perforin mediates the entrance of granzymes into the target cell, whereas granzymes activate apoptosis (programmed cell death) through the cleavage of caspases and other mechanisms (87). Upon NK cell activation, NK cells will degranulate – releasing their cytotoxic contents – and begin to translate and secrete cytokines (84). In addition to target cell interactions, NK cell activity can be shaped via the cytokine environment (e.g., type I interferons, IL-12, IL-15, IL-18, IL-21), and through cell-cell interactions with other immune cells (12).

As mentioned previously, NK cells have traditionally been considered to be part of the innate immune system and therefore to lack adaptive immune response features such as clonal expansion and memory (87). In recent years, however, further NK cell complexity has been uncovered, wherein these cells have been demonstrated to possess both clonal/subset expansion and memory features (88). For example, Ly49H+ (a virus-specific NK cell receptor) NK cell subsets in mice that readily proliferate upon exposure to cytomegalovirus, and self-renewing Ly49H+ NK cells that persist in lymphoid and non-lymphoid organs post the initial infection and exhibit superior degranulation and cytokine responses upon reactivation, have been identified (88). Furthermore, a variety of tissue-specific NK cell subsets with unique phenotypes such as lung, liver, and uterine decidua NK cells have been recently identified, expanding the perceived utility of NK cells from cytotoxic cells to cells that play an important regulatory role in maintenance of immune homeostasis in various tissues (89).
Finally, NK cells are not only critical to host defences, but are also being developed as a potential therapeutic approach to targeting malignancies, for example, in the use of expanded allogeneic NK cells or allogeneic NK cell lines for adoptive cellular immunotherapy (90).

2.2.2 History of identification

NK cells were first identified over 30 years ago as large granular lymphocytes that could kill syngeneic or allogeneic tumour cell targets in vitro without prior sensitization (i.e., exposure to the target) and without MHC restriction, thereby earning the name of “natural killers” (91-93). Observations of higher NK cell cytotoxicity towards target cells with low MHC class I expression and decreased NK cell lysis of targets with type I IFN-mediated up-regulation of MHC class I expression, led to the formulation of Kärre’s “Missing-Self Hypothesis” wherein Kärre and colleagues postulated that NK cells recognize the absence of “self” molecules such as MHC class I (94). Kärre’s hypothesis was soon followed by discoveries of NK cell inhibitory receptors with specificity for certain MHC class I molecules in both mice and humans (95-98). Today, NK cell:target cell recognition is known to be a complex process mediated by a multitude of receptors and co-stimulatory molecules (12, 84). In addition, NK cells are known to possess a variety of functions beyond cytotoxicity against abnormal cells (12, 84).

2.2.3 Natural killer cell functions and subtypes

NK cells are found in a multitude of lymphoid and non-lymphoid tissues in the body (12). However, typically, NK cells represent only a small fraction of lymphocytes within each tissue; for example, only ~2-5% of the lymphocytes in the mouse spleen are NK cells (12). Although previously underappreciated, NK cells execute a variety of tasks beyond cytotoxicity against abnormal cells in the host (12). Other NK cell functions include cytokine (IFN-γ, TNF-α, GM-CSF [granulocyte-macrophage colony-stimulating factor]) and chemokine (M1P-1α
[macrophage inflammatory protein 1], MIP-1β) production, which contributes to and shapes the adaptive immune response, antibody-dependent cellular cytotoxicity (ADCC) via the Fc receptor, CD16, as well as a multitude of regulatory roles, such as cytotoxicity against activated immune effector cells and cytokine production (12).

In recent years, NK cells have been identified to represent a subset of a larger group of leukocytes referred to as innate lymphoid cells (ILCs) (99). The ILC designation encompasses leukocytes which do not exhibit somatic immunoglobulin gene rearrangement, nor myeloid cell and dendritic cell phenotypic markers (99). In addition, these cells typically function as effector cells of innate immunity, in maintenance of immune homeostasis, and/or in modelling of lymphoid tissues (99). ILCs are thought to derive from the same precursor cells, but are sub-classified into three main groups, based on their functional properties and the specific cytokine environment and transcription factors required for their development (99). Group 1 ILCs include NK cells, produce IFN-γ, and require the transcription factors T-bet (T-box 21 transcription factor) and/or Eomes (eomesodermin) for development and function (99). Group 2 ILCs include cells that produce T_{H}2 cell associated cytokines, require the transcription factors GATA3 (GATA-binding protein 3) and RORα (retinoic acid receptor related orphan receptor-α), and are implicated in defense against parasites (helminths), airway hyper-responsiveness, and other atopic conditions (99). Group 3 ILCs produce IL-17 and/or IL-22, require the transcription factor RORγT, and include lymphoid tissue inducer cells and cells implicated in maintenance of gut-immune homeostasis among other functions (99).

Furthermore, a variety of NK cell subtypes have been identified in both humans and mice – these subtypes typically differ both by the tissue in which they reside and by their functional properties. In humans, the two main NK cell subtypes include CD56^{dim}CD16+ and
CD56^{bright}CD16- NK cells (12). CD56^{dim}CD16+ NK cells are found mainly in the peripheral circulation and spleen, express perforin and are primarily cytotoxic, whereas CD56^{bright}CD16-NK cells are found mainly in lymph nodes, lack perforin and are primarily cytokine-producing (12). In mice, three main NK cell subtypes exist and are listed here by order of maturity: CD11b-CD27+ cells, CD11b+CD27+ cells and CD11b+CD27- cells (12, 100). CD11b-CD27+ NK cells are found mainly in the bone marrow and lymph nodes, whereas the most mature CD11b+CD27-NK cells are found mainly in the blood, spleen, lung and liver and CD11b+CD27+ NK cells are more homogenously distributed (12, 100). CD11b-CD27+ NK cells are functionally immature, whereas CD11b+CD27- NK cells are terminally-differentiated and have a limited capacity for proliferation (12, 100). In addition, relative to CD11b+CD27+ NK cells, CD11b+CD27- NK cells are reported to display lower cytotoxicity but similar IFN-γ production when stimulated with cancer cells in vitro, and lower IFN-γ production when stimulated with cytokines in vitro (100-102).

Tissue-specific NK cells such as those of the uterus, lung, and liver exhibit varied phenotypes and play largely regulatory roles. Of interest is that NK cells, with respect to the peripheral circulation or lymphoid organs, are found in relatively higher frequencies in these “immunotolerant” organs, where tolerance to foreign but innocuous substances is of high importance (89). For example, the majority of leukocytes within the uterine decidua are NK cells (89). This highlights the importance of NK cells in regulation of immune homeostasis in addition to their cytotoxic functionality. NK cells also appear to possess a multitude of previously under-appreciated phenotypes and these phenotypes appear to be highly influenced by the specific microenvironment (89). For example, uterine decidua-resident NK cells in humans play an
important role in both trophoblast invasion and spiral artery remodeling – two processes critical to establishment of a successful pregnancy (103, 104).

2.2.4 Natural killer cell receptors

NK cells possess germ-line encoded receptors that allow them to distinguish between abnormal cells, which must be eliminated, and healthy cells to which they must remain tolerant (84). As described earlier, it is an integration of antagonistic activating and inhibitory signals that determines the functional outcome of the NK cell (84). Ligands for inhibitory NK cell receptors typically include MHC class I or “self” molecules; whereas ligands for NK cell activating receptors are less well-characterized but include, among others, some self-molecules that are upregulated on abnormal cells, “altered-self”, but also foreign ligands (105). The major families of receptors which recognize classical or non-classical MHC class I molecules include the killer immunoglobulin-like receptors (KIRs) in humans, the Ly49 receptor family in mice and the CD94/NKG2 family in both humans and mice (84). The inhibitory leukocyte immunoglobulin-like receptors (LIRs) expressed on human NK cells also bind MHC class I molecules (84, 105). The activating NKG2D receptor found in both humans and mice recognizes MHC class I-related molecules that are upregulated on abnormal cells and in situations of cellular stress (84, 105, 106). NK cell receptors that recognize ligands unrelated to MHC class I molecules include the activating natural cytotoxicity receptor (NCR) family expressed in humans and mice, which appears to recognize a variety of host-encoded and microbe-associated molecules, the multi-functional 2B4 receptor (CD2 family of Ig related proteins) in humans and mice, and the inhibitory killer cell lectin-like receptor G1 (KLRG1) in humans and mice that recognizes classical cadherins expressed in healthy tissues (84, 105, 107). Other NK cell receptors that may function in a co-stimulatory manner include: the NKR-P1 family of receptors, which is more
prominent in mice than humans, contains both activating and inhibitory members, and recognizes host encoded lectin-like ligands; the activating DNAM-1 receptor (Ig superfamily) in humans and mice; and the paired Ig-like 2 receptors (PILRs) in mice (84, 105, 108). These receptors and their major features are summarized in Table 2.1.

Inhibitory NK cell receptors generally contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic region of the receptor (84). Upon ligation of the receptor, phosphorylation of tyrosine within the ITIM occurs and results in the recruitment of tyrosine-specific phosphatases such as SHP-1/-2 (Src homology 2 (SH2)-containing protein-tyrosine phosphatase) and SHIP (SH2-containing inositol polyphosphate 5-phosphatase) (84). Recruitment and activation of these phosphatases results in abrogation of signalling events required for NK cell activation; i.e., dephosphorylation of signalling molecules required for NK cell activation (Zap70, Zeta-chain associated protein kinase 70; Syk, spleen tyrosine kinase; VAV1; a guanine nucleotide exchange factor, among others) and inactivation of Ca++-dependent signalling processes involved in NK cell activation (84). In contrast to inhibitory NK cell receptors, several activating NK cell receptors rely on adapter molecules, such as FcεRIγ, CD3ζ, and DAP12, which contain immunoreceptor tyrosine-based activation motifs (ITAMs) for transduction of activating signals. These adapter molecules are associated with a charged residue in the transmembrane component of the activating receptor (84). Phosphorylation of the ITAM tyrosine residues in the adapter molecules results in the recruitment of the tyrosine kinases, Syk and Zap70, which mediate downstream signalling events that result in activation of NK cell effector functions (84).

The major families of NK cell receptors that recognize classical MHC class I molecules are the KIR family in humans and the Ly49 family of receptors in mice (84, 105). The KIR and
the Ly49 receptor families contain both inhibitory and activating members and are functionally analogous, but differ structurally (84, 105). The KIRs are derived from the immunoglobulin (Ig) superfamily and are type-I transmembrane glycoproteins which contain two or three Ig-like domains in their extracellular region; whereas, the Ly49 receptors are type-II transmembrane anchored glycoprotein (C-type lectin-like) receptors and are expressed as homodimers (84, 105). NK cell expression of KIR and Ly49 receptors is stochastic and variegated (84, 105). In addition, KIRs and Ly49 receptors are both polygenic and polymorphic, and as such NK cell subsets exhibit substantial diversity in repertoire of KIR or Ly49 receptor expression (84, 105).

The NKG2D receptor and the NCRs among others have been implicated as important activating receptors in host defence against malignancy. The NKG2D receptor recognizes ligands that resemble MHC class I molecules structurally, but may not be encoded in the MHC complex and are not similar functionally. These include MIC (MHC class I polypeptide related sequence)-A/B and ULBP (cytomegalovirus UL-16 binding proteins) molecules in humans and the RAE-1 α/β/γ/δ/ε (retinoic acid early inducible 1), H60a/b/c (histocompatibility 60) and MULT-1 (murine ULBP-like transcript 1) molecules in mice (84). These ligands may be upregulated in abnormal cells or in situations of cellular stress (106). For example, MIC-A/B is frequently upregulated on cancer cells and in response to stressors such as heat shock (106). Ligands for NCRs include a variety of viral, bacterial, and parasite derived molecules, such as viral hemagglutinins, cell-derived ligands, such as heparan sulphates and proteins that are upregulated in stressed/tumour cells, and presumably some unidentified ligands expressed on several hematopoietic cell types (105, 107).
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Type</th>
<th>Genetic parameters</th>
<th>Ligands</th>
<th>Major Features</th>
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<tr>
<td>Ly49</td>
<td>Mouse</td>
<td>-Type II transmembrane anchored glycoprotein receptors (C-type lectin-like) -Expressed as homodimers -Activating (Ly49D, Ly49H) and inhibitory members</td>
<td>-Multiple genes; number depends on mouse strain -Extensive allelic polymorphism -Located: NK complex (NKC) on Chr 6</td>
<td>-Classical MHC-I: H2-K, -D, -L -Murine cytomegalovirus (MCMV) m157 glycoprotein (Ly49H)</td>
<td>-Recognition of MHC-I molecules -Ly49H implicated in defense against (MCMV) infection -Stochastic and variegated expression; mediated by bidirectional Pro1 promoter (functions as a &quot;probabilistic switch&quot; in Ly49 receptor expression)</td>
</tr>
<tr>
<td>KIR</td>
<td>Human</td>
<td>-Type I transmembrane glycoproteins with 2 (KIR2D) or 3 (KIR3D) Ig domains in the extracellular region (Ig superfamily) -Activating (short cytoplasmic domains: KIR2DS/KIR3DS) and inhibitory (long cytoplasmic domains: KIR2DL/KIR3DL) members</td>
<td>-Multiple genes and pseudogenes -Extensive allelic polymorphism -Located: Leukocyte receptor complex (LRC) on Chr 19</td>
<td>-Classical MHC-I: HLA-A, -B, -C -Activating KIRs also bind MHC-I but with weaker affinity than inhibitory counterparts</td>
<td>-Recognition of MHC-I molecules -Stochastic and variegated expression; maintained by DNA methylation in KIR CpG promoters</td>
</tr>
<tr>
<td>Mouse</td>
<td>-2 genes similar to KIR3DL -Located: X Chr</td>
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<td>-</td>
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<tr>
<td>CD94 &amp; NKG2</td>
<td>Mouse and Human</td>
<td>-Type II transmembrane anchored glycoprotein receptors (C-type lectin-like) -CD94 is expressed as a heterodimer with NKG2A (inhibitory) or NKG2C (activating) -Activating and inhibitory members</td>
<td>-Several genes -Located: NKC (mouse &amp; human)</td>
<td>-Non-classical MHC class I b molecules (human HLA-E and mouse Qa1b)</td>
<td>-Prevention of inappropriate NK cell activation -Human HLA-E and mouse Qa1b molecules bind and present peptides from leader fragments of classical MHC-I molecules; believed to allow for NK cell monitoring of MHC- I expression</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Mouse and Human</td>
<td>-Type II transmembrane anchored glycoprotein receptor (C-type lectin-like) -Activating receptor -Expressed as a homodimer</td>
<td>-One gene -Little homology to NKG2 genes -Located: NKC (mouse and human)</td>
<td>-MHC class I related molecules: -MIC-A/B and ULBP1 in humans -RAE, H60 and MULT-1 in mice</td>
<td>-Implicated in defense against malignant cells; recognition of molecules upregulated in abnormal cells -Associates with the adapter molecules DAP10 in humans and DAP10 or DAP12 in mice</td>
</tr>
<tr>
<td>Receptor</td>
<td>Species</td>
<td>Type</td>
<td>Genetic parameters</td>
<td>Ligands</td>
<td>Major Features</td>
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| LILR          | Human       | -Leukocyte immunoglobulin-like receptors (type I glycoproteins) aka CD85, ILT  
- Inhibitory receptors                           | -Located: LRC        | -Classical MHC-I                                                       | -Capable of inhibiting NK cell activation  
-One LILR binds UL18 (a human cytomegalovirus protein that functions as a MHC-I “decoy” molecule) |
| NKR-P1 Receptors | Mouse       | -Type II transmembrane anchored glycoprotein receptor (C-type lectin-like)  
- Activating (ex. NKR-P1C) and inhibitory members (ex. NKR-P1B/D)   | -Several genes       | -Lectin-like molecules  
- NKR-P1B/D recognizes Clr-b aka Ocil  
- NKR-P1G recognizes Clr-g                         | -NKR-P1C aka NK1.1 is used in flow cytometric identification of NK cells in select inbred mouse strains (C57BL/6)  
-NKR-P1B is important to recognition of a “missing” indication of cell health; Clr-b is expressed in hematopoietic and other cells and is down-regulated in cancerous and infected cells and in cells undergoing genotoxic or cellular stress |
|               | Human       | -Type II transmembrane anchored glycoprotein receptor (C-type lectin-like)  
- NKR-P1A aka CD161, KLRB1                                | -Single gene         | -LLT1 (lectin-like transcript-1) – human orthologue of mouse Clr       | -Inhibitory receptor; may be functionally analogous to the NKR-P1B receptor in mice |
| NCR           | Mouse and Human | -Type I transmembrane Ig-like receptors with 1-2 Ig domains in the extracellular region  
- Activating receptors (NKp46, NKp44, NKp30 in humans and NKp46 in mice) | -Located: NKp46: LRC (Chr 7 in mice), NKp44 and NKp30: human Chr 6 (class III region of MHC locus) | -Viral, bacterial and parasite-associated ligands  
- Cellular ligands such as heparan sulphates                              | -First identified based on their ability to activate NK cells and induce killing of cancer cells in vitro  
-Believed to play a major role in induction of cytotoxicity against malignant cells |
| 2B4           | Mouse and Human | -Aka CD244  
- Member of the CD2 family of Ig-related proteins  
- 2 isoforms in humans                                      | -Located: Chr 1 (human and mouse) | -CD48 (expressed on hematopoietic cells)                                   | -Generally activating but may also be inhibitory (multi-functional): modulated by ratio of activating to inhibitory adapter molecules and extent of ligand binding |
| DNAM-1        | Mouse and Human | -Aka CD226  
- Member of Ig superfamily  
- Activating                                                      | -Located: Chr 18 (humans and mice) | -CD155 (Polio virus receptor) and CD112 (Nectin-2)                           | -Important to anti-cancer defenses; ligands may be upregulated in some tumour cells  
- Possible roles in NK cell migration and target cell ligation |
| PILR          | Mouse       | -Paired Ig-like 2 receptor (type 1 glycoproteins)  
- Activating/inhibitory isoforms                               | -Located: Chr 5      | -PILR-L (CD99)                                                          | -Possible role in recognition of carbohydrate chains                                 |
| KLRG-1        | Mouse and Human | - Killer cell lectin-like receptor G1  
- Inhibitory                                                      | -Located: NKC        | -Classical cadherins (E-, N-, R-cadherins)                                 | -Important to prevention of damage to healthy tissues and to recognition of malignant epithelial cells with downregulated E-cadherin expression |
2.2.5 Mechanism of natural killer cell cytotoxicity

NK cells eliminate abnormal cells through a cytotoxic process that relies on the directed release of NK cell cytotoxic contents towards the target cell through the formation of an “immunological synapse” (Figure 2.4) (116). The NK cell immunological synapse, formed through cytoskeletal rearrangement of the NK cell, is the site of aggregation of various receptor and adhesion molecules referred to as the supramolecular activation cluster (SMAC) (86). F-actin and adhesion molecules aggregate in the peripheral SMAC (pSMAC) to form a ring-like structure, through which cytotoxic granules are delivered to the target cell (86). The process of cytotoxic granule release is tightly regulated in NK cells to prevent unnecessary cell damage, as in contrast to cytotoxic CD8+ T cells, NK cells contain preformed cytotoxic granules (86).

Initial NK cell recognition of an abnormal cell and initial activating events lead to the activation of adhesion molecules such as LFA-1 (leukocyte functional antigen-1; a heterodimer composed of the integrins CD11a and CD18) and MAC-1 (macrophage receptor 1; a heterodimer composed of the integrins CD11b and CD18), which mediate high affinity NK cell-target cell adhesion and facilitate further NK cell-target cell interaction and NK cell activation (86, 116). A common result of various activating receptor signalling pathways and of signalling downstream of adhesion molecule engagement is the phosphorylation and consequent activation of the guanine exchange factor, VAV1, which functions as an actin regulator (116). In addition, VAV1 is a SHP-1 substrate; thus, inhibition of VAV1 function may represent a target of NK cell inhibitory signalling (116). NK cell activation-associated actin reorganization is dependent on the Wiskott-Aldrich syndrome protein (WASP; involved in F-actin branching), and facilitates the structural changes necessary for cytotoxic granule polarization and directed release (86, 116). Cytotoxic granule polarization towards the NK cell-target cell interface involves granule
convergence at the microtubule organizing centre (MTOC) via dynein-dependent trafficking, and subsequent polarization of the MTOC towards the NK cell-target cell interface (86). The ATP-dependent actin motor protein myosin IIa has been hypothesized to mediate delivery of cytotoxic granules through the actin network to the plasma membrane (86, 116). Exocytosis of cytotoxic granules or “degranulation” is dependent on activation-induced intracellular Ca++ mobilization, and is mediated by docking of these specialized secretory lysosomes at the NK cell-target cell interface, followed by SNARE (soluble N-ethylmaleimide-sensitive-factor accessory-protein receptor) protein-mediated fusion with the NK cell plasma membrane (86).

Cytotoxic granules contain apoptosis-inducing components such as perforin, granzymes and FasL among others (86). Perforin, a pore-forming protein, facilitates the entry of cytotoxic molecules such as granzymes into the target cell (117). Granzymes are a family of serine proteases homologous to the enzyme trypsin; where granzyme B and A are the most abundant members (118). Granzyme B cleaves proteins after aspartic residues and activates target cell apoptosis both by cleaving caspases such as caspase-3 and also through directly cleaving caspase substrates (118). Granzyme A cleaves proteins after either arginine or lysine residues and activates an apoptosis-like caspase-independent target cell death through mitochondrial and DNA damage pathways (118). Pro-apoptotic molecules such as FasL and TRAIL (TNF-related apoptosis inducing ligand) also localize to cytotoxic granules within NK cells and induce target cell apoptosis through engagement of target cell surface death receptors (117, 119). Granulysin, a cytolytic (i.e., membrane-disrupting) protein, may also localize to cytotoxic granules and function as a cytotoxic effector molecule (119).
Figure 2.4: Formation and function of the natural killer cell lytic synapse. Formation of the lytic synapse between an NK cell and a target cell can be divided into 3 main stages: initiation, effector (shown) and termination. The initiation stage is characterized by NK cell recognition of a target cell, adhesion, and initiation of activating signalling events. The effector stage involves a multitude of processes including actin reorganization, clustering of receptors, polarization of the microtubule organizing centre (MTOC) and lytic granules towards the NK cell-target cell interface, lytic granule fusion with the cytoplasmic membrane, and directed release of cytotoxic contents towards the target cell resulting in target cell death. The termination stage is proposed to involve a period of inactivity followed by detachment of the NK cell and recycling of its cytotoxic potential. Adapted and reprinted by permission from the publisher (Macmillan Publishers Ltd.): (86). Copyright © 2008 Macmillan Publishers Ltd.

2.2.6 Factors implicated in regulation of natural killer cell function

NK cell function is highly regulated not only by NK cell receptors but also by the cytokine milieu and through NK cell interaction with other cell types (12). For example, cytokines such as Type I IFNs, IL-2, IL-12, IL-15, IL-18 and IL-21 promote NK cell effector functions and/or proliferation (12). The cross-talk between NK cells and dendritic cells (DCs) is also very important to regulation of NK cell functions. For example, activated DCs will stimulate NK cells by producing IL-12 and IL-18, which together potently induce IFN-γ production in NK cells; in turn, IFN-γ promotes DC maturation and these DCs then prime further pro-inflammatory responses (120). Furthermore, DC production and subsequent trans-presentation of IL-15 to resting NK cells has been demonstrated to prime NK cell cytotoxic functions (121). Type I IFNs, which are commonly produced by DCs and other cells types in the context of viral infection, also
potentiate NK cell effector functions (120). Interactions with Foxp3+CD25+ T-regulatory cells are also important to NK cell activity, as NK cell effector function may be suppressed via T-regulatory cell mediated TGF (transforming growth factor)-β-dependent mechanisms (122).

2.2.7 Natural killer cell development and education

Generally, NK cells arise from the common lymphoid progenitor (CLP), which in turn derives from bone marrow-resident self-renewing pluripotent hematopoietic stem cells (HSCs) (102). Commitment to the NK cell lineage occurs through a multitude of differentiation and maturation steps and therefore involves several major cellular intermediates (102). Although NK cells were originally thought to develop predominantly in the bone marrow, NK cell precursor cells have also been identified in the thymus, liver and secondary lymphoid organs such as the lymph nodes, implying that NK cell maturation also takes place in other tissues (87).

IL-15, a cytokine that signals through an IL-2Rγ (common γ chain) containing receptor, is of significant importance to NK cell differentiation, maturation, and survival, wherein deficiency in IL-15 and its signalling components is associated with impaired NK cell development (102). Thus, NK cell precursors are identified on the basis of expression of IL-15 receptor components, in addition to the absence of markers signifying commitment to other lymphocyte lineages, and the absence of a functional phenotype and cell surface markers associated with mature NK cells (102). Furthermore, NK cells are reliant on the expression of transcription factors, T-bet and Eomes, for their development and maturation (99). Notch signalling mediated by binding of ligands from the Delta and jagged families has also been implicated in NK cell development (87). NK cell maturation is characterized by a sequential acquisition of functional receptors and capabilities (87, 102). Acquisition of functional capacities, such as secretion of cytokines (IFN-γ and TNF-α) and perforin-mediated cytotoxicity,
follows acquisition of functional receptors, and is typically the last stage of NK cell maturation (87). This maturational scheme is in alignment with NK cell inhibitory receptor-mediated “education”; i.e., tuning of functional responsiveness of NK cells in a manner that facilitates prevention of inappropriate NK cell responses and maintenance of tolerance to self (102).

NK cell education is the process by which NK cells are rendered functionally responsive based on their ability to recognize “self” MHC class I molecules; as such, educated NK cells are capable of both “missing self” recognition and of tolerance towards healthy cells (87). NK cell education thus represents a mechanism by which NK cells lacking inhibitory receptors for “self” molecules are prevented from causing damage to healthy cells, in that these NK cells are functionally hyporesponsive in regard to cytotoxicity and cytokine secretion (123). Indeed, hyporesponsive NK cells lacking inhibitory receptors for self MHC class I molecules have been identified in both mice and humans (123). NK cells are educated through interactions with “self” MHC class I molecules and although this process is not fully understood mechanistically, it appears to be quantitative; i.e., NK cell functional responsiveness is tuned with respect to the strength of the inhibitory input received by the NK cell (123-125).

An important caveat to the functional distinction between uneducated (NK cells lacking inhibitory receptors for self MHC class I molecules) and educated NK cells is that uneducated NK cells can respond normally in an inflammatory environment (87). In addition, uneducated NK cells may have a more robust response in the context of viral infection than their educated counterparts, as observed in a mouse model of cytomegalovirus infection (126). It is also possible that uneducated NK cells may respond more effectively to certain malignancies, such as leukemia (87). For example, improved patient outcomes have been observed to be correlated with the number of missing KIR ligands in leukemia patient recipients of HLA-matched and
KIR-mismatched bone marrow transplants (127). Finally, NK cell education also appears to be a dynamic and reversible process, wherein adaptation of NK cells has been observed post adoptive transfer into an alternate MHC class I environment (128).

2.2.8 Importance of natural killer cells to health

NK cells represent a primary line of defense against intracellular microbial infections and malignancy, due to both their cytotoxicity against abnormal cells and their capacity to produce inflammatory cytokines, such as IFN-γ and TNF-α, and thus to promote and shape a subsequent adaptive immune response (12).

NK cells function in initial containment of viral replication and elimination of virally-infected cells through cytotoxicity and inflammatory cytokine production, as well as in priming of an inflammatory adaptive immune response through activation of antigen-presenting dendritic cells and T-cell polarization (12, 129). Viral immune-evasion mechanisms often involve downregulation of MHC class I molecules on infected cells; NK cells are however able to respond to and eliminate these cells via “missing-self” recognition (12, 129). The significance of NK cell function to anti-viral defenses is further demonstrated by the evolution of viral “decoy” molecules, which resemble MHC class I and thus function to abrogate NK cell activation and cytotoxicity (12, 129).

NK cells function in tumour immunosurveillance in that they exhibit cytotoxicity against cancerous cells, which often have upregulated expression of NK cell activating ligands (for example, NKG2D ligands such as MIC-A/B and ULBPs in humans) and loss of MHC class I expression; i.e., NK cell cytotoxicity as a result of “induced-self” and “missing-self” recognition (106). NK cells are believed to function in early elimination of neoplastic cells and thus in prevention of tumour development (106). NK cells may exert a protective effect against
malignancy not only via cytotoxicity, but also through the induction of an adaptive immune response involving; for example, tumour-specific T cell responses (12).

A collection of experimental animal studies support a role for NK cells in defence against the growth and progression of several cancer types (12). Depletion of NK cells or abrogation of their functions results in outcomes such as increased tumour incidence or compromised tumour rejection (12). The importance of NK cell function to defense against malignancies is further supported by epidemiological evidence. For example, an 11-year follow up study in a general Japanese population revealed an association between lower relative NK cell activity and an increased cancer risk in older adults (130). Perforin, TRAIL and IFN-γ mediated defenses have been shown to be important to tumour immunosurveillance, although these mechanisms are not exclusive to NK cells (106). Furthermore, NK cell receptors such as NCRs, 2B4, DNAM-1 and NKG2D have been implicated in tumour cell elimination, although expression of these receptors in not entirely exclusive to NK cells, and other immune cell types may also be implicated in receptor-mediated defense against cancerous cells (106).

NK cell receptor mediated defenses against malignancy have been most thoroughly studied in regard to NKG2D, where NKG2D mediated elimination of cancer cells expressing NKG2D ligands has been observed in vitro, and where lack of expression of NKG2D has been shown to result in poorer outcomes in some but not all animal cancer models (106). Furthermore, certain polymorphisms in the NKG2D gene in humans have been associated with cancer susceptibility (131). It is of importance to note that NK cell tumour immunosurveillance may inadvertently cause selection of cells that are more immune-evasive and therefore more difficult to eliminate (106). In addition, cancerous cells have been documented to utilize certain
mechanisms, such as shedding of NKG2D ligands, in order to evade NK-cell mediated elimination (106).

NK cells also play an important role in hematopoietic stem cell transplantation, owing to their potential cytotoxicity against allogeneic cells in the case of donor KIR repertoire and recipient MHC class I mismatch; as such, NK cells can have a beneficial graft-versus-leukemia effect. This is best described in regard to acute myeloid leukemia, where “self” MHC class I inhibitory input is limited, whereas activating signals are mediated by detection of ligands on leukemic cells associated with abnormality, resulting in donor NK cell killing of recipient leukemic cells (132). NK cell function in solid organ transplantation is less well-characterized; wherein both graft tolerance and graft rejection have been reported to be modulated by NK cell activity (133).

Finally, the significance of NK cell function in many other aspects of human health, such as pregnancy (as previously described) and autoimmune disorders, in which NK cells have been implicated in both protective and exacerbating roles (134), has come to light in more recent years and is currently under active investigation (12).

2.2.9 Aging and nutritional factors in natural killer cell function

Aging is associated with a general decline and dysregulation in immune function referred to as immunosenescence. Although immunosenescence is a universal process, it presents with great heterogeneity among individuals and is believed to be influenced by a variety of factors, including genetics, environment, life style and nutrition (135). Common features of immunosenescence include reduced effectiveness of both cell-mediated and humoral immunity in the context of defense against infection and malignancy (135). However, immune function in aging is also characterized by upregulated generalized inflammation (135).
In humans, NK cell effector functions such as cytotoxicity and cytokine production are documented to decline on a per cell basis during aging; however, typically NK cell numbers remain the same or are increased (13). In addition, shifts in NK cell subset distribution, with a decrease in CD56^{bright}CD16^- NK cells and an increase in CD56^{dim}CD16+ NK cells, are also documented in elderly individuals (13). CD56^{bright}CD16^- NK cells decrease not only in frequency, but also in capacity to produce cytokines and chemokines upon stimulation (13). The age-related reduction in NK cell cytotoxic function has been shown to be mediated at least partially by impaired secretion of perforin, via defective polarization of cytotoxic granules towards the immunological synapse (136). In mice, similar phenomena have been observed, with decreased NK cell cytotoxicity and cytokine production in response to stimulation with type I IFNs and other cytokine combinations, as well as with reduced NK cell migration, cytotoxicity and cytokine production in viral infection models (13).

Several nutritional parameters including but not limited to zinc status, caloric restriction, vitamin E status, vitamin A status and high fat consumption have been implicated in modulation of NK cell function (135, 137-142). Furthermore, in some cases nutrient status has been shown to exacerbate parameters of immunosenescence, such as reduced NK cell function in aging (135).

Zinc is an important factor in the structural and catalytic stability of various enzymes and may also function in signal transduction through transient stabilization or inhibition of signalling molecules (137). As such, zinc deficiency is associated with a reduction in many aspects of immune function, including NK cell cytotoxicity, and concomitantly with an increased risk of infection (137). In contrast, zinc supplementation in elderly individuals with inadequate zinc status has been generally linked to improved immune function (137). Zinc has been shown to
function in several NK cell related processes such as promotion of NK cell differentiation and multimerization of KIRs, which in turn modulates ligand binding (137). It is hypothesized that zinc may also modulate NK cell function through its inhibitory action on protein tyrosine phosphatases, which are major players in mediating NK cell receptor signalling (137).

Caloric restriction has been implicated in ameliorating certain aspects of immunosenescence in experimental models (138). While caloric restriction was shown to reduce NK cell cytotoxicity in influenza infection in both young and aged mice, in the absence of infection in young mice, caloric restriction was associated with changes in NK cell subset distribution in the spleen (reduced CD11b+CD27- NK cells), that in turn were associated with functional changes (138). These functional changes included reduced IFN-γ production in response to cytokine and activating receptor stimulation, and increased degranulation and IFN-γ production in response to cancer cell (YAC-1) stimulation (138).

Supplementation with vitamin E, a lipid soluble anti-oxidant, has been generally associated with improved NK cell activity (135). For example, a study examining the effects of micronutrient status on NK cell function in a healthy elderly population showed a significant positive correlation between plasma vitamin E concentration and NK cell cytotoxicity in women (139). Vitamin E-associated improved NK cell activity in the elderly could potentially be mediated by mechanisms such as increased IL-2 production and reduced production of the NK cell-inhibitory molecule, prostaglandin E₂ (140).

Vitamin A or retinol is important to the maintenance of immune homeostasis and has also been implicated in regulation of NK cells and other ILCs (141). Macrophages and DCs, as well as other cell types, express retinol dehydrogenase enzymes that convert vitamin A to its main active metabolite, retinoic acid (RA), which signals through nuclear retinoic acid receptors.
RARα, RARβ, RARγ) and nuclear rexinoid receptors (RXRα, RXRβ, RXRγ) (141). RA has been recently implicated in the differentiation of DCs as well as modulation of their functions. In steady state conditions in the intestine, RA derived from DCs can inhibit T_{H17} cell differentiation, whereas RA in combination with TGF-β can promote Foxp3+ T-regulatory cell differentiation (141). In contrast, in the context of inflammation RA promotes pro-inflammatory cytokine secretion by T_{H1} and T_{H17} CD4+ T cells (141). As NK cell-DC cross-talk is important to regulation of NK cell function, it is speculated that RA may potentially regulate NK cell function via this pathway. Indeed, vitamin A deficiency has been associated with reduced splenic NK cell cytotoxicity in the context of viral infection and in un-infected rats (143). However, whether cytotoxicity was reduced on a per cell basis or as a result of reduced NK cell numbers was not examined in these studies (143). Furthermore, circulating NK and NK-T cell numbers have been shown to be positively correlated with retinol stores (141). RA has also been implicated in promotion of IL-22 secretion by Group 3 ILCs in the intestine and as such to promote gut-immune homeostasis (141). Finally, RA enhances expression of NKG2D ligands, such as MICA/B and the RAE proteins, on tumour cells, which may augment NK cell cytotoxic functions (141).

In addition, a recent study using a MCF-7 xenograft-model of breast cancer in mice showed that a high fat diet without body weight gain was associated with increased tumour growth and reduced NK cell cytotoxicity against the YAC-1 cancer cell line (142). Interestingly, NK cell cytotoxicity has also been shown to be modulated by the appetite-regulating hormone, leptin, which is increased in its free-form in obesity (144). Signalling via leptin receptors (Ob-R) on NK cells was demonstrated to potentiate NK cell cytotoxicity against the YAC-1 cell line in lean but not obese mice (144). Defective signalling and not reduced Ob-R expression was
determined to be the mechanism behind the absence of a cytotoxicity promoting effect in obese mice (144).

2.2.10 Characterization of natural killer cells and natural killer cell function in the laboratory

In the laboratory, human and mouse NK cells are identified via detection of expression and absence of specific cell surface markers by flow cytometry. Human NK cells are commonly identified as CD56+CD3- cells, whereas in mice, NK cells are identified as CD49b/DX5+CD3- cells, NK1.1+CD3- cells in mouse strains expressing the NK1.1 receptor (for example, C57BL/6 mice), or NKp46+CD3- cells (although this population may also include other ILCs). NK cells can then be further sub-classified based on detection of expression or the level of expression of NK cell subset specific markers as previously described.

The “gold standard” in assessing NK cell cytotoxicity in vitro is the radioactive chromium (51Cr) release assay. Target cells are first labeled with 51Cr and then incubated with NK cells or other cytotoxic lymphocytes for several hours; subsequently, cytotoxicity or cell lysis is assessed by measurement of radioactivity released into the supernatant, which is indicative of the degree of target cell lysis by the effector population (145). Percent lysis is calculated as: [experimental release – spontaneous release]/[maximum release – spontaneous release] x 100%. The 51Cr release assay is the method of choice in assessing cytotoxicity as it is highly reproducible; however, alternative cytotoxicity assays have been developed in order to limit the use of biohazardous radioisotopes, as well as complications associated with their disposal (146). Alternatives to the 51Cr release assay include colourimetric or fluorometric assays that rely on a similar principle as the 51Cr release assay; i.e., release of molecules from dead cells that are readily identifiable. For example, the calcein-acetoxyethyl (calcein-AM) release cytotoxicity assay measures the release of the fluorescent dye calcein-AM from lysed cells (147). Other
assays include flow cytometry based assays for detection of cell death (146) and bioluminescent assays which rely on transfection of target cells with light emitting enzymes such as luciferase; reduction in luciferase activity (which is ATP-dependent) reflects target cell death and thus cytotoxicity (148).

Another commonly employed method in assessment of NK cell activity in response to target cell and other stimulations is the measurement of NK cell degranulation by flow cytometry. The process of degranulation involves the release of cytotoxic granules from the NK cell upon activation, and as such assessment of degranulation can be considered a measure of NK cell cytotoxic potential (149). However, a major caveat is that degranulation is not always synonymous with cytotoxicity. Defects in, for example, the components of cytotoxic granules such as perforin-deficiency may not alter degranulation, but could result in reduced cytotoxicity (146). Regardless, measurement of NK cell degranulation is a useful assessment of NK cell function as it provides information on the activation status of NK cells within the study population, whereas traditional cytotoxicity assays only provide information concerning target cell death (149). Cell surface expression of the molecule CD107a (LAMP-1, lysosomal-associated membrane protein-1) is used as a marker of degranulation as CD107a is rapidly upregulated on the surface of cytotoxic lymphocytes post release of cytotoxic granules (150). CD107a is a component of cytotoxic granules, which release their active components via fusion with the cell membrane (exocytosis), which in turn results in appearance of CD107a on the cell surface (150). CD107a has been reported to act in prevention of damage to the cytotoxic lymphocyte post release of its cytotoxic contents (150).

NK cell cytokine production in response to stimulation with target cells or cytokines, etc., can be assessed using flow cytometry-based detection of intracellular cytokines, provided that
protein transport inhibitors are used in the experiment (149). Alternatively, NK cell cytokine production can be assessed \textit{in vitro} via measurement of secreted cytokines, using enzyme-linked immunosorbent assays.

In the laboratory, the intrinsic responsiveness of NK cells i.e., the end-product of NK cell education, is assessed using functional assays, as markers denoting NK cell educational state have not been identified (123). Various types of assays may be used in assessment of NK cell education, such as \textit{in vivo} rejection studies and cell-cell interaction assays, but most commonly used is the technique of stimulating activating receptors (such as NK1.1 and NKp46) \textit{in vitro} by the use of activating receptor directed-antibody crosslinking and assessment of effector outputs such as degranulation and cytokine production (123).
2.3 Folate and natural killer cell function

2.3.1 Folate and immune function

There exists limited data in regard to the effects of folate deficiency and FA supplementation on immune function and in particular on NK cell activity.

Animal and human studies of folate deficiency from the late 70s and early 80s reported reduced humoral and cell mediated immunity, including modified T-lymphocyte proliferative responses (151). Dhur et al. observed reduced thymus size and T-lymphocyte thymus content in post-weaning folate-deficient C57BL/6 mice, which were normalized when examined 10 days post folate repletion at the level equivalent to the basal dietary requirement (BDR) (i.e., 2 mg FA/kg diet) (151).

In a study conducted by Courtemanche et al., folate deficiency was shown to impair phytohaemagglutinin (PHA)-activated human T-lymphocyte proliferation in vitro (152). In addition, folate deficiency was observed to induce S phase cell cycle arrest and apoptosis, as well as to increase DNA uracil misincorporation (152). An altered CD4+ to CD8+ T-lymphocyte ratio was also observed in this study due to greater impairment in CD8+ T-cell proliferative response, and supplementation with either FA or nucleosides ameliorated the observed effects (152). Therefore, this study highlights the importance of adequate folate as a co-factor in DNA synthesis to rapidly proliferating cells, such as those of the immune system.

Recently, a role for folate in survival and maintenance of Foxp3+CD25+ T-regulatory cells (T-regs) in the peripheral circulation, as well as within the small intestine and colon, has been identified (153-155). Examination of the impact of folate on Foxp3+CD25+ T-regs was prompted by observations made in a study by Yamaguchi and colleagues in 2007, in which these investigators identified constitutive and high expression of folate receptor 4 (FR4, also known as
FRδ or folate binding protein 3) as a cell surface marker distinguishing Foxp3+CD25+ T-regs from other activated CD4+ T-lymphocytes (153). As Foxp3+CD25+ T-regs are more proliferative in antigen-naïve mice relative to other CD4+ T-lymphocytes, these investigators hypothesized that folate, as a co-factor in DNA synthesis, may be critical to their maintenance in the peripheral circulation and tissues, thereby explaining the high expression of FR4 (153). Indeed, Yamaguchi and colleagues demonstrated that FR4 was functionally essential for maintenance of Foxp3+CD25+ T-regs in vivo (153). Antibody-mediated blocking of FR4 resulted in specific reductions in Foxp3+CD25+ T-regs in the peripheral circulation and potentiated an anti-tumour response in a tumour-afflicted BALB/C mouse model, while symptoms of auto-immune disease arose in a healthy young BALB/C mouse model (153).

Kunisawa and colleagues further demonstrated that in vitro culture in folate-reduced conditions resulted in impaired Foxp3+CD25+ T-reg survival and reduced expression of the anti-apoptotic molecule, Bcl-2 (154). In addition, using a 7-9 week old BALB/c mouse model, these investigators demonstrated that a 3-month dietary intervention with a folate-deficient diet resulted in reduced Foxp3+CD25+ T-reg numbers in the small intestine, whereas Foxp3+CD25+ T-reg numbers in the spleen and colon were not significantly altered (154). In contrast, using a BALB/c model in which dietary intervention was commenced during gestation and effects were examined in 4-6 week old offspring, Kinoshita et al demonstrated that a folate-deficient diet reduced Foxp3+CD25+ T-reg numbers in the colon, but not in the small intestine (155). A possible explanation for the discrepancy in these findings may involve the modulation of folate status achieved in each of these studies – for example, in the study conducted by Kunisawa et al. folate content in luminal washes of the small intestine was significantly reduced in the folate-deficient mice, but was not significantly altered in the colon, whereas the opposite was observed
in the study conducted by Kinoshita et al.. Furthermore, the dietary interventions in both studies differed not only in duration, but also in the dietary intervention strategies. For example, the study conducted by Kinoshita et al. was trans-generational, where folate status in utero and in later life, and combinations thereof have been shown to modulate certain phenotypes in animal models such as cancer risk (57, 156).

Similarly to the Kunisawa et al. study, the Kinoshita et al. study demonstrated that in folate-deficient animals expression of the anti-apoptotic molecules Bcl-2 and Bcl-xL was reduced in Foxp3+CD25+ T-reg from the colon (155). Furthermore, the authors observed that intervention with a folate-deficient diet was associated with increased colonic inflammation in a chemically-induced model of colitis in BALB/c mice, whereas transfer of FR4+ T-reg prior to colitis induction in these animals resulted in improved survival (155).

Collectively, the findings from these three studies suggest a possible role for folate in maintenance of gut immune homeostasis via regulation of Foxp3+CD25+ T-reg, which in turn is responsible for suppressing unwarranted inflammatory responses and protecting tissues from unnecessary damage. This process is particularly important in the gut where the interplay between commensal bacteria, food-derived antigens and the immune response must be tightly controlled (155). In addition, it is of importance to note that dysregulation in the T-reg response in the context of cancer may result in suppression of immune elimination of tumours (122). As folate is known to have dual modulatory effects on colorectal cancer development and progression (8), the influence of folate on T-regulatory cells in this context may provide additional insight into the mechanism of the folate-mediated colorectal carcinogenesis.
2.3.2 Folate deficiency and natural killer cell function

Kim et al. examined the effects of both moderate and severe folate deficiency on NK cell cytotoxicity in post-weaning (3 week old) male Sprague-Dawley rats using the $^{51}$Cr release cytotoxicity assay (157). The effects of severe folate deficiency, which was induced by administering the antibiotic succinylsulfathiazole (10 g/ kg of diet) concurrently with a folate deficient diet, were measured at 4 and 5 weeks of the dietary intervention, as severe illness and death were observed beyond these time points (157). NK cell cytotoxicity was significantly lower in the severely folate deficient group relative to a control (2 mg FA/kg) and a moderate FA supplementation group (8 mg FA/kg) at week 5 (157). Furthermore, NK cell cytotoxicity was correlated directly with plasma ($r = 0.64–0.70, p = 0.015$) and liver ($r = 0.66–0.73, p = 0.008$) folate concentrations at week 5 but not week 4 (157). However, no differences in cytotoxicity were observed between the control and supplemented groups at either time point (157). The observation of reduced NK cell cytotoxicity in the severely folate deficient group at week 5, however, was confounded by factors including significant growth retardation and lymphopenia, where NK cell cytotoxicity was also correlated with body weight ($r = 0.54–0.64, p < 0.04$) and absolute lymphocyte counts ($r = 0.58–0.63, p < 0.03$) at week 5 in the severe folate deficiency experiment (157).

In a study using a moderate degree of dietary folate deficiency, comparable to that seen in humans, reduced NK cell cytotoxicity was not observed in moderately folate deficient rats relative to a moderately supplemented group (8 mg FA/kg), measured at week 24 of the dietary intervention (157). An important consideration regarding this study is that NK cell cytotoxicity in the moderate folate deficiency group was not assessed relative to a control group, where effects of FA supplementation on NK cell function are not well characterized. Finally, a study of
the effects of micronutrient deficiency on NK cell activity in a population of elderly Italians (90+ years old) revealed no associations between folate deficiency and NK cell activity (139).

2.3.3 Folic acid supplementation and reduced natural killer cell activity

The effects of FA supplementation on NK cell function are not well-defined. However, a possible adverse effect of high FA intake was reported in a study of post-menopausal women (n = 105) by Troen et al., in which an inverse U-shaped relationship was observed between combined dietary folate and FA-containing supplement intake and NK cell cytotoxicity against the cancer cell line K562, as measured using a flow cytometry based cytotoxicity assay (11). Although NK cell cytotoxicity was significantly higher in women consuming FA supplements at levels ≤ 400 µg/day relative to women consuming low dietary folate without FA supplements, NK cell cytotoxicity was significantly reduced in women consuming high dietary folate (≥ 233 µg/day) combined with FA supplement intake at levels > 400 µg/day relative to women consuming low dietary folate without FA supplements (11).

In addition, UMFA was detected in 78% of the study population at an average concentration of 2.31 ± 1.91 nmol/L (mean ± SD) after an overnight fast in this study (11). NK cell cytotoxicity was significantly lower (by ~23%) in women with detectable plasma UMFA compared to those without detectable plasma UMFA (11). Furthermore, NK cell cytotoxicity was strongly and inversely associated with plasma UMFA concentrations in the subset of women aged 60-75 years (11). Although the same trend was observed in women aged 50-59 years, the association was not statistically significant (11). NK cell cytotoxicity was not associated with plasma total folate or other folate metabolite concentrations. In addition, UMFA concentrations were not associated with peripheral blood NK cell absolute numbers, suggesting a functional impairment in the observed reduced cytotoxicity (11). Presence and concentration of plasma
UMFA were not correlated with plasma folate concentrations (11), which may result from the high inter-person variability observed in DHFR activity (9).

The association between high FA intake and UMFA and reduced NK cell cytotoxicity reported by Troen et al. (11), prompted interest in further examination of this relationship as reduced NK cell cytotoxicity could potentially contribute mechanistically to the purported tumour progression-promoting effect of high FA intake. Hirsch et al. examined the relationship between serum total folate concentrations and NK cell cytotoxicity (via the $^{51}$Cr release cytotoxicity assay) in a healthy Chilean population aged 20+ years ($n = 227$); however, no significant correlation between serum folate concentrations and NK cell cytotoxicity, adjusted for sex, age, and body mass index, was observed (158). One major limitation of this study is the absence of examination of plasma UMFA concentrations. Indeed, an association between plasma total folate concentrations and NK cell cytotoxicity was not observed in the Troen et al. study either (11). In addition, FA supplementation was not documented among participants in this study, and female participants represented a minority of the study population (32%), limiting assessment of gender-specific effects.

In a further study conducted by Hirsch and colleagues, human peripheral blood mononuclear cells (PBMCs), or purified NK cells, were incubated in varying concentrations of FA (PBMCs: 0, 30, 100 nM; purified NK cells: 0, 15, 30, 75 or 100 nM) in vitro for 72-96 hours (159). No significant differences in NK cell cytotoxicity as examined using the $^{51}$Cr release cytotoxicity were observed in either an NK cell-purified or PBMC population at different levels of FA in the culture media (159). The limitations of this study include: 1) a short duration of exposure to FA, as a reduction in NK cell cytotoxicity may result from a more chronic exposure to high FA; 2) folate status and supplemental FA intake of the PBMC/NK cell donors were not
examined in this study, and could potentially confound the results; and 3) reduced NK cell cytotoxicity in response to high FA intake could result from alterations in NK cell developmental processes or functional maturation, the effects of which would not be captured within this study.
Chapter 3: Rationale, hypothesis and objectives

3.1 Rationale

Dietary and supplemental intake and blood levels of folate and FA have significantly increased over the past 15 years in the North American population, owing to mandatory FA fortification and widespread supplemental use (2, 3, 19). Although folate and FA have largely been considered safe and may provide several health benefits, including the prevention of NTDs, an emerging body of evidence has suggested that high folate and FA intake may have adverse health effects with potentially serious consequences. In particular, high FA intake and plasma UMFA were reported to be associated with reduced NK cell cytotoxicity in a study of post-menopausal women (11). NK cells are cytotoxic towards cancerous and microbe-infected cells, and therefore play an important role in host defense against malignancy and pathogens (12). To date, however, this purported adverse effect of FA on NK cell function has not been unequivocally established; evidence regarding this relationship remains limited and inconsistent. As such, studies are warranted to examine this potential deleterious effect of high FA intake and circulating UMFA on NK cell function, which may be associated with serious adverse health outcomes. Furthermore, a reduction in NK cell function mediated by exposure to excessive FA could provide an additional mechanistic explanation for the purported tumour-promoting effect of FA supplementation observed in animal and some clinical studies.

3.2 Hypothesis

As per the observations made by Troen and colleagues (11), FA supplementation at a dose sufficient to saturate DHFR and to elicit circulating UMFA is hypothesized to impair NK cell function, potentially through mechanisms relating to dysregulation of folate-dependent processes, such as DNA methylation-dependent regulation of gene expression. Persistent
exposure to high levels of circulating UMFA could theoretically lead to disruptions or preferential shifts in folate-dependent one-carbon transfer reactions and metabolism, through competition between FA and the appropriate folate substrates for folate-dependent enzymes, or through modulation of folate transporter and folate-dependent enzyme expression (7). Due to the multitude of physiological processes that may be influenced by dysregulation in folate-dependent mechanisms, it is difficult to hypothesize the mechanism by which excessive FA may impair NK cell cytotoxicity. However, given the variety of factors that can modulate NK cell responses, potential mechanisms may include, but are not limited to, dysregulation in NK cell developmental or maturation processes, interactions with other immune cells, and impairment of NK cell-target cell recognition mechanisms.

Nonetheless, one such possible mechanism could involve FA-mediated DNA-methylation-dependent epigenetic modification of NK cell receptor expression. KIRs in humans and Ly49 receptors in mice are expressed on subsets of NK cells in a stochastic, monoallelic, and highly variegated fashion (84, 105). DNA methylation has been implicated in maintenance of NK cell receptor expression; while increased DNA methylation at CpG-rich sites within KIR promoters suppresses KIR expression, demethylation increases KIR expression (113). Although the Ly49 family of receptors in mice does not contain CpG rich promoters, a similar mechanism has been implicated in the monoallelic expression of the Ly49A receptor (160). In addition, Gao et al. demonstrated that expression of KIRs in human NK cells in vitro, and the consequent cytotoxic activity of these cells, can be modulated using a CpG DNA methyltransferase inhibitor (5-Azacytidine) (161). Therefore, high FA intake could potentially lead to perturbations in DNA methylation, resulting in modulation of NK cell receptor expression, which in turn could affect NK cell target cell recognition and reduce NK cell cytotoxicity.
3.3 General Objectives

The main objective of this thesis was to examine the effects of FA supplementation on NK cell function using an animal model and hence, to either provide support for or refute the previously reported inverse association between high FA intake/UMFA levels and NK cell cytotoxicity. A secondary objective of this thesis was to elucidate potential mechanisms behind the deleterious impact of FA supplementation on NK cell function.
Chapter 4: The effects of folic acid supplementation on natural killer cell activity in an animal model

4.1 Abstract

**Background:** Intake and blood levels of folate and its synthetic form, folic acid (FA), have significantly increased in the North American population, due to FA fortification and widespread use of FA supplements. Excessive FA appears to saturate the key enzyme (dihydrofolate reductase, DHFR) involved in FA biotransformation, and unmetabolized FA (UMFA) enters the circulation. An emerging body of evidence has suggested that high folate status, in particular high FA intake and plasma UMFA, may have adverse effects on human health. High intake of FA and plasma UMFA were associated with reduced natural killer (NK) cell cytotoxicity in a study of post-menopausal women, but this observation has not yet been confirmed. Impaired NK cell function as a result of FA supplementation is a significant concern, as NK cells represent a primary line of defense against malignancies and infection. Given this consideration, we examined the relationship between FA supplementation and NK cell activity in a mouse model.

**Methods:** Post-weaning C57BL/6 mice were randomized to receive diets containing 2 (control) or 20 (high FA; HFA) mg FA/kg diet in Study I and 0 (moderate folate deficiency; MFD), 2 or 20 mg FA/kg diet in Study II for 3 months. Plasma and liver folate and plasma UMFA concentrations were determined in both studies. In Study I, splenic NK cell function was assessed using a flow cytometry-based assay for detection of NK cell degranulation and IFN-γ production, in response to stimulation with the murine lymphoma cell line, YAC-1. In Study II, generalized responsiveness of splenic NK cells was characterized via plate-bound anti-NK1.1 antibody stimulation, using a similar flow cytometry-based assay as in Study I. As a corollary,
hepatic *Dhfr* gene expression and its correlation to parameters of folate status were determined in Study II.

**Results:** Plasma folate and plasma UMFA concentrations were significantly higher in the HFA group relative to the control in Study I \( (p < 0.0001) \) and differed significantly among the dietary groups in Study II \( (p < 0.0001) \). Significant linear trends in plasma folate and plasma UMFA concentrations were observed with increasing FA content in the diet \( (p\text{-trend} < 0.0001) \) in Study II. Hepatic folate concentrations were significantly higher in the HFA group relative to control in Study I \( (p = 0.021) \) and differed significantly among the dietary groups in Study II \( (p < 0.0001) \). A significant linear trend in hepatic folate concentrations was observed with increasing FA content in the diet \( (p\text{-trend} < 0.0001) \) in Study II. In Study I, NK cell degranulation in response to stimulation with the YAC-1 cancer cell line and cytokines was significantly lower \( (p = 0.044) \) in the HFA group relative to the control; furthermore, plasma folate \( (r_p = -0.54, p = 0.024) \) and plasma UMFA \( (r_p = -0.56, p = 0.019) \) concentrations were significantly and inversely correlated with NK cell degranulation in response to stimulation with YAC-1 and cytokines. In study II, neither NK cell degranulation nor IFN-γ production in response to plate-bound anti-NK1.1 antibody stimulation differed significantly among the dietary groups, nor were plasma folate or plasma UMFA concentrations correlated with NK cell activity. Hepatic expression of *Dhfr* did not differ significantly among the MFD, control, and HFA dietary groups. However, plasma folate \( (r_p = 0.50, p = 0.094) \) and UMFA \( (r_p = 0.56, p = 0.058) \) concentrations were positively, albeit non-significantly, correlated with hepatic *Dhfr* gene expression.

**Conclusions:** These data suggest that high FA intake impairs the NK cell cytotoxic response to cancerous cells. These data also suggest that dysregulation in generalized NK cell responsiveness is not a probable mechanism behind this observation. Our findings corroborate the previously
observed inverse relationship between NK cell cytotoxicity and high FA intake, as well as plasma UMFA, in a human pilot study. Finally, our data suggest that impaired tumour immunosurveillance as a result of high FA intake may be a mechanism behind the purported tumour-promoting effect of high FA intake.

4.2 Introduction

Folate and its synthetic form, folic acid (FA), are critical to human health, due to their essential role in one-carbon transfer reactions involved in nucleotide biosynthesis and biological methylation reactions (1). Intake and blood levels of folate and FA have significantly increased in the North American population, due to mandatory FA fortification and prevalent supplement use (2, 3). Although the benefits of FA supplementation in regard to neural tube defect prevention are well established (4), an emerging body of evidence has linked high FA intake to certain adverse health effects, such as promotion of tumour progression (8). Excessive FA intake saturates the enzyme dihydrofolate reductase (DHFR), responsible for FA biotransformation, and unmetabolized FA (UMFA) enters into the circulation (9). It has been suggested that UMFA may be responsible for the adverse health effects associated with high folate status (10). Possible mechanisms by which UMFA may adversely affect health outcomes include disruptions or preferential shifts in folate related processes, mediated by competition between FA and the appropriate substrates for folate-dependent enzymes, or by altered gene expression of folate transporters/receptors and folate-dependent enzymes (7, 61).

A possible adverse effect of high FA intake on immune function was reported in a study of post-menopausal women by Troen et al., where an inverse U-shaped relationship was observed between combined dietary folate and supplemental FA intake and natural killer (NK) cell cytotoxicity, such that NK cell cytotoxicity was lower in women consuming high dietary
folate (≥233 µg/day) combined with supplemental FA at levels ≥ 400 µg/day, relative to women consuming low dietary folate (<233 µg/day) without FA supplementation (11). NK cell cytotoxicity was also strongly and inversely associated with detectability of plasma UMFA in all study participants and with plasma UMFA concentrations in the subset of women aged 60+ (11). However, this intriguing observation has not yet been unequivocally confirmed, and conflicting results regarding the inverse relationship between excessive FA intake and NK cell cytotoxicity have recently been reported (158, 159). For example, null findings were reported in a study examining the relationship between serum folate concentrations and NK cell cytotoxicity in a healthy Chilean population (158), and in a study examining the effects of high levels of FA on human NK cell cytotoxicity in vitro (159).

NK cells are an important component of host defense against malignancies and infection, due to cytotoxic activity against abnormal cells (12). Although previously underappreciated, NK cells also function in a variety of regulatory roles in the body, including but not limited to, shaping adaptive immune responses (12). Furthermore, reduced NK cell function in response to FA supplementation may constitute an additional mechanistic explanation behind the purported tumour progression promoting effect of high FA intake.

In light of the high FA intake in the North American population, the importance of NK cell function to human health, and the paucity of studies investigating the relationship between folate/FA status and NK cell cytotoxicity, we undertook two animal studies to examine the effects of FA supplementation on NK cell function. The objectives of Study I were to determine whether high FA intake impairs the NK cell functional response to malignant cells, including degranulation, as a surrogate marker of cytotoxic potential, and cytokine (IFN-γ) production, and to examine whether plasma folate and/or plasma UMFA concentrations are inversely correlated
with these parameters. The objectives of Study II were to determine whether FA supplementation and folate/plasma UMFA concentrations are associated with impairment in the generalized or intrinsic ability of NK cells to respond via effector functions, such as degranulation and cytokine production. The impact of FA supplementation on hepatic Dhfr gene expression and correlation between parameters of folate status and Dhfr gene expression were also assessed to investigate whether excessive FA intake may exert negative health effects via dysregulation of the folate metabolic pathway through modulation of folate-dependent enzyme expression.

4.3 Materials and methods

4.3.1 Animals and dietary intervention

This study was carried out in strict accordance with the Regulations of the Animals for Research Act in Ontario and the Guidelines of the Canadian Council on Animal Care. The study protocol was approved by the Animal Care Committee of St. Michael’s Hospital (Toronto, ON). Post-weaning (3 week old) male and female C57BL/6 mice were purchased from Charles River Laboratories (St. Constant, QC). Upon arrival, the mice were randomized to receive amino-acid defined diets (Dyets, Bethlehem, PA) containing 2 (control) or 20 (high FA; HFA) mg FA/kg diet in Study I and 0 (moderate folate deficiency; MFD), 2, or 20 mg FA/kg diet in Study II for a duration of 3 months. Water and diets were provided ad libitum. Diets were replaced twice weekly to ensure palatability of the diet. Diet consumption was monitored weekly to ascertain similar dietary consumption across dietary treatment groups. Animal health was monitored daily and body weight was recorded weekly.
4.3.2 Experimental diets

The aforementioned amino-acid defined diets containing varying concentrations of FA (Dyets, Bethlehem, PA) are the well-established method to induce a moderate degree of folate deficiency and to provide different supplemental levels of FA in a predictable manner in rodents. These diets have been used extensively in studies of FA intervention in rodent models (57, 58, 68, 156, 157, 162-164). The detailed compositions of these diets are shown in Table 4.1 and Table 4.2. The 2 mg FA/kg diet is generally accepted to represent the basal dietary requirement (BDR) for folate in rodents (165). The 0 mg FA/kg diet induces moderate folate deficiency in rodents and does not result in significant growth retardation or premature death as the host intestinal microbiota provides folate that can be incorporated into host tissues via de novo folate synthesis (157). The 20 mg FA/kg diet represents FA supplementation at 10X the BDR. Although folate intake levels at 10X the RDA (4.0 mg/day) are not commonly observed in the general North American population except in certain medical conditions where high supplemental levels of FA are given to reduce the adverse effects of antifolates, this supplemental level of FA was chosen to account for reported differences in efficiency of FA biotransformation between rodents and humans (9). FA biotransformation is more efficient in rodents than in humans because of significantly higher DHFR activity (9) and therefore a higher dose of FA is likely required to elicit similar physiological effects of FA in rodents relative to humans.
Table 4.1: Nutrient compositions of experimental L-amino acid defined diets

<table>
<thead>
<tr>
<th>Nutrient (g/kg of diet)</th>
<th>0 mg FA/kg Cat # 517751 (MFD)</th>
<th>2 mg FA/kg Cat # 517774 (control)</th>
<th>20 mg FA/kg Cat # 517801 (HFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Arginine free base</td>
<td>11.2</td>
<td>11.2</td>
<td>11.2</td>
</tr>
<tr>
<td>L-Asparagin.H₂O</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
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<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>35.0</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>23.3</td>
<td>23.3</td>
<td>23.3</td>
</tr>
<tr>
<td>L-Histidine free base</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>8.2</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>11.1</td>
<td>11.1</td>
<td>11.1</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>14.4</td>
<td>14.4</td>
<td>14.4</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>8.2</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>11.6</td>
<td>11.6</td>
<td>11.6</td>
</tr>
<tr>
<td>L-Proline</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Serine</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Threonine</td>
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<td>8.2</td>
<td>8.2</td>
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<tr>
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<td>1.74</td>
<td>1.74</td>
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<tr>
<td>L-Tyrosine</td>
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<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Valine</td>
<td>8.2</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td><strong>Total L-amino acid</strong></td>
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<td><strong>171.44</strong></td>
<td><strong>171.44</strong></td>
</tr>
<tr>
<td>Dextrin</td>
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<td>407</td>
<td>407</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>194.6</td>
<td>191</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Corn Oil (w/0.015% BHT)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Salt Mix #210006</td>
<td>57.96</td>
<td>57.96</td>
<td>57.96</td>
</tr>
<tr>
<td>Vitamin Mix #317756 (no Folate)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>6.6</td>
<td>6.6</td>
<td>6.6</td>
</tr>
<tr>
<td>Folic Acid/ sucrose premix</td>
<td>0.4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1000</strong></td>
<td><strong>1000</strong></td>
<td><strong>1000</strong></td>
</tr>
</tbody>
</table>
Table 4.2: Salt mix and vitamin mix compositions of experimental L-amino acid defined diets

<table>
<thead>
<tr>
<th>Salt Mix # 210006</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients (g/kg of diet)</td>
</tr>
<tr>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>Calcium phosphate, dibasic</td>
</tr>
<tr>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Potassium phosphate, dibasic</td>
</tr>
<tr>
<td>Magnesium sulfate, anhydrous</td>
</tr>
<tr>
<td>Manganese sulfate, monohydrate</td>
</tr>
<tr>
<td>Ferric citrate</td>
</tr>
<tr>
<td>Zinc carbonate</td>
</tr>
<tr>
<td>Cupric carbonate</td>
</tr>
<tr>
<td>Potassium iodide</td>
</tr>
<tr>
<td>Sodium selenite</td>
</tr>
<tr>
<td>Chromium potassium sulfate</td>
</tr>
<tr>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>Molybdic acid, ammonium salt</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamin Mix # 317756</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients (g/kg of diet)</td>
</tr>
<tr>
<td>Thiamin HCl</td>
</tr>
<tr>
<td>Riboflavin</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
</tr>
<tr>
<td>Nicotinic acid</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
</tr>
<tr>
<td>Vitamin A palmitate (500 000 IU/g)</td>
</tr>
<tr>
<td>Vitamin D3 (400 000 IU/g)</td>
</tr>
<tr>
<td>Vitamin E acetate (500 IU/g)</td>
</tr>
<tr>
<td>Menadiolone sodium bisulfate</td>
</tr>
<tr>
<td>Biotin</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
</tbody>
</table>
4.3.3 Sample collection

Mice were killed at 3 months of dietary intervention by 5% isoflurane inhalation followed by cardiac puncture and cervical dislocation. Mice were fasted for 2 hours prior to sacrifice, by removal of diet from the cages, to reduce variation in plasma UMFA concentration measurements resulting from variation in consumption of the FA diets in the time period prior to sacrifice and sample collection. Blood was collected by cardiac puncture and stored in EDTA-treated vacutainer tubes (BD Biosciences) on ice and protected from light. Plasma was collected by centrifuging the blood at 2000 x g for 15 minutes at 4°C. Plasma was stored at -80°C with 5% ascorbic acid for plasma folate analysis and undiluted for plasma UMFA analysis. Livers were harvested at necropsy. Liver lobes were immediately snap frozen in liquid nitrogen and stored at -80°C for hepatic folate content analysis (left lateral lobe) and RNA extraction (right and left medial lobes).

4.3.4 Determination of plasma and hepatic folate concentrations

Plasma and hepatic folate concentrations were determined by the standard microbiological microtitre plate assay using Lactobacillus rhamnosus (previously named Lactobacillus casei) (166, 167). This assay produces consistent results and is considered to be the “gold standard” in assessment of folate concentrations (166, 167). L. rhamnosus is incapable of synthesizing folate and is therefore dependent on external sources of folate for growth; over a certain range of folate concentrations, bacterial growth will be proportional to folate concentrations in medium (166). Therefore, the folate concentration of an unknown sample can be determined based on the growth of the bacteria in culture, as measured by turbidity of the media. The microbiological microtitre plate assay quantifies “total” folate content in a given sample as L. rhamnosus is equally effective in using all folate vitamers for growth (166).
Folic acid standard preparation: 10 mg of FA was dissolved in 10 mL of double-distilled water (ddH₂O) with 5 µL of 10M NaOH, to give a final concentration of 1 mg FA/ml. The solution was adjusted to a pH of 7-8 using HCl; this pH was verified using spectrophotometry (282 nm). The solution was then diluted to 50 µg/mL using methanol. Aliquots were stored at -80°C for use.

Lactobacillus rhamnosus stock preparation: L. rhamnosus ATCC 7649 stock was incubated with Lactobacillus MRS Broth (Difco™, BD Biosciences) (200 µL in 200 mL) for 18 hours at 37°C. The cells were centrifuged and the supernatant was decanted under aseptic conditions. The cell pellet was then resuspended in 180 mL of Lactobacillus MRS Broth and 20 mL of autoclaved 100% cold glycerol. The solution was mixed thoroughly and aliquots of stock bacteria were stored at -80°C for use.

Chicken pancreas conjugase preparation: Chicken pancreas acetone powder (Difco™, BD Biosciences) was dissolved in 0.1M KPO₄ buffer (1.05 g KH₂PO₄, 0.4 g K₂HPO₄, 0.1 g C₆H₇NaO₆ and 100 mL ddH₂O; sterile filtered) after which the solution was incubated under a blanket of toluene for 6 hours at 37°C. Post removal of the toluene the solution was centrifuged at 10,000 x g for 15 minutes; the supernatant was collected and an equal volume of tricalcium phosphate (BioRad Gel HTP was rehydrated: 1 part HTP to 6 parts 0.1M KPO4 buffer per 10 g HTP) was added. The solution was mixed at 4°C for 30 minutes and centrifuged at 10,000 x g for 30 minutes at 4°C; the supernatant was collected. An equal volume of 95% ethanol was added to the cooled supernatant, mixed, and stored overnight at -20°C. The following day, the solution was centrifuged at 10,000 x g for 30 minutes; the recovered supernatant was resuspended in 50 mL of cold 1.0M KPO4 buffer. Dowex-1 (BioRad AG1-X8) (10 g) was added
to the solution which was then mixed for 1 hour at 4°C. Finally, the solution was filtered using Whatman #1 filter paper at 4°C. Aliquots were stored at -80°C for use.

Hepatic tissue folate extraction: The left lateral hepatic lobe was used for the determination of hepatic folate concentrations. The tissue samples were weighed and 1 mL of extraction buffer (0.1M C₆H₇NaO₆, 0.1M Bis-Tris, and 5mM β-mercaptoethanol) per 0.1 g of tissue was added to each sample. The samples were boiled for 15-20 minutes and then cooled on ice. The mixture was homogenized and centrifuged at 5000 rpm at 4°C for 20 minutes. The supernatant was collected and stored at -80°C. The tissue folate extract was incubated with chicken pancreas conjugase and 0.1M KPO₄ buffer at a 4:1:15 ratio. Samples were thoroughly mixed and incubated at 37°C for 2 hours. The samples were further diluted by a factor of 10 with 0.1M KPO₄ buffer, aliquoted and stored at -80°C.

Determination of plasma and hepatic folate concentrations: Three µL of *L. rhamnosus* stock was inoculated into 3 ml of *Lactobacillus* MRS Broth; the inoculum was then incubated in a shaker at 250 rpm and 37°C for 16-18 hours. 500 µL of the resulting culture was added to 2.5 mL of *Lactobacillus* MRS Broth and incubated for a further 6-6.5 hours in a shaker at 250 rpm and 37°C. Bacterial growth of this culture was confirmed via optical density measurement at 650 nm using a spectrophotometer.

150 µL of freshly-prepared KPO₄ buffer was added to each well of a sterile and clear 96-well flat-bottom plate. An eight point standard curve was generated in duplicate for each plate by adding 150 µL of FA standard (diluted to 2 ng/mL) to the starting wells and then 2-fold serially diluting the standard. The concentration of each sample was assessed in duplicate by adding 5 µL of sample to a designated starting well wherein the volume was adjusted to 300 µL by adding KPO₄ buffer; the samples were then 2-fold serially diluted across 3 additional wells resulting in
four measurements. The *L. rhamnosus* culture was washed thoroughly with and then resuspend in 3 mL of freshly-prepared folic acid casei media (9.4 g Folic Acid Casei Medium [Difco™, BD Biosciences] 0.05 g C₆H₇NaO₆ and 100 mL ddH₂O; boiled and sterile-filtered) as to remove traces of folates from the *Lactobacillus* MRS Broth. The culture was diluted step-wise by a cumulative factor of 1000X with folic acid casei medium and 150 µl of this diluted culture was added to each well of the 96-well plate. The plate was sealed using Mylar and incubated for 16-18 hours at 37°C to allow for bacterial growth. Optical density at 650 nM was then determined using a spectrophotometer. Sample folate concentrations were interpolated from the standard curve of folate concentrations plotted against optical densities using SoftMax® Pro V5.4.1 software (Molecular Devices, CA).

4.3.5 Determination of plasma unmetabolized folic acid concentrations

Plasma UMFA concentrations were assessed using the isotope-dilution tandem mass spectrometric method coupled to liquid chromatography (LC/MS/MS) described by Pfeiffer *et al.* (168) by Dr. Susanne Aufreiter in the laboratory of Dr. Deborah O’Connor and by Hailey Craig-Barnes at the Analytical Facility for Bioactive Molecules of the Hospital for Sick Children in Toronto, ON. The limit of detection (LOD) for FA was approximately 0.23 nmol/L.

A preliminary assessment of plasma UMFA concentrations was conducted to determine an optimal fasting period which would result in a reduction in variation of plasma UMFA concentrations without eliminating detectability of plasma UMFA. Based on the data (Table 4.3) obtained from this preliminary experiment, a fasting period of 2 hours prior to sacrifice was determined to be the most optimal time for fasting.
Table 4.3: Preliminary analysis of plasma unmetabolized folic acid concentrations for determination of optimal fasting time

<table>
<thead>
<tr>
<th>Dietary Group</th>
<th>Plasma UMFA (nmol/L)</th>
<th>2 hour fast</th>
<th>4 hour fast</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg FA/kg</td>
<td>-</td>
<td>0.24, 0.52*</td>
<td>-</td>
</tr>
<tr>
<td>2 mg FA/kg</td>
<td>0.21</td>
<td>-</td>
<td>0.24</td>
</tr>
<tr>
<td>20 mg FA/kg</td>
<td>-</td>
<td>2.11</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Female mice (n = 2/group) sacrificed at 5 weeks completion of dietary intervention. * Plasma volume collected was insufficient for analysis (< 300 µL) and therefore the sample was diluted prior to analysis, the concentration reported is adjusted for this dilution.

4.3.6 Lymphocyte isolation

Mouse spleens were collected aseptically and were immediately placed in FA-free RPMI-1640 medium with 2.1 mM L-glutamine (Gibco®, Life Technologies) supplemented with 10% FBS (Gibco®, Life Technologies), 1% penicillin/streptomycin (Gibco®, Life Technologies) and 0.1% Fungizone (Gibco®, Life Technologies) (referred to as FA-free cRPMI hereafter) on ice. Processing of spleens began immediately after isolation; all lymphocyte isolation steps were conducted on ice and all cell washes were performed with FA-free cRPMI. Spleens were homogenized to a single cell suspension via mechanical separation using a cell dissociation wire screen (mesh size 50; Sigma Aldrich) and passage through a 70 µm mesh size cell strainer (Fisher Scientific). Contaminating red blood cells were removed using an ammonium chloride lysis buffer (155 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA in ddH₂O; pH 7.2; sterile-filtered). The obtained splenocytes were filtered once more through a 70 µm cell strainer as to remove cell clumps and ensure a “single-cell” suspension. Isolated splenocytes were enumerated using a ViCell automated cell counting instrument (Beckman-Coulter).

4.3.7 Natural killer cell frequency analysis

Splenocytes (5 x 10⁵ cells/sample) were stained with fluorochrome-conjugated antibodies directed against cell surface markers of NK cell identity, where NK cells were characterized as NK1.1+CD3- cells. Splenic NK cell frequency was calculated as the % of NK1.1+CD3-
cells/total splenocytes. Samples assessed for splenic NK cell frequency were derived from Study I C57BL/6 mice.

4.3.8 Natural killer cell activity analysis

NK cell function was assessed using a flow cytometry-based assay for detection of degranulation (cell surface CD107a expression) and IFN-γ production in response to stimulation with the cancer cell line, YAC-1, and the cytokines IL-12, IL-15 and IL-18 in Study I, and in response to stimulation with plate-bound anti-NK1.1 antibody in Study II. The utilized flow cytometry-based assay for simultaneous detection of degranulation and IFN-γ production is based on methodology described by Betts et al., in 2003 and Alter et al., in 2004 (149, 169).

4.3.8.1 Study I: Stimulation with the murine lymphoma cell line YAC-1

In Study I, experimental assessment of splenic NK cell function was conducted via in vitro stimulation with the YAC-1 cell line and the cytokines IL-12, IL-15, and IL-18. The YAC-1 cell line (ATCC #TIB-160) is a murine T cell lymphoma traditionally used as the cancer cell target in assessment of murine NK cell cytotoxic activity. IL-12, IL-15 and IL-18 were included in the stimulation to potentiate NK cell activity, as naive mouse splenic NK cells possess poor cytotoxic activity in vitro (12). Unstimulated NK cells were used as a negative control and phorbol 12-myristate 13-acetate (PMA) and ionomycin-stimulated NK cells (mitogenic stimulation) were used as a positive control.

All steps in the following described protocol were conducted under aseptic conditions. NK cells were enriched from the splenocyte population by use of a magnetic NK cell separation kit (IMag™ BD Biosciences) as per manufacturer’s instructions. Briefly, the splenocyte population was stained with a fluorochrome (PE; phycoerythrin)-conjugated primary antibody directed against the NK cell antigen CD49b (aka DX5) and a magnetic bead-conjugated
secondary antibody directed against PE. The cells were then separated by use of a specialized magnet. NK cells were enriched within the splenocyte population to facilitate interaction between the NK cells and the target YAC-1 cells by removal of bystander cells within the splenocyte population, as NK cells typically represent a small fraction (2-5%) of splenocytes in mice (12).

The NK cell-enriched splenocyte population was incubated with an anti-CD16/32 antibody [clone 93] (Tru-Stain fcX™, Biolegend) at 1 µg per 10⁶ cells in 100 µL for 15 minutes on ice in order to block IgG Fc receptors and prevent unspecific binding of antibodies used in identification of NK cell activation markers. The NK cell-enriched-splenocyte population was then plated with YAC-1 cells in a 96-well round-bottom tissue culture plate at an effector to target ratio of 10:1 (1.5 x 10⁵ NK cell enriched splenocytes: 1.5 x 10⁴ YAC-1 cells per well) in the presence of 10 ng/mL of IL-12, IL-15 (Biolegend) and IL-18 (MBL International) in FA-free cRPMI media as the experimental condition. The YAC-1 cells used in all experiments conducted were from subcultures 9-11 and exhibited > 95% viability prior to the assay as per assessment by Trypan blue staining. 1.5 x 10⁵ NK cell-enriched splenocytes were plated per well in FA-free cRPMI media as the negative control, and 1.5 x 10⁵ NK cell-enriched splenocytes were plated per well in FA-free cRPMI media containing 0.05 µg/mL of PMA and 2 µg/mL of ionomycin as the positive control. All wells also contained APC (allophycocyanin)-conjugated anti-CD107a antibody at 2.5 µg/mL for detection of CD107a cell surface expression; the anti-CD107a antibody was included during the NK cell stimulation, as CD107a can be re-internalized post cell activation (149).

The 96-well plate was incubated for 1 hour at 37°C and 5% CO₂, after which the protein transport inhibitors monesin (GolgiStop; BD Biosciences) and brefeldin A (GolgiPlug; BD
Biosciences) were added to each well at a volume resulting in a final dilution of 1:1000 of each reagent. Monesin and brefeldin A were utilized to prevent release of synthesized cytokines (IFN-γ), as well as to prevent degradation of re-internalized CD107a (149). The plate was then incubated at 37°C and 5% CO₂ for a further 6-7 hours. Post cell stimulation, the plate was stored in the dark at 4°C for approximately 4-5 hours, at which point the cells were stained with fluorochrome-conjugated antibodies directed against markers of NK cell identity and NK cell activation including degranulation (CD107a cell surface expression) and intracellular IFN-γ production. Enumeration of NK cells (characterized as CD49b+CD3- cells) expressing surface CD107a or intracellular IFN-γ over the total number of NK cells was used as a measure of NK cell activity. Negative controls were used to ascertain the specificity of activation marker detection and to ensure that observed activation in response to stimulation was resultant from that stimulation and not exposure to an alternative factor. Positive controls were used to ascertain the capacity of NK cells to respond to stimulation in our experimental system.

**Figure 4.1** shows NK cell degranulation and IFN-γ production in response to varying stimulation conditions; increased frequency of NK cell degranulation and IFN-γ production in response to increased intensity of stimulus validates the use of this assay in detecting differences in NK cell response between dietary FA intervention groups.
Figure 4.1: Representative flow cytometry density plots of natural killer cell activation showing degranulation (CD107a cell surface expression) and IFN-γ production in response to stimulation in Study I: unstimulated negative control [A]; stimulation with YAC-1 cells at an effector to target ratio of 10:1 in addition to IL-12, IL-15 and IL-18 at concentrations of 10 ng/mL each [B]; mitogenic stimulation with PMA and ionomycin at concentrations of 0.05 µg/mL and 2 µg/mL, respectively, positive control [C]. Each point in the plot represents a NK cell (NK cells were characterized as CD49b+CD3- cells); each point in the upper two quadrants (Q1, Q2) represents an NK cell positive for IFN-γ production; whereas each point in the right-most two quadrants (Q2, Q3) represents an NK cell positive for cell surface expression of CD107a which is indicative of degranulation. The numbers shown in the quadrants of the plots represent the % of NK cells within each quadrant.
4.3.8.2 Study II: Plate-bound anti-NK1.1 antibody stimulation as an assessment of generalized NK cell responsiveness

In Study II, experimental assessment of splenic NK cell function was conducted by *in vitro* crosslinking of the NK cell activating receptor, NK1.1, via plate-bound anti-NK1.1 antibody; degranulation and IFN-γ production were assessed as NK cell activation parameters. As in Study I, unstimulated NK cells were used as a negative control and mitogenic stimulation was used as a positive control.

All steps in the subsequent described protocol were conducted under aseptic conditions. High-affinity protein-binding 96-well plates (Thermo Scientific) were incubated with 50 µL of LEAF™ anti-NK1.1 antibody [clone PK136] (Biolegend) at concentrations of 15, 5 or 3 µg/mL in protein-free Dulbecco’s phosphate buffered saline (DPBS) (HyClone™, Fischer Scientific) per well for 16 hours at 4°C. The concentrations of anti-NK1.1 antibody used were chosen based on conducted preliminary screens to assess which anti-NK1.1 antibody concentrations would result in optimal NK cell stimulation. Concentrations between 0.3 and 30 µg of antibody per mL were tested; the 15 µg/mL and 5 µg/mL concentrations were chosen for use based on observed high frequencies of NK cell degranulation and IFN-γ production, whereas the 3 µg/mL concentration was chosen to assess NK cell activation between dietary groups at non-saturated activation levels as it produced significantly lower, and yet sufficiently detectable degranulation and IFN-γ production frequencies relative to other antibody concentrations.

Following the 16 hour incubation, the plate was washed 5 times with protein free-DPBS without allowing the wells to dry. Splenocytes were incubated with anti-CD16/32 [clone 93] (Tru-Stain fcX™, Biolegend) at 1 µg per 10^6 cells in 100 µL for 15 minutes on ice. 1.5 x 10^6 splenocytes in FA-free cRPMI were plated per anti-NK1.1 antibody treated well as the
experimental condition, whereas $1.5 \times 10^6$ splenocytes were plated in untreated wells in media alone as the negative control and with media containing 0.05 µg/mL of PMA and 2 µg/mL of ionomycin as the positive control. The wells for all conditions also contained APC-conjugated anti-CD107a antibody at a concentration of 2.5 µg/mL. As in Study I, the plate was incubated for 1 hour at 37°C and 5% CO$_2$, after which the protein inhibitors monesin and brefeldin A were added to a final dilution of 1:1000 of each reagent. The plate was then incubated for a further 5 hours at 37°C and 5% CO$_2$, after which cells were immediately stained with fluorochrome-conjugated antibodies directed against markers of NK cell identity and NK cell activation, including CD107a cell surface expression and intracellular IFN-γ production. As in Study I, enumeration of NK cells (characterized as CD49b+CD3- cells) expressing surface CD107a or intracellular IFN-γ over the total number of NK cells was used as a measure of NK cell activity.

Figure 4.2 shows representative flow cytometry density plots of NK cell activation (degranulation and IFN-γ production) in response to stimulation with plate-bound anti-NK1.1 antibody.
Figure 4.2: Representative flow cytometry density plots of natural killer cell activation showing degranulation (CD107a cell surface expression) [A,B,C] and IFN-γ production [D,E,F] in response to stimulation in Study II: unstimulated negative control [A,D]; stimulation with plate-bound anti-NK1.1 antibody (15 µg/mL) [B,E]; mitogenic stimulation with PMA and ionomycin at concentrations of 0.05 µg/mL and 2 µg/mL, respectively, positive control [C,F]. Each point in the plot represents a NK cell (NK cells were characterized as CD49b+CD3- cells); each point within the displayed gate represents an NK cell positive for either CD107a cell surface expression or IFN-γ production.
4.3.9 Antibodies, cell staining, and flow cytometry

All monoclonal antibodies utilized were purchased from Biolegend and included: FITC (fluorescein isothiocyanate) anti-mouse NK1.1 [clone PK136], PE-anti-mouse CD49b [clone DX5], PE/Cy7 (PE/cyanin7) anti-mouse CD3ε [clone 145-2C11], PerCP/Cy5.5 (peridinin chlorophyll/Cy5.5) anti-mouse CD3ε [clone 145-2C11], APC anti-mouse CD107a [clone 1D4B], FITC anti-mouse IFN-γ [clone XMG1.2] and FITC rat IgG1 κ isotype control [clone RTK2071] which was used as an isotype control for intracellular IFN-γ staining. In the study I NK cell activity analysis, cells were pre-stained with PE anti-mouse CD49b [clone DX5] as part of the IMag™ mouse NK cell separation set (BD Biosciences) protocol.

Cells were stained with BD Horizon™ Fixable Viability Stain 450 (FV450) (BD Biosciences) as per manufacturer’s instructions to allow for dead cell exclusion. Cells were stained for cell surface markers (FITC anti-NK1.1 antibody at 1.5 µg/mL and PE/Cy7 anti-CD3ε antibody at 2 µg/mL for NK cell frequency staining, PE/Cy7 anti-CD3ε antibody at 2 µg/mL for Study I NK cell activity analysis and PerCP/Cy5.5 anti-CD3ε antibody and PE anti-CD49b antibody at 2.5 µg/mL each for Study II NK cell activity analysis) in 100 µL of staining buffer (DPBS with 2% bovine serum albumin (BSA) and 0.1% NaN₃) on ice and in the dark for 20 minutes. For NK cell frequency analysis, cells were fixed with 4% paraformaldehyde (15 minutes at room temperature), and samples were stored in staining buffer at 4°C overnight prior to analysis. For NK cell activity analyses, cells were fixed using 4% paraformaldehyde (15 minutes at room temperature) and permeabilized using 0.15% Triton-X (10 minutes at room temperature) in Study I and using Fixation Buffer (Biolegend) (20 minutes at room temperature) and Permeabilization Wash Buffer (Biolegend) (15 minutes at room temperature), respectively, in Study II to allow for intracellular staining for IFN-γ. Cells were stained with FITC anti-IFN-γ.
antibody at a concentration of 4.5 µg/mL in 100 µL of staining buffer for 20 minutes on ice and in the dark in Study I, and in 100 µL of Permeabilization Wash Buffer for 20 minutes at room temperature and in the dark in Study II. Cells were stored in staining buffer at 4°C overnight prior to analysis.

Stained cells were analyzed on a MACSQuant (Miltenyi Biotec) or a FACSCanto II (BD Biosciences) instrument and obtained data were assessed using FlowJo Version 10 (Treestar) software. OneComp eBeads (eBioscience) were used for single-stain controls with the exception of the FV450 single-stain control for which splenocytes were used. Single-stain controls were used as compensation controls in instrument set-up. Unstained cells and appropriate fluorescence-minus-one (FMO) controls were used in the set-up of analysis gates; an isotype control corresponding to the IFN-γ stain was used to ascertain the specificity of intracellular staining (Figures S1 and S2). Both unstimulated cells and PMA and ionomycin stimulated cells were used for FMO and isotype controls in the NK cell activity assays. Generally, gating strategy involved: gating on cell events, followed by exclusion of cell clumps via time vs. side scatter analysis, followed by gating on single cell events, followed by gating on live cell events (Figures S3 and S4). In both Studies I and II, further gating strategy involved exclusion of CD3+ cells, followed by gating on CD49b+ cells and assessment of frequency (i.e., %) of CD107a or IFN-γ positivity within the CD49b+CD3- cell population (Figures S3 and S4). At least 10,000 live cell events were collected in the splenic NK cell frequency analysis. At least 10,000 live cell events and on average ~6,000 (range: 2971 to 11,522) live NK cell events were collected in the NK cell activity experiments in Study I, whereas at least 60,000 (on average ~250,000) live cell events and on average ~10,000 (range: 2527 to 21,378) NK cell events were collected in the NK cell activity experiments in Study II.
4.3.10 Gene expression analysis by quantitative real-time reverse transcriptase PCR

Total RNA was extracted from snap-frozen hepatic medial lobes using the RNeasy® Microarray Tissue Mini Kit (Qiagen) according to the manufacturer’s instructions. In brief, the RNeasy® Microarray Tissue Mini kit protocol involves mechanical homogenization of tissues in a phenol/guanidine-based lysis buffer followed by guanidine/phenol/chloroform-based RNA extraction, after which RNA is recovered using a RNA-binding-specific silica membrane within a spin-column. On-column DNA digestion using the RNase-free DNase set (Qiagen) was also performed to ascertain prevention of DNA contamination within the samples. RNA purity was confirmed via spectrophotometric measurement of pertinent absorbance values and calculation of absorbance ratios ($A_{260/280} \approx 2.0$, $A_{260/230} \approx 2.0-2.2$) using a NanoDrop 2000 (Thermo Scientific) instrument. RNA quality and concentration were determined using the Agilent RNA 6000 Nano Kit and the 2100 Bioanalyzer Instrument with 2100 Expert Software (Agilent Technologies) as per manufacturer’s instructions; only samples with RNA integrity numbers (RINs) > 8 were utilized for gene expression analysis.

cDNA was synthesized using the QuantiTect® Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. Real-time quantitative PCR was performed using the ViiA7™ Real-Time PCR System (Applied Biosystems, Life Technologies) according to the manufacturer’s instructions. PCR reactions were performed in a 10 µL volume with Fast SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies), 2 µL of cDNA template, and 200 µM ($Dhfr$) or 300 µM ($Rpl13a$) of primer. The cycling conditions included: 2 min at 50°C and 10 min at 95°C which was followed by 40 cycles involving 15 second denaturation at 95°C and 1 min primer annealing and fragment elongation at 60°C. Samples were run in six replicates per plate. Relative gene expression was calculated using the delta delta C$_T$
(ΔΔCT) method (170) where expression levels are expressed relative to an endogenous control (housekeeping gene) and normalized to an internal calibrator (control dietary group). The ribosomal protein L13a (Rpl13a) gene was selected for use as an endogenous control based on its low variation in a preliminary assessment of housekeeping gene primers. Primer quality was assessed using standard curve analysis where primer efficiencies [efficiency (%) = \((10^{(-1/slope of standard curve)} - 1) \times 100\)] between 80 and 110% were accepted and using melt-curve analysis for primer specificity (observation of a single amplification product). Real-time PCR standard curves were generated for each primer by plotting C_T values of serial dilutions of cDNA starting material (0.016-50 ng of cDNA) versus the logarithm of cDNA amount in ng. Primer sequences as well as primer associated real-time PCR standard curve slopes and linearity (R^2) are listed in Table 4.4.

### Table 4.4: Gene-of-interest and housekeeping gene primer sequences and primer associated real-time PCR standard curve parameters

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Standard curve slope and linearity (R^2)</th>
</tr>
</thead>
</table>
| Rpl13a(171) | Forward: 5’-GCTCTCAAGGGTTGTTCCGCTGA-3’ | Slope: -3.47 ± 0.04  
R^2: 0.99 |
|       | Reverse: 5’-AGATCTGCTTTCTTCTTCCGATA-3’       |                                        |
| Dhfr(172)      | Forward: 5’-GTAGAGAACTCAAAGAACCACCG-3’      | Slope: -3.66 ± 0.07  
R^2: 0.99 |
|      | Reverse: 5’-TTTCTCCTGGACCTCAGAGG-3’         |                                        |

1Real-time PCR standard curves were generated using C_T values corresponding to 4-6 serial dilutions of cDNA in the amount of 0.016-50 ng. Standard curves were established using cDNA corresponding to a sample from the control dietary group.
4.3.11 **Statistical analysis**

The distribution of each variable was assessed both graphically and utilizing the D’Agostino and Pearson Omnibus K² normality test where \( n > 8 \) as to ascertain consistency with a normal (Gaussian) distribution. The D’Agostino and Pearson Omnibus K² normality test assesses normality using skewness and kurtosis and has been reported to possess good power properties over a wide range of non-normal distributions (173). Variables which deviated from normality were log-transformed prior to analysis; appropriate non-parametric tests were utilized where log-transformation did not result in a normal distribution of the variable in question. One-Way Analysis of Variance (ANOVA) was used to assess significance of differences between dietary groups in Study II; Bartlett’s test was used to assess differences in standard deviations of the variables where sample size was not equal among the three dietary groups.

Statistical significance of differences in growth curves (body weight in g) between dietary groups was assessed using repeated measures Two-Way ANOVA (mixed-model Two-Way ANOVA) followed by Sidak’s multiple comparisons test to assess differences between dietary groups during each week of dietary intervention.

In Study I, significance of differences in plasma folate concentrations were assessed using the Student’s T test with Welch’s correction while significance of differences in plasma UMFA concentrations and hepatic folate concentrations were assessed using the non-parametric Mann Whitney U test. In Study II, significance of differences in plasma folate, plasma UMFA and liver folate concentrations were assessed using One-Way ANOVA followed by Tukey’s multiple comparisons test and a post-test for linear trend. Correlation between parameters of folate status (plasma and hepatic folate and plasma UMFA concentrations) was assessed using the Pearson correlation method; however, the Spearman correlation method was used for correlations.
involving hepatic folate concentrations in Study I because the distribution of the values was not symmetrical and was not transformed to symmetry.

Statistical significance of differences in splenic NK cell frequency in Study I was assessed using Student’s Unpaired T Test. Analysis of NK cell activity parameters such as frequency of degranulation (% CD107a+ NK cells) was conducted by pooling data from three separate experiments as sample size within each experiment was limiting in detecting significance of a small effect size; sample size in each experiment was kept to 3 mice per dietary group due to temporal feasibility of the NK cell activity assays conducted. Significance of differences between dietary groups observed in NK cell activity parameters was therefore assessed using 2-factor ANOVA with experiment as a covariate, where a certain degree of variability in the data was attributable to day-to-day variation in the experiment itself. Significance of differences in NK cell activity parameters between dietary groups within each experiment were assessed using Student’s Unpaired T test in Study I and One-Way ANOVA in Study II. Correlations between plasma folate/ plasma UMFA concentrations and NK cell activity parameters were assessed using the Pearson correlation method; the Spearman correlation method was employed where transformation did not result in a symmetric distribution of the data.

Gene expression data were collected for 5 samples per dietary group; values falling outside of the 95% confidence interval of the mean within each dietary group were removed prior to statistical analysis. Statistical testing of differences in gene expression between dietary groups was conducted at the ΔC_T level (C_T value of the gene of interest – C_T value of a housekeeping gene) using One-Way ANOVA followed by Tukey’s multiple comparisons test. Assessment of correlation between relative mRNA expression (relative to the mean of the control dietary group)
and plasma and hepatic folate and plasma UMFA concentrations was performed using the Pearson correlation method.

Statistical tests were conducted using GraphPad Prism Version 6 software (La Jolla, CA). All statistical tests were two sided and were considered statistically significant if the observed significance level ($p$-value) was less than 0.05.

4.4 Results

4.4.1 Animal health and body weight

In both Studies I and II, C57BL/6 animal health was assessed daily, and animals remained in good health throughout the dietary intervention, without any evidence of growth retardation and premature death. Body weight was recorded weekly and growth curves in both studies were consistent with the standard growth curves of C57BL/6 mice provided by the mouse vendor (Charles River Laboratories).

In Study I, growth curves were not significantly different between the dietary groups in both genders (female: $p = 0.85$, $n = 6$/dietary group, male: $p = 0.36$, $n = 2-3$/dietary group) (Figure 4.3A). Furthermore, body weight did not differ significantly ($p > 0.05$) between the dietary groups during each week of dietary intervention, although a trend towards higher body weight was observed in the male HFA group relative to the male control group ($p = 0.074$) in week 1. As a difference in body weight was no longer apparent in later weeks of the dietary intervention, the observed trend in week 1 was most likely a result of randomization of the mice and not an effect of the diets. A significant interaction effect of diet*time ($p = 0.0087$) on body weight was also observed in the male mice in Study I, where this effect was most likely mediated by the more pronounced variation in body weight between the control and HFA groups in weeks 1 and 2 relative to the rest of the dietary intervention.
In Study II, growth curves did not differ significantly among the three dietary groups ($p = 0.97$) (Figure 4.3B). Body weight did not differ significantly ($p > 0.05$) among the three dietary groups during each week of dietary intervention. However a significant interaction effect of diet*time ($p < 0.0001$) on body weight was observed, where again this was most likely mediated by the more pronounced variation in body weight among the three dietary groups in week 1 of the dietary intervention relative to the rest of the dietary intervention.
Figure 4.3: Effects of dietary folic acid intervention on C57BL/6 body weight. Growth curve for C57BL/6 mice in Study I; n = 6/dietary group (F: female), n = 2-3/dietary group (M: male) [A]. Growth curve for C57BL/6 mice in Study II; n = 9/dietary group (female) [B]. Growth curves were not significantly different by dietary group in Study I (female: \( p = 0.85 \), male: \( p = 0.36 \)) nor in Study II (\( p = 0.97 \)). Body weight did not differ significantly (\( p > 0.05 \)) between dietary groups during each week of dietary intervention in Studies I and II, as assessed by Sidak’s multiple comparisons test.
4.4.2 Plasma folate, plasma unmetabolized folic acid, and hepatic folate concentrations

The effects of FA supplementation on plasma folate, plasma UMFA, and hepatic folate concentrations in the C57BL/6 mouse model were assessed in both Study I (Table 4.5A) and Study II (Table 4.5B).

In Study I, supplementation with 20 mg of FA per kg of diet resulted in significantly higher plasma folate ($p < 0.0001$), plasma UMFA (~2-fold; $p < 0.0001$), and hepatic folate ($p = 0.022$) concentrations, compared with the control group (Table 4.5A). The difference in plasma folate concentrations between the HFA and control groups was more pronounced than that in hepatic folate concentrations; this observation is consistent with those made in other FA supplementation studies conducted in rodents (68, 163). Furthermore, plasma UMFA concentrations observed in the control and HFA groups were comparable to those documented in the North American population (11, 14, 42). Plasma folate concentrations were significantly correlated with plasma UMFA concentrations ($r_p = 0.63, p = 0.0068$), and plasma folate concentrations were positively, albeit non-significantly, correlated with hepatic folate concentrations ($r_s = 0.48, p = 0.053$) (Table 4.6). However, plasma UMFA concentrations were not correlated with hepatic folate concentrations ($r_s = 0.33, p = 0.20$) (Table 4.6).

In Study II, significant linear trends in plasma folate ($R^2 = 0.88, p$-trend $< 0.0001$), hepatic folate ($R^2 = 0.74, p$-trend $< 0.0001$), and plasma UMFA ($R^2 = 0.75, p$-trend $< 0.0001$) concentrations were observed with increasing FA content in the diet. Plasma folate and UMFA concentrations differed significantly among the three dietary groups (MFD < control < HFA) ($p < 0.0001$) (Table 4.5B). Hepatic folate concentrations differed significantly among the three dietary groups ($p < 0.0001$), and were significantly lower in the MFD group with respect to both the control and the HFA group, but did not differ significantly between the control and HFA
groups (Table 4.5B). Plasma folate, plasma UMFA and hepatic folate concentrations observed in the control and HFA groups were comparable to those observed in the corresponding dietary groups in Study I. Plasma folate concentrations were significantly correlated with plasma UMFA concentrations ($r_p = 0.88, p < 0.0001$) and with hepatic folate concentrations ($r_p = 0.83, p < 0.0001$). Furthermore, plasma UMFA concentrations were correlated with hepatic folate concentrations ($r_p = 0.81, p < 0.0001$) (Table 4.6). The discrepancy between Study I and Study II in regard to correlations involving hepatic folate concentrations have likely resulted from the inclusion of data from the MFD group in the Study II analysis.
Table 4.5A: Effects of dietary folic acid intervention on plasma folate, plasma unmetabolized folic acid, and hepatic folate concentrations in Study I

<table>
<thead>
<tr>
<th>Dietary Group (n)</th>
<th>2 mg FA/kg (8) (control)</th>
<th>20 mg FA/kg (9) (HFA)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma Folate</strong> (ng/mL)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Mean +/- SD</td>
<td>28.6 ± 6.4</td>
<td>47.6 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>Geometric mean [95% CI]</td>
<td>28.0 [23.6, 33.4]</td>
<td>47.1 [41.6, 53.3]</td>
</tr>
<tr>
<td><strong>UMFA (nmol/L)</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Mean +/- SD</td>
<td>1.21 ± 0.20</td>
<td>2.36 ± 1.04</td>
</tr>
<tr>
<td></td>
<td>Median (95% CI)</td>
<td>1.24 (0.81, 1.47)</td>
<td>2.08 (1.64, 3.38)</td>
</tr>
<tr>
<td><strong>Hepatic Folate (ng/g tissue)</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Mean +/- SD</td>
<td>12.9 ± 2.2</td>
<td>17.5 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>Median (95% CI)</td>
<td>12.1 (10.1, 16.7)</td>
<td>16.2 (12.5, 21.3)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Significantly different by Student’s T test with Welch’s correction.

<sup>2</sup>Significantly different by Mann Whitney U test.
Table 4.5B: Effects of dietary folic acid intervention on plasma folate, plasma unmetabolized folic acid, and hepatic folate concentrations in Study II

<table>
<thead>
<tr>
<th>Dietary Group</th>
<th>0 mg FA/kg (MFD)</th>
<th>2 mg FA/kg (control)</th>
<th>20 mg FA/kg (HFA)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Plasma Folate (ng/mL)(^1)</td>
<td>Mean +/- SD</td>
<td>9.4 ± 4.4</td>
<td>32.5 ± 5.5</td>
<td>60.6 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>Geometric mean [95% CI]</td>
<td>8.7 [6.4, 11.8](^a)</td>
<td>32.1 [28.3, 36.3](^b)</td>
<td>60.1 [54.0, 67.0](^c)</td>
</tr>
<tr>
<td>UMFA (nmol/L)(^1)</td>
<td>Mean +/- SD</td>
<td>0.48 +/- 0.31</td>
<td>1.02 +/- 0.20</td>
<td>2.25 +/- 0.61</td>
</tr>
<tr>
<td></td>
<td>Geometric mean [95% CI]</td>
<td>0.41 [0.25, 0.64](^a)</td>
<td>1.00 [0.85, 1.17](^b)</td>
<td>2.19 [1.80, 2.66](^c)</td>
</tr>
<tr>
<td>Hepatic Folate (ng/g tissue)(^1)</td>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Mean +/- SD</td>
<td>6.1 +/- 1.2(^a)</td>
<td>11.6 +/- 1.4(^b)</td>
<td>12.8 +/- 1.6(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Significantly different by One-Way ANOVA (reported p-value); \(^ab\)significantly different (p < 0.05) by Tukey’s multiple comparisons test.
Table 4.6: Correlation of plasma and hepatic folate and plasma unmetabolized folic acid concentrations

<table>
<thead>
<tr>
<th></th>
<th>Study I [2 and 20 mg FA/kg groups]</th>
<th>Study II [0, 2, and 20 mg FA/kg groups]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Correlation coefficient (r)</td>
</tr>
<tr>
<td>Plasma Folate (ng/mL) vs. Plasma UMFA (nmol/L)</td>
<td>17</td>
<td>0.63</td>
</tr>
<tr>
<td>Plasma Folate (ng/mL) vs. Hepatic Folate (ng/g of tissue)</td>
<td>17</td>
<td>0.48†</td>
</tr>
<tr>
<td>Plasma UMFA (nmol/L) vs. Hepatic Folate (ng/g of tissue)</td>
<td>17</td>
<td>0.33†</td>
</tr>
</tbody>
</table>

1Correlation was assessed using the Pearson correlation method unless otherwise indicated.
†Correlation was assessed using the Spearman correlation method.
4.4.3 Splenic natural killer cell frequency

Splenic NK cell frequency, defined as the percentage of cells within the splenocyte population identified as NK cells (NK1.1+CD3- cells), was assessed as a reduction in NK cell numbers could potentially result in impaired NK cell defenses. However, splenic NK cell frequency did not differ significantly between the control and HFA groups (\( p = 0.46 \)) (Figure 4.4).

Figure 4.4: Effects of dietary folic acid intervention on natural killer cell frequency in the spleen.
Representative flow cytometry density plot of NK cell frequency analysis [A]; NK cells were defined as NK1.1+CD3- cells; splenic NK cell frequency was defined as the % of NK1.1+CD3- cells/total splenocytes. Splenic NK cell frequency in the control (2 mg FA/kg) and HFA dietary groups (20 mg FA/kg) [B]; n = 8-9/dietary group, data are pooled from 3 experiments; data are presented as mean +/- SD; significance of difference (shown \( p \)-value) was determined using the Student’s unpaired T test.

4.4.4 Splenic natural killer cell activity in response to stimulation with the murine lymphoma cell line, YAC-1 (Study I)

Frequency of NK cell degranulation (% CD107a+ NK cells) in response to stimulation with the murine lymphoma cell line, YAC-1, in addition to IL-12, IL-15 and IL-18 was significantly lower (\( p = 0.044 \)) in the HFA group (19.3 ± 3.9%, \( n = 9 \)) relative to the control
(21.7 ± 4.3%, n = 8), where an ~10% reduction in degranulation frequency was observed in the HFA group (Figure 4.5A). Furthermore, frequency of NK cell degranulation in response to YAC-1 and cytokine stimulation was significantly and inversely correlated with plasma folate ($r_p = -0.55, p = 0.024$) and plasma UMFA ($r_p = -0.56, p = 0.019$) concentrations (Figures 4.6A and 4.7A). Similarly, frequency of multifunctional NK cells (% CD107a+IFN-γ+ NK cells; simultaneous degranulation and IFN-γ production) in response to YAC-1 and cytokine stimulation was significantly lower ($p = 0.042$) in the HFA group (13.0 ± 2.6%, n = 9) relative to the control (14.6 ± 3.0%, n = 8) (Figure 4.5C), and was significantly and inversely correlated with plasma folate ($r_p = -0.56, p = 0.020$) and UMFA ($r_p = -0.49, p = 0.044$) concentrations (Figures 4.6E and 4.7E).

Frequency of NK cell IFN-γ production (% IFN-γ+ NK cells) in response to YAC-1 and cytokine stimulation did not differ significantly between the dietary groups ($p = 0.55$) (Figure 4.5B), nor did the amount of IFN-γ production per NK cell, as assessed by median fluorescence intensity (MFI) of the IFN-γ+ NK cell population, although trends towards increased IFN-γ production per cell were observed in the HFA group in two of three conducted experiments (Table 4.7). Furthermore, frequency of NK cell IFN-γ production in response to stimulation with YAC-1 and cytokines was not correlated with plasma folate concentrations ($r_p = -0.082, p = 0.75$) (Figure 4.6C). However, a trend suggesting a positive correlation between plasma UMFA concentrations and the frequency of NK cell IFN-γ production in response to stimulation with YAC-1 and cytokines was observed ($r_p = 0.42, p = 0.093$) (Figure 4.7C).

NK cell degranulation, NK cell IFN-γ production, and multifunctional NK cell frequency in response to mitogenic stimulation (PMA and ionomycin) did not differ significantly between the dietary groups (degranulation: $p = 0.13$; IFN-γ production: $p = 0.82$; multifunctional NK
cells: $p = 0.20$) (Figure 4.5D-F, Table 4.8), nor were these parameters correlated with plasma folate concentrations (degranulation: $r_p = -0.10, p = 0.69$; IFN-$\gamma$ production: $r_p = -0.024, p = 0.93$; multifunctional NK cells: $r_p = -0.12; p = 0.66$) (Figure 4.6B,D,F). Similarly, frequency of NK cell IFN-$\gamma$ production in response to mitogenic stimulation was not correlated with plasma UMFA concentrations ($r_p = -0.38; p = 0.14$) (Figure 4.7D). However, plasma UMFA concentrations were significantly and inversely correlated with frequency of NK cell degranulation ($r_p = -0.59; p = 0.013$) (Figure 4.7B), and a trend suggesting a positive correlation between plasma UMFA concentrations and multifunctional NK cell frequency in response to mitogenic stimulation was observed ($r_p = -0.48; p = 0.052$). (Figure 4.7F).
Table 4.7: Effects of dietary folic acid intervention on natural killer cell degranulation and IFN-γ production in response to YAC-1 + cytokine stimulation in Study I: results by experiment\(^1,2\)

<table>
<thead>
<tr>
<th>Experiment # (gender)</th>
<th>2 mg FA/kg (control)</th>
<th>20 mg FA/kg (HFA)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>% CD107a+ NK Cells</td>
<td>17.8 ± 1.9</td>
<td>18.0 ± 2.1</td>
<td>0.95</td>
</tr>
<tr>
<td>% IFN-γ+ NK Cells</td>
<td>44.4 ± 3.1</td>
<td>45.0 ± 5.1</td>
<td>0.52</td>
</tr>
<tr>
<td>% CD107a+IFN-γ+ NK Cells</td>
<td>11.7 ± 1.4</td>
<td>11.5 ± 1.2</td>
<td>0.83</td>
</tr>
<tr>
<td>IFN-γ+ NK MFI</td>
<td>301.0 ± 12.5</td>
<td>320.2 ± 12.7</td>
<td>0.13</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>% CD107a+ NK Cells</td>
<td>26.2 ± 2.3</td>
<td>23.8 ± 0.8</td>
<td>0.16</td>
</tr>
<tr>
<td>% IFN-γ+ NK Cells</td>
<td>49.9 ± 1.4</td>
<td>49.0 ± 0.8</td>
<td>0.37</td>
</tr>
<tr>
<td>% CD107a+IFN-γ+ NK Cells</td>
<td>17.7 ± 1.4</td>
<td>16.1 ± 0.4</td>
<td>0.14</td>
</tr>
<tr>
<td>IFN-γ+ NK MFI</td>
<td>386.7 ± 14.1</td>
<td>387.0 ± 23.4</td>
<td>0.98</td>
</tr>
<tr>
<td>n</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>% CD107a+ NK Cells</td>
<td>20.7 ± 1.3</td>
<td>16.2 ± 2.9</td>
<td>0.14</td>
</tr>
<tr>
<td>% IFN-γ+ NK Cells</td>
<td>49.7 ± 7.3</td>
<td>53.3 ± 2.3</td>
<td>0.45</td>
</tr>
<tr>
<td>% CD107a+IFN-γ+ NK Cells</td>
<td>14.4 ± 1.9</td>
<td>11.3 ± 2.0</td>
<td>0.19</td>
</tr>
<tr>
<td>IFN-γ+ NK MFI</td>
<td>404.5 ± 12.0</td>
<td>441.0 ± 33.1</td>
<td>0.25</td>
</tr>
</tbody>
</table>

\(^1\)Results are presented as mean ± SD; significance of differences was assessed using Student’s Unpaired T test.
\(^2\)\% CD107a+ NK cells: frequency of NK cell degranulation, \% IFN-γ+ NK cells: frequency of NK cell IFN-γ production, \% CD107a+IFN-γ+ NK Cells: frequency of multifunctional NK cells, IFN-γ+ NK MFI: median fluorescence intensity of the IFN-γ+ NK cell population; indicative of the median amount of IFN-γ production per NK cell.
# Table 4.8: Effects of dietary folic acid intervention on natural killer cell degranulation and IFN-γ production in response to mitogenic stimulation in Study I: results by experiment¹,²

<table>
<thead>
<tr>
<th>Experiment # (gender)</th>
<th>2 mg FA/kg (control)</th>
<th>20 mg FA/kg (HFA)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>1 (F)</td>
<td>% CD107a+ NK Cells</td>
<td>65.1 ± 2.1</td>
<td>59.1 ± 5.5</td>
</tr>
<tr>
<td>1 (F)</td>
<td>% IFN-γ+ NK Cells</td>
<td>66.6 ± 1.2</td>
<td>63.4 ± 1.0</td>
</tr>
<tr>
<td>1 (F)</td>
<td>% CD107a+IFN-γ+ NK Cells</td>
<td>45.5 ± 1.9</td>
<td>40.1 ± 3.4</td>
</tr>
<tr>
<td>1 (F)</td>
<td>IFN-γ+ NK MFI</td>
<td>355.7 ± 7.6</td>
<td>357.0 ± 7.6</td>
</tr>
<tr>
<td>2 (F)</td>
<td>% CD107a+ NK Cells</td>
<td>60.4 ± 3.0</td>
<td>62.0 ± 3.7</td>
</tr>
<tr>
<td>2 (F)</td>
<td>% IFN-γ+ NK Cells</td>
<td>65.5 ± 1.5</td>
<td>66.9 ± 4.1</td>
</tr>
<tr>
<td>2 (F)</td>
<td>% CD107a+IFN-γ+ NK Cells</td>
<td>43.3 ± 2.4</td>
<td>45.0 ± 5.2</td>
</tr>
<tr>
<td>2 (F)</td>
<td>IFN-γ+ NK MFI</td>
<td>386.7 ± 14.1</td>
<td>387.0 ± 23.4</td>
</tr>
<tr>
<td>3 (M)</td>
<td>% CD107a+ NK Cells</td>
<td>54.8 ± 6.0</td>
<td>48.9 ± 4.9</td>
</tr>
<tr>
<td>3 (M)</td>
<td>% IFN-γ+ NK Cells</td>
<td>49.7 ± 7.3</td>
<td>53.3 ± 2.3</td>
</tr>
<tr>
<td>3 (M)</td>
<td>% CD107a+IFN-γ+ NK Cells</td>
<td>33.6 ± 3.3</td>
<td>28.5 ± 7.4</td>
</tr>
<tr>
<td>3 (M)</td>
<td>IFN-γ+ NK MFI</td>
<td>355.5 ± 30.4</td>
<td>363.7 ± 19.6</td>
</tr>
</tbody>
</table>

¹Results are presented as mean ± SD; significance of differences was assessed using Student’s Unpaired T test.

²% CD107a+ NK cells: frequency of NK cell degranulation, % IFN-γ+ NK cells: frequency of NK cell IFN-γ production, % CD107a+IFN-γ+ NK Cells: frequency of multifunctional NK cells, IFN-γ+ NK MFI: median fluorescence intensity of the IFN-γ+ NK cell population; indicative of the median amount of IFN-γ production per NK cell.
Figure 4.5: Effects of dietary folic acid intervention on natural killer cell degranulation and IFN-γ production in response to stimulation with YAC-1 + cytokines [A,B,C] and mitogenic stimulation [D,E,F] in Study I. 2 mg FA/kg (control), 20 mg FA/kg (HFA). Frequency of degranulation (% CD107a+ NK cells) [A,D], IFN-γ production (% IFN-γ+ NK cells) [B,E] and multifunctional NK cells (% CD107a+ IFN-γ+ NK cells) [C,F]; data are expressed as mean ± SD; n = 8-9/group, data are pooled from 3 separate experiments [● Experiment # 1 ▼ Experiment # 2 △ Experiment # 3]; statistical significance of differences was assessed using 2-factor ANOVA with experiment as a covariate.
Figure 4.6: Correlation of plasma folate concentrations (ng/mL) and natural killer cell activation parameters in response to stimulation with YAC-1 + cytokines [A, C, E] and in response to mitogenic stimulation [B, D, F] in Study I. x-axis: plasma folate concentrations (ng/mL); y-axis: frequency of degranulation (% CD107a+ NK cells) [A,B], IFN-γ production (% IFN-γ+ NK cells) [C,D] and multifunctional NK cells (% CD107a+ IFN-γ+ NK cells) [E,F]. n = 17 [A,B,C,E,F], n = 16 [D], data are pooled from 3 separate experiments; significance of correlation was assessed using the Pearson correlation method ($r_p$: Pearson correlation coefficient); ● 2 mg FA/kg, ▲ 20 mg FA/kg; linear regression line is shown in the graphs.
Figure 4.7: Correlation of plasma unmetabolized folic acid concentrations (nmol/L) and natural killer cell activation parameters in response to stimulation with YAC-1 + cytokines [A, C, E] and in response to mitogenic stimulation [B, D, F] in Study I. x-axis: plasma UMFA concentrations (nmol/L); y-axis: frequency of degranulation (% CD107a+ NK cells) [A,B], IFN-γ production (% IFN-γ+ NK cells) [C,D] and multifunctional NK cells (% CD107a+ IFN-γ+ NK cells) [E,F]. n = 17 [A,B,C,E,F], n = 16 [D], data are pooled from 3 separate experiments; data were log-transformed prior to statistical analysis and significance of correlation was assessed using the Pearson correlation method ($r_p$: Pearson correlation coefficient); ● 2 mg FA/kg, ▲ 20 mg FA/kg; linear regression line is shown in the graphs.
4.4.5 Splenic natural killer cell activity in response to stimulation with plate-bound anti-NK1.1 antibody (Study II)

Splenic NK cell activity in response to stimulation with plate-bound anti-NK1.1 stimulation was assessed, as in vitro crosslinking of an activating NK cell receptor is a technique by which generalized NK cell responsiveness is assessed, where the strength of the effector response (i.e., degranulation or cytokine production) is considered a measure of the end product of NK cell education processes (123). The association between excessive FA intake and reduced NK cell cytotoxicity observed in the study conducted by Troen et al. (11) and the reduced NK cell degranulation in response to high FA supplementation observed in Study I could potentially be mediated by dysregulation in the process of NK cell development and education, and thus the intrinsic capability of NK cells to respond to stimuli via cytotoxicity or cytokine secretion. The frequency of splenic NK cell degranulation, IFN-γ production and multifunctional NK cells did not differ significantly among the three dietary groups under all anti-NK1.1 antibody stimulation conditions tested (15, 5 and 3 µg of antibody/ml) (p > 0.05; Table 4.9, Tables S1A-C). Furthermore, the amount of IFN-γ production per NK cell (MFI of the IFN-γ+ NK cell population) did not differ significantly among the dietary groups for all three anti-NK1.1 antibody stimulation conditions (p > 0.05; Tables S1A-C). Consistent with observations made in Study I, the frequency of splenic NK cell degranulation, IFN-γ production and multifunctional NK cells in response to mitogenic stimulation did not differ significantly (p > 0.05) among the dietary groups (Table 4.9, Tables S1A-C), nor did the amount of IFN-γ production per NK cell (Table S1A-C).

Analysis of correlation between plasma folate and UMFA concentrations and parameters of NK cell function was conducted for variables derived from the 0 and 2 mg FA/kg dietary
groups in one analysis, and for variables derived from the 2 and 20 mg FA/kg dietary groups in a separate analysis, due the previously reported inverse U-shaped relationship between combined folate and FA intake and NK cell cytotoxicity (11). The frequencies of NK cell degranulation, IFN-γ production, and multifunctional NK cells were not significantly ($p > 0.05$) correlated with plasma folate or UMFA concentrations for all anti-NK1.1 antibody stimulation conditions tested in either low or high ranges of dietary FA (Table 4.10, Table 4.11). As observed in Study I, the frequencies of NK cell degranulation, IFN-γ production and multifunctional NK cells in response to mitogenic stimulation were not significantly ($p < 0.05$) correlated with plasma folate concentrations (Table 4.10), nor was the frequency of IFN-γ production correlated with plasma UMFA concentrations (Table 4.11). In contrast to Study I, the frequencies of NK cell degranulation and multifunctional NK cells in response to mitogenic stimulation were not significantly correlated with plasma UMFA concentrations (Table 4.11).
Table 4.9: The effects of dietary folic acid intervention on natural killer cell function in response to stimulation with plate-bound anti-NK1.1 antibody in Study II – pooled analysis\(^1,2\)

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Function Parameter</th>
<th>0 mg FA/kg (MFD)</th>
<th>2 mg FA/kg (control)</th>
<th>20 mg FA/kg (HFA)</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 µg Anti-NK1.1</td>
<td>% CD107a+</td>
<td>30.3 ± 3.0</td>
<td>29.9 ± 4.8</td>
<td>29.93 ± 5.8</td>
<td>0.97</td>
</tr>
<tr>
<td>Antibody</td>
<td>% IFN-γ+</td>
<td>9.3 ± 1.2</td>
<td>9.5 ± 2.7</td>
<td>9.6 ± 2.4</td>
<td>0.96</td>
</tr>
<tr>
<td>% CD107a+IFN-γ+</td>
<td>8.7 ± 1.3</td>
<td>8.8 ± 2.6</td>
<td>8.9 ± 2.3</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>5 µg Anti-NK1.1</td>
<td>% CD107a+</td>
<td>30.2 ± 3.4</td>
<td>29.3 ± 5.0</td>
<td>28.6 ± 4.7</td>
<td>0.74</td>
</tr>
<tr>
<td>Antibody</td>
<td>% IFN-γ+</td>
<td>11.1 ± 2.9</td>
<td>11.2 ± 3.3</td>
<td>11.1 ± 2.5</td>
<td>0.99</td>
</tr>
<tr>
<td>% CD107a+IFN-γ+</td>
<td>10.5 ± 2.9</td>
<td>10.5 ± 3.1</td>
<td>10.4 ± 2.5</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>3 µg Anti-NK1.1</td>
<td>% CD107a+</td>
<td>15.0 ± 5.6</td>
<td>15.2 ± 5.8</td>
<td>14.1 ± 5.8</td>
<td>0.74</td>
</tr>
<tr>
<td>Antibody</td>
<td>% IFN-γ+</td>
<td>4.9 ± 2.9</td>
<td>5.0 ± 2.7</td>
<td>4.5 ± 2.7</td>
<td>0.84</td>
</tr>
<tr>
<td>% CD107a+IFN-γ+</td>
<td>4.6 ± 2.7</td>
<td>4.7 ± 2.6</td>
<td>4.3 ± 2.6</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>PMA + Ionomycin</td>
<td>% CD107a+</td>
<td>42.6 ± 2.9</td>
<td>41.5 ± 3.4</td>
<td>41.8 ± 2.4</td>
<td>0.63</td>
</tr>
<tr>
<td>% IFN-γ+</td>
<td>70.0 ± 4.4</td>
<td>69.0 ± 6.4</td>
<td>70.7 ± 2.0</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>% CD107a+IFN-γ+</td>
<td>32.4 ± 3.6</td>
<td>30.9 ± 4.5</td>
<td>31.3 ± 3.4</td>
<td>0.72</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Data are pooled from 3 separate experiments; \(n = 9\)/dietary group; significance of differences was assessed using 2-factor ANOVA with experiment as a covariate.

\(^2\) % CD107a+: frequency of NK cell degranulation, % IFN-γ+: frequency of NK cell IFN-γ production, % CD107a+IFN-γ+: frequency of multifunctional NK cells.
Table 4.10: Correlation of plasma folate concentrations and natural killer cell function parameters in Study II$^{1,2}$

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Function Parameter</th>
<th>Correlation coefficient ($r$)</th>
<th>$P$-value</th>
<th>Correlation coefficient ($r$)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 µg Anti-NK1.1</td>
<td>% CD107a+</td>
<td>-0.047</td>
<td>0.85</td>
<td>-0.045</td>
<td>0.86</td>
</tr>
<tr>
<td>Antibody</td>
<td>% IFN-γ+</td>
<td>0.035</td>
<td>0.89</td>
<td>-0.10</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>0.027</td>
<td>0.92</td>
<td>-0.091</td>
<td>0.72</td>
</tr>
<tr>
<td>5 µg Anti-NK1.1</td>
<td>% CD107a+</td>
<td>-0.071</td>
<td>0.78</td>
<td>-0.065</td>
<td>0.80</td>
</tr>
<tr>
<td>Antibody</td>
<td>% IFN-γ+</td>
<td>0.12</td>
<td>0.64</td>
<td>-0.14</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>0.10</td>
<td>0.68</td>
<td>-0.13</td>
<td>0.60</td>
</tr>
<tr>
<td>3 µg Anti-NK1.1</td>
<td>% CD107a+</td>
<td>-0.058</td>
<td>0.82</td>
<td>0.058</td>
<td>0.82</td>
</tr>
<tr>
<td>Antibody</td>
<td>% IFN-γ+</td>
<td>-0.030</td>
<td>0.91</td>
<td>0.044</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>-0.037</td>
<td>0.89</td>
<td>0.046</td>
<td>0.85</td>
</tr>
<tr>
<td>PMA + Ionomycin</td>
<td>% CD107a+</td>
<td>-0.10</td>
<td>0.68</td>
<td>0.15</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>% IFN-γ+</td>
<td>0.11†</td>
<td>0.66</td>
<td>0.21†</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>-0.097</td>
<td>0.70</td>
<td>0.092</td>
<td>0.72</td>
</tr>
</tbody>
</table>

$^1$ Correlation was assessed using the Pearson correlation method unless otherwise indicated; $n = 18$, data are pooled from 3 separate experiments. ($^\dagger$) Correlation was assessed using the Spearman correlation method.

$^2$ % CD107a+: frequency of NK cell degranulation, % IFN-γ+: frequency of NK cell IFN-γ production, % CD107a+IFN-γ+: frequency of multifunctional NK cells.
Table 4.11: Correlation of plasma unmetabolized folic acid concentrations and natural killer cell function parameters in Study II$^{1,2}$

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Function Parameter</th>
<th>0 and 2 mg FA/kg groups</th>
<th>2 and 20 mg FA/kg groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation coefficient ($r$)</td>
<td>$P$-value</td>
<td>Correlation coefficient ($r$)</td>
</tr>
<tr>
<td>15 µg</td>
<td>% CD107a+</td>
<td>0.12</td>
<td>0.64</td>
</tr>
<tr>
<td>Anti-NK1.1</td>
<td>% IFN-γ+</td>
<td>0.049</td>
<td>0.85</td>
</tr>
<tr>
<td>Antibody</td>
<td>% CD107a+IFN-γ+</td>
<td>0.076</td>
<td>0.76</td>
</tr>
<tr>
<td>5 µg</td>
<td>% CD107a+</td>
<td>-0.021</td>
<td>0.93</td>
</tr>
<tr>
<td>Anti-NK1.1</td>
<td>% IFN-γ+</td>
<td>-0.12</td>
<td>0.65</td>
</tr>
<tr>
<td>Antibody</td>
<td>% CD107a+IFN-γ+</td>
<td>0.20</td>
<td>0.43</td>
</tr>
<tr>
<td>3 µg</td>
<td>% CD107a+</td>
<td>0.34</td>
<td>0.16</td>
</tr>
<tr>
<td>Anti-NK1.1</td>
<td>% IFN-γ+</td>
<td>0.30</td>
<td>0.23</td>
</tr>
<tr>
<td>Antibody</td>
<td>% CD107a+IFN-γ+</td>
<td>0.30</td>
<td>0.23</td>
</tr>
<tr>
<td>PMA + Ionomycin</td>
<td>% CD107a+</td>
<td>-0.26</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>% IFN-γ+</td>
<td>0.068†</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>-0.21</td>
<td>0.41</td>
</tr>
</tbody>
</table>

$^1$Correlation was assessed using the Pearson correlation method unless otherwise indicated; $n = 18$, data are pooled from 3 separate experiments. ($^†$) correlation was assessed using the Spearman correlation method.

$^2$% CD107a+: frequency of NK cell degranulation, % IFN-γ+: frequency of NK cell IFN-γ production, % CD107a+IFN-γ+: frequency of multifunctional NK cells.
4.4.6 Hepatic dihydrofolate reductase gene expression

Hepatic dihydrofolate reductase (DHFR) gene expression was assessed using liver tissue derived from mice in Study II and thus was assessed in the MFD, control, and HFA dietary groups. Hepatic Dhfr gene expression did not differ significantly among the dietary groups \( (p = 0.56) \) (Figure 4.8A). However, high variation in mRNA levels was observed in the HFA group. Furthermore, trends suggesting a positive correlation between plasma folate concentrations and hepatic Dhfr gene expression (expressed as relative mRNA level) \( (r_p = 0.50, p = 0.094) \) (Figure 4.8B), and between plasma UMFA concentrations and hepatic Dhfr gene expression \( (r_p = 0.56, p = 0.058) \) (Figure 4.8C) were observed. A significant correlation between hepatic folate concentrations and hepatic Dhfr gene expression was not observed (Figure 4.8D). However, the distribution of relative mRNA levels across hepatic folate concentrations prompted a hepatic folate concentration tertile based analysis of Dhfr gene expression. In this analysis, Dhfr gene expression differed significantly amongst the tertiles of hepatic folate concentrations, suggesting an upside down U-shaped relationship \( (p = 0.043) \) (Figure 4.8E).
Figure 4.8: Effects of dietary folic acid intervention on hepatic dihydrofolate reductase gene expression. Hepatic Dhfr gene expression expressed as relative mRNA level [A]; values are mean ± SD; n = 3-5/group; differences were not significant (p > 0.05) as assessed at the ΔC_T level (C_T_DHFR – C_T_HOUSEKEEPING GENE) using One-Way ANOVA. Correlation of relative mRNA levels and parameters of folate status [B,C,D]; plasma folate [B], plasma UMFA [C], and hepatic folate [D]; ● 0 mg FA/kg (MFD) ▲ 2 mg FA/kg (control) ▼ 20 mg FA/kg (HFA); n = 12; correlation analysis was conducted using the Pearson correlation method.; linear regression line is shown in the graphs. Relative Dhfr mRNA levels by tertile of hepatic folate [E]; n = 12; significance of differences was assessed at the ΔC_T level (C_T_DHFR – C_T_HOUSEKEEPING GENE) using One-Way ANOVA followed by Tukey’s multiple comparisons test.
4.5 Discussion

The reported inverse association between high intake and blood levels of FA and NK cell cytotoxicity in post-menopausal women (11) represents a potentially highly significant health concern, due to the current folate status of the North American population, and the importance of NK cell function to host defense. As such, we assessed the effects of FA supplementation on NK cell activity using a 3-month dietary FA intervention in a C57BL/6 mouse model. Our primary aim was to determine whether high FA intake and biomarkers of high FA intake, such as elevated plasma folate and UMFA concentrations, are indeed associated with impairment in NK cell function. Our secondary aim was to elucidate potential mechanisms behind an inhibitory effect of FA supplementation and high circulating UMFA on NK cell function. Therefore, we performed two studies. In Study I, we assessed the effects of FA supplementation on NK cell function in response to cancer cell (YAC-1) stimulation, and in Study II, we assessed the effects of FA supplementation on the generalized or intrinsic responsiveness of the NK cell population (an end-product of NK cell education processes).

Dietary FA intervention with amino-acid defined diets containing either 2 (control), 20 (HFA; 10X BDR), or 0 (MFD) mg FA/kg diet effectively modulated folate status in our animals. We observed significantly higher plasma folate and plasma UMFA concentrations in the HFA group relative to control in both Studies I and II, and significantly higher plasma folate and plasma UMFA concentrations in the HFA and control groups relative to the MFD group in Study II. Hepatic folate concentrations were significantly, albeit modestly, higher in the HFA group relative to control in Study I and non-significantly higher in the HFA group relative to control in Study II. However, hepatic folate concentrations were significantly higher in the HFA group and control with respect to the MFD group and a significant linear trend in hepatic folate
concentrations was observed with increasing FA content in the diet in Study II. The discrepancy in regard to significance of differences in hepatic folate concentrations between the HFA and control groups in Studies I and II may reflect a small effect size that was not detectable in Study II, based on sample-to-sample variation in hepatic folate concentrations (Type II error), potentially resulting from the section of liver lobe assayed. Alternatively, this discrepancy may reflect a Type I error in Study I, suggesting that hepatic folate concentrations did not differ between the control and HFA groups. Indeed, a saturation of hepatic folate concentrations has been previously reported in a few rodent studies of FA supplementation at levels similar to the present study (68, 163).

Mean plasma folate, plasma UMFA, and hepatic folate concentrations in the HFA and control groups were similar in Studies I and II. Furthermore, the range of plasma folate and hepatic folate concentrations observed in our studies were consistent with observations made in previous studies of dietary FA supplementation at similar levels in mice (162, 164).

To our knowledge, our study is among the first to assess plasma UMFA concentrations in a rodent model of FA supplementation. As mentioned previously, we observed significantly higher plasma UMFA concentrations in the HFA group relative to control in both Studies I and II. In addition, the plasma UMFA concentrations observed in the control and HFA groups were comparable to those observed in human studies conducted in North America (11, 14, 42). Mean plasma UMFA concentrations reported in these studies ranged from 0.50 to 2.31 nmol/L (11, 14, 42). The mean plasma UMFA concentration among post-menopausal women in the NK cell cytotoxicity study conducted by Troen and colleagues was 2.31 ± 1.91 nmol/L (mean ± SD) (11). Similarly, mean plasma UMFA concentrations in the HFA groups in our studies were 2.36 ± 1.04 and 2.25 ± 0.61 nmol/L (mean ± SD) in Study I and Study II, respectively. We used a 20
mg FA/kg diet, representative of 10X the BDR for folate in rodents, as a high FA supplementation dose; although folate intake levels at 10X the RDA (4 mg /day) may be recommended to individuals with certain medical conditions, intakes of this magnitude are not commonly observed in the general North American population. However, this supplemental level was employed in our mouse model to compensate for considerably higher efficiency of DHFR-mediated FA biotransformation in rodents relative to humans (9). Therefore, a higher dose of FA is likely required in rodents to elicit similar physiological consequences associated with a lower dose of FA in humans. The similarity between plasma UMFA concentrations observed in our studies and those observed in the North American population validates the relevancy of the level of FA supplementation employed in our model.

Although published data regarding UMFA in animal models are limited, preliminary data presented in an abstract form by Sawaengsri et al. reported mean plasma UMFA concentrations of 0.6 nmol/L and 29 nmol/L in aged (16+ months) female C57BL/6 mice supplemented with 2 and 40 mg FA/kg diet for 3 months, respectively (174). Our control group UMFA concentrations are similar to those observed in this study. However, the plasma UMFA concentrations observed in the 40 mg FA/kg diet supplemented group are markedly higher than those observed in mice supplemented with 20 mg FA/kg in our studies, suggesting that UMFA may increase exponentially in response to higher levels of FA supplementation. Alternatively, variables which differed between the study designs, such as fasting time prior to blood collection (not reported in Sawaengsri’s study) and the age of the mice, could have potentially contributed to the magnitude of the observed difference in plasma UMFA concentrations. Indeed, in humans, increased folate turnover after FA supplementation is observed in young but not elderly adults (175).
Finally, the observation of UMFA in plasma samples from the MFD group in Study II was unexpected, as mice in the MFD group were fed a FA-free diet, and as such, plasma from these mice should not contain UMFA. The limit of detection (LOD) for FA in the LC/MS/MS technique employed to quantify plasma UMFA was approximately 0.23 nmol/L. The LOD for FA, therefore, falls within one standard deviation of the mean plasma UMFA concentration observed in the MFD group (0.48 ± 0.31 nmol/L), and as such, the majority of the UMFA readings made in samples from the MFD group are most likely representative of experimental noise. A possible explanation for the observation of concentrations markedly higher than the LOD in a few of the MFD samples may involve artificial production of FA during sample collection and processing; for example, DHF and THF have been observed to undergo interconversion to FA in acidic conditions in vitro (176).

Our examination of the effects of FA supplementation on NK cell function is among the first to corroborate the previously reported association between high intake and blood levels of FA and a reduced NK cell cytotoxic response to cancerous cells (11). In Study I, splenic NK cell degranulation frequency in response to stimulation with the murine lymphoma cell line (YAC-1) and cytokines was significantly lower (by ~ 10%) in the HFA group relative to the control. Furthermore plasma folate and plasma UMFA concentrations were significantly and inversely correlated with splenic NK cell degranulation in response to YAC-1 and cytokine stimulation. NK cell degranulation represents the release of cytotoxic contents from the NK cell in response to activation (149). Although degranulation cannot be considered completely synonymous with NK cell cytotoxicity, which is typically assessed by measuring target cell death, the reduced NK cell degranulation observed in our study reflects reduced activation of granule-directed cytotoxic
function within the NK cell population in response to cancer cell target stimulation, and therefore likely translates to reduced NK cell cytotoxicity.

Our finding of an inverse correlation between plasma folate and NK cell degranulation is interesting in light of the observations made by Troen and colleagues (11), in which plasma UMFA but not plasma folate concentrations were inversely associated with NK cell cytotoxicity. This difference in findings may reflect the fact that in our study, mice were fed only FA and not naturally occurring folates from dietary sources, and as such plasma FA and plasma folate concentrations were highly correlated. In contrast, in the study by Troen and colleagues (11), the combined intake of dietary folate and FA consumption was likely highly variable in the post-menopausal women. Furthermore, DHFR activity in regard to FA reduction is reported to be highly variable in humans but not in rodents (9). As such, similar FA consumption among the post-menopausal women could have resulted in varied exposure to circulating UMFA, an event unlikely in our mouse model. Indeed, a correlation between plasma folate and plasma UMFA was not observed in the study conducted by Troen and colleagues (11), whereas a highly significant positive correlation was observed between these two biomarkers of folate/FA intake in our study.

As observed with frequency of splenic NK cell degranulation, frequency of multifunctional splenic NK cell degranulation and IFN-γ production in response to YAC-1 and cytokine stimulation was significantly lower in the HFA group relative to the control, and this parameter was significantly and inversely associated with both plasma folate and UMFA concentrations. Concurrent NK cell degranulation and IFN-γ production was assessed, as this measure likely reflects a highly accurate representation of multifunctional NK cell activation, due to a requirement for the presence of two activation markers in flow cytometric analysis.
Notwithstanding potential differences inherent to the specific experimental model, our data contrast previous studies that did not demonstrate an association between high intake and blood levels of FA/folate and NK cell activity. Hirsch et al. reported null findings in examination of the association between serum folate concentrations and NK cell cytotoxicity in a healthy Chilean population (158), and in examination of the effects of high FA concentrations on human NK cell cytotoxicity in an in vitro model (159). Our study obviates several limitations associated with these two reports including the lack of assessment of FA intake and UMFA concentrations in the study population in the former human study (158) and a short duration of exposure (96 hours) to FA in a non-physiological environment in the latter in vitro study (159). Effects on NK cell function may be mediated by a more chronic exposure to high levels of FA, and may result from alterations in external physiological factors implicated in regulation of NK cell function.

Our findings are in line with data reported in an abstract form by Sawaengsri et al., where reduced NK cell cytotoxicity in response to YAC-1 stimulation was observed in aged (16+ months) female C57BL/6 mice supplemented with 40 mg FA/kg diet relative to a 2 mg FA/kg diet control group (174). This study also reported a reduction in splenic NK cell frequency in the FA supplemented group, which may have accounted for the observed reduction in splenic NK cell cytotoxicity, as unsorted splenocytes were used as the effector population (i.e., a reduction in cytotoxicity mediated by a lower number of NK cells in the effector cell population) (174). In contrast, we did not observe alterations in splenic NK cell frequency between dietary groups in our study; this discrepancy may reflect an aging-associated effect. However, NK cell function on a per cell basis is typically reduced with aging whereas NK cell numbers stay the same or are increased (13).
In regard to the mitogenic stimulation condition in our studies, a significant inverse correlation between splenic NK cell degranulation frequency and plasma UMFA concentrations, and a trend suggesting an inverse correlation between splenic multifunctional NK cell degranulation/IFN-γ production frequency and plasma UMFA concentrations, were observed in Study I. However, splenic NK cell degranulation and/or IFN-γ production frequencies did not differ between dietary groups and were not correlated with plasma folate levels in Study I in response to mitogenic stimulation, and in Study II, in which a similar mitogenic stimulation was conducted. Furthermore, data from the mitogenic stimulation in Study II did not show a significant inverse correlation between these parameters and plasma UMFA. Collectively, these data may suggest that NK cell activation in response to mitogenic stimulation is not influenced by FA supplementation. However, further clarification of these discrepant findings is necessary.

As mitogenic stimulation using PMA and ionomycin activates NK cell effector functions by inducing non-specific signalling events such as PKC activation, and thus bypasses NK cell receptor recognition processes, an observation of reduced NK cell degranulation in response to target cell stimulation, but not in response to mitogenic stimulation, may reflect impairment in NK cell target cell recognition. Indeed, dysregulation in NK cell receptor expression represents a hypothesized mechanism by which excessive FA may modulate NK cell function. The variegated and stochastic expression of NK cell KIRs in humans and Ly49 receptors in mice has been demonstrated to be in part maintained by epigenetic mechanisms such as DNA methylation (113, 160, 177). A study by Gao et al., demonstrated that treating human NK cells with a methyltransferase inhibitor (5-azacytidine) in vitro modulated KIR expression and altered NK cell cytotoxicity (161). As such, it is possible that FA-induced alteration in DNA methylation could reduce the NK cell cytotoxic response to cancerous cells through dysregulation in NK cell
receptor expression. In fact, in a study by Li et al., incubation of human CD4+ and CD8+ T cells in folate-deficient conditions in vitro (10 nM FA) for 72 hours resulted in increased surface expression of the KIRs, KIR2DL2/2DL3/2DS2, increased mRNA levels of KIR2DL2, and reduced KIR2DL2 promoter methylation, relative to a folate-replete (40 nM FA) condition in cells from elderly donors, but not in cells from young donors (178). This effect was shown to be dependent on age-related reductions in expression of the maintenance DNA methyltransferase DNMT1 in T-cells from elderly donors (i.e., folate deficiency synergized with reduced DNMT1 expression in T-cells from elderly donors to increase KIR2DL2 expression via reduced promoter methylation) (178). However, in a study conducted by Hirsch et al., incubation of purified human NK cells from young donors in either 0, 30, or 100 nM FA in vitro for 96 hours did not result in significantly altered expression of KIR2DL3, KIR2DL4, nor the activating receptor NKG2D; although insignificant trends towards increased expression of the KIRs and decreased expression of NKG2D were observed with increasing FA concentration in the media (159). Our data do not refute the hypothesis that FA modifies NK cell function through modulation of NK cell receptor expression. However, further clarification, perhaps through analysis of the effects of FA supplementation on NK cell receptor expression in a C57BL/6 model in a study of similar design, using a methodology such as RNA sequencing or high-throughput flow cytometry, is required. The expression of activating receptors implicated in cytotoxicity against tumour cells such as NKG2D may be of particular interest. NKG2D expression is correlated with promoter CpG demethylation and histone H3K9 acetylation in human NK cells (179).

Generally, we did not observe alterations in IFN-γ production in response to FA supplementation in splenic NK cells stimulated with YAC-1 cells and the cytokines IL-12, IL-15 and IL-18; this absence of an effect may reflect the concentrations of IL-12, IL-15 and IL-18
utilized in the stimulation. In contrast to the previous understanding of “natural” activity within NK cells, NK cells require priming for optimal induction of their effector functions, which can be achieved through cytokine stimulation. As such, “un-primed” splenic NK cells from mice respond quite poorly in vitro (12), and therefore, we included cytokines in our YAC-1 stimulation condition. However, the concentration of IL-12, IL-15 and IL-18 (10 ng/mL of each cytokine) used in this stimulation may have saturated NK cell IFN-γ production capacities, as evidenced by the observation that the frequency of NK cell IFN-γ production in response to this stimulation was quite high. As such, the high concentrations of the cytokines may have inadvertently masked a possible effect of FA on IFN-γ production. In this regard, we observed a trend of a positive correlation between plasma UMFA concentrations and frequency of IFN-γ production in response to YAC-1 and cytokine stimulation. In addition, trends towards an increased amount of IFN-γ production per NK cell in the HFA group were observed in two of three YAC-1 + cytokine stimulation experiments conducted. Furthermore, trends towards increased frequency of IFN-γ production in response to high FA supplementation in NK cells stimulated with YAC-1 and cytokines were also noted in some preliminary observations made during assay optimization.

Concurrent observations of increased IFN-γ production and decreased degranulation in assayed NK cells may reflect a subset shift within the NK cell population towards NK cells that are less cytotoxic, but more cytokine-producing (12). Moreover, in a study of women with recurrent spontaneous abortion, FA supplementation at 0.5 mg per day for 2 months was associated with a significantly increased frequency of CD56+CD16- NK cells, but a constant frequency of NK cells (CD56+CD3-) within the PBMC population, such that the ratio of CD56+CD16- NK cells:CD56+CD16+ NK cells increased from 0.25 to 0.55 with FA.
supplementation (180). In humans, the CD56+(CD56\textsubscript{dim})\text{CD16+} NK cell subset is more cytotoxic whereas the CD56+(CD56\textsubscript{bright})\text{CD16-} subset is more cytokine-producing (12). A shift towards an increased proportion of CD56\textsubscript{bright}CD16- NK cells could potentially result in reduced NK cell cytotoxicity. Indeed, an insignificant trend towards reduced NK cell cytotoxicity in response to FA supplementation was observed in the study of women with recurrent spontaneous abortion (180). As such, FA-mediated alteration of NK cell subset proportions may represent a mechanism behind the FA-associated impairment in NK cell cytotoxicity. Our observations of FA-supplementation-associated reduced degranulation and trends towards increased IFN-\gamma production in NK cells stimulated with target cells and cytokines are in line with this hypothesis. However, it is also important to consider that enhanced cytotoxicity or cytokine effector functions are not as clearly attributable to the main identified NK cell subsets in mice as they are in those of humans. For example, the CD11b+CD27+ NK cells in mice have been demonstrated to be more cytotoxic and more or similarly cytokine-producing, relative to their CD11b+CD27- counterparts (100, 101). Nonetheless, analysis of NK cell subset distribution in mice supplemented with high FA represents an approach to further characterize the effects of FA supplementation on NK cell activity. Indeed, the nutritional intervention of caloric restriction in C57BL/6 mice was shown to modify splenic NK cell subset distribution and to result in altered splenic NK cell function, including reduced IFN-\gamma production in response to cytokine stimulation and increased degranulation/IFN-\gamma production in response to YAC-1 stimulation (138).

In Study II, we assessed splenic NK cell response to antibody-mediated crosslinking of the activating receptor NK1.1, as a measure of generalized NK cell responsiveness. The association between excessive FA intake and reduced NK cell cytotoxicity observed in the Troen et al. study (11) and reduced NK cell degranulation in response to high FA supplementation
observed in Study I could potentially be mediated by dysregulation in the process of NK cell education and thus the intrinsic capability of NK cells to respond to stimuli via cytotoxicity. However, our data suggest that dysregulation in intrinsic responsiveness of NK cells is not a likely mechanism behind the association between high intake and blood levels of FA and reduced NK cell cytotoxic response to cancerous cells, as we did not observe significant differences in splenic NK degranulation or IFN-γ production under all anti-NK1.1 antibody stimulation conditions tested, nor were plasma folate nor plasma UMFA concentrations correlated with these splenic NK cell effector functions under all anti-NK1.1 antibody stimulation conditions tested. The lack of a FA-mediated effect on generalized NK cell responsiveness may reflect but does not necessarily imply that NK cell inhibitory receptor expression is not dysregulated by FA-dependent mechanisms. NK cell functional responsiveness has been demonstrated to be quantitatively tuned by the strength and number of NK cell inhibitory receptor-“self” ligand interactions (125), and as such one could hypothesize that FA-mediated dysregulation in NK cell inhibitory receptor expression could result in differences in NK cell intrinsic functional responsiveness, as tested by activating receptor stimulation.

To the best of our knowledge, our study is the first to assess the effects of FA supplementation on generalized responsiveness of NK cells to antibody-mediated crosslinking of an activating receptor. We also demonstrate that moderate folate deficiency is not associated with defects in generalized NK cell responsiveness, nor was NK cell cytotoxicity against cancerous cells affected by moderate folate deficiency in Sprague-Dawley rats in a previous study conducted by Kim and colleagues (157).

Another potential mechanism by which high levels of FA could impair NK cell cytotoxic function, which warrants investigation but was not assessed within our study, involves FA-
mediated modulation of T-regulatory cells. A role of folate in maintenance of regulatory (Foxp3+CD25+) T lymphocytes in the peripheral circulation, small intestine and colon has recently been reported (153-155), but has not been fully characterized, and the effect of FA supplementation on these cells has not been examined. T-regulatory cells can downregulate NK cell cytotoxicity and NK cell expression of activating receptors such as NKG2D through TGF-β-dependent mechanisms (122). Indeed, in the context of cancer, an increase in T-regulatory cell numbers is generally inversely correlated with NK cell function, whereas depletion of T-regulatory cells has been reported to increase NK cell cytotoxicity (122). It is plausible that chronic exposure to high levels of circulating FA could result in an overrepresentation of T-regulatory cells and/or their immune-suppressive functions, which in turn could downregulate NK cell cytotoxic activity. As such, the effects of FA supplementation on T-regulatory cells and the potential for this to influence NK cell functions should be investigated in future studies. It is also plausible that oversupplementation with FA could modulate, through DNA-methylation-associated epigenetic mechanisms, the expression of factors involved in NK cell regulation; e.g., the prostaglandin E2 E-prostanoid (EP) receptor 2 is epigenetically regulated (181) and plays an important role in regulation of NK cell function (182). As such, exploration of the effects of FA oversupplementation on expression of genes involved in regulation of NK cell function represents a potential approach to mechanistically elucidate the NK cell cytotoxicity impairing effects of FA.

Our findings suggest that a FA-associated impairment in the NK cell cytotoxic response to cancer cells may be an additional mechanism behind the purported tumour-promoting effect associated with high FA supplementation (8). Folate appears to possess dual modulatory effects on colorectal carcinogenesis (8). Folate deficiency promotes, whereas FA supplementation at
modest levels protects against, CRC development in normal tissues (8). However, in the presence of (pre)neoplastic lesions or at supraphysiological doses in normal tissues, FA supplementation promotes tumour progression (8). A reduction in NK cell cytotoxicity in response to FA supplementation could promote the progression of (pre)neoplastic lesions via impaired tumour immunosurveillance and elimination of these abnormal cells. FA supplementation has also been implicated in promoting the progression of breast and prostate cancers (74, 163, 183). For example, in a study by Manshadi et al., FA supplementation was associated with increased progression of established mammary tumours in Sprague-Dawley rats (163). Furthermore, in the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial, an increased breast cancer risk was reported in postmenopausal women with a high total folate intake, mainly from FA supplements, relative to women with low folate intake (183). In the Aspirin/Folate Polyp Prevention Study (AFPPS) trial, an increased risk of prostate cancer was documented in men receiving FA supplementation at 10 years of follow-up (74). A tumour-promoting effect of FA supplementation in various cancers suggests a global mechanism of action, and impaired NK cell cytotoxic function against cancerous cells represents a plausible mechanism.

In summary, our data corroborate the previously observed inverse association between high intake and blood levels of FA and NK cell cytotoxicity (11) and suggest that dysregulation in NK cell generalized responsiveness is not a likely mechanism mediating the effects of FA supplementation on NK cell function. The use of a young C57BL/6 mouse model in our studies may be considered a limitation in examining the observations made by Troen et al. (11). However, our aim was to assess the effects of FA supplementation on NK cell activity in the absence of confounding factors, such as the decline in immune function associated with aging. Nonetheless, future assessment of the effects of FA supplementation on NK cell function in an
aged C57BL/6 model and comparison to our current studies should provide insight into potential synergistic FA and aging-associated effects on NK cell functional impairment (i.e., the effects of FA supplementation on NK cell activity may be exacerbated by aging). An additional limitation of our study was that we did not assess gender-specific effects, only a small subset of mice used in Study I were male, and therefore our results cannot be interpreted by gender, where gender-specific factors could potentially interact with the effect of FA on NK cell function. Furthermore, sample size in each experiment was limited, due to the labour intensive nature of the experiments conducted, and a moderate degree of experimental noise was observed in each experiment, limiting the power of our analysis in detecting significance of certain outcomes. As such, careful examination of non-significant trends observed in our studies is warranted.

Finally, as a corollary to our NK cell study, we also examined the impact of FA supplementation on expression of the enzyme responsible for its biotransformation, DHFR, to test the hypothesis that excessive FA may mediate adverse health effects through dysregulation in expression of folate-dependent enzymes and folate receptors/transporters. Due to the close association of hepatic DHFR activity with circulating UMFA, we decided to assess the impact of FA on Dhfr gene expression, and to correlate parameters of folate status, such as plasma UMFA concentrations, with hepatic gene expression of this enzyme. We did not observe significant differences in hepatic Dhfr mRNA levels between the assayed dietary groups (MFD, control, HFA). However, we observed high variation in gene expression from sample-to-sample within the dietary groups – particularly in the HFA group. Furthermore, we observed trends towards a positive correlation between plasma folate and UMFA concentrations and relative Dhfr mRNA levels, suggesting that DHFR may be upregulated with increasing FA intake. This observation is consistent with a potential adaptation effect to high FA exposure. For example, in a study by
Tam and colleagues, women of child-bearing age were supplemented with either 1.1 mg or 5 mg of FA per day as part of a multi-vitamin, for a period of 30 weeks (20). Although an increase in plasma UMFA concentrations was observed initially, UMFA concentrations returned to baseline over time (20). Although other factors such as a potential decline in adherence over the course of the study may have contributed to this observation, an up-regulation of DHFR could also mediate such an effect (20). Furthermore, in a study by Gao and colleagues, incubation of bovine endothelial cells in vitro in a high concentration of FA (50 µM) for 24 hours resulted in increased protein expression of DHFR, and supplementation of 6-9 week old C57BL/6J mice with 15 mg FA/kg diet for 16 days resulted in increased expression of DHFR in aortic endothelial cells relative to mice fed a control diet (184).

In contrast to our observation of a trend towards decreased hepatic Dhfr gene expression with parameters indicating folate deficiency, an intuitive response to folate deficiency would involve upregulation of DHFR expression and activity, in order to enable improved recycling of DHF to THF which could then be further modified to 5,10-methylene-THF and 10-formyl THF and used in nucleotide biosynthesis necessary for cell replication (185). Indeed, increased DHFR protein expression and activity have been reported in Chinese hamster lung fibroblasts cultured in low-folate conditions (185). However, the observation of folate-deficiency associated reduced Dhfr gene expression in the liver may reflect a mechanism to increase the flux of folates into the methionine cycle (185). In a study by Hayashi et al., incubation of the human colon adenocarcinoma cell lines HCT116 and Caco-2 cell lines in FA-deficient (0.6 nmol/L) media for 20 days resulted in significantly lower DHFR gene expression, 61% and 29% lower, respectively, relative to cells cultured in folate sufficient media (2.3 µM FA) (186). Finally, we
also observed an inverse U-shaped relationship between hepatic folate concentrations and Dhfr relative mRNA levels which warrants further investigation.

Limitations of our assessment of the effects of FA supplementation on hepatic Dhfr gene expression include high sample-to-sample variability; in future analyses a larger sample size may be used for clarification of observed effects. In addition, the relevance of any differences in gene expression should be assessed by examining protein levels and activity of DHFR. Although clarification of our findings is necessary, our data provide some support for the hypothesis suggesting that high FA can modulate gene expression of folate dependent enzymes, and as such could potentially contribute to dysregulation of folate dependent processes; alternatively modulation of expression may reflect homeostatic mechanisms.

4.6 Conclusion

Our data corroborate the previously observed (11) inverse association between high intake and blood levels of FA and NK cell cytotoxicity towards cancerous cells in a human study. Further studies are warranted to unequivocally establish the impairing effect of high FA intake on NK cell function, which is a significant health concern given the current folate status of the North American population, and the importance of NK cell function to host defenses against malignancies and infection. Our data also suggest that dysregulation in generalized NK cell responsiveness is not a likely mechanism underlying the relationship between FA supplementation and decreased NK cell cytotoxic function. Our observations of trends suggesting altered hepatic Dhfr gene expression in response to FA supplementation provide limited support for the hypothesis that chronic or prolonged exposure to excessive FA may potentially exert unfavourable health effects through dysregulation in enzymes involved in folate
and one-carbon metabolism. Finally, our findings suggest that reduced NK cell activity may be an additional mechanism behind the purported tumour promoting effect of FA supplementation on established (pre)neoplastic foci (8), a finding of particular importance as the prevalence of vitamin and mineral supplement use is high among cancer patients, and is often initiated by the patient after the cancer diagnosis (39).
Chapter 5: General discussion, future directions and conclusion

5.1 Summary and general discussion

Two dietary intervention studies were conducted to investigate the relationship between FA supplementation and NK cell function in a C57BL/6 mouse model. The effects of FA supplementation on splenic NK cell function in response to stimulation with cancer cell targets (YAC-1 cell line) and cytokines, and in response to stimulation with plate-bound antibody directed against the activating NK cell receptor NK1.1, as a measure of generalized NK cell responsiveness, were assessed in Studies I and II, respectively. The dietary FA intervention employed in our studies effectively modulated folate status in our animals as assessed by plasma folate, plasma UMFA and hepatic folate concentrations. Plasma UMFA concentrations in the high FA supplementation dietary group (supplemented with 20 mg FA/kg diet, 10X the BDR for rodents), were comparable to those observed in the general North American population, which validates the level of FA selected to reflect high FA supplementation in our study.

Significantly lower NK cell degranulation in response to stimulation with the YAC-1 cell line and cytokines was observed in mice supplemented with a high level of FA, relative to control. Additionally, plasma folate and UMFA concentrations were significantly and inversely correlated with NK cell degranulation in response to YAC-1 and cytokine stimulation. As such, these data corroborate the inverse association between high FA intake/UMFA and NK cell cytotoxicity observed previously in a study of post-menopausal women conducted by Troen and colleagues (11). Significant differences among the dietary groups were not observed in the assessed NK cell functional parameters in response to cross-linking of the NK cell activating receptor anti-NK1.1, nor was NK cell activity correlated with plasma folate or UMFA concentrations in response to this stimulation. Therefore, the data suggest that dysregulation in
generalized NK cell responsiveness is not a probable mechanism behind the reduced NK cell cytotoxic response to cancerous cells observed with high FA supplementation.

Finally, as a corollary, the effects of FA supplementation on hepatic Dhfr gene expression were examined, in order to investigate the hypothesis that adverse health effects associated with high FA intake may be mediated by dysregulation in folate-dependent processes such as DNA methylation through modulation of the expression of folate-dependent enzymes (7). Although significant differences in hepatic Dhfr gene expression among the dietary groups supplemented with 0, 2 (control) or 20 mg FA/kg diet were not observed, the findings were confounded by high sample-to-sample variation. In addition, non-significant positive correlations between plasma folate and UMFA concentrations and relative Dhfr mRNA level and an inverse U-shaped relationship between hepatic folate concentrations and relative Dhfr mRNA level were observed. Thus, the data suggest that exposure to excessive FA may modulate expression of folate-dependent enzymes such as DHFR and hence, may lead to dysregulation of folate dependent processes.

Our findings highlight the necessity for additional studies examining the effects of FA supplementation on NK cell function. A reduction in NK cell cytotoxic activity in response to high FA intake may be of particular importance in the context of cancer as cancer patients often initiate supplement use post-diagnosis. Indeed, the prevalence of vitamin and mineral supplement use is reported to be between 64 and 81% among cancer survivors, relative to approximately 50% in the general North American population (39). Our data demonstrating reduced NK cell degranulation in response to stimulation with cancerous cells suggest that reduced NK cell tumour immunosurveillance may be an additional mechanism behind the purported tumour promoting effect of FA supplementation. Our study therefore highlights the
need for optimized public health recommendations regarding FA intake, particularly among the highly susceptible population of cancer patients in whom reduced NK cell function may have significantly adverse consequences. A FA-supplementation-mediated impairment in NK cell function may also have important implications in cancer therapy, as NK cell based therapies for malignant disease are currently under development (90); appropriate FA intake may be important to maximizing efficacy of these treatments.

Finally, our identification of an impaired NK cell response to cancerous cells in response to high FA supplementation adds to the growing body of evidence that suggests a dual modulatory role for folate status and FA intake in health and disease. Although folate deficiency is associated with a multitude of disorders such as megaloblastic anaemia, development of certain cancers, incidence of NTDs, cardiovascular disease and stroke among others, the efficacy of FA supplementation has only been unequivocally demonstrated in regard to treatment of megaloblastic anaemia and in prevention of NTDs (7, 8, 16). On the contrary, high FA intake has been associated with adverse health effects such as masking of vitamin B12 deficiency in the elderly; potential for development of tolerance or resistance to anti-folate drugs; impaired cognition when combined with low vitamin B12 status; epigenetic modifications resulting in altered disease risk; and promotion of tumour progression (7). Our study, in combination with these other lines of evidence, brings to light the need for careful examination of the potential adverse health effects of FA supplementation on human health prior to recommendations of supplemental FA intake, either in a direct or indirect fashion via further FA fortification, among individuals in whom FA supplementation does not have a clear health benefit.
5.2 Future directions

Future studies should delineate the mechanisms behind the effects of FA supplementation on NK cell function. Potential mechanisms which may be of interest to assess include the effects of FA supplementation on NK cell receptor expression, particularly in regard to activating receptors implicated in the NK cell response to malignant cells such as NKG2D (106, 179), and on NK cell subset distribution where modification of NK cell subset frequencies may result in a global alteration of NK cell effector functions (12, 138, 180). As a role for folate in maintenance of T-regulatory lymphocytes has recently been identified (153-155), it may be of interest to assess the effects of FA supplementation on this cell population in order to infer whether T-regulatory cell-NK cell interactions may be at play in modulating NK cell function in this context. A screen of differentially expressed genes (RNA sequencing) or a proteomics-approach in an animal model of FA supplementation may be useful in identifying potentially altered expression of factors involved in regulation of NK cell function.

The impact of high FA intake on NK cell function should also be assessed in an aged animal model, as FA-mediated impairment in NK cell activity may be exacerbated by aging. The trans-generational effect of maternal FA supplementation on NK cell function in the offspring represents an additional study with relevance to human health. It may also be of interest to assess the effects of FA supplementation on NK cell activity in the context of a high-fat diet or in obesity; leptin-signalling which may be dysregulated in obesity and consumption of a high fat-diet have been linked to modulation of NK cell cytotoxicity (142, 144). Furthermore, in the study conducted by Troen and et al., participants had a mean BMI of 30.3 ± 3.9 25 kg/m² (mean ± SD), and all participants had a BMI > 25 kg/m² or > 24 kg/m² if combined with a body fat percentage > 33% as assessed by bioelectrical impedance (11). As such, the effects of FA supplementation
on NK cell cytotoxicity may have been exacerbated by obesity or related factors in this study. An understanding of the mechanisms behind the impairment of NK cell cytotoxic function mediated by high FA intake will allow for optimal design of, and elimination of confounding factors in, studies examining the impact of FA supplementation on NK cell function in humans. The effects of FA supplementation on NK cell function in humans could be examined using a cross-sectional clinical study where NK cell function could be compared among individuals who are not consuming FA-supplements and individuals consuming multivitamins containing 400 µg of FA and higher levels of FA supplementation (e.g., 1 mg, 5 mg), after adjusting for potential confounding factors including dietary intake of folate. The definitive approach to elucidate the effect of FA supplementation on NK cell function is a randomized clinical trial with treatments including 1) no supplementation; 2) supplementation with 250 µg of FA/day (~RDA); and 3) supplementation with 1 mg of FA/day, in young and aged adults and by gender. Furthermore, conditions such as duration of supplementation and differences between FA supplementation, 5-methylTHF supplementation, and very high intakes of natural dietary folate, and their impact on NK cell function could be explored in these trials.

Finally, in order to fully characterize the effects of FA supplementation on NK cell defenses against malignancies, the effects of FA supplementation on the expression of ligands associated with activation of NK cytotoxicity should be investigated. McGilvray et al. reported an association between high expression of MIC ligands (recognized by NKG2D) and improved colorectal cancer patient survival (187), whereas Chavez-Blanco et al. reported an up-regulation of these ligands in several human cancer cell lines (DU145: prostate cancer cell line, SW480: colon cancer cell line, T47D: breast cancer cell line, and CaSki: cervical cancer cell line) and consequent increased NK cell cytotoxicity against these cells, with the administration of DNA
methylation and histone deacetylase inhibiting drugs (188). Therefore, high FA intake-mediated reduced expression of NK cell activating ligands in transformed cells, via epigenetic effects, could represent yet another mechanism behind the promoting effect of high FA intake on cancer progression in transformed tissues.

5.3 Conclusion

Our data are among the first to corroborate an inverse association between high intake and blood levels of FA and reduced NK cell cytotoxicity against malignant cells. Furthermore, our data support the hypothesis that impaired tumour immunosurveillance may be an additional mechanism behind the tumour progression promoting effect associated with high FA intake. Our data will serve as a platform for future mechanistic and human studies examining this relationship and aid in development of improved public health recommendations for FA intake particularly among cancer patients and elderly individuals in whom reduced NK cell function may have particularly deleterious consequences.
REFERENCES


117. Krzewski K, Coligan JE. Human NK cell lytic granules and regulation of their exocytosis. Frontiers in Immunology 2012;3:


## Appendix 1

### Supplementary tables and figures

*Table S1A: The effects of dietary folic acid intervention on natural killer cell function in response to stimulation with plate-bound anti-NK1.1 antibody (Study II, Experiment 1)*

<table>
<thead>
<tr>
<th>Experiment (n)</th>
<th>Stimulation</th>
<th>Function Parameter</th>
<th>0 mg FA/kg (MFD)</th>
<th>2 mg FA/kg (control)</th>
<th>20 mg FA/kg (HFA)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (3/group)</td>
<td>15 µg Anti-NK1.1 Antibody</td>
<td>% CD107a+</td>
<td>28.5 ± 1.6</td>
<td>26.7 ± 3.2</td>
<td>27.0 ± 3.5</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% IFN-γ+</td>
<td>8.5 ± 0.4</td>
<td>8.0 ± 2.5</td>
<td>8.0 ± 1.6</td>
<td>0.91</td>
</tr>
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<td></td>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>7.9 ± 0.5</td>
<td>7.4 ± 2.3</td>
<td>7.4 ± 1.4</td>
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<td></td>
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<td>IFN-γ+ NK MFI</td>
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<td></td>
<td>5 µg Anti-NK1.1 Antibody</td>
<td>% CD107a+</td>
<td>28.5 ± 3.0</td>
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<td>27.7 ± 4.3</td>
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<td></td>
<td></td>
<td>% IFN-γ+</td>
<td>8.9 ± 1.5</td>
<td>9.7 ± 3.8</td>
<td>9.2 ± 1.7</td>
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<tr>
<td></td>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>8.3 ± 1.4</td>
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<td></td>
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<td>IFN-γ+ NK MFI</td>
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<td>3 µg Anti-NK1.1 Antibody</td>
<td>% CD107a+</td>
<td>22.1 ± 0.9</td>
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<td>% IFN-γ+</td>
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<td>% CD107a+IFN-γ+</td>
<td>8.1 ± 1.0</td>
<td>7.4 ± 2.1</td>
<td>7.1 ± 1.9</td>
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<td></td>
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<td>IFN-γ+ NK MFI</td>
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<td>482.7 ± 10.1</td>
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<td>PMA + Ionomycin</td>
<td>% CD107a+</td>
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<td>% IFN-γ+</td>
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<td>% CD107a+IFN-γ+</td>
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<td>IFN-γ+ NK MFI</td>
<td>628.7 ± 66.6</td>
<td>625.0 ± 9.17</td>
<td>631.0 ± 33.9</td>
<td>0.99</td>
</tr>
</tbody>
</table>

1 Data are presented as mean ± SD; n = 3/group; significance of differences between dietary groups was assessed using One-Way ANOVA.
2 % CD107a+: frequency of NK cell degranulation, % IFN-γ+: frequency of NK cell IFN-γ production, % CD107a+IFN-γ+: frequency of multifunctional NK cells, IFN-γ+ NK MFI: median fluorescence intensity of the IFN-γ+ NK cell population; indicative of the median amount of IFN-γ production per NK cell.
Table S1B: The effects of dietary folic acid intervention on natural killer cell function in response to stimulation with plate-bound anti-NK1.1 antibody (Study II, Experiment 2)\(^1,2\)

<table>
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<tr>
<th>Experiment (n)</th>
<th>Stimulation</th>
<th>Function Parameter</th>
<th>0 mg FA/kg (MFD)</th>
<th>2 mg FA/kg (control)</th>
<th>20 mg FA/kg (HFA)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (3/group)</td>
<td>15 µg Anti-NK1.1 Antibody</td>
<td>% CD107a+</td>
<td>29.4 ± 1.9</td>
<td>30.3 ± 6.1</td>
<td>26.2 ± 3.8</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% IFN-γ+</td>
<td>9.3 ± 0.9</td>
<td>10.2 ± 2.7</td>
<td>9.3 ± 3.0</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>8.4 ± 0.9</td>
<td>9.3 ± 2.6</td>
<td>8.3 ± 2.5</td>
<td>0.80</td>
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<td>IFN-γ+ NK MFI</td>
<td>447.3 ± 8.7</td>
<td>452.3 ± 8.7</td>
<td>463.3 ± 37.5</td>
<td>0.69</td>
</tr>
<tr>
<td>5 µg Anti-NK1.1 Antibody</td>
<td></td>
<td>% CD107a+</td>
<td>29.0 ± 2.7</td>
<td>28.8 ± 8.5</td>
<td>25.5 ± 4.5</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
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<td>% IFN-γ+</td>
<td>10.8 ± 0.9</td>
<td>11.8 ± 4.5</td>
<td>11.0 ± 2.9</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>9.9 ± 1.0</td>
<td>10.9 ± 4.2</td>
<td>9.8 ± 2.5</td>
<td>0.89</td>
</tr>
<tr>
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<td></td>
<td>IFN-γ+ NK MFI</td>
<td>472.0 ± 27.5</td>
<td>470.3 ± 18.5</td>
<td>504.0 ± 43.9</td>
<td>0.40</td>
</tr>
<tr>
<td>3 µg Anti-NK1.1 Antibody</td>
<td></td>
<td>% CD107a+</td>
<td>10.1 ± 2.4</td>
<td>9.9 ± 3.1</td>
<td>8.9 ± 2.9</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% IFN-γ+</td>
<td>2.6 ± 0.8</td>
<td>3.0 ± 1.8</td>
<td>2.7 ± 1.5</td>
<td>0.94</td>
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<td>% CD107a+IFN-γ+</td>
<td>2.4 ± 0.7</td>
<td>2.8 ± 1.7</td>
<td>2.4 ± 1.4</td>
<td>0.92</td>
</tr>
<tr>
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<td>IFN-γ+ NK MFI</td>
<td>474.0 ± 3.60</td>
<td>484.3 ± 21.1</td>
<td>454.7 ± 76.2</td>
<td>0.73</td>
</tr>
<tr>
<td>PMA + Ionomycin</td>
<td></td>
<td>% CD107a+</td>
<td>41.9 ± 3.7</td>
<td>42.3 ± 4.2</td>
<td>40.7 ± 0.5</td>
<td>0.82</td>
</tr>
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<td></td>
<td></td>
<td>% IFN-γ+</td>
<td>71.4 ± 1.6</td>
<td>71.7 ± 1.9</td>
<td>70.7 ± 2.2</td>
<td>0.81</td>
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<tr>
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<td></td>
<td>% CD107a+IFN-γ+</td>
<td>31.8 ± 2.9</td>
<td>32.5 ± 4.5</td>
<td>30.4 ± 1.0</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFN-γ+ NK MFI</td>
<td>688.0 ± 8.7</td>
<td>699.3 ± 13.4</td>
<td>676.0 ± 19.3</td>
<td>0.22</td>
</tr>
</tbody>
</table>

\(^1\) Data are presented as mean ± SD; n = 3/group; significance of differences between dietary groups was assessed using One-Way ANOVA.

\(^2\) % CD107a+: frequency of NK cell degranulation, % IFN-γ+: frequency of NK cell IFN-γ production, % CD107a+IFN-γ+: frequency of multifunctional NK cells, IFN-γ+ NK MFI: median fluorescence intensity of the IFN-γ+ NK cell population; indicative of the median amount of IFN-γ production per NK cell.
Table S1C: The effects of dietary folic acid intervention on natural killer cell function in response to stimulation with plate-bound anti-NK1.1 antibody (Study II, Experiment 3)\(^1,2\)

<table>
<thead>
<tr>
<th>Experiment (n)</th>
<th>Stimulation</th>
<th>Function Parameter</th>
<th>0 mg FA/kg (MFD)</th>
<th>2 mg FA/kg (control)</th>
<th>20 mg FA/kg (HFA)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (3/group)</td>
<td>15 µg Anti-NK1.1 Antibody</td>
<td>% CD107a+</td>
<td>32.9 ± 3.8</td>
<td>32.7 ± 4.2</td>
<td>36.6 ± 3.1</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% IFN-γ+</td>
<td>10.1 ± 1.8</td>
<td>10.2 ± 3.2</td>
<td>11.6 ± 1.3</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>9.6 ± 1.8</td>
<td>9.7 ± 3.2</td>
<td>11.1 ± 1.2</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFN-γ+ NK MFI</td>
<td>425.0 ± 34.0</td>
<td>461.0 ± 12.5</td>
<td>444.0 ± 16.6</td>
<td>0.24</td>
</tr>
<tr>
<td>5 µg Anti-NK1.1 Antibody</td>
<td>% CD107a+</td>
<td>33.0 ± 3.3</td>
<td>31.2 ± 2.1</td>
<td>32.7 ± 3.3</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>% IFN-γ+</td>
<td>13.7 ± 3.6</td>
<td>12.2 ± 1.7</td>
<td>13.0 ± 1.6</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>13.3 ± 3.4</td>
<td>11.6 ± 1.7</td>
<td>12.6 ± 1.6</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFN-γ+ NK MFI</td>
<td>458.0 ± 11.3</td>
<td>488.3 ± 26.2</td>
<td>469.0 ± 22.5</td>
<td>0.28</td>
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<tr>
<td>3 µg Anti-NK1.1 Antibody</td>
<td>% CD107a+</td>
<td>12.8 ± 1.6</td>
<td>14.5 ± 4.8</td>
<td>13.2 ± 4.3</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>% IFN-γ+</td>
<td>3.6 ± 1.3</td>
<td>4.0 ± 1.4</td>
<td>3.4 ± 1.9</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>3.4 ± 1.3</td>
<td>3.9 ± 1.3</td>
<td>3.3 ± 1.9</td>
<td>0.90</td>
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<tr>
<td></td>
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<td>IFN-γ+ NK MFI</td>
<td>454.3 ± 10.8</td>
<td>461.0 ± 45.2</td>
<td>479.3 ± 27.4</td>
<td>0.62</td>
</tr>
<tr>
<td>PMA + Ionomycin</td>
<td>% CD107a+</td>
<td>44.7 ± 2.9</td>
<td>38.7 ± 2.3</td>
<td>40.8 ± 2.6</td>
<td>0.072</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>% IFN-γ+</td>
<td>69.8 ± 2.6</td>
<td>66.4 ± 11.1</td>
<td>70.2 ± 1.6</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% CD107a+IFN-γ+</td>
<td>35.1 ± 3.5</td>
<td>28.4 ± 6.0</td>
<td>31.6 ± 2.5</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFN-γ+ NK MFI</td>
<td>613.0 ± 25.2</td>
<td>601.0 ± 66.1</td>
<td>628.7 ± 25.5</td>
<td>0.75</td>
</tr>
</tbody>
</table>

\(^1\) Data are presented as mean ± SD; n = 3/group; significance of differences between dietary groups was assessed using One-Way ANOVA.
\(^2\) % CD107a+: frequency of NK cell degranulation, % IFN-γ+: frequency of NK cell IFN-γ production, % CD107a+IFN-γ+: frequency of multifunctional NK cells, IFN-γ+ NK MFI: median fluorescence intensity of the IFN-γ+ NK cell population; indicative of the median amount of IFN-γ production per NK cell.
Figure S1: Representative flow cytometry density plots of isotype controls and fluorescence-minus-one controls used for gating boundary set-up in the Study I natural killer cell activity assay. An FMO control incorporates every fluorochrome conjugated antibody utilized in a specific experiment with the exception of the fluorochrome conjugated antibody of interest. Both unstimulated (not shown) and PMA + ionomycin stimulated samples (shown above) were used for FMO controls; FV450 stain FMO [A]; PE/Cy7 anti-mouse CD3ε antibody FMO [B]; APC anti-mouse CD107a antibody FMO [C]; FITC anti-mouse IFN-γ antibody FMO [D]; FITC isotype control – used to ascertain specificity of intracellular staining [E]. A PE anti-mouse CD49b FMO was not included as NK cells were stained with PE anti-mouse CD49b during NK cell enrichment, an unstained control was used for gating boundary set up in this case.
Figure S2: Representative flow cytometry density plots of isotype controls and fluorescence-minus-one controls used for gating boundary set-up in the Study II natural killer cell activity assay. An FMO control incorporates every fluorochrome conjugated antibody utilized in a specific experiment with the exception of the fluorochrome conjugated antibody of interest. Both unstimulated (shown above) and PMA + ionomycin stimulated samples (not shown) were used for FMO controls; FV450 stain FMO [A]; PerCP/Cy5.5 anti-mouse CD3ε antibody FMO [B]; PE anti-mouse CD49b antibody FMO [C] APC anti-mouse CD107a antibody FMO [D]; FITC anti-mouse IFN-γ antibody FMO [E]; FITC isotype control – used to ascertain specificity of intracellular staining [F].
Figure S3: Gating strategy used in analysis of flow cytometry data in the Study I natural killer cell activity assay. Analysis of data involved gating on cell events i.e., exclusion of debris [A], followed by exclusion of cells clumps in a time vs. side scatter analysis [B], followed by gating on single cell events [C]; followed by gating on live cell events (FV450- cells); followed by gating on CD3- cells (PE/Cy7- cells) [E]; followed by gating on CD49b+ cells (PE+ cells) [F]; frequency of degranulation (CD107a+ [APC+] cells; x-axis) and IFN-γ production (IFN-γ+ [FITC+, y-axis]) was assessed in a CD49b+CD3- (NK) cell population, data plot shown corresponds to an unstimulated sample [G].
Figure S4: Gating strategy used in analysis of flow cytometry data in the Study II natural killer cell activity assay. Analysis of data involved gating on cell events i.e., exclusion of debris [A], followed by exclusion of cells clumps in a time vs. side scatter analysis [B], followed by gating on single cell events [C]; followed by gating on live cell events (FV450- cells); followed by gating on CD3- cells (PerCP/Cy5.5- cells) [E]; followed by gating on CD49b+ cells (PE+ cells) [F]; frequency of degranulation (CD107a+ [APC+] cells) and IFN-γ production (IFN-γ+ [FITC+]) was assessed in a CD49b+CD3- (NK) cell population, plots shown correspond to an unstimulated sample [G,H].