GENETIC AND NUTRITIONAL DISTURBANCES OF FOLATE METABOLISM MODULATE THE OUTCOME OF CEREBRAL MALARIA

Danielle Nichole Meadows

Department of Human Genetics

McGill University

Montreal, Quebec, Canada

May 2015

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

This thesis is dedicated to my family, those I share genes with and those of the heart.

To Mima and Daddy for teaching me that I could do anything I put my mind to, for always "making it work", and for their unwavering support, reassurance, and belief in me;

To Pop-pop for always encouraging and embracing his granddaughter's obsession with (dinosaurs/mummies/the Titanic/theatre/sea urchins/etc.) genetic inheritance;

To Kevin for laughter, fierce loyalty, surprise visits and the best hugs;

To Gramma, Ronnie, Meagan, Allie and everyone else for their love, support and encouragement.

ACKNOWLEDGEMENTS

In the Rozen lab, it has oft been said that "it takes a village to raise a successful PhD candidate", but I'll see that and raise it. President Obama once said, "If you were successful, somebody along the line gave you some help." While working on my PhD I have found help, advice and support from a wide cast of characters and from many different places. I am grateful to each and every one for their contributions.

First, to my supervisor, Professor Rima Rozen, thank you for giving me the opportunity to join your lab and for allowing me to tackle an interesting project that combined a little bit of everything- genetics, biochemistry, immunology, nutrition, etc. Thank you for being a tough but compassionate mentor, for your shared ideas and your excitement and enthusiasm to bring new techniques into the lab's repertoire.

To my supervisory committee members, Professors Jacquetta Trasler and Silvia Vidal, thank you for your comments, critiques and suggestion. A special thank you to Professor Vidal for sharing lab space and expertise, and for allowing her lab to adopt me when visiting Bellini.

Graduate school requires an immense amount of paperwork, particularly for visiting international students. To Aileen Lebofsky and Thomas Leslie, thank you for helping make my move to Montreal as painless as possible and for always helping to find solutions when problems arose. To Kandace Springer for being a cheerleader and always knowing how to calm my nerves. To Ross Mackay, thank you for always having documents ready almost before I ask for them and thank you for always being up for a few minutes of detective work. To the support staff at Place Toulon: Matthew Hunter, Melanie Cotiangco, Angela Russo and Marlene Aardse, thank you for day to day support and answers to so, so, many answers.

To the members of the Rozen Lab, past and present, thank you for all of your advice and support over the years. To Qing Wu and Liyuan Deng: thank you both for sharing your skilled hands with me, for countless hours of genotyping and for being so helpful and willing to teach me how to do new techniques. To Karen Christensen: thank you for being my friend, my editor, my "translator", and my sanity more often than you know. To Renata Bahous: my lab "little sister", thank you for baked goods and for being my go-to immunology buddy. To Daniel Leclerc: for a diligence and thoroughness that provides the backbone to this cast of women. To Leonie Mikael for teaching me how to do a mean Western Blot for helping troubleshoot problems with at least a few dozen of them. To the other members of the lab: Nancy Levesque, Diep Pham, and Yvan Cao; thank you all for celebrating the highs, for troubleshooting the lows, and for enabling a chocoholic.

To the members of the Vidal Lab, past and present, thank you for adopting me during nomadic experiment weeks at the GCC. To Jen Marton: thanks for checking on mice and saving me countless treks

from the Children's. To Michal Pyzik: thank you for teaching me everything I know about staining cells and flow sorting. To the others in the lab- Gab Levia, Mathieu Mancini, Greg Bovin, and Sean Wiltshire- thank you for beers, late night and early morning chats in TC, and some of our more off-the-wall conversations. Stay away from tea ceremonies and Listerine!

To Ana Best: thank you for being a good friend and the best stats guru ever (oh, and for explaining stats in English, so important).

To Julie Knowles, Nancy Laughren, Jeff Miles and all of the other animal technicians at the MGH and GCC, thank you for helping to breed, feed and otherwise take care of my mice.

To the all those who have come and gone at Place Toulon and Bellini: you have all made my time at McGill memorable and have helped to brighten even the most repetitive weeks. To Murielle Akpa: thank you for tea time, lab Saturdays/Sundays, and road trips. To Sabrina Torre: thank you for teaching me how to work with *P. berghei* ANKA and for helping to monitor mice on passage days. To Rebekah van Brugger and Jimmy Kennedy: thank you for helping with passages and inoculums. To Brian Meehan: thank you for so many hours of help with the microscopes. To Lauren Van Der Kraak: thank you for coffee and cake breaks, for falling down the science rabbit hole with me, and for reminding me when it's time for fun distractions. To Erik Larson: thank you for providing an extra set of hands when they were needed most and for many laughs and moments of silliness.

And finally to my fellow graduate students that I have had the pleasure of serving with and during my tenures with the HGSS and the PGSS, thank you for making my personal time as a graduate student better, thank you all for your hard work and dedication to making this experience for others better, thank you for many memories and countless friendships.

ABSTRACT

Folates are B vitamins that are required for the synthesis of nucleotides and amino acids, and for methylation reactions. A common polymorphism in methylenetetrahydrofolate reductase (*MTHFR*, 677C>T), results in enzymatic deficiency and increased risk for several disorders. This polymorphism exists at high frequencies in many populations. Malaria is a parasitic infection that affects nearly 200 million people per year. The folate pathway has been a popular pharmacological target for anti-malarial therapies for many years and, more recently, there has been increased folate intake due to food fortification and vitamin supplementation in many countries. We questioned whether genetic or nutritional disturbances in folate metabolism would affect the response to malarial infection in mice.

Mthfr$^{+/-}$ mice fed standard mouse chow were protected against *Plasmodium berghei* ANKA infection (cerebral malaria). These mice survived longer than *Mthfr*$^{+/+}$ mice and had tissue-specific changes in expression of interferon-γ (IFNγ), peroxisome proliferator-activated receptor-γ (PPARγ) and interleukin-10 (IL-10). *Mthfr*$^{+/-}$ mice also had increases in total splenocytes, total T cells, CD4^{+} and CD8^{+} T cells, and in CCR4^{+} NK cells. Uninfected *Mthfr*$^{+/-}$ mice had changes in immune cell distribution and cytokine expression.

Mice that over-express MTHFR (*MTHFR*^{Tg}) were more susceptible to *P. berghei* ANKA infection when fed standard mouse chow. They died more quickly and had decreases in their NK and CCR4^{+} NK cells, compared with their wild-type littermates. Uninfected *MTHFR*^{Tg} mice had decreases in total splenocytes, total T cells, and CD8^{+} T cells.

Mthfr$^{+/-}$ and *Mthfr*$^{+/+}$ mice fed amino acid-defined control diets (CD, recommended folate level) or folic acid-supplemented diets (FASD, 10-fold higher than recommended) had no survival differences. However, FASD mice died more quickly and had higher parasitemia compared with CD mice. Infected FASD mice had lower numbers of total splenocytes, total T cells, and lower numbers of specific T and NK cell sub-populations. These differences due to diet are similar to those seen in the study of genotype differences. FASD mice also had increased brain TNFα and increased *Bcl-xl/Bak* ratios in the liver that suggested they were more susceptible to *P. berghei* ANKA infection.

We conclude that genetic or dietary disturbances in folate metabolism result in decreased parasite replication, an altered immune response and increased resistance to malaria in mice. These findings support our hypothesis that *MTHFR* 677C>T may have reached high frequencies in human populations as a result of the selective advantage it may have conferred against malaria. This work also has relevance for malaria-endemic regions when considering folic acid fortification or supplementation programs.

RESUMÉ

Les folates sont des vitamines B nécessaires pour la synthèse des nucléotides et des acides aminés, ainsi que pour les réactions de méthylation. Un polymorphisme fréquent chez plusieurs de la méthylènetétrahydrofolate réductase (MTHFR, 677C> T) résulte en un déficit enzymatique et un risque accru pour plusieurs problèmes de santé. Cette variante est très fréquente parmi plusieurs populations. Le paludisme est une infection parasitaire qui touche près de 200 millions de personnes annuellement. Le métabolisme de l'acide folique est une cible pharmacologique habituelle pour les thérapies antipaludiques depuis de nombreuses années. Plus récemment, une augmentation de l'apport en folate dans de nombreux pays a été observée en raison des suppléments vitaminés et de l'enrichissement des aliments. Nous nous sommes alors demandé si des altérations génétiques ou nutritionnelles du métabolisme des folates auraient une incidence sur la réponse à l'infection paludéenne chez la souris.

Des souris *Mthfr*$^{+/-}$ nourries avec une diète standard ont été protégées contre l'infection par *Plasmodium berghei* ANKA (paludisme cérébral). Ces souris ont survécu plus longtemps que les souris *Mthfr*$^{+/+}$ et nous avons noté, dans certains tissus, des changements d'expression pour l'interféron-γ (IFN-γ), le récepteur-γ activé par les prolifé rateurs de peroxysomes (PPAR-γ) et l'interleukine-10 (IL-10). Les souris *Mthfr*$^{+/-}$ ont également une augmentation des splénocytes totaux, des cellules T, des cellules CD4^{+} et CD8^{+}, ainsi que des cellules NK CCR4^{+}. D'autre part, la distribution des cellules immunitaires et l'expression des cytokines étaient perturbées chez les souris *Mthfr*$^{+/-}$ non infectées.

Les souris surexprimant MTHFR (*MTHFR*^{Tg}) étaient plus sensibles à l'infection par *P. berghei* ANKA lorsqu'elles recevaient une diète standard. Elles survivaient moins longtemps et avaient moins de cellules NK et CCR4^{+} que leurs congénères *Mthfr*$^{+/+}$. Les souris non infectées *MTHFR*^{Tg} ont moins de splénocytes totaux, de cellules T totales et de cellules T CD8^{+}.

Les souris *Mthfr*$^{+/-}$ et *Mthfr*$^{+/+}$ qui recevaient une diète d'acides aminés contrôlée (CD, taux de folate recommandé) ou un régime supplémenté en acide folique (FASD, 10 fois supérieure à la norme) montraient un taux de survie similaire. Cependant, les souris FASD sont décédées plus rapidement et avaient une parasitémie supérieure aux souris CD. Les souris FASD infectées avaient une diminution du nombre de splénocytes totaux, des cellules T totales, et du nombre de cellules T spécifiques et des sous-populations de cellules NK. Ces différences dues à l'alimentation sont similaires à celles liées au génotype. Les cerveaux des souris FASD avaient également une augmentation du niveau de TNFα et du rapport *Bcl-xl/Bak* dans le foie, suggérant qu'elles sont plus sensibles à l'infection par *P. berghei* ANKA.

Nous concluons que des perturbations héréditaires ou alimentaires du métabolisme du folate causent : 1) une réduction de la réplication du parasite, 2) une réponse immunitaire modifiée et 3) une résistance accrue au paludisme chez les souris. Ces résultats favorisent notre hypothèse selon laquelle la mutation *MTHFR* 677 C>T est si fréquente dans les populations humaines en raison d'un avantage sélectif contre le paludisme. Nos résultats pourraient aussi influencer les programmes d'enrichissement en acide folique et les programmes de supplémentation dans les zones d'endémie.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

THESIS FORMAT

This thesis is comprised of VI chapters. Chapter I is a review of the literature that is pertinent to the content of this thesis. Chapter II is a combined materials and methods section and Chapters III-V are original research chapters. Chapters III and IV contain portions of a single manuscript published in *Human Mutation* (Meadows, et al., 2014); both chapters have been supplemented with additional data not included in the published manuscript. Chapter V has been submitted for publication as a complete manuscript. Chapter VI is a general discussion about the role of folates in malaria infection and future directions for the project.

Standard naming conventions have been used. Human gene names are capitalized and italicized, murine genes have the first letter capitalized and are italicized. Proteins are capitalized with no italics.

CONTRIBUTIONS OF THE AUTHORS

For chapters III, IV and V the candidate designed the experiment, interpreted the data, and wrote the manuscripts in collaboration with her supervisor RR. QW and LD helped with genotyping and animal manipulations.

In chapters III and IV, the candidate carried out all experiments. MP helped the candidate design the flow cytometry panel and with analyses. QW assisted with all animal manipulations, performed initial genotyping and measured homocysteine levels. ST helped the candidate prepare parasite inoculums and monitor infected mice.

In chapter V, the candidate carried out all animal experiments, flow sorting experiments and immunoblots. RHB performed quantitative PCR experiments and helped with necropsies.

ABBREVIATIONS

Abca1 (ATP-binding cassette sub-family A, member 1)

ANOVA (analysis of variance)

APC (allophycocyanin)

Bak (BCL2-agonist/killer 1)

Bcl-xl (B cell lymphoma-extra-large)

BHMT (betaine homocysteine methyltransferase)

CCR4 (chemokine receptor 4)

CD (control diet)

CO_2 (carbon dioxide)

Cy7 (cyanine 7)

DNA (deoxyribonucleic acid)

DHF (dihydrofolate)

DHFR (dihydrofolate reductase)

DHPS (dihydropteroate synthase)

dpi (days post-infection)

dTMP (deoxythymidine monophosphate)

dUMP (deoxyuridine monophosphate)

FASD (folic acid- supplemented diet)

FITC (fluorescein isothiocyanate)

Gapdh (glyceraldehyde-3-phosphate dehydrogenase)

HLA (human leukocyte antigen)

H^s (sickle hemoglobin)

IFNγ (interferon gamma)

IL-10 (interleukin 10)

IL-12 (interleukin 12)

MC (mouse chow)

mCMV (murine cytomegalovirus)

MTHFR (5,10-methylenetetrahydrofolate reductase)

NK cells (Natural Killer cells)

PCR (polymerase chain reaction)

PE (phycoerythrin)

PerCP (Peridinin chlorophyll protein)

PPARα (peroxisome proliferator-activated receptor alpha)

PPARγ (peroxisome proliferator-activated receptor gamma)

pRBCs (parasite-infected red blood cells)

RBC (red blood cells)

RNA (ribonucleic acid)

SAM (S-adenosyl methionine)

SAH (S-adenosyl homocysteine)

SEM (standard error of the mean)

SST (succinylsulfathiazole)

THF (tetrahydrofolate)

TS (thymidylate synthase)

TNFα (tumor necrosis factor alpha)

WBCSs (White Blood Cells)

WHO (World Health Organization)

Ywhaz (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta)

CHAPTER I
LITERATURE REVIEW

1.1.Folate metabolism

1.1.1. Roles and biological functions of folate metabolism

Folate metabolism is an umbrella term used to describe a complex interplay of one-carbon transfers that result in many of the basic biological building blocks. Figure 1.1 diagrams basic folate metabolism. In mammals, folates and/or folate derivatives from food are absorbed through the intestine and metabolized (Ueland and Rozen, 2005). Gut bacteria can synthesize folates *de novo,* and these folates can then be absorbed by the mammalian host.

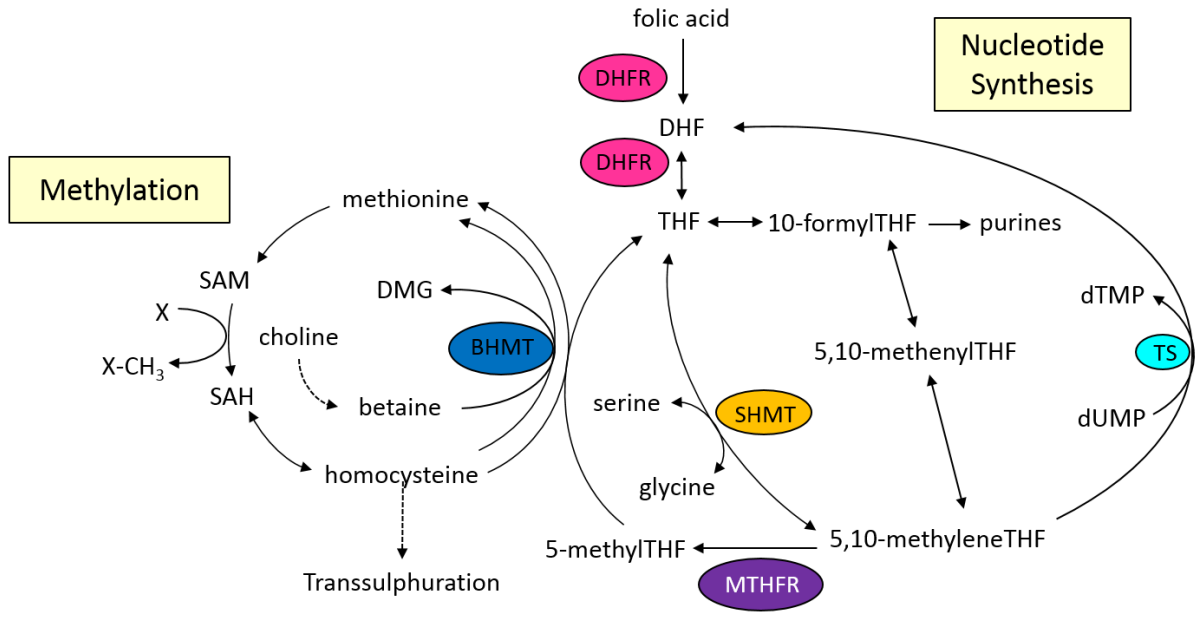


Figure 1.1 Folate Metabolism
A simplified schematic of folate metabolism in mammals. Enzymes are in colored circles. Broken arrows indicate that a given change has more than one step in the process. Arrows point in the direction of the reaction. DHFR (dihydrofolate reductase); DHF (dihydrofolate); THF (tetrahydrofolate); dTMP (deoxythymidine monophosphate); TS (thymidylate synthase); dUMP (deoxyuridine monophosphate); MTHFR (5,10-methyleneTHF reductase); BHMT (betaine homocysteine methyltransferase); DMG (dimethylglycine); SAM (S-adenosyl methionine); SAH (S-adenosyl homocysteine); SHMT (serine hydroxymethyltransferase).

In the body, all folates must be converted into the useable tetrahydrofolate (THF) form for all subsequent reactions. THF and its derivatives, taken from food or scavenged from gut bacteria synthesis, are absorbed in the intestine and further metabolized into needed forms for methylation reactions, nucleotide synthesis, etc. Upon consumption, synthetic folic acid is reduced by dihydrofolate reductase (DHFR), first to dihydrofolate, and then it is further reduced to tetrahydrofolate.

Once reduced to THF, there are several ways that the THF can be used depending on current metabolic requirements. THF can be converted into 10-formylTHF. 10-formylTHF can be used for purine synthesis, or it can be converted to 5,10-methenylTHF and then reduced to 5,10-methyleneTHF. 5,10-methyleneTHF can donate a carbon to thymidylate synthase (TS) for thymidine synthesis, or aid in serine synthesis by serine hydroxymethyltransferase (SHMT), or be reduced by 5-10 methyleneTHF reductase (MTHFR) to 5-methylTHF, the primary circulating form of folate. 5-methylTHF can reduced by methionine synthase (MTR) and the residual methyl group used to re-methylate homocysteine into methionine. Methionine can be used in protein production or it can be converted to S-adenosylmethionine (SAM) and used for methylation reactions. There are additional metabolites such as choline and vitamin B_{12} that can feed into the pathway. Methionine can also be derived from choline metabolism. Betaine, a choline metabolite, can also provide a methyl group to re-methylate homocysteine into methionine.

The products of folate metabolism are key for in cellular growth and proliferation. Folate derivatives are cofactors in the synthesis of nucleotides, which are necessary for the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Folate derivatives are needed to convert

the toxic amino acid homocysteine into methionine for use in protein synthesis and SAM-dependent methylation. Other amino acids used in protein synthesis, such as serine and glycine, are also products of folate metabolism.

1.1.2. Dietary sources of folates

Fruits, vegetables, legumes, meats and dairy products are all great sources of dietary folates (NIH, 2012). Of these, leafy greens and liver have some of the highest naturally occurring folate levels, and yeast is also quite high. By 1998, in an effort to minimize the incidence of neural tube defects, the United States (USFDA, 1996) and Canada (Canada, 1998) had fortified cereal and grain products with folic acid to boost dietary intake (Boulet, et al., 2008; De Wals, et al., 2007); other countries have followed suit (EUROCAT, 2015).

1.2.Dietary folate

1.2.1. Dietary fortification and folic acid supplementation

As mentioned, there has been considerable effort in the last few decades to increase folate intake to minimize the incidence of neural tube defects and other disorders (reviewed in (Smith, et al., 2008)). In 1996 and 1998 respectively, the United States (USFDA, 1996) and Canada (Canada, 1998) implemented mandatory fortification of cereals and grains to boost folate intake in the population. From studies done in the nearly two decades since implementation, it seems that the incidence of neural tube defects has decreased (Boulet, et al., 2008; De Wals, et al., 2007). Today, folic acid fortification of food is routine in many countries worldwide (EUROCAT, 2015).

Periconceptional folic acid supplementation and supplementation throughout pregnancy is also recommended (CDC, 1991). Folic acid is a synthetic form of the vitamin that is used in food fortification and vitamin supplements. Supplementation during pregnancy may also have other benefits such as minimizing anemia during pregnancy (WHO, 2012). Around the world, the daily recommended intake of folic acid ranges from 0.2mg - 0.4mg (Krawinkel, et al., 2014), but most countries adhere to the WHO's recommendation of 0.4mg daily (WHO, 2012). Higher doses, such as 4- 5 mg/day (approximately 10-fold higher than the recommended daily intake) are sometimes recommended for women with a high-risk pregnancy (CDC, 1991; Wilson, et al., 2007).

1.2.2. Biological consequences of high levels of folic acid

Many have questioned whether fortification or supplementation with folic acid is actually a good idea (reviewed by Crider, et al. (2011) and Smith, et al. (2008)). Unmetabolized folic acid appears at greater levels in the circulation, compared to pre-fortification values (Kalmbach, et al., 2008). Fortification and supplementation has led to increased blood folate in some North American sub-groups and in other countries that fortify with folic acid (Britto, et al., 2014; Kalmbach, et al., 2008; McDowell, et al., 2008; Morris, et al., 2010; Troen, et al., 2006).

Studies are emerging that allude to potential negative consequences of high folate intake. Though, it is not clear whether the concerns relate to the increase in the total folate pool or to the unmetabolized folic acid. Concerns have been raised about immune dysfunction (Troen, et al., 2006) and increased inflammation (Protiva, et al., 2011). Lower performance in cognitive tests in older women has also been reported (Morris, et al., 2010). In a recent paper, it has been suggested

that too much folic acid during pregnancy may have an impact on the psychomotor development of offspring (Valera-Gran, et al., 2014).

It has also been suggested that high levels of folic acid in the diet may interact with B_{12} metabolism. High levels of serum folate in older patients with B_{12} deficiency leads to exacerbated symptoms (Miller, et al., 2009; Morris, et al., 2007). Subsequent work has demonstrated that high serum folate in these elderly individuals impairs the function of at least two B_{12}-dependent enzymes (Selhub, et al., 2009). Studies in India have shown that B_{12} deficiency and high blood folate *in utero* result in higher levels of adiposity and insulin resistance in offspring (Yajnik, et al., 2008).

In our laboratory we have worked with folic acid supplemented diets that are either 10x or 20x the recommended daily intake values (Reeves, 1997). In murine models, both 20x (Pickell, et al., 2011) and 10x (Mikael, et al., 2013a) folic acid intake negatively impact embryonic development. Embryonic delays, increases in resorptions, and higher susceptibility to birth defects in offspring were observed in mothers fed 10x and 20x folic acid supplemented diets. Our lab has also reported that 10x levels of folic acid in the diet lead to liver injury through alterations of lipid metabolism (Christensen, et al., 2015). Others have suggested that *in utero* exposure to folic acid supplementation (2.5x level of the control diet) may increase the risk of offspring later developing mammary tumors (Ly, et al., 2011), but may decrease their risk of developing colorectal tumors (Sie, et al., 2011). The 10x folic acid diet used in our lab (Christensen, et al., 2015; Mikael, et al., 2013a), results in a 3-fold increase in plasma folate (Mikael, et al., 2013a), the same degree of

increase that has been reported in the United States since mandatory fortification was implemented (Pfeiffer, et al., 2012).

1.3. Methylenetetrahydrofolate reductase (MTHFR)

1.3.1. Mutations and polymorphisms in *MTHFR*

MTHFR (EC 1.5.1.20) is the enzyme responsible for the reduction of 5,10-methyleneTHF to 5-methylTHF, the primary circulating form of folate in mammals. 5-MethylTHF will then go on to re-methylate homocysteine into methionine, which can in turn be used for protein synthesis or converted to the ubiquitous methyl donor SAM.

Deficiency in MTHFR results in the build-up of excess homocysteine in the blood (hyperhomocysteinemia) and global DNA hypomethylation in humans and mice (Chen, et al., 2001; Friso, et al., 2013). Hyperhomocysteinemia is associated with pro-inflammatory responses, oxidative stress and other cellular disturbances (Lazzerini, et al., 2007; Mikael, et al., 2013b; Papatheodorou and Weiss, 2007). Altered nucleotide ratios have also been reported in MTHFR deficiency (Lawrance, et al., 2009).

In humans, *MTHFR* is located on chromosome 1p36.3 (Goyette, et al., 1994). Since its mapping in 1994, mutations of varying severities have been reported (Goyette, et al., 1996; Goyette, et al., 1995; Leclerc, et al., 2005). Mutations in *MTHFR* are the most common inborn errors in folate metabolism (Rosenblatt, et al., 1992). As with most enzymes, the severity of the phenotype is a function of where the mutation occurs in the enzyme. Those mutations in

functional domains or those that truncate the protein tend to cause more severe phenotypes than minor changes in non-functional domains.

There are many clinical reports of patients with severe *MTHFR* mutations. Severe deficiency is classified as mutations that lead to less than 20% residual enzyme activity and result in excess homocysteine in the blood (homocysteinemia) and the urine (homocystinuria) (Rosenblatt and Fenton, 2001). Each clinical presentation varies, but in general homocystinuria is associated with neurological symptoms including developmental delays, motor and gait abnormalities, seizures and other psychiatric features (Thomas and Rosenblatt, 2005). There are a few reported cases of asymptomatic patients with severe mutation in *MTHFR* (Goyette, et al., 1994; Haworth, et al., 1993).

In addition to the mutations known to cause severe *MTHFR* deficiency, there are several known polymorphisms (Ueland and Rozen, 2005). Each polymorphism has associated clinical implications, and their frequencies vary across populations.

1.3.2. *MTHFR* 677 C→ T in humans

Of the human polymorphisms, the 677 C→T mutation (c.665C→T, rs1801133) is the best characterized (Frosst, et al., 1995). This mutation is a base pair change from a cytosine (C) to a thymine (T). The DNA change results in an amino acid change at the 222nd amino acid position in the MTHFR protein, substituting an alanine for a valine (p.Ala222Val). This amino acid change result in a thermolabile enzyme with reduced activity (Frosst, et al., 1995; Kang, et al., 1988).

The frequency of the 677TT genotype is high in southern Europe along the Mediterranean (15-20%), most notably in Spain and Italy. The highest frequencies have been recorded in

populations of Hispanic descent in the United States, Mexico and Colombia (~25-30%) (Figure 1.2) (Gueant-Rodriguez, et al., 2006; Leclerc, et al., 2005; Meadows, et al., 2014). Although limited, there are some data available for African populations. Algeria (Houcher, et al., 2010) and Tunisia (Rouissi, et al., 2009) in northern Africa have higher frequencies (~14% and ~11% respectively) than Benin (Gueant-Rodriguez, et al., 2006), Burkina Faso (Angius, et al., 2007) and Ghana (Rosenberg, et al., 2002) (all <1%) in sub-Saharan Africa. The polymorphism is part of a common haplotype shared among diverse populations, suggesting a shared ancestral history; selective advantage could have assisted in spreading the variant through populations (Rosenberg, et al., 2002).

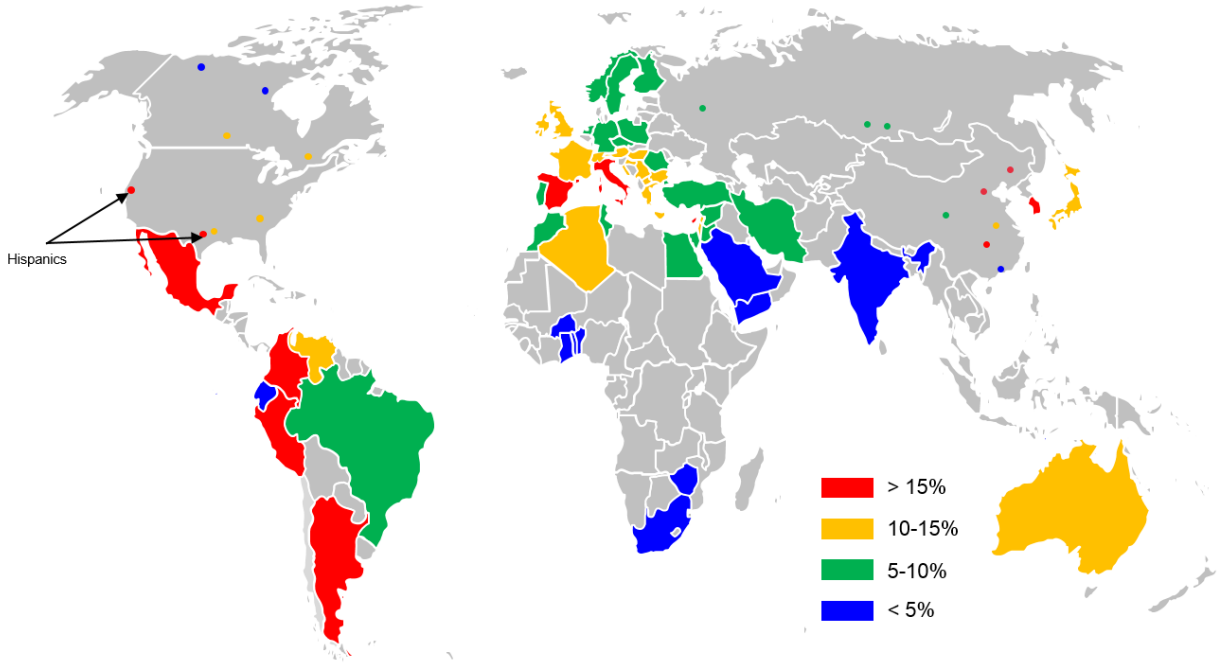


Figure 1.2 *MTHFR* 677 TT frequencies around the world
Reported *MTHFR* 677TT frequencies from around the world (Alfthan, et al., 2003; Meadows, et al., 2014). Circles indicate the frequencies reported for sub-populations or regions in a given country. Uncolored countries have no reported data. (Meadows, et al., 2014)

MTHFR 677C→T results in mild hyperhomocysteinemia, and it was identified as the first genetic risk factor for spina bifida (van der Put, et al., 1995). This polymorphism has also been linked to many other disorders including other birth defects, pregnancy complications and adverse reproductive outcomes (Bozovic, et al., 2011; Guan, et al., 2005; Hobbs, et al., 2000; Naidu, et al., 2007; Rah, et al., 2012; Rajkovic, et al., 2000; Shotelersuk, et al., 2003). There is evidence that it increases risk for some neuropsychiatric disorders (Bjelland, et al., 2003; Joober, et al., 2000) and Alzheimer's disease (Bi, et al., 2009). Increased risk for vascular and blood disorders are also reported (Choi, et al., 2003; Frosst, et al., 1995).

The role of MTHFR677C→T in cancer is complicated and risk varies between populations and studies, and is dependent on individual folate status as well. MTHFR677C→T has also been reported to increase the risk for some types of cancers, including breast (Ergul, et al., 2003; Gao, et al., 2009; Hosseini, et al., 2011), pancreatic (Li, et al., 2005a), and esophageal (Langevin, et al., 2009) cancers. In contrast, the 677 variant may reduce the risk of other cancers, including colorectal cancer (Chen, et al., 1996; Cohen, et al., 2003) and certain types of leukemia (Skibola, et al., 1999) when dietary folate is adequate.

Despite its known negative consequences on human health, the high frequency of the *MTHFR* 677C→T allele in many populations has been largely unexplained. One report suggested that during periods when there is low availability of dietary folate, 677TT may have provide protection against megaloblastic anemia of pregnancy (Green and Miller, 1999). Additionally, it has been suggested that, with sufficient folate levels, mutation in MTHFR diverts methylenetetrahydrofolate towards nucleotide synthesis increasing nucleotide pools for DNA

synthesis or repair (Ueland, et al., 2001). There is a clinical report that young people in West Africa with the *MTHFR* 677C→T allele are protected against chronic hepatitis B (Bronowicki, et al., 2008). This report suggests that positive selection for protection against infection (such as hepatitis B) may be a potential selection mechanism. In any case, there is little experimental evidence to support these hypotheses for the persistence of 677T in human populations.

1.3.3. MTHFR mouse models

1.3.3.1. *Mthfr*-deficient mice

To study the effects of *Mthfr* deficiency, a murine model has been developed. In mice, the *Mthfr* gene maps to chromosome 4 (Frosst, et al., 1996). A *neo*^{r} insertion into the *Mthfr* gene was used to create a null *Mthfr* allele (Chen, et al., 2001). The *Mthfr* knockout mouse model has been backcrossed onto both a BALB/c (Chen, et al., 2001; Schwahn, et al., 2004) and C57Bl/6J (Knock, et al., 2008) background. Both heterozygous offspring (*Mthfr*$^{+/-}$) and homozygous knockout offspring (*Mthfr*$^{-/-}$) are viable, but there are strain differences in the survival rate of the homozygous knockout mice (Lawrance, et al., 2011). *Mthfr*$^{-/-}$ mice are more viable on a C57Bl/6J background.

In humans, 677TT results in partial deficiency of MTHFR and mild hyperhomocysteinemia (Frosst, et al., 1995). In mice, *Mthfr*$^{+/-}$ animals experience a similar enzymatic deficiency and elevated plasma homocysteine (Chen, et al., 2001). *Mthfr*$^{-/-}$ mice are a model for severe MTHFR deficiency (Rosenblatt, et al., 1992).

1.3.3.2. *MTHFR*-overexpressing mice

To comparatively study the effect of MTHFR expression, whether over-expression or under-expression, a transgenic murine model has also been developed by the Rozen lab (Celtikci, et al., 2008). The *MTHFR* transgenic mice ($MTHFR^{Tg}$) have a copy of human *MTHFR* inserted into the *HPRT* locus on the X-chromosome. $MTHFR^{Tg}$ mice have been back-crossed onto a C57Bl/6J background (Celtikci, et al., 2008). $MTHFR^{Tg}$ mice express both human and murine MTHFR. As their expression of murine MTHFR is similar to $Mthfr^{+/+}$ mice, the total MTHFR expression in $MTHFR^{Tg}$ mice is approximately 2-fold higher than $MTHFR^{Wt}$ littermates. $MTHFR^{Wt}$ mice have similar MTHFR levels to $Mthfr^{+/+}$ mice. Due to the incorporation of the *MTHFR* gene on the X-chromosome, only male mice are used in experiments to ensure that the over-expression of *MTHFR* is uniform across all mice.

1.3.4. Diet models

There is a complex relationship between dietary folate status and mild MTHFR deficiency (reviewed by Jacques and Choumenkovitch (2005)). Defined diets with variable amounts of folic acid and other one-carbon metabolites have been used extensively in our lab and others. In the Rozen lab, we regularly use folic acid deficient diets (Li, et al., 2005b), 20x high (Pickell, et al., 2011) and 10x high (Mikael, et al., 2013a) folic acid diets, and choline deficient diets (Chan, et al., 2010b). All diets are based on the recommended daily intake guidelines (Reeves, 1997). Typically the dietary formulas used in our lab contain succinylsulfathiazole, and antibiotic that targets bacteria residing in the gut, to ensure that the folates being consumed are taken from the diet and not scavenged from gut bacterial production *de novo* (Sepehr, et al., 2003).

1.3.5. Studies using the *Mthfr*, *MTHFR* and dietary mouse models

Using the *Mthfr*$^{+/-}$ mouse model, the role of *Mthfr* deficiency and how it may be affected by folates and one-carbon metabolites in the diet has been studied for many of the disorders linked to MTHFR 677C→T. Many of the murine studies of *Mthfr*-deficiency and/or high or low dietary folate support the observations of human clinical studies. Murine studies also allow for exploration of potential mechanisms.

In pregnancy studies, *Mthfr*-deficient mice on folate deficient diets (7x lower than recommended daily intake (Reeves, 1997)) experienced more embryonic losses, birth defects, and other pregnancy complications than their wild-type littermates or those mice on folate sufficient diets (Li, et al., 2005b; Pickell, et al., 2009). It has also been shown that high levels of folic acid in the diet (either 10x or 20x over the recommended daily intake (Reeves, 1997)) results in embryonic losses, pregnancy delays and cardiac defects (Mikael, et al., 2013a; Pickell, et al., 2009). Deficiency of choline, another one-carbon metabolite, also resulted in cardiac defects and restriction of embryonic growth (Chan, et al., 2010b). In another study, it was demonstrated that high levels of folic acid *in utero* results in glucose intolerance and insulin resistance in male offspring (Huang, et al., 2014).

In studies of coronary artery disease, *Mthfr*–deficient mice were shown to be more at risk for atherosclerosis, likely due to changes in cholesterol metabolism (Mikael, et al., 2006). High folic acid (10x over the recommended daily intake (Reeves, 1997)) has also been shown to alter lipid metabolism in *Mthfr*-deficient mice (Christensen, et al., 2015). Studies of brain morphology and cognitive development have shown that mice with severe *Mthfr*-deficiency have behavioral

anomalies, memory impairments and structural changes in their brains (Jadavji, et al., 2012). High levels of folic acid in pregnancy have been shown to affect brain-development genes, and offspring exhibit anxiety-like behavior and hyperactivity (Barua, et al., 2014).

Mthfr-deficiency may also affect fertility as in fertility studies, strain differences in *Mthfr*-deficient mice have been described. BALB/c mice who are *Mthfr*-deficient are infertile, but *Mthfr*-deficient C57Bl/6J mice are not, despite having decreased sperm counts and changes in testicular morphology (Chan, et al., 2010a). Treatment with betaine, the one-carbon donor for folate-independent homocysteine remethylation partially rescues infertility in *Mthfr*-deficient BALB/c mice (Kelly, et al., 2005). Paternal folate deficiency has been shown to result in cranio-facial birth defects (Lambrot, et al., 2013).

Murine studies have also been used to investigate the effects of *Mthfr*-deficiency and/or low dietary folate on cancer incidence and progression. In colon cancer studies, the Rozen lab has shown that low dietary folate results in spontaneous tumor development and that *Mthfr*-deficiency enhances the tumor development (Knock, et al., 2006). Others have shown that folic acid supplementation had little to no effect on inflammation or tumorgenesis in a colitis-induced cancer model (MacFarlane, et al., 2013). The Rozen lab also showed that in mice that are predisposed to colon cancer, low dietary folate in combination with *Mthfr*-deficiency resulted in lower tumor numbers (Lawrance, et al., 2009). Others, working with the same genetically predisposed mice, have made similar observations. They have found that feeding these mice methyl donor deficient diets (including folate) also results in lower tumorgenesis (Kadaveru, et al., 2012). These studies suggest that as in humans, murine cancer is quite a complex phenotype.

These differences may be related, in part, to the high metabolic requirement of folates for tumor growth and proliferation. Dietary folate seems to be most important once tumorgenesis has already occurred (Lawrance, et al., 2009).

The *MTHFR*^{Tg} mice were generated to study the effect of MTHFR dose on methotrexate response; methotrexate is a DHFR inhibitor used to treat certain cancers and inflammatory disorders (Celtikci, et al., 2008). The *MTHFR*^{Tg} mice were shown to have similar RBC changes after treatment with methotrexate that *Mthfr*-deficient mice did, but they were protected from methotrexate-induced hyperhomocysteinemia. Due to the close relationship between host and metabolism and that of different pathogens, the mice were also used to investigate the hypothesis that the 677C→T variant may increase resistance to murine cytomegalovirus (mCMV) infection (Fodil-Cornu, et al., 2009). In these experiments, *Mthfr*$^{-/-}$ mice were shown to be more resistant to mCMV infection than wild-type or *Mthfr*$^{+/-}$ mice. *Mthfr*$^{-/-}$ and *Mthfr*$^{+/-}$ mice had significantly lower viral titers in the spleen than wild-type animals. The numbers of immune cells were significantly increased in *Mthfr*$^{-/-}$ mice as compared to wild-type. *MTHFR*^{Tg} had higher viral titers and were more susceptible to mCMV infection than the wild-type. Together, these results suggested that low levels of *Mthfr* activity provide protection against this viral pathogen.

1.4.Malaria

1.4.1. Global burden of malaria

Malaria, caused by *Plasmodium* parasites, is endemic to wet environments near the equator. The parasites have complex life cycles with part of the cycle in the gut of *Anopheles*

mosquitoes and other stages in the bloodstream and organs of infected mammalian hosts. A mammalian host is infected when an infected female *Anopheles* mosquito takes a blood meal. Once in the host, parasites infect the red blood cells (RBCs) where they replicate, grow, mature, and infect other RBCs (Fortin, et al., 2002b). During this process of growth and replication, the host's RBCs are destroyed. During the blood stage, hosts are able to infect other *Anopheles* hosts during a blood meal. At maturity, parasites are able to infect other organs, especially the liver. In some *Plasmodium* strains, the parasite also sequesters itself inside various tissues (Franke-Fayard, et al., 2010; Longley, et al., 2011). Malaria is also a complex disease; its presentation (phenotype) is influenced by interactions between the host's genes and the parasite's genes. The infection outcome is also dependent upon the social and political environment when the infection occurs; access to quality medical care vastly improves the outcome of infection (Fortin, et al., 2002b; WHO, 2014).

In 2013 (the most recent year for which complete data are available), there were 198 million reported cases of malaria, and of those there were approximately 584,000 fatal cases (WHO, 2014). These numbers are an improvement from earlier reports, but malaria's impact on economic growth and its relationship to poverty still exists (Sachs and Malaney, 2002). Malaria transmission regularly occurs in the 97 countries that have been identified as being malaria-endemic; leaving an estimated 3.2 billion people at risk of being infected with malaria and developing disease (WHO, 2014). Children, pregnant women, and poverty-stricken individuals are at greatest risk for poor outcomes (Nzila, et al., 2014; WHO, 2014). Organizations such as the WHO are working to protect these poor and vulnerable communities, but the more the disease is understood, the more effective interventions will be.

1.4.2. Malaria in humans

There are five strains of malaria that can infect humans: *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi.* Of these strains, *P. falciparum* and *P. vivax* are most common, with the majority of complications in patients with *P. falciparum* infection. Uncomplicated cases of malaria results in cyclical fevers, mild anemia and the aches, pains and "flu-like" symptoms that typically accompany febrile illnesses (WHO, 2015). Diagnosis is confirmed when a thin-layer smear of blood is Giemsa stained and parasites identified within patient's red blood cells (RBCs).

Complications may arise when the infection is left untreated particularly in patients with *P. falciparum* infection, or in pregnant women and small children. In pregnancy, maternal death, miscarriage, stillbirth and low birth weight are additional complications that may arise (WHO, 2015). Normal malaria complications include severe anemia, splenomegaly or possible spleen rupture, kidney and liver failure, respiratory failure, cerebral malaria, and death. Cerebral malaria encompasses such symptoms as paralysis, seizure, brain hemorrhage and/or death.

1.4.3. Malaria and genetics

Pathogens such as malaria, often kill quickly and in the prime of life. Due to this nature, they are thought to be one of the leading selective forces that have helped to shape the human genome. Several genetic mutations with negative health consequences have persisted in human populations because they confer protection against infections, which exerts positive selection on these negative traits.

Malaria in particular has often been reported to be a positive selection factor, to the extent that it has been called "the greatest selective pressure on the human genome in recent history"

(Kwiatkowski, 2005). The classic example of such a mutation is sickle cell hemoglobin, the gene responsible for sickled hemoglobin (H^S). Originally observed and hypothesized based on lower infection rates in patients with H^S (Allison, 1954), it was later shown experimentally (Beutler, et al., 1955) that H^S offers protection from malaria infection. Other hemaglobinopathies have also been shown to offer protection against malaria including α-thalassemia (Flint, et al., 1986), β-thalassemia (Willcox, et al., 1983), and glucose-6-phosphate dehydrogenase deficiency (Ruwende and Hill, 1998). It has been proposed that these genes have maintained prevalence in the Mediterranean, a once malaria-rich region, because they protect against the disease (Fortin, et al., 2002b). Other factors such as the Duffy surface antigen on RBCs (Miller, et al., 1976), HLA type (Hill, et al., 1991), and pyruvate kinase deficiency (Berghout, et al., 2012) have also been shown to improve the outcome of malaria infections. Results of genome-wide studies of malaria susceptibility in humans have been mixed (Culleton, et al., 2005; Griffiths, et al., 2005; Timmann, et al., 2007); many of the mutations, such as sickle cell trait, classically associated with protection from malaria have not shown up in most genome-wide studies. Instead, significant findings from these genome-wide studies have helped us to start unraveling the complex interactions between the host and pathogen (reviewed in Khor and Hibberd (2011)).

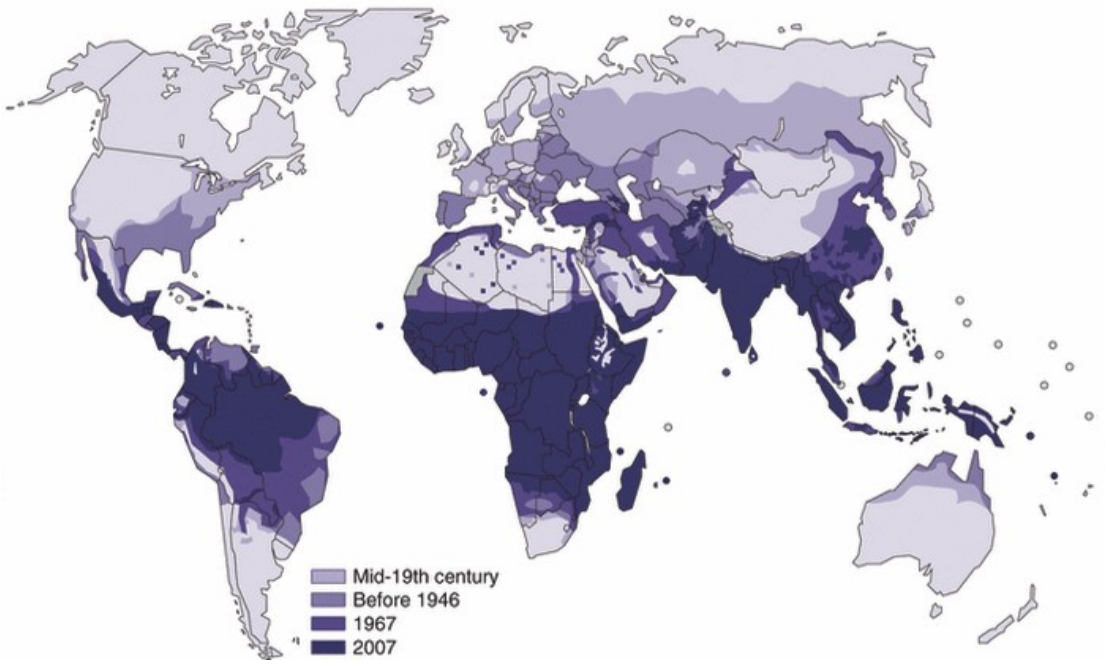


Figure 1.3 World distribution of malaria, from mid-19th century to 2007
World map of the changing distribution of malaria as the WHO and others have moved towards control and eradication of the parasite (Mendis, et al., 2009)

Many of the genes associate with malaria protection have high frequencies in the Mediterranean region. Historically, the malaria endemic region was much wider than it is today (Figure 1.3) and included the Mediterranean region. Improvements in medical care and sanitation, combined with targeted efforts to control and eradicate mosquito carriers in specific regions have helped to narrow the malaria endemic region (Mendis, et al., 2009). In the last few generations, most severe cases of malaria are found in sub-Saharan Africa and Southeast Asia, where the parasite still kills hundreds of thousands each year (Mendis, et al., 2009; WHO, 2014). Despite its currently more contained region, the malaria parasite has left lasting effects on the genomes of those from the Mediterranean and in other regions where it once thrived.

1.4.4. Malaria mouse models

Many of the human diseases thought to persist in populations due to positive selection have been confirmed using murine genetic studies. Confirming human studies in sickle cell (Allison, 1954; Beutler, et al., 1955), malaria survival positively correlates with the level of H^s expression in transgenic H^s mice (Hood, et al., 1996). Mice that express H^s are also protected against cerebral malaria (Ferreira, et al., 2011). Murine studies in β-thalassemic mice (Roth, et al., 1988) also confirm human studies (Willcox, et al., 1983). Conversely, murine studies originally identified pyruvate kinase deficiency as protective in mice (Min-Oo, et al., 2004; Min-Oo, et al., 2003) and human studies later confirmed this finding (Ayi, et al., 2008; Berghout, et al., 2012).

To better understand malaria and its variable phenotypes, mouse models have been developed to study them experimentally. *P. falciparum,* the malaria infection that most commonly causes complications in humans, is studied using two different murine parasites, *P. chabaudi* AS and *P. berghei* ANKA. The susceptibility of the mouse to infection is dependent upon the strain of the mouse and the parasite; the phenotype (outcome of infection) is similarly strain dependent. Murine studies have identified many resistance and susceptibility loci to these parasites, but not all of the mechanisms have been elucidated (reviewed by Fortin, et al. (2002a) and Longley, et al. (2011)).

In C57Bl/6 mice, the outcome of *P. berghei* ANKA infection results in cerebral malaria, a severe complication of *P. falciparum.* In such susceptible mouse strains, cerebral malaria symptoms may include paralysis, seizures, brain hemorrhages and death. The onset of cerebral symptoms is between 5 and 7 days post-infection (dpi), and most susceptible mice succumb to

infection between 8 and 10dpi (Hood, et al., 1996; Min-Oo, et al., 2003; Roth, et al., 1988). If infected mice survive the cerebral phase, they will eventually die from severe anemia (around 21 dpi). The prevailing theory from experimental evidence is that cerebral malaria results, at least in part, from the dysregulation of the immune response mounted by the host (Hunt and Grau, 2003; Lamb, et al., 2006; Patel, et al., 2008). The immune response is characterized by extremely high levels of pro-inflammatory cytokines such as interferon-γ (IFNγ) and tumor necrosis factor α (TNFα), while expression of anti-inflammatory cytokines such as interleukin-10 (IL-10) and transcription growth factor β (TGFβ) are often decreased (Hunt and Grau, 2003).

Genetic manipulation and neutralization of these cytokines have been used to help elucidate their relationships and how the immune response leads to cerebral malaria symptoms. High levels of TNFα are present in early stages of infection and *in vivo* neutralization of TNFα can also prevent cerebral malaria symptoms by down-regulating the expression of endothelial receptors (Grau, et al., 1987). As might be expected, mice with a genetic knockout of TNFα-signaling were also resistant to cerebral malaria (Rudin, et al., 1997a). *In vivo* neutralization of IFNγ, in mice that are susceptible to infection, protects susceptible mice by helping to neutralize downstream expression of TNFα (Grau, et al., 1989a). Experiments with *P. berghei* ANKA in IFNγ knockout mice, showed that IFNγ was required to induce cerebral pathology (Rudin, et al., 1997b). IL-10-deficiency in resistant strains rendered deficient mice susceptible to *P. berghei* ANKA infection, and administration of recombinant IL-10 in susceptible mice protected against cerebral symptoms by down-regulating IFNγ, but not TNFα, expression (Kossodo, et al., 1997).

Modulation of other cytokines and immune regulators have also been shown to protect against cerebral malaria. Treatment of patients with rosiglitazone, a PPARγ agonist, not only improves the clearance of parasitized red blood cells (pRBCs) and the outcome of *P. chabaudi chabaudi* infection, but it also decreases the inflammatory response and improves the outcome of *P. berghei* ANKA infection (Serghides, et al., 2009). A genome-wide analysis of inbred mouse strains following *P. berghei* ANKA infection also points to *Pparγ* as a locus important in malaria survival (Bopp, et al., 2010). Knockouts of ATP-binding cassette sub-family A, member 1 (*Abca1*), *Abca1*$^{-/-}$, have fewer pro-inflammatory microparticles that have been shed from endothelial cells and are circulating in the plasma. They also have decreased brain TNFα compared with wild-type littermates and are protected from cerebral malaria (Combes, et al., 2005). In general, it appears that disruption of many of the pathways that drive inflammation will lead to a more preferable outcome in cerebral malaria (Berghout, et al., 2010; Campanella, et al., 2008; Lu, et al., 2006; Patel, et al., 2008; Schmidt, et al., 2011).

1.4.5. Folate metabolism in malaria

As in mammals, folate metabolism in the parasite is a requisite to synthesize nucleotides and amino acids and for methylation reactions (Muller and Hyde, 2013), and involves many similar enzymes. One of the most striking differences between malaria and its hosts is that, unlike its hosts, the malaria parasite can, if necessary, synthesize folate *de novo* from other precursors (Muller and Hyde, 2013; Nzila, et al., 2005a; Nzila, et al., 2005b). The parasite's dihydrofolate reductase (DHFR) enzyme also has a synthetase domain that the mammalian host does not have.

For many years, anti-folate drugs have been used to inhibit growth of pathogens, including the malarial parasite (reviewed in (Muller and Hyde, 2013)). Two folate-dependent enzymes, DHFR and dihydropteroate synthase (DHSP) are popular targets in malaria (Muller and Hyde, 2013; Nzila, et al., 2005a). Mammals do not have a DHSP enzyme, so it is also an ideal target for anti-malarial drugs. Blocking these enzymes inhibits *de novo* folate synthesis in the parasite and these drugs are used in combination therapy with other drugs derived from artemisinin to target parasites within the RBCs (WHO, 2014). Unfortunately, resistance to drugs that target folate synthesis has developed in some malaria-endemic regions. The latest reports of drug efficacy and resistant malaria strains is included in the WHO's annual malaria report (WHO, 2014).

1.4.6. Folic acid supplementation and anti-malarial drugs

The WHO recommends that the anti-malarial drugs targeting folate metabolism be prescribed in combination therapy with an artemisinin derivative (WHO, 2014). To limit the impact of this disease, one preventative strategy has been the prescription of folate-blocking anti-malarials to late-term pregnant women and to children during routine health care screenings (Nzila, et al., 2014; WHO, 2014). In these preventative strategies, a combination therapy of sulfadoxine and pyrimethamine (SP) is administered. Both drugs target folate metabolism, but at different parts of the pathway; sulfadoxine is a competitive inhibitor in the synthesis of the folate backbone, while pyrimethamine directly targets DHFR function. The latest reports show that these prevention programs are not reaching the desired level of efficacy and dissemination (WHO, 2014). This may be due, in part, to increased frequency of folic acid fortification (EUROCAT, 2015) and the folic acid supplementation also recommended by the WHO during pregnancy (WHO,

2012). Fortification and supplementation rely on folic acid, a stable, synthetic folate with high bioavailability. Folic acid may actually inhibit folate-dependent enzymes and transporters (Smith, et al., 2008). Several malaria-endemic countries such as Ghana and Cote d'Ivoire have already implemented dietary fortification programs (EUROCAT, 2015). There is considerable over-lap of malaria-endemic countries and those that have implemented mandatory folic acid fortification (Figure 1.4). There is a growing body of clinical evidence that suggests folic acid fortification and/or supplementation may interfere with malaria treatment (and subsequent eradication efforts) (Carter, et al., 2005; Dzinjalamala, et al., 2005; Mulenga, et al., 2006; Nzila, et al., 2014). Further studies are needed to help better inform these practices. '

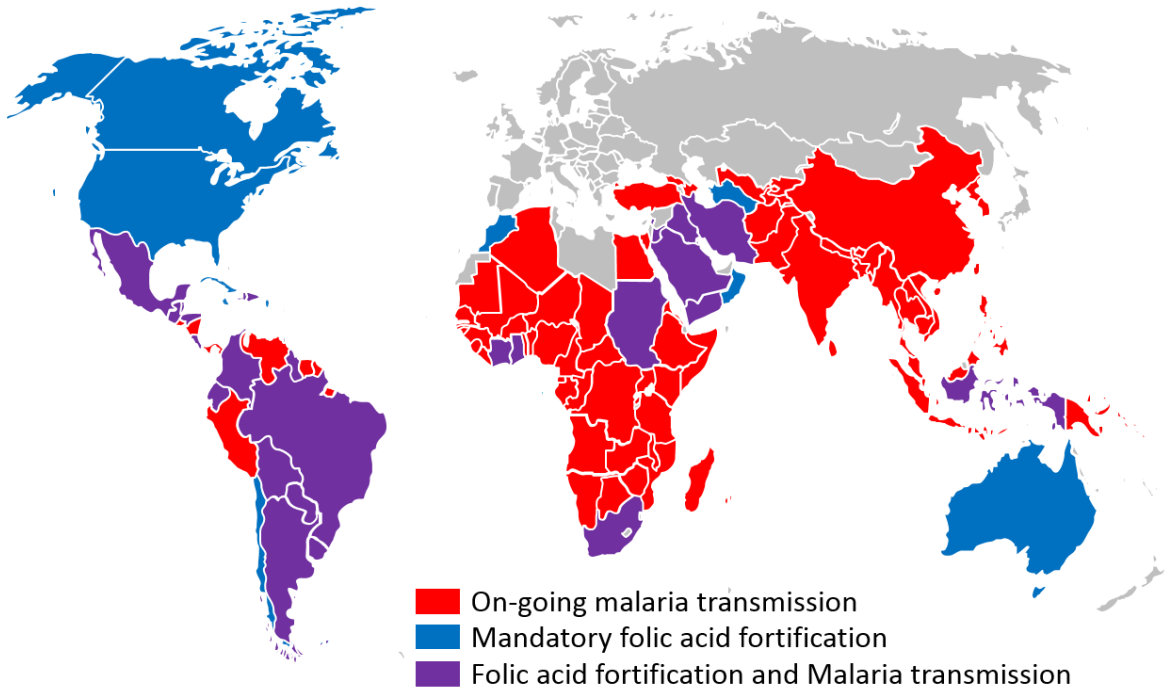


Figure 1.4 On-going malaria transmission and folic acid fortification
Map comparing over-lap of countries with on-going malaria transmission (WHO, 2014) and mandatory folic acid fortification (EUROCAT, 2015) around the world. Countries in grey do not have reported malaria transmission nor mandatory folic acid fortification.

THESIS RATIONALE

Although the importance of folate metabolism during malaria infection has been known for many years, the effect of a genetic disturbance in folate metabolism during infection has not been studied. A common polymorphism in an important enzyme in folate metabolism, *MTHFR* 677C→T, exists at high frequencies in many populations. Despite association with many health consequences, a mechanism for positive selection of this polymorphism has not been illustrated. It is possible that mild *Mthfr*-deficiency, as experienced in *MTHFR* 677TT individuals, can provide protection against pathogens, suggesting a possible mechanism of positive selection. Clinical studies have recently pointed to extensive folate supplementation as having a negative impact on the outcome of malaria infection, but experimental evidence is slim. The use of murine studies avails us the opportunity to explore potential mechanisms involved.

In humans, *P. falciparum* infection causes the majority of malaria-related mortality and morbidity in humans. In mice, this infection is studied using two different murine parasites, *P. chabaudi* AS (models blood-stage infection) and *P. berghei* ANKA (models the cerebral malaria complication), because each parasite models different aspects of the human infection. In this thesis, the *P. berghei* ANKA parasite will be used because of its easy availability from collaborators and because its pathology is known to be related to inflammation and host immune response. Both inflammation and immune function have been shown to be altered by mild *Mthfr*-deficiency and dietary folate, so a parasite also sensitive to such changes provides an interesting avenue of study.

The objectives of this thesis are to explore the effect of *Mthfr*-deficiency during *P. berghei* ANKA infection using *Mthfr*$^{+/-}$mice (mice with a single null allele in *Mthfr*) and measuring immunological parameters. We will also explore immune changes in uninfected *Mthfr*$^{+/-}$mice. We will then explore the effect of *MTHFR*-overexpression during *P. berghei* ANKA infection using *MTHFR*^{Tg} mice (mice with an additional human *MTHFR* gene) and measuring immune cell populations. We will also explore immune changes in uninfected *MTHFR*^{Tg} mice. Finally, we will then explore the effect of high levels of folic acid during *P. berghei* ANKA infection using *Mthfr*$^{+/-}$ mice fed folic acid supplemented diets, and measuring various immunological parameters.

CHAPTER II

MATERIALS AND METHODS

2.1.Animals

All animal use protocols were approved by the Montreal Children's Hospital Animal Care Committee and in accordance with the guidelines adopted by the Canadian Council on Animal Care. We used *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice (Chen, et al., 2001) backcrossed on a C57BL/6 background (Charles River Laboratory, St. Constant, Quebec, Canada) for at least 15 generations (Knock, et al., 2008). We also used *MTHFR* transgenic (*MTHFR*^{Tg}) C57BL/6 mice with an extra copy of human *MTHFR* (Celtikci, et al., 2008) and their wild-type littermates. *Mthfr*$^{+/+}$ and *MTHFR*^{Wt} mice are both wild-type C57BL/6 mice, although they are differentially designated based on their littermates. Genotypes were determined by PCR (Celtikci, et al., 2008; Chen, et al., 2001) prior to infection and confirmed after death. All animals were housed in clean facilities with 12 hours light/dark cycles and fed *ad libitum.*

2.2.Diets

At birth and post-weaning, all mice were fed a diet of standard mouse chow diets (2920x Irradiated Teklad Global Soy Protein-Free Extruded Rodent Diet, Harlan Laboratories Inc., Madison, WI, USA). Mouse chow is a diet made from soy and other natural food products, so the folates are naturally-occurring derivatives. As it is not an amino acid-defined diet, it contains higher quantities of several other methyl donors including B_{12}, choline and methionine. Prior to irradiation, it reportedly contains approximately 4mg/kg folate. Irradiation leads to loss of approximately 15% of folate (personal communication, supplier). In genotype experiments, the mice were fed mouse chow diet from weaning until death.

For dietary experiments, five weeks prior to infection, 2-3-month-old mice were randomly assigned to groups fed mouse chow or one of the folate-defined diets: 1) CD + SST, an amino acid-based Control Diet that contained succinylsulfathiazole (SST), an antibiotic that prevents folate synthesis by intestinal flora. This diet contains the recommended amount of folic acid for rodents (2mg/kg diet) (TD.090704 Folic Acid Control Diet, a modified version of TD01369 (Li, et al., 2005b), Harlan Laboratories, Inc., Madison, WI, USA); 2) CD, the same Control Diet without SST (TD.130565 Folic Acid Control Diet, Harlan Laboratories, Inc., Madison, WI, USA); 3) FASD (Folic Acid-Supplemented Diet), a diet identical to CD without SST but supplemented with folic acid at 10 times the amount in the CD diet (20 mg folic acid/kg diet, TD.130998, a modified version of TD.09258 (Mikael, et al., 2013a), Harlan Laboratories, Inc., Madison, WI, USA). All diets had their vitamin levels (including folic acid) adjusted to allow for irradiation (as recommended by the supplier), so that vitamin levels were at the desired levels post-irradiation.

2.3. *Plasmodium berghei* ANKA infection

After 5 weeks on diets or at 2-3 months of age, mice were randomly administered an inoculum of 200µl phosphate-buffered saline with 10^5 parasite-infected red blood cells (pRBCs), delivered by intravenous tail injection. Parasites were maintained through infection of naïve mice and inoculums prepared fresh on the day of infection.

The parasite dosage best suited for these experiments was determined experimentally. Three dosages were tested (10^4, 10^5 and the standard 10^6) to determine which was useful in elucidating the differences between our genotype groups (data not shown). The dosage that

showed the best separation between genotypes was the 10^5 dose, so this was the dose used for all subsequent experiments. Prior to infection, animals were genotyped by PCR (Celtikci, et al., 2008; Chen, et al., 2001), and genotypes were confirmed after death.

Following infection, animals were closely monitored several times a day until death or until they reached a pre-defined endpoint (loss of ability to use hind limbs, complete loss of mobility and/or difficulty breathing). At the endpoint, animals were euthanized by CO_2 asphyxiation. For the purpose of tracking survival, if an animal was euthanized, the recorded time of death was rounded to the next time the animal would have been monitored.

2.4.Measurement of blood parasites

At various time points during infection, blood was collected via tail pricks from infected mice and smeared into a very thin layer on microscope slides. Slides were stained with the Hemacolor© kit (Harleco, EMD Millipore, USA) to differentiate pRBCs and normal RBCs. Parasitemia was determined by calculating the ratio of pRBCs to total RBCs.

2.5.Tissue collection

Animals were infected as described above and sacrificed as noted, at 6 or 7 days post infection (dpi). Uninfected animals were age-matched at time of sacrifice. In random order, animals were asphyxiated in a CO_2 chamber, and blood was collected via cardiac puncture and processed in serum separation tubes (Sarstedt, St. Léonard, Quebec, Canada). Whole brain

hemispheres, liver and spleen were harvested by dissection and tissues were frozen at -70°C until use. Bloods were sent to the McGill Animal Resource Centre for complete blood counts and differentials.

2.6.Measurement of serum homocysteine and IFNγ

Serum homocysteine was measured using a colorimetric enzyme assay (A/C Enzymatic HCY Assay Kit, A/C Diagnostics, San Diego, CA, USA). The kit was used as suggested by the manufacturer. Serum IFNγ was measured using an ELISA kit (BD OptEIA™ Mouse IFN-gamma ELISA Kit, BD Biosciences, CA, USA). The kit was used as suggested by manufacturer, with samples diluted 1:2.5.

2.7.Assessment of immune cell populations

Infected animals were sacrificed at 6 or 7 dpi and spleens were harvested. Uninfected animals were sacrificed when age-matched to the infected mice. Spleens were macerated using a 70μm strainer and strained again through a clean 70μm strainer to remove debris. RBCs were lysed using ammonium-chloride-potassium (ACK) lysis buffer. Splenocytes were counted using a hemocytometer and trypan blue solution, and then seeded at $5.0x10^6$ for incubation with GolgiStop™ (BD Biosciences, Mississauga, ON, Canada) for 3 hours. $1x10^6$ cells were stained for viability with either eFluor®780 viability dye (eBioscience, San Diego, CA, USA) or Live/Dead® Fixable Aqua Stain (LifeTechnologies, Burlington, ON, Canada). After washing, cells were stained

with a mix of surface antibodies: APC-eFluor® 780 anti-CD4 (eBioscience, San Diego, CA, USA) or V500 anti-CD4 (BD Biosciences, Mississauga, ON, Canada), FITC anti-DX5 (BioLegend, San Diego, CA, USA), PE anti-CD194/CCR4 (BioLegend, San Diego, CA, USA), APC anti-CD183/CXCR3 (BioLegend, San Diego, CA, USA), and eFlour®450 anti-CD8 (eBioscience, San Diego, CA, USA) or APC-eFlour®780 anti-CD8 (eBioscience, San Diego, CA, USA).

Cells were fixed and permeabilized (BD Cytofix/ Cytoperm™ Fixation and Permeablization Kit, BD Biosciences, Mississauga, ON, Canada). After permeabilization, cells were intracellularly stained with a mix of antibodies: PE/Cy7 anti-IFNγ (BioLegend, San Diego, CA, USA) and PerCP-eFluro®710 anti-CD3 (eBioscience, San Diego, CA, USA). Samples were run on a BD Canto Flow Cytometer (BD Biosciences, Mississauga, ON, Canada) and $2.0x10^5$ events were recorded. Data were analyzed with FlowJo software (Treestar Inc, Ashland, OR, USA).

2.8.Immunoblot measurement of tissue cytokines

Cytokines in tissues were measured by immunoblotting. Protein lysates were prepared for electrophoresis, and immunoblotting was performed as previously described (Mikael, et al., 2006). Primary antibodies against β-actin (Sigma-Aldrich, Oakville, ON, Canada), PPARγ (Abcam, Cambridge, MA, USA), IFNγ (Sigma-Aldrich, Oakville, ON, Canada), IL-12 (Abbiotech, San Diego, CA, USA) , TNFα (Abcam, Cambridge, MA, USA) and IL-10 (Abbiotech, San Diego, CA, USA) were used. Secondary antibodies were horseradish peroxidase-coupled anti-rabbit IgG (Amersham, GE Healthcare Life Sciences, Piscataway, NJ, USA) and horseradish peroxidase-coupled anti-goat IgG (Santa Cruz Biotechnology Inc., Dallas, TX, USA), as prescribed. The ECL Plus chemiluminescence

system (Amersham, GE Healthcare Life Sciences, Piscataway, NJ, USA) was used for signal detection. The signal was visualized by film exposure. Proteins were quantified by densitometry using Quantity One 4.1.0 software (Bio-Rad Laboratories, Mississauga, ON, Canada); β-actin was used as internal control. Wild-type or CD mice were used to generate a mean value that was designated as 1. Values for mutant or FASD mice were calculated as a ratio of the mean wild-type or CD value for each blot.

2.9.RNA extraction and quantitative reverse-transcriptase-PCR

RNA was extracted from frozen liver (~15mg) using the RNeasy Tissue Mini Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. Reverse transcription was performed as previously described (Knock, et al., 2006). Quantitative PCR reactions were performed using Platinum SYBR® Green master mix (Invitrogen™, Burlington, ON, Canada), with primers described in Table 2.1, using the Roche Lightcycler® 480 II (Roche Life Science, Laval, QC, Canada). Primers were designed using NCBI PrimerBLAST (Ye, et al., 2012). Expression of target genes was normalized to *Gapdh* and *Ywhaz* using a normalization factor calculated by geNorm v.3.4 (Vandesompele, et al., 2002).

Table 2.1: Primers for quantitative real-time RT-PCR

Gene	Direction	Primer Sequence	Amplicon Size	T_m (°C)	Reference
Gapdh	Forward	CAGGAGCGAGACCCCACTAACAT	74	62	(Leclerc, et al., 2013)
	Reverse	AAGACACCAGTAGACTCCACGAC			
Ywhaz	Forward	TGCTGGTGATGACAAGAAAGGA	119	60	(Garcia-Crespo, et al., 2009)
	Reverse	TGAGGGCCAGACCCAGTCT			
Bak	Forward	TATTAACCGGCGCTACGACAC	109	60	(Garcia-Crespo, et al., 2009)
	Reverse	CTTAAATAGGCTGGAGGCGATCTT			
Bcl-xl	Forward	GGTAGTGAATGAACTCTTTCGGGAT	131	60	(Garcia-Crespo, et al., 2009)
	Reverse	TCCGACTCACCAATACCTGCAT			
Abca1	Forward	CGTTTCCGGGAAGTGTCCTA	78	60	(Brunham, et al., 2006)
	Reverse	CTAGAGATGACAAGGAGGATGGA			

The table includes expected amplicon size, melting temperature (T_m) and reference source. Housekeeping genes (*Gapdh* and *Ywhaz*) were used to generate the normalization factor.

2.10. Statistical analyses

Statistical analyses, including log-rank analysis of survival, analysis of variance (ANOVA), correlations and unpaired t-tests were performed using GraphPad v5 or v6 (GraphPad Software Inc, La Jolla, CA, USA). Following ANOVA, Tukey post-hoc tests were used for pair-wise comparisons. Grubb's test was used to identify potential outliers; outliers, if they were present, were removed. In comparative graphs, data are presented as mean ± standard error of the mean (SEM). When combining experiments, wild-type or CD mice in each experiment were standardized to a mean expression level or intensity ratio of 1, and the values for mutant or FASD mice were calculated as a proportion of the value for wild-type or CD mice.

CHAPTER III

INCREASED RESISTANCE TO MALARIA IN MICE WITH METHYLENETETRAHYDROFOLATE REDUCTASE (*MTHFR*) DEFICIENCY SUGGESTS A MECHANISM FOR SELECTION OF THE *MTHFR 677C>T (C.665C>T) V*ARIANT

Published in part as:

Increased Resistance to Malaria in Mice with Methylenetetrahydrofolate Reductase (Mthfr) Deficiency Suggests a Mechanism for Selection of the MTHFR 677C>T (c. 665C>T) Variant

Danielle N. Meadows, Michal Pyzik, Qing Wu, Sabrina Torre, Philippe Gros, Silvia M. Vidal and Rima Rozen

Human Mutation (2014) 35(5): 594-600

3.1.Summary

A polymorphism in methylenetetrahydrofolate reductase (*MTHFR*, 677C>T (c.665C>T, p.Ala222Val, rs1801133)) results in mild enzymatic deficiency and increased risk for several complex traits including adverse reproductive outcomes, birth defects, and heart disease. Despite these deleterious effects, homozygosity is high (5 – 15%) in many populations, and among the highest in Mediterranean regions, where malaria was historically endemic and may have conferred a selective advantage for other mutations. We infected *Mthfr*-deficient (*Mthfr*$^{+/-}$) mice with *Plasmodium berghei* ANKA to induce cerebral malaria. *Mthfr*$^{+/-}$ mice survived longer ($p<0.02$, log-rank test) after infection compared with wild-type littermates. IFNγ and PPARγ immunoreactive protein was increased in brain of *Mthfr*$^{+/-}$ mice and IFNγ was increased in spleen as well. Immunoreactive IL-10 proteins was decreased in brain of *Mthfr*$^{+/-}$ mice compared to wild-type littermates. Flow cytometry revealed increased lymphocyte populations and increased CCR4^{+} natural killer (NK) cells in spleen of infected *Mthfr*$^{+/-}$ mice. Uninfected *Mthfr*$^{+/-}$ mice had changes in distribution of immune cell populations, specifically increases in the proportion of CCR4^{+} NK cells and IFNγ^{+} NK cells. They also have changes in their brain IFNγ and IL-10 immunoreactive proteins, with decreases in IFNγ and increases in the inactive form of IL-10. These findings suggest that MTHFR deficiency alone may alter immune response. We suggest that mild MTHFR deficiency protects against malarial infection and that this phenomenon may have led to the high frequency of the 677 C→T variant in human populations.

3.2.Introduction

Methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20,) synthesizes methyltetrahydrofolate, the primary circulatory form of folate. Several common variants of *MTHFR* (607093) have been reported (Leclerc, et al., 2005). Of these, the 677C→T polymorphism (c.665C>T, p.Ala222Val, 607093.003, rs1801133) has been studied the most extensively, with over 3600 citations for our original report (Frosst, et al., 1995). The 677TT genotype results in an approximate 70% loss of enzyme activity and mild hyperhomocysteinemia (Frosst, et al., 1995).

This polymorphism is the first identified genetic risk factor for spina bifida (van der Put, et al., 1995). Other reported associations include increased risk for adverse reproductive outcomes, birth defects, vascular disease, and some neuropsychiatric disorders (Ueland and Rozen, 2005). Despite its known negative consequences on human health, the high frequency of the *MTHFR* 677TT genotype in many populations has been largely unexplained. Some hypotheses have been proposed (Green and Miller, 1999) (Ueland, et al., 2001), but none of these hypotheses have been investigated. In this study, we endeavored to find a mechanism that might explain the high frequency of this polymorphism.

Pathogens are often cited as one of the selective forces that help shape the human genome through positive selection. Several mutations with negative health consequences have persisted in human populations because they confer protection against infections, malaria in particular. Hemoglobinopathies and other factors such as the Duffy-coat antigen, HLA type, and pyruvate kinase deficiency (Berghout, et al., 2012; Fortin, et al., 2002b) have also been shown to improve the outcome of malaria.

The frequency of the 677TT genotype is high in southern Europe (15-20%), notably Spain and Italy, with the highest frequencies in populations of Hispanic descent in the United States, Mexico and Colombia (~25-30%) (Figure 1.2) (Gueant-Rodriguez, et al., 2006; Leclerc, et al., 2005). Although data are limited for African populations, Algeria (Houcher, et al., 2010) and Tunisia (Rouissi, et al., 2009) in northern Africa have higher frequencies (~14% and ~11% respectively) than other nations in sub-Saharan Africa (all <1%) (Angius, et al., 2007; Gueant-Rodriguez, et al., 2006; Rosenberg, et al., 2002). The polymorphism is part of a common haplotype shared among diverse populations, suggesting a shared ancestral history; selective advantage could have assisted in spreading the variant through populations (Rosenberg, et al., 2002).

Malaria is caused by *Plasmodium* parasites that are carried in the saliva of *Anopheles* mosquitoes. Several strains of *Plasmodium* that infect humans, but most severe infections in humans are caused by *Plasmodium falciparum.* Uncomplicated malaria results in cyclical fevers, mild anemia and the aches and pains that typically accompany fever. Complications may arise, including severe anemia, splenomegaly and cerebral malaria. Symptoms of cerebral malaria include paralysis, seizure, brain hemorrhage and death. To study cerebral malaria in mice, *P. berghei* ANKA is used (Fortin, et al., 2002b). Mouse models of infection have been used to show that mutations in hemoglobin offer protection against malaria; these models include mice with sickle cell hemoglobin (Hood, et al., 1996) or with β-thalassemia (Roth, et al., 1988).

In earlier work, we generated an *Mthfr*-deficient mouse (Chen, et al., 2001). The heterozygous knockout mouse (*Mthfr*$^{+/-}$) serves as a model for the 677TT genotype, since both 677TT humans and *Mthfr*$^{+/-}$ mice have a mild enzyme deficiency. *Mthfr*$^{-/-}$ mice serve as a model

for the inborn error of metabolism, homocystinuria (Rosenblatt, et al., 1992), and were not studied here since they manifest many other abnormalities, including changes in brain morphology (Chen, et al., 2001; Lawrance, et al., 2011).

To determine whether the 677 polymorphism may have conferred a selective advantage against malaria, we used mice with decreased MTHFR expression and infected them with *P. berghei* ANKA to induce cerebral malaria. We observed increased resistance, and demonstrate some changes in immune cell populations and cytokines that could explain the increased resistance in MTHFR deficiency. We also examined some of the same parameters in uninfected mice with decreased MTHFR to determine whether some of the immune changes observed were independent of the infection model.

3.3.Results

3.3.1. Survival and parasitemia of *Mthfr*$^{+/-}$ mice during *Plasmodium berghei* ANKA infection

Male *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice were infected intravenously with 10^5 *P. berghei* ANKA parasites. Animals were monitored over a two-week period until death or until they lost the ability to use hind limbs, experienced complete loss of mobility and/or difficulty breathing. Survival was recorded. *Mthfr*$^{+/-}$ mice survived longer than their wild-type littermates, with onset of mortality in both groups at the end of 6 dpi (Figure 3.1 A; $p<0.02$, log-rank test).

The day before the onset of mortality in the majority of animals, thin-layer blood smears were collected from each animal. Slides were stained to determine percent parasitemia by

calculating the ratio of infected RBC to total RBC. There was a trend for *Mthfr*$^{+/-}$ mice to have lower percent parasitemia compared to wild-type littermates at 6 dpi (Figure 3.1B; p=0.067, t-test), but it is not clear whether this difference has biologic significance. Nevertheless, the lower parasitemia is consistent with the increased resistance to infection observed in mutant mice.

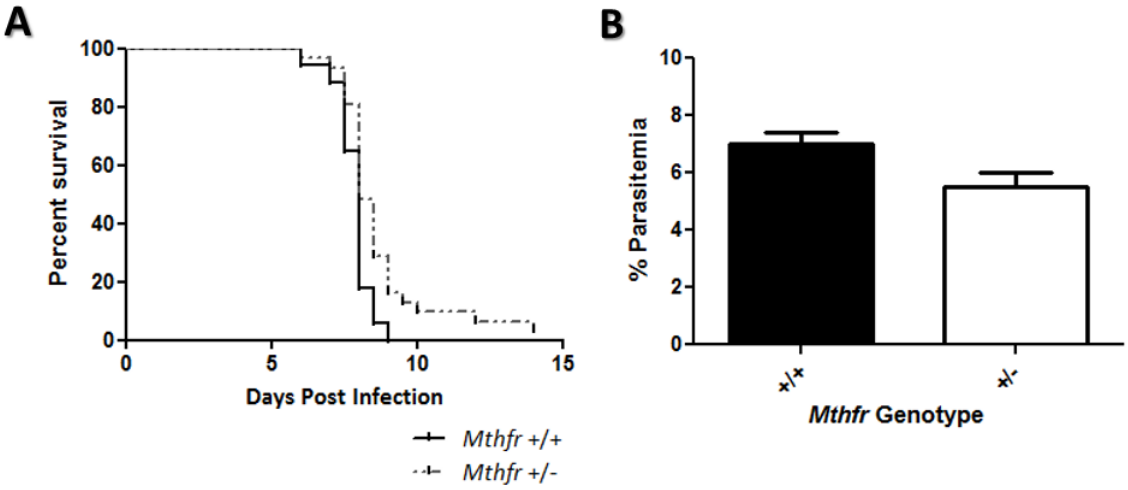


Figure 3.1 Survival and parasitemia in *Mthfr*$^{+/-}$ mice intravenously infected with 10^5 *P. berghei* ANKA. (A) *Mthfr*$^{+/-}$ mice (dotted line) survived longer than *Mthfr*$^{+/+}$ mice (solid line) (n=17 *Mthfr*$^{+/+}$, n=30 *Mthfr*$^{+/-}$, 3 combined infections; p<0.02, log-rank test). (B) *Mthfr*$^{+/-}$ mice (white bar) had lower percent parasitemia compared with *Mthfr*$^{+/+}$ mice (black bar) (n=17 *Mthfr*$^{+/+}$; n=37 *Mthfr*$^{+/-}$, 3 combined infections; borderline significant by t-test, p=0.067).

3.3.2. Complete blood counts in infected *Mthfr*$^{+/-}$ mice

Blood was collected from mice at 5 and 6 dpi. They were sent for complete blood counts and differential counts. Results are summarized in Table 3.1. There were no differences of note between the *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice at 5 or 6dpi.

Table 3.1 Complete blood counts and differentials from *Mthfr*$^{+/-}$ mice 5 and 6 dpi[1, 2]

DPI:	5		6	
GENOTYPE:	*Mthfr*$^{+/+}$	*Mthfr*$^{+/-}$	*Mthfr*$^{+/+}$	*Mthfr*$^{+/-}$
RBCs (x 10^{12}/L)	13.06 ± 0.09	12.09 ± 0.50	12.56 ± 0.70	12.21 ± 0.30
Hematocrit (L/L)	0.595 ± 0.004	0.554 ± 0.021	0.569 ± 0.032	0.552 ± 0.010
Hemoglobin (g/L)	191 ± 1	178 ± 6	193 ± 8	188 ± 3
MCV (fL)	45.5 ± 0.3	45.7 ± 0.3	45.3 ± 0.3	45.4 ± 0.2
MCH (pg)	14.7 ± 0.1	14.9 ± 0.1	15.4 ± 0.4	15.4 ± 0.2
MCHC (g/L)	321 ± 1	325 ± 1	339 ± 6	340 ± 3
WBCs (x 10^9/L)	2.5 ± 0.3	2.5 ± 0.2	2.6 ± 0.2	2.0 ± 0.2
neutrophils %	15 ± 1	14 ± 2	13 ± 8	11 ± 2
lymphocytes %	85 ± 1	85 ± 3	86 ± 7	89 ± 2
neutrophils (x 10^9/L)	0.37 ± 0.06	0.36 ± 0.08	0.29 ± 0.10	0.22 ± 0.04
lymphocytes (x 10^9/L)	2.10 ± 0.26	2.18 ± 0.18	1.78 ± 0.33	1.80 ± 0.15
platelets (x 10^9/L)	215 ± 26	254 ± 50	291 ± 150	102 ± 17

[1] n=4 *Mthfr*$^{+/+}$ and n=6 *Mthfr*$^{+/-}$
[2] Mean ± SEM

3.3.3. Serum homocysteine and IFNγ in infected *Mthfr*$^{+/-}$ mice

To confirm that the infected *Mthfr* $^{+/-}$ mice had moderate increases in homocysteine levels, we measured level at 6 dpi. *Mthfr* $^{+/-}$ mice have increased homocysteine levels after infection (Figure 3.2A; p=0.07, borderline significant, t-test). Homocysteine levels were only measured on a small number of mice since these results are consistent with previous reports of moderate elevation in uninfected *Mthfr*$^{+/-}$ mice (Chen, et al., 2001; Devlin, et al., 2004; Mikael, et al., 2012; Schwahn, et al., 2003).

Cerebral malaria is caused by dysregulation of the immune response mounted by the host in an effort to eliminate *Plasmodium* pathogens (Spence and Langhorne, 2012). A pro-inflammatory response that includes IFNγ is required for the development of cerebral malaria (Grau, et al., 1989a; Rudin, et al., 1997b). However, mice that are more resistant to malaria have been reported

to have lower levels of IFNγ in serum compared with susceptible mice (Ishih, et al., 2008; Schmidt, et al., 2011). To confirm that our resistant mice had lower levels of serum IFNγ, as suggested in the literature, we measured IFNγ by ELISA. We found that *Mthfr*$^{+/-}$ mice had lower serum IFNγ concentrations than *Mthfr*$^{+/+}$ mice: 88.7±27.3 pg/mL (*Mthfr*$^{+/-}$) compared with 217.1±49.6 pg/mL (*Mthfr*$^{+/+}$) (Figure 3.2B; p<0.05, t-test).

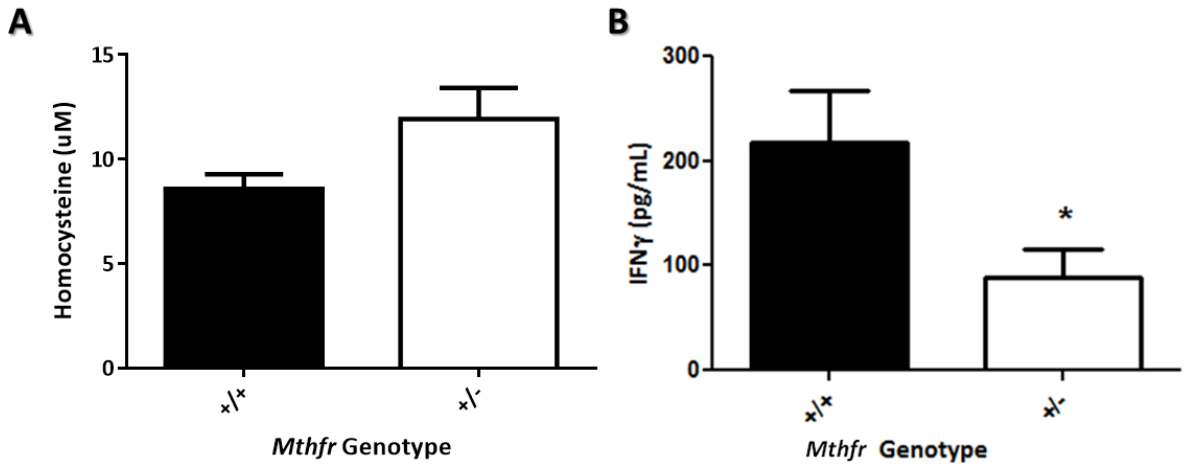


Figure 3.2: Homocysteine and IFNγ concentrations in serum of *Mthfr*$^{+/-}$ mice intravenously infected with 10^5 *P. berghei* ANKA at 6 dpi Values are presented as mean ± SEM. (A) Homocysteine levels are moderately increased in *Mthfr*$^{+/-}$ mice (white bar) compared with *Mthfr*$^{+/+}$ mice (black bar) (n=5 *Mthfr*$^{+/+}$, n=5 *Mthfr*$^{+/-}$; one infection; p=0.07, t-test). (B) *Mthfr*$^{+/-}$ mice had significantly decreased IFNγ concentrations compared with *Mthfr*$^{+/+}$ mice (n= 10 *Mthfr*$^{+/+}$, n= 10 *Mthfr*$^{+/-;}$ two combined infections; *p<0.05, t-test).

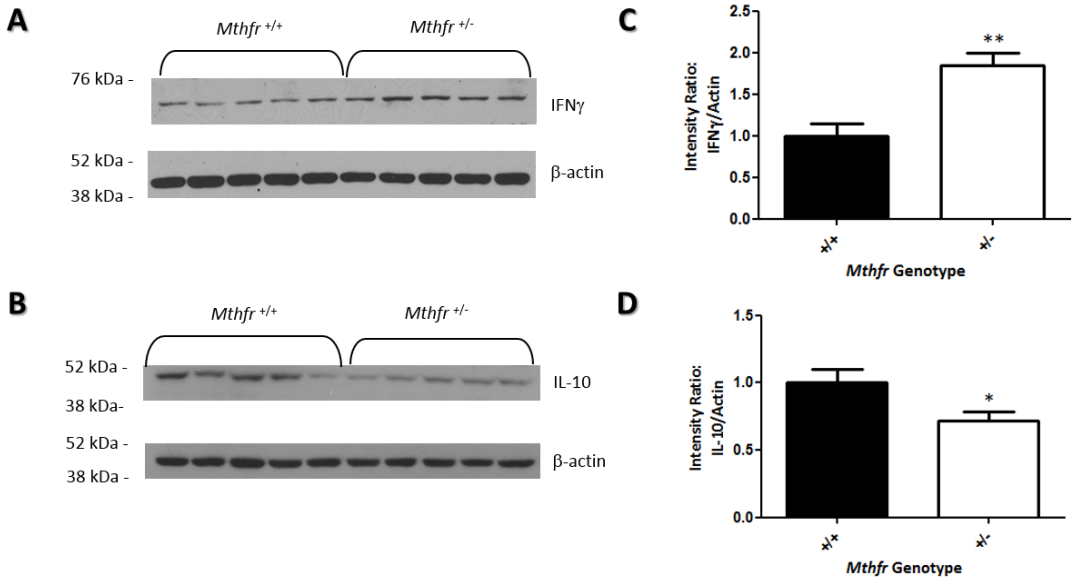


Figure 3.3: IFNγ and IL-10 immunoreactive protein in brain of *Mthfr*$^{+/-}$ mice intravenously infected with 10^5 *P. berghei* ANKA at 6 dpi. Representative Western blots are shown for IFNγ (A) and IL-10 (B). (C) *Mthfr*$^{+/-}$ animals (white bars) had significantly increased levels of IFNγ in brain compared with wild-type littermates (black bars) (3 infections combined, n=10 mice per genotype, **p<0.01, t-test). (D) *Mthfr*$^{+/-}$ mice had significantly reduced levels of IL-10 (2 infections combined, n=7 *Mthfr*$^{+/+}$, n=8 *Mthfr*$^{+/-}$, *p<0.05, t-test).

3.3.4. Brain cytokines during infection: TNFα, IFNγ, IL-10, IL-12, and PPARγ

Since the brain is the primary tissue of interest in a cerebral malaria infection, we performed immunoblotting in whole brain to measure levels of cytokines that may be of interest during infection. High levels of TNFα have been described as detrimental in cerebral malaria (Grau, et al., 1987; Grau, et al., 1989a; Rudin, et al., 1997a). TNFα levels were the same in *Mthfr*$^{+/-}$ animals compared with *Mthfr*$^{+/+}$ mice (data not shown).

We measured brain IFNγ (Figure 3.3A) and IL-10 (Figure 3.3B). *Mthfr*$^{+/-}$ animals had an IFNγ level of 1.84±0.16 compared with 1.00±0.15 in *Mthfr*$^{+/+}$ mice (Figure 3.3C, p<0.01, t-test). *Mthfr*$^{+/-}$

animals had an IL-10 level of 0.73±0.07 compared with 1.00±0.10 in *Mthfr*$^{+/+}$ mice (Figure 3.3D, p<0.05, t-test). These observations revealed an approximate 2-fold increase of IFNγ and an approximate one-third decrease of IL-10 in *Mthfr*$^{+/-}$ mice compared with wild-type animals. Expression levels of IL-12, a cytokine related to IFNγ (Rudin, et al., 1997b), were similar in *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice (data not shown).

A genome-wide association study of mouse strains that were resistant and susceptible to *P. berghei* infection, identified a protective locus that contained *Ppar-gamma* (Bopp, et al., 2010). In addition, other studies have shown that therapeutic administration of an agonist of PPARγ, rosiglitazone, is protective and improves the outcome of cerebral malaria (Serghides, et al., 2009). With this in mind, and the knowledge that a similar molecule, PPARα, can be affected by disturbances in folate metabolism (Leclerc, et al., 2013; Mikael, et al., 2012), we measured brain PPARγ using immunoblot (Figure 3.4). We found that *Mthfr*$^{+/-}$ animals had a PPARγ level of 1.67±0.15 compared with 1.00±0.11 in *Mthfr*$^{+/+}$ mice (Figure 3.4, p<0.01, t-test).

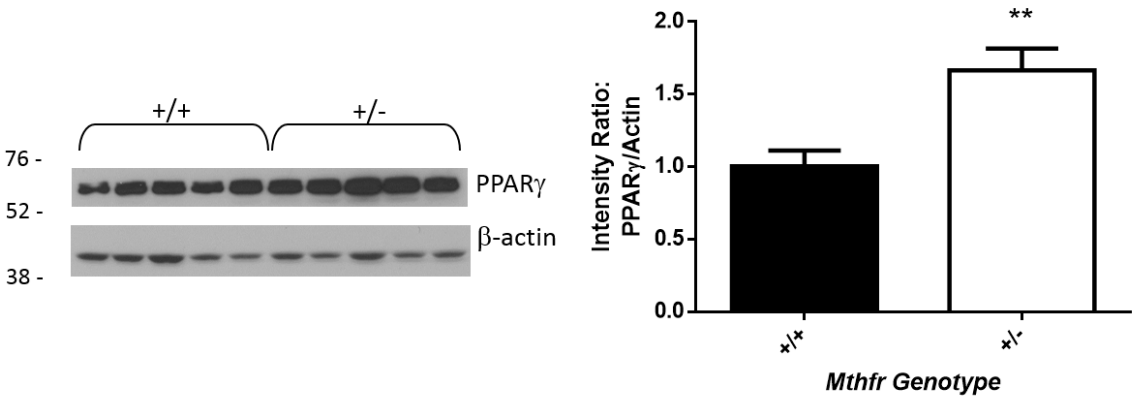


Figure 3.4: PPARγ immunoreactive protein in the brain of *Mthfr*$^{+/-}$ mice intravenously infected with 10^5 *P. berghei* ANKA 6dpi Representative Western blot are shown for PPARγ in brain (A) and quantification (B). Values represent means ± SEM. *Mthfr*$^{+/-}$ animals had significantly increased levels of PPARγ in brain compared with wild-type littermates (2 combined infections; n=10 mice per genotype; **p<0.01, t-test).

3.3.5. Spleen cytokines during infection: IFNγ and IL-10

In the spleen of *P. berghei* ANKA infected mice, IFNγ expression increases in *Mthfr*$^{+/-}$ mice (Figure 3.5A,B; $p<0.01$, t-test) while ELISA measurement showed that expression of total IL-10 also had a trend to increase in the spleen of *Mthfr*$^{+/-}$ mice (Figure 3.5C; $p=0.081$,t-test). The inconsistencies between measurements of IL-10 in the brain and spleen may be due to an ELISA's inability to differentiate between active and inactive forms of the cytokine. Active IL-10 is a homodimer, while the inactive form is a monomer of the protein (Mikael, et al., 2013b). IL-10 immunoblots in spleen could not be interpreted. The IFNγ changes in brain and spleen are consistent with one another, but distinct from those in serum. This may suggest that inflammation in tissue is important for resistance. IL-10 and IFNγ are thought to act in concert with one another in cerebral malaria with IL-10 acting in an immunomodulatory capacity.

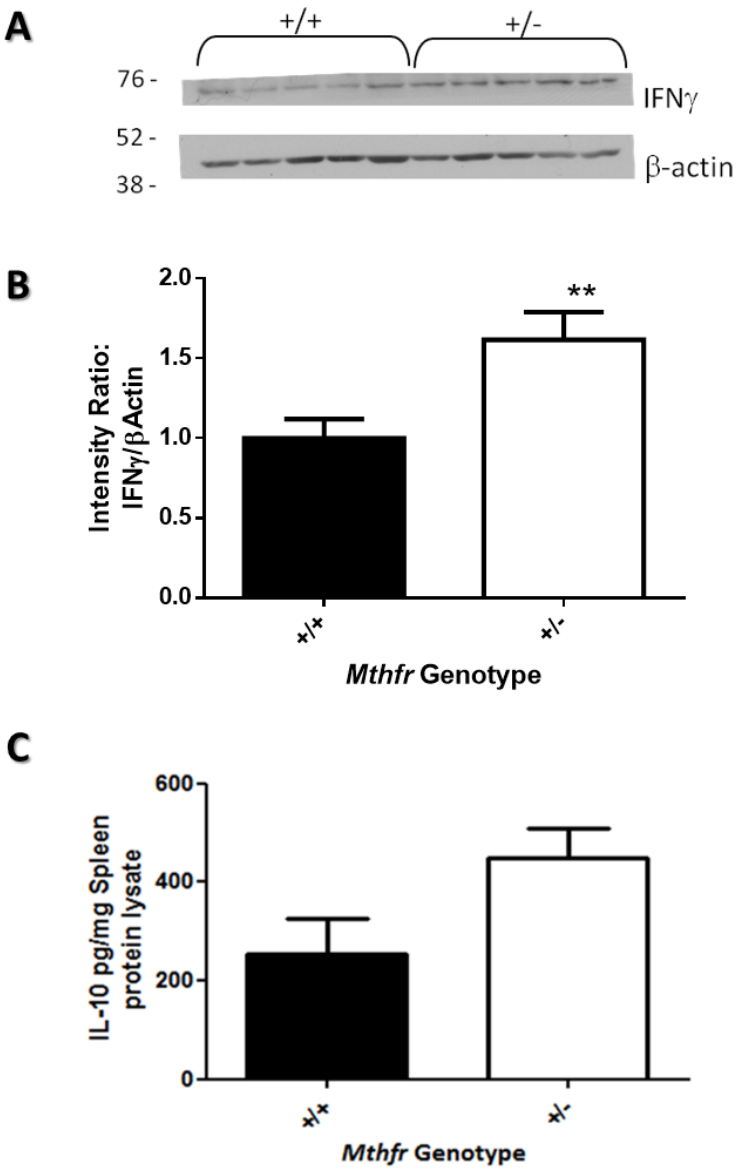


Figure 3.5: IFNγ and IL-10 immunoreactive protein in the spleen of *Mthfr*$^{+/-}$ mice intravenously infected with 10^5 *P. berghei* ANKA at 6dpi Representative Western blots are shown for IFNγ (A). Values are presented as mean ± SEM. (B) *Mthfr*$^{+/-}$ animals had significantly increased levels of IFNγ in spleen compared with wild-type littermates (2 infections combined, n=10 mice per genotype, **p<0.01, t-test). (C) ELISA measurements show *Mthfr*$^{+/-}$ mice had a trend toward increased levels of IL-10 (1 infection, n=4 *Mthfr*$^{+/+}$, n=4 *Mthfr*$^{+/-}$, p=0.081, t-test).

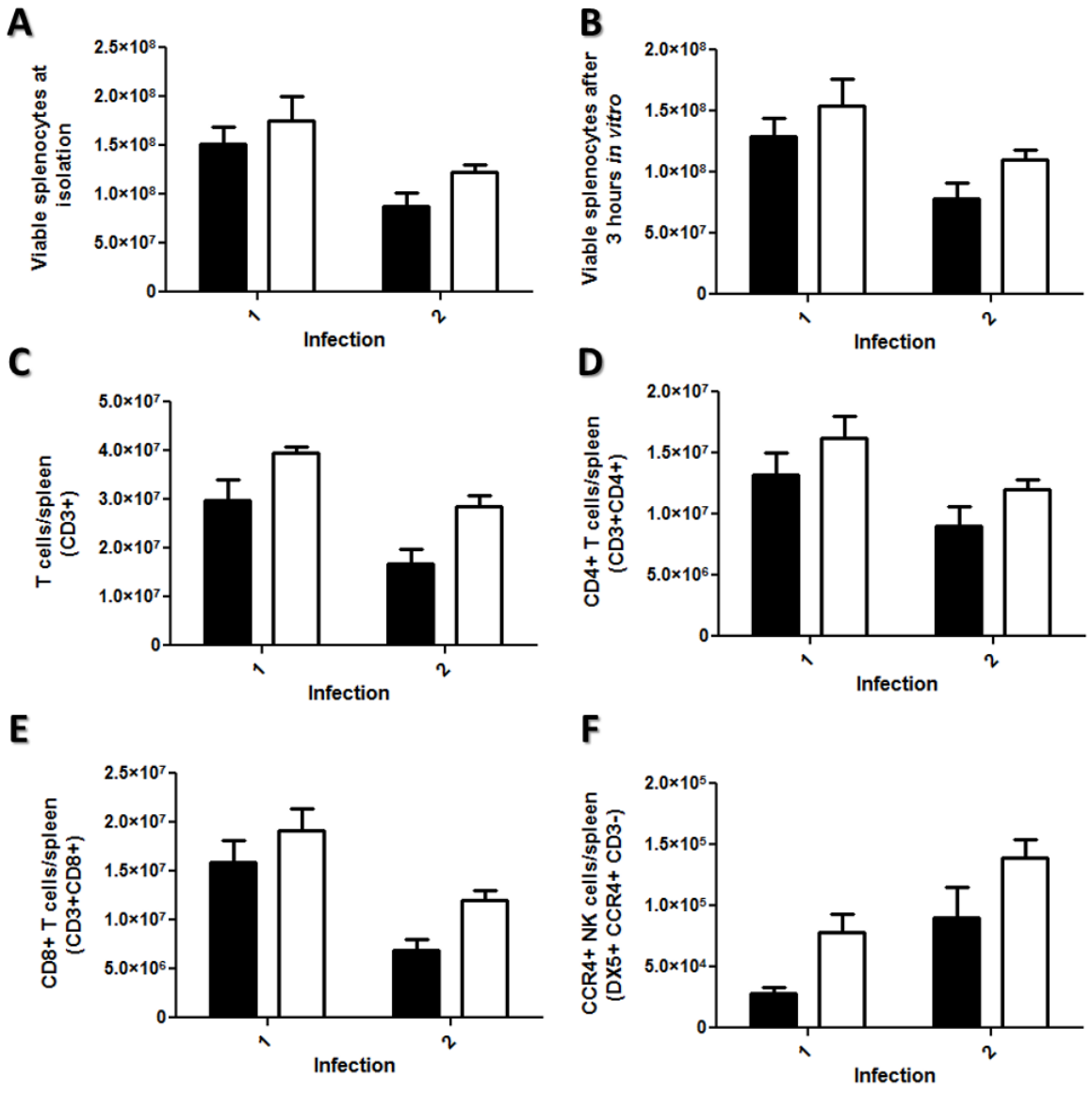


Figure 3.6: Cell population changes at 6 dpi in *Mthfr*$^{+/-}$ mice infected with 10^5 *P. berghei* ANKA. Two different infections are shown. (A). *Mthfr*$^{+/-}$ animals (white bar) had a trend toward increased viable splenocyte counts compared with *Mthfr*$^{+/+}$ (black bar) littermates (p=0.072, ANOVA). (B) Viable splenocyte numbers were higher in *Mthfr*$^{+/-}$ cultures after incubation with GolgiStop™ (p=0.058, ANOVA). *Mthfr*$^{+/-}$ mice had significantly increased (C) total T cells/spleen (p<0.05, ANOVA), increased (D) CD4+ T cells/spleen (p=0.054, ANOVA), significantly increased (E) CD8+ T cells/spleen (p<0.05; ANOVA), and significantly increased (F) CCR4+ NK cells /spleen (p<0.05; ANOVA), compared to *Mthfr*$^{+/+}$ littermates.

3.3.6. Immune cell population changes in infected *Mthfr*$^{+/-}$ mice

To identify potential mechanisms for increased resistance in *Mthfr*$^{+/-}$ mice, we isolated splenocytes and first counted the number of viable cells. *Mthfr*$^{+/-}$ mice had a trend toward higher splenocyte numbers compared with wild-type littermates (Figure 3.6A; p=0.072, ANOVA). Equal numbers of viable splenocytes (5.0 x 10^6 cells) were then incubated for 3 hours with GolgiStop™ to inhibit cytokine secretion and stained for viability. Again, *Mthfr*$^{+/-}$ mice had a trend toward increased numbers of viable cells (Figure 3.6B; p=0.058, ANOVA).

Surface staining (CD4, CD8, DX5 (CD49b), CXCR3 (CD183), CCR4 (CD194)) and intracellular staining (CD3 and IFNγ) were performed to identify various immune cell populations (T cells, NK cells, NK T cells). Cells were analyzed by flow cytometry; only significant population differences are shown (Figure 3.6C-F). *Mthfr*$^{+/-}$ mice had increased T lymphocyte populations (Figure 3.6C; $p<0.05$, ANOVA), and increased numbers of $CD4^+$ (Figure 3.6D; p=0.054, ANOVA) and $CD8^+$ (Figure 3.6E; $p<0.05$, ANOVA) T cells. They also had increased numbers of $CCR4^+$ NK cells (Figure 3.6F; $p<0.05$, ANOVA).

3.3.7. Brain IFNγ and IL-10 in uninfected *Mthfr*$^{+/-}$ mice

In whole brain of uninfected and age-matched mice, IFNγ expression is decreased in *Mthfr*$^{+/-}$ mice (Figure 3.7A, C; $p<0.001$, t-test) while expression of the IL-10 monomer was increased in brains of *Mthfr*$^{+/-}$ mice (Figure 3.7B, D; $p<0.01$, t-test). There was no genotype difference in expression of the active homodimer (data not shown). IL-10, when active, is a homodimer (Mikael, et al., 2013b) and at 6dpi when the infection is well-underway, brain of

Mthfr$^{+/-}$ mice show decrease in the active homodimer (Figure 3.3). The increase in the inactive IL-10 monomer seen in uninfected *Mthfr*$^{+/-}$ mice, may suggest that have a greater potential to express active protein when challenged.

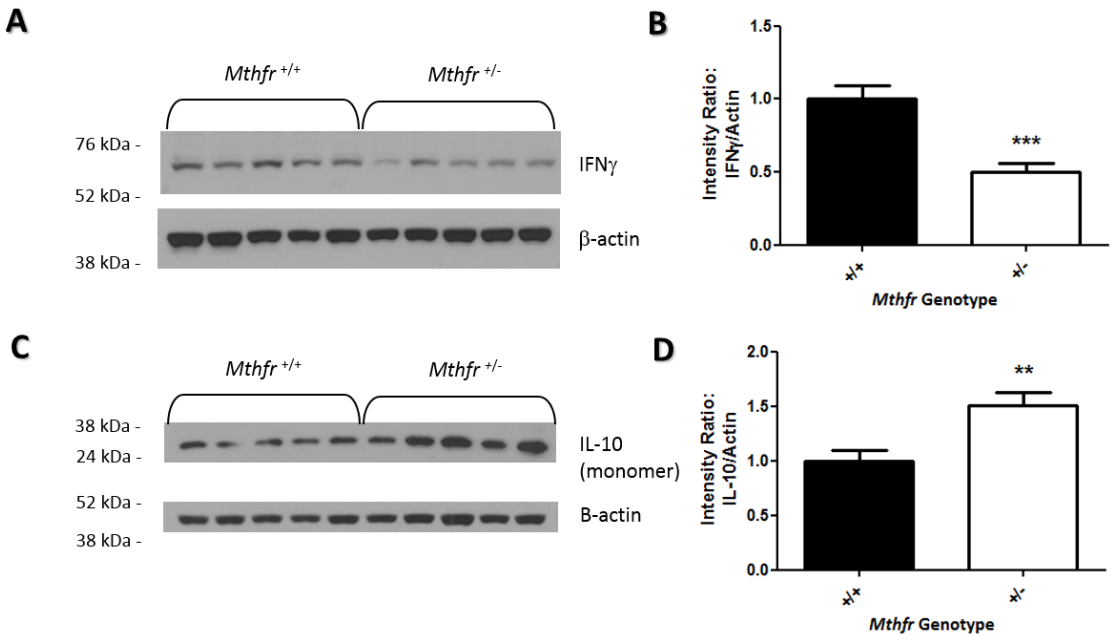


Figure 3.7: IFNγ and IL-10 immunoreactive protein in the brain of uninfected *Mthfr*$^{+/-}$ mice Representative Western blots are shown for IFNγ (A) and IL-10 (C). (B) *Mthfr*$^{+/-}$ animals (black bars) had significantly decreased levels of IFNγ in brain compared with wild-type (white bars) littermates (2 experiments combined, n=10 mice per genotype, ***p<0.001, t-test). For each experiment, the wild-type mice (n=10) were used to generate a mean value that was designated as 100%. Values for each *Mthfr*$^{+/-}$ mouse were calculated as a percentage of the mean wild-type value. (D) *Mthfr*$^{+/-}$ mice had significantly increased levels of IL-10 monomer (1 infection, n=5 *Mthfr*$^{+/+}$, n=5 *Mthfr*$^{+/-}$, **p<0.01, t-test). The values were calculated as described for (C).

3.3.8. Immune cell population changes in uninfected *Mthfr*$^{+/-}$ mice

Having found so many immune changes in the malaria-infected *Mthfr*$^{+/-}$ mice, we wanted to see whether there were any baseline changes in uninfected *Mthfr*$^{+/-}$ mice. Using the same flow cytometry panel, we stained the splenocytes of age-matched *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice. There

were no differences in total cell numbers before or after incubation with GolgiStop™. Of interest though, while the total number of cells was not increased, in uninfected *Mthfr*$^{+/-}$ mice there was an increased percentage of CCR4^{+} NK cells (Figure 3.8A, $p<0.05$, ANOVA) in the total population. Uninfected *Mthfr*$^{+/-}$ mice also had increased percentages of IFNγ^{+} NK cells (Figure 3.8B, $p<0.01$, ANOVA) in the total population.

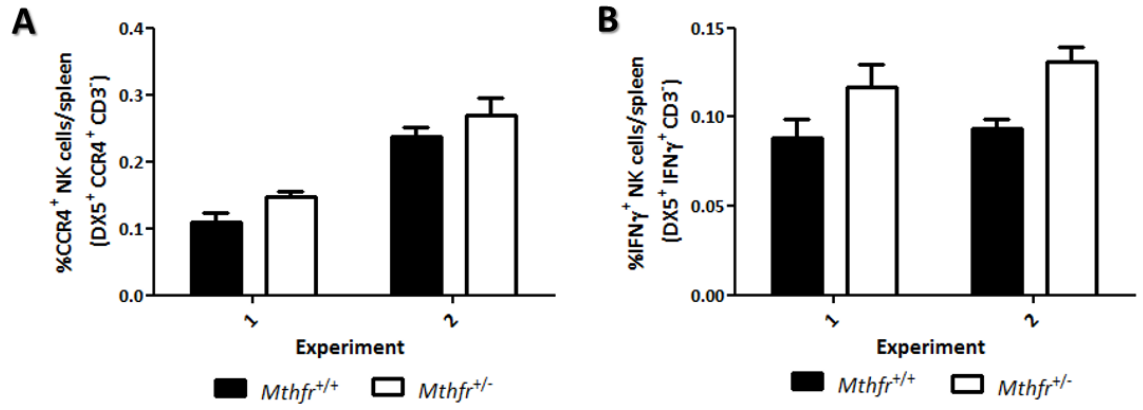


Figure 3.8: Cell population changes in uninfected *Mthfr*$^{+/-}$ mice Male *Mthfr*$^{+/+}$ (black bar) and *Mthfr*$^{+/-}$ (white bar) mice age-matched to those used in infection experiments (~3 months) were sacrificed and splenocytes stained for flow sorting. n=10 *Mthfr*$^{+/+}$ and n=10 *Mthfr*$^{+/-}$. Two different infections are shown. Means ± SEM are indicated for each group. (A) *Mthfr*$^{+/-}$ mice had an increased population percentage of CCR4^{+} NK cells ($p<0.05$, ANOVA) and (B) they also had an increased population percentage of IFNγ^{+} NK cells ($p<0.01$, ANOVA)

3.4.Discussion

An interaction between folate metabolism and the malarial parasite has been known for some time; anti-folate drugs have been used as anti-malarial treatments for many years (Goodwin, 1956; Muller and Hyde, 2013). Folates are required for synthesis of nucleotides, conversion of the toxic amino acid homocysteine into methionine and the supply of one-carbons for methylation

reactions. MTHFR deficiency results in hyperhomocysteinemia and global DNA hypomethylation in humans and mice (Chen, et al., 2001; Friso, et al., 2013). Hyperhomocysteinemia is associated with pro-inflammatory responses, oxidative stress and other cellular disturbances (Lazzerini, et al., 2007; Mikael, et al., 2013b; Papatheodorou and Weiss, 2007). A variety of these afore-mentioned folate-related functions could affect the response to an infection.

Several murine models have been used to study survival in the context of cerebral malaria and genetic protection. The survival curves we observed for wild-type animals were similar to those reported for wild-type mice in other studies examining transgenic mouse models infected with cerebral malaria, including sickle cell (Hood, et al., 1996), pyruvate kinase (Min-Oo, et al., 2003) and β-thalassemia (Roth, et al., 1988). Parasite levels for our wild-type mice were also in the expected range. These observations suggest that our model was effective, even with the slightly lower parasite dose. We did not see any changes in the RBCs or the WBCs in the complete blood counts or the blood differentials of *Mthfr*$^{+/-}$ mice at 5 or 6 dpi, but this may be due to the sensitivity of such a clinical assay.

We observed increased total splenocyte populations in infected mice with *Mthfr* deficiency. *Mthfr* deficiency would result in increased levels of the substrate, methylenetetrahydrofolate, which is required for thymidine production; consequently enhanced thymidine synthesis could lead to increased numbers of viable splenocytes in *Mthfr*$^{+/-}$ mice. We also observed increased populations of $CD4^+$ and $CD8^+$ T cells, which are critical for the response to cerebral malaria. The host relies on a delicate interplay between $CD4^+$ and $CD8^+$ cells to control infection, as they have both protective and pathogenic roles (Hafalla, et al., 2006; Spence and

Langhorne, 2012; Villegas-Mendez, et al., 2011). The increase in these populations may have also been due to increased thymidine production, but additional studies to determine specific T cell sub-types would be useful to determine their role in protection.

We observed increased $CCR4^+$ NK cell numbers in *Mthfr*-deficient mice. In humans, $CCR4^+$ NK cells have been shown to be IFNγ producers, but their exact function in mice is unknown (Pandya, et al., 2011). However, the findings of increased $CCR4^+$ NK cell numbers in *Mthfr*-deficient mice are consistent with the increase in immunoreactive IFNγ in brain.

IFNγ and IL-10 are thought to act in concert, with IL-10 acting in an immunomodulatory capacity (Hunt and Grau, 2003). Although IL-10 can be involved in suppression of cerebral pathology, IL-10 deficiency in a murine *P. berghei* ANKA model prolonged survival in infected mice (Kossodo, et al., 1997). Despite the many studies with cytokine levels in murine serum, there are limited reports on IFNγ and IL-10 levels in infected brains. One study using ELISA did not find differences in IL-10 (de Miranda, et al., 2011), nor did we in our spleen IL-10 ELISA. Our analyses using immunoblotting should be more specific. Our observations suggest that there may be enhanced production or migration of IFNγ-containing lymphocytes into brain and spleen that could contribute to increased resistance in mutant mice. The decrease in IL-10 might facilitate a stronger or longer-acting IFNγ response. This proposed increase in IFNγ -containing lymphocytes in tissues could contribute to the decreased IFNγ in the circulation. This concept has been suggested for several different infections i.e. the “spill-over” of cytokines into peripheral blood is not indicative of the interactions that occur within primary tissues (reviewed in (Tisoncik, et al., 2012)).

We also see baseline immune changes in the uninfected *Mthfr*$^{+/-}$ mice. Increases in the population percentage of CCR4^{+} NK cells in uninfected mice are consistent with our findings of increased numbers of these cells during infection. This suggests that the role and function of these cells in *Mthfr*-deficiency should be further explored. These cells are known to be IFNγ-producing cells in humans (Pandya, et al., 2011), and perhaps they are contributing to the pro-inflammatory responses, oxidative stress and other cellular disturbances (Lazzerini, et al., 2007; Mikael, et al., 2013b; Papatheodorou and Weiss, 2007) that are known to be associated with hyperhomocysteinemia. The finding of increases in the population of IFNγ^{+}-producing cells is also consistent with the finding of increased CCR4^{+} NK cells. There is also differential expression of brain IFNγ and IL-10. In uninfected *Mthfr*$^{+/-}$ mice, brain IFNγ is decreased and the IL-10 inactive monomer is increased. These findings suggest that uninfected *Mthfr*$^{+/-}$ mice may have a greater potential to respond to infection when challenged.

Taken together, our data suggest that *Mthfr*$^{+/-}$ deficiency confers resistance to malaria. The differences between genotypes in our mice are subtler than those seen in most other murine models of malarial resistance, since the latter are often mice with null alleles. In our model, there is a mild enzymatic deficiency in *Mthfr*$^{+/-}$ mice, with a similar decrease in 677TT individuals. Hood et al, using a different cerebral malaria parasite, showed that even a modest increase in sickle cell hemoglobin resulted in longer survival and obtained a survival curve that looked similar to that in our model (Hood, et al., 1996).

There are several mechanisms related to MTHFR disturbances that can contribute to resistance. MTHFR deficiency leads to DNA hypomethylation in humans (Friso, et al., 2013) and

mice (Chen, et al., 2001), which can modify gene expression. It is also associated with increased methyleneTHF, required for thymidine synthesis, as mentioned above; this increase could potentially enhance DNA synthesis and replication of lymphocytes (Bagley and Selhub, 1998; Celtikci, et al., 2009). MTHFR variants are known to be modulated by folate intake and there are several nutrients that interact at various steps in folate metabolism eg. choline, vitamin B_{12} (Ueland and Rozen, 2005). It is therefore possible that the outcome of infection is also influenced by the intake of these nutrients.

Our findings are consistent with our earlier report, in which we demonstrated increased resistance of mice with a complete knockout of *Mthfr* (*Mthfr*$^{-/-}$ mice) to murine cytomegalovirus (Fodil-Cornu, et al., 2009). However, this work is the first demonstration that mild MTHFR deficiency, as seen in *MTHFR* 677TT individuals, confers a degree of protection against a pathogen associated with selection. We suggest that a selective advantage against malaria may have maintained the 677 polymorphism in human populations.

3.5.Acknowledgements

This work was supported by a Canadian Institutes of Health Research grant to RR (MOP#43232). DNM was supported by a Foundation of Stars Studentship at the Montreal Children's Hospital (McGill University Health Centre) and by a McGill University Faculty of Medicine Studentship. The McGill University Health Centre Research Institute is supported by a Centres grant from the Fonds de recherche du Québec-Santé.

CHAPTER IV

DECREASED RESISTANCE TO MALARIA IN MICE THAT OVER-EXPRESS METHYLENETETRAHYDROFOLATE REDUCTASE (*MTHFR)*

Taken in part from:

Increased Resistance to Malaria in Mice with Methylenetetrahydrofolate Reductase (Mthfr) Deficiency Suggests a Mechanism for Selection of the MTHFR 677C>T (c. 665C>T) Variant

Danielle N. Meadows, Michal Pyzik, Qing Wu, Sabrina Torre, Philippe Gros, Silvia M. Vidal and Rima Rozen

Human Mutation (2014) 35(5): 594-600

4.1.Summary

MTHFR is the enzyme that catalyzes the breakdown of 5, 10-methyleneTHF to 5-methyTHF. Genetic deficiency in this enzyme due to a common polymorphism at the 667th base pair (677C→T (c.665C→T, p.Ala222Val, rs1801133)) has been linked to increased risk for several complex traits. Despite these associated risks, homozygosity is high (5 – 15%) in many populations, particularly in Mediterranean regions where malaria was historically endemic, and may have conferred a selective advantage for other mutations. In the previous chapter, we infected *Mthfr*-deficient (*Mthfr*$^{+/-}$) mice with *Plasmodium berghei* ANKA to induce cerebral malaria. *Mthfr*$^{+/-}$ mice survived longer after infection compared with wild-type littermates. They also had changes in their lymphocyte populations and increased CCR4^{+} NK cells in the spleen. Uninfected mice had changes in distribution of immune cell populations as well. To confirm that MTHFR expression affected the outcome of malaria infection, we infected mice that over-express MTHFR, *MTHFR*^{Tg}, with *Plasmodium berghei* ANKA. These mice were more susceptible to infection ($p<0.05$, log-rank test). They also had decreases in total NK cells ($p<0.05$, t-test) and in CCR4^{+} NK cells ($p<0.01$, t-test). Uninfected *MTHFR*^{Tg} mice had decreased total splenocytes and decreases in total T cells and in CD8+ subsets ($p<0.05$, t-test). Our findings, in light of our observations in *Mthfr*-deficient mice, are consistent with our hypothesis that mild MTHFR deficiency protects against malarial infection.

4.2. Introduction

Plasmodium parasites are responsible for malaria infection. Though several strains can infect humans, most severe infections are caused by *Plasmodium falciparum.* Cerebral malaria is

a complication of malaria infection and its symptoms may include paralysis, seizure, brain hemorrhage and death.

Pathogens help shape the human genome by positive selection. Alleles that confer protection against infections persist in populations, despite also causing negative health consequences under other circumstances. Hemoglobinopathies and other factors have also been shown to improve the outcome of malaria (Berghout, et al., 2012; Fortin, et al., 2002). In murine studies *P. berghei* ANKA for the cerebral stage of malaria in C57Bl/6 mice (Fortin, et al., 2002). In earlier studies, we found that mice deficient in methylenetetrahydrofolate reductase (*Mthfr)* also have protection against cerebral malaria.

The primary circulating form of folate, methylTHF, remethylates homocysteine into methionine. It is synthesized by the enzyme methyleneTHF reductase (MTHFR, EC 1.5.1.20). *MTHFR* (607093) has several common variants (Leclerc, et al., 2005), though the 677C→T polymorphism (c.665C→T, p.Ala222Val, 607093.003, rs1801133) has been studied most extensively. *MTHFR*677C→T results in an approximate loss of approximately 70% enzyme activity and mild hyperhomocysteinemia (Frosst, et al., 1995). The frequency of the 677TT genotype is high in southern Europe (15-20%), but widely varies in population around the world (Figure 1.2) (Angius, et al., 2007; Gueant-Rodriguez, et al., 2006; Houcher, et al., 2010; Leclerc, et al., 2005; Rosenberg, et al., 2002). The polymorphism is part of a common haplotype shared among diverse populations, suggesting a shared ancestral history; selective advantage could have assisted in spreading the variant through populations (Rosenberg, et al., 2002).

In earlier work, we generated transgenic mice that over-express *MTHFR* (*MTHFR*^{Tg}) to study the effect of MTHFR expression on methotrexate. *MTHFR*^{Tg} mice have a copy of human *MTHFR* inserted on their X chromosome. Human *MTHFR* is approximately 90% homologous with murine *Mthfr* (Goyette, et al., 1999). This gene insertion results in an approximately 2-fold increase in MTHFR protein and enzyme activity; consistent increases in RNA, protein levels and enzyme activity suggest that there is little to no post-translational modification of the enzyme (Celtikci, et al., 2008). Overall, there is little information on regulation of MTHFR in humans or mice.

MTHFR^{Tg} mice did not have changes in the distribution of folate intermediates in their plasma, where most of the folates are methylTHF, but they did have changes in the brain, intestine and liver. In the liver and intestine they had decreased levels of 10-formylTHF, and brain had increased methionine. These observations are consistent with higher MTHFR activity driving nonmethylTHF conversion into methylTHF, which would in turn drive methionine synthesis. There is also evidence that the conversion of dUMP and dTMP is compromised in *MTHFR*^{Tg} mice (Celtikci, et al., 2008).

In our initial study of *Mthfr*-deficient mice, we endeavored to find a mechanism that might explain the high frequency of this polymorphism. In this study, we endeavored to confirm our findings in the *Mthfr*-deficient mice by determining whether mice that over-express *MTHFR* would be more susceptible to infection. *MTHFR*^{Tg} mice were infected with *P. berghei* ANKA to induce cerebral malaria. We observed decreased resistance and we found decreases in some cell populations that were increased in the protected *Mthfr*-deficient mice. We also demonstrated some changes in immune cell populations of uninfected mice that over-express MTHFR.

4.3.Results

4.3.1. Survival and parasitemia of *MTHFR^{Tg}* mice during *Plasmodium berghei* ANKA infection

After our earlier experiments in which mice deficient in *Mthfr* survived longer than wild-type mice, we wanted to confirm that MTHFR expression level influences survival. We infected *MTHFR^{Tg}* and *MTHFR^{Wt}* mice intravenously with 10^5 *P. berghei* ANKA parasites and monitored their survival. *MTHFR^{Tg}* mice were more susceptible to *P. berghei* ANKA infection than wild-type littermates (Figure 4.1A; $p<0.05$, log-rank test). Mortality was first observed at 5 dpi and 6 dpi in *MTHFR^{Tg}* and *MTHFR^{Wt}* mice, respectively.

As in earlier experiments, thin-layer blood smears were collected from each animal at different days. Slides were stained to determine percent parasitemia by calculating the ratio of infected RBC to total RBC. *Mthfr$^{+/+}$* and *MTHFR^{Wt}* mice have comparable parasite levels at 4 dpi, as expected (data not shown). *MTHFR^{Tg}* mice and their wild-type littermates have similar parasite levels at 4 and 5 dpi (Figure 4.1B; $p>0.05$, ANOVA), but they have a trend toward higher levels of parasite at 6 dpi (Figure 4.1C; $p=0.090$; t-test). Whether this difference has biologic significance is unclear. Nevertheless, the higher parasitemia is consistent with the increased susceptibility to infection observed in *MTHFR^{Tg}* mice.

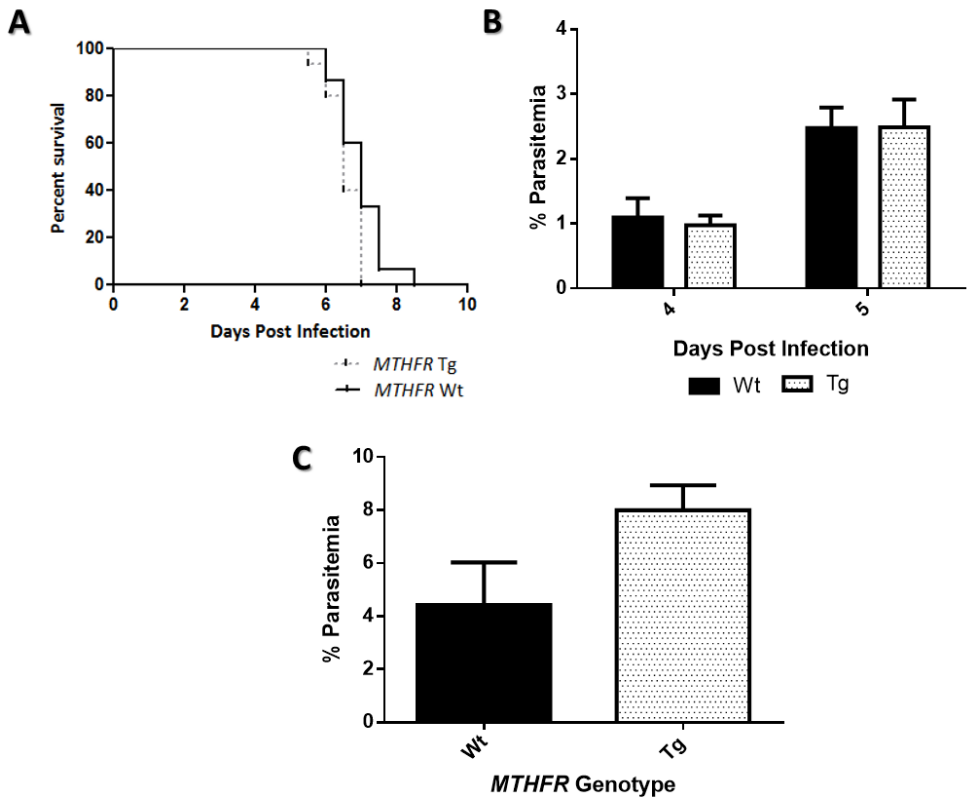


Figure 4.1: Survival and parasitemia of *MTHFR*$^{Tg-}$ mice following 10^5 *P. berghei* ANKA infection. (A) *MTHFR*^{Tg} mice (dotted line) did not survive infection as long as *MTHFR*^{Wt} mice (solid line); n=15 *MTHFR*^{Wt} and n=15 *MTHFR*^{Tg}; $p<0.05$, Log-rank test. (B) Parasitemia in *MTHFR*^{Tg} mice at 4 and 5 dpi, n=5/day *MTHFR*^{Wt} (black bar) and n=5/day *MTHFR*^{Tg} (dotted bar); $p>0.05$, t-test. (C) Parasitemia in *MTHFR*^{Tg} mice at 6 dpi, n=5 *MTHFR*^{Wt} (black bar) and n=5 *MTHFR*^{Tg} (dotted bar); p=0.090, t-test.

4.3.2. Complete blood counts in infected *MTHFR*^{Tg} mice

Blood was collected from mice at 4 and 5 dpi. They were sent for complete blood counts and differential counts. Results are summarized in Table 4.1. There were no differences of note between the *MTHFR*^{Wt} and *MTHFR*^{Tg} mice at 4 or 5dpi.

Table 4.1 Complete blood counts and differentials in infected *MTHFR*^{Tg} mice 4 and 5 dpi[1, 2]

DPI:	4		5	
GENOTYPE:	*MTHFR*^{Wt}	*MTHFR*^{Tg}	*MTHFR*^{Wt}	*MTHFR*^{Tg}
RBCs (x 10^{12}/L)	12.11 ± 0.27	11.75 ± 0.10	12.44 ± 0.45	12.10 ± 0.22
Hematocrit (L/L)	0.542 ± 0.014	0.523 ± 0.006	0.558 ± 0.019	0.541 ± 0.010
Hemoglobin (g/L)	176 ± 4	172 ± 2	179 ± 5	176 ± 4
MCV (fL)	44.8 ± 0.2	44.4 ± 0.2	44.8 ± 0.2	44.8 ± 0.2
MCH (pg)	14.6 ± 0.1	14.6 ± 0.1	14.4 ± 0.2	14.5 ± 0.1
MCHC (g/L)	329 ± 2	326 ± 3	321 ± 3	326 ± 4
WBCs (x 10^9/L)	4.3 ± 0.5	4.5 ± 0.5	2.1 ± 0.4	1.9 ± 0.3
neutrophils %	14 ± 3	16 ± 2	21 ± 3	15 ± 2
lymphocytes %	85 ± 3	82 ± 2	68 ± 8	84 ± 2
neutrophils (x 10^9/L)	0.59 ± 0.16	0.71 ± 0.08	0.46 ± 0.12	0.28 ± 0.06
lymphocytes (x 10^9/L)	3.64 ± 0.47	3.71 ± 0.50	1.40 ± 0.27	1.59 ± 0.23
platelets (x 10^9/L)	1143 ± 230	1007 ± 33	401 ± 12	414 ± 112

[1] n=5 *MTHFR*^{Wt} and *MTHFR*^{Tg}-
[2] Mean ± SEM

4.3.3. Immune cell population changes in infected *MTHFR*^{Tg} mice

As in experiments with *Mthfr*- deficient mice, we isolated and stained splenocytes from *MTHFR*^{Tg} and *MTHFR*^{Wt} mice at 6 dpi to determine whether any of the cell population changes found in *Mthfr*-deficient mice would also be found in the reverse direction in *MTHFR*^{Tg} mice after infection. We isolated splenocytes and counted the number of viable cells. Equal numbers of viable splenocytes (5.0 x 10^6 cells) were then incubated for 3 hours with GolgiStop™ to inhibit cytokine secretion and stained for viability. Surface staining (CD4, CD8, DX5 (CD49b), CXCR3 (CD183), CCR4 (CD194)) and intracellular staining (CD3 and IFNγ) were performed to identify various immune cell populations (T cells, NK cells, NK T cells). Cells were analyzed by flow cytometry; only significant population differences are shown (Figure 4.2 A-B). We did not find changes in total splenocytes or in lymphocyte numbers (data not shown), but we did find

decreased numbers of NK cells (Figure 4.2A; p<0.5, t-test) in *MTHFR*^{Tg} mice. We also found decreased numbers of CCR4^{+} NK cells (Figure 4.2B; p<0.01, t-test) compared with *MTHFR*^{Wt} mice.

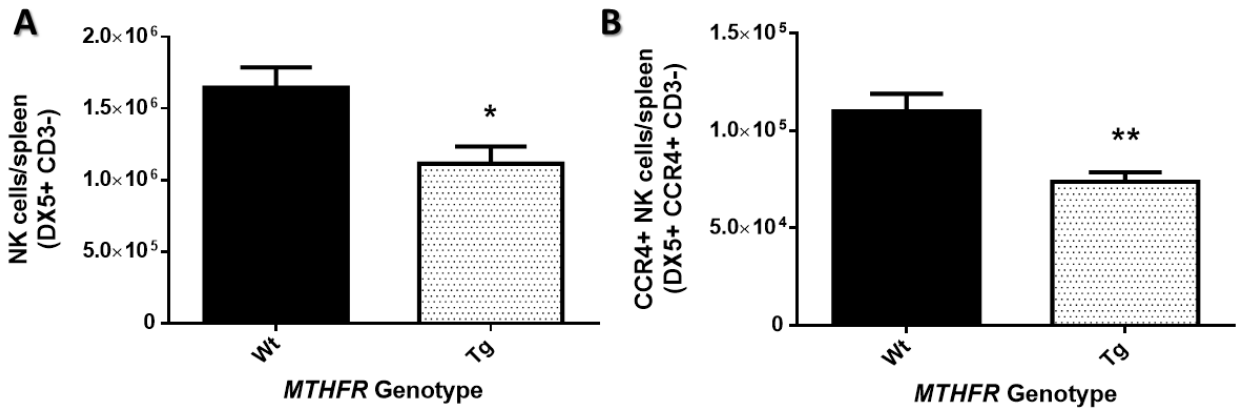


Figure 4.2: Cell population changes at 6 dpi in *MTHFR*^{Tg} mice intravenously infected with 10^5 *P. berghei* ANKA. Male *MTHFR*^{Tg} mice and wild-type littermates were sacrificed at 6 dpi. Spleens were harvested and processed for flow cytometry as in Figure 3.6. Means ± SEM are indicated for each group. *MTHFR*^{Tg} mice (striped bar) had significantly lower numbers of (A) NK cells/spleen (*p<0.05, t-test) and lower numbers of CCR4^{+} (CD194^{+}) NK cells/spleen (**p<0.01, t-test) compared with *MTHFR*^{Wt} (black bars) littermates.

4.3.4. Immune cell population changes in uninfected *MTHFR*^{Tg} mice

We then isolated and stained splenocytes from uninfected *MTHFR*^{Tg} mice to see if any of the changes post-infection were observed prior to infection. *MTHFR*^{Tg} mice had decreased numbers of total splenocytes at isolation (Figure 4.3A; p<0.05, t-test) and after 3 hours incubation with GolgiStop™ (Figure 4.3B; p<0.05, t-test). They also had decreased numbers of total T cells (Figure 4.3C; p<0.05, t-test) and CD8^{+} T cells (Figure 4.3D; p<0.05, t-test). There was a trend towards a decrease in CD4^{+} T cells (Figure 4.3E; p=0.071, t-test). Interestingly uninfected *MTHFR*^{Tg} mice showed no changes in total NK cells or in CCR4^{+} NK cells.

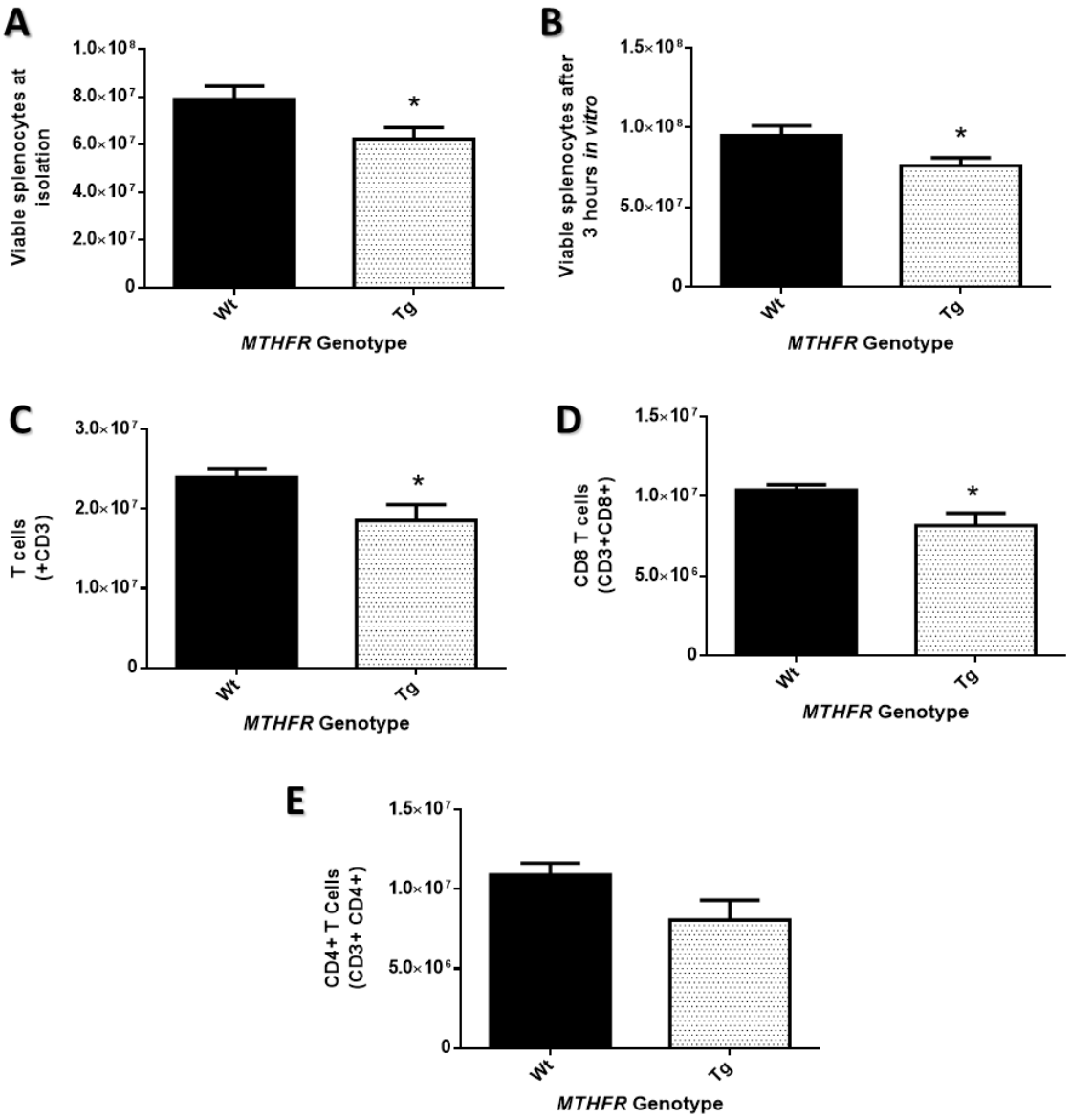


Figure 4.3: Cell population changes in uninfected *Mthfr^{Tg}* mice Male *Mthfr^{Tg}* (striped bar) and *MTHFR^{Wt}* (black bar) mice age-matched to those used in infection experiments (~3 months) were sacrificed and splenocytes stained for flow sorting. n=8 *MTHFR^{Tg}* and n=8 M*THFR^{Wt}*, means ± SEM are indicated for each group. (A) *MTHFR^{Tg}* mice had decreased cells at (A) isolation (*p<0.05, t-test) and (B) after 3 hours *in vivo* (*p<0.05, t-test). *MTHFR^{Tg}* mice also has decreased numbers of (C) T cells (*p<0.05, t-test) and (D) CD8+ T cells (*p<0.05, t-test). There was also a trend towards less (E) CD4^{+} T cells (p=0.071, t-test).

4.4.Discussion

In our earlier infection experiments, we found that *Mthfr*-deficiency protects mice against *P. berghei* ANKA infection. *Mthfr*$^{+/-}$ mice survived longer and had lower parasitemia than wild-type mice. They also had higher numbers of total splenocytes, T lymphocytes and CCR4^{+} NK cells. In these experiments, we found that MTHFR-over-expression had a reverse effect. *MTHFR*^{Tg} mice were more susceptible to infection and died earlier. *MTHFR*^{Tg} mice also had fewer immune cells, but in infected *MTHFR*^{Tg} mice, there were only changes in the number of total NK cells and in CCR4^{+} NK cells. Like in *Mthfr*$^{+/-}$ mice, we did not find any differences in the complete blood counts or differential counts of *MTHFR*^{Tg} mice. This clinical test may not be sensitive enough to see the cellular changes we saw in the spleen.

The uninfected *MTHFR*^{Tg} mice had changes in many of the same immune cell populations that the infected *Mthfr*$^{+/-}$ mice did, except in the opposite direction. Instead of increases in total viable splenocytes and T lymphocytes, the *MTHFR*^{Tg} mice had decreases in the same populations. We hypothesized in our earlier work that the increases seen in infected *Mthfr*$^{+/-}$ mice may be due to increased levels of the substrate, methyleneTHF, which is required for thymidine production. The enhanced thymidine synthesis could then lead to more cell proliferation. In the liver and the spleen, the levels of 10-formylTHF are significantly decreased in *MTHFR*^{Tg} mice (Celtikci, et al., 2008). As the immediate folate precursor of purine synthesis, less 10-formylTHF would indicate that purine synthesis is limited. With fewer purines for proliferation, the decreases in total splenocytes and T cells that we see in the uninfected *MTHFR*^{Tg} mice may be due to their metabolic constraints. There is also evidence that the conversion of dUMP and dTMP is compromised in

MTHFR^{Tg} mice (Celtikci, et al., 2008), which would also limit the nucleotides available for proliferation and likely lead to the decreases we see in cell number.

The CCR4^{+} NK cells are of interest and should be the subject of further study. In *Mthfr*$^{+/-}$ mice they are elevated in the spleens of both naïve and *P. berghei* ANKA infected mice, and in *MTHFR*^{Tg} mice these cells are decreased. Human studies suggest that these cells are potent IFNγ producers and pro-inflammatory (Pandya, et al., 2011). It is possible that the elevation of these cells in *Mthfr*$^{+/-}$ mice and the decrease in *MTHFR*^{Tg} mice may be related to the inflammation and oxidative stress that accompanies elevated homocysteine levels (Lazzerini, et al., 2007; Mikael and Rozen, 2008; Papatheodorou and Weiss, 2007).

Our findings in *MTHFR*^{Tg} mice are not only consistent with, and supporting of our hypothesis that *Mthfr* deficiency is protective against cerebral malaria, but they are also consistent with the findings in mCMV studies. *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice had lower viral titers than wild-type mice, while *MTHFR*^{Tg} mice had higher viral titers than wild-type mice did (Fodil-Cornu, et al., 2009). Higher titers in mCMV infection experiments are indicative of higher infection susceptibility to mCMV. Though in those experiments the results were not significant, they are consistent our findings that a change in *Mthfr*$^{+/-}$ mice, goes the opposite way in *MTHFR*^{Tg} mice. These results are also consistent with clinical reports that West African MTHFR 677T carriers are protected from chronic hepatitis B infection (Bronowicki, et al., 2008). Taken together these results suggest that the level of MTHFR expression can influence resistance to several pathogens.

4.5.Acknowledgements

This work was supported by a Canadian Institutes of Health Research grant to RR (MOP#43232). DNM was supported by a Foundation of Stars Studentship at the Montreal Children's Hospital (McGill University Health Centre) and by a McGill University Faculty of Medicine Studentship. The McGill University Health Centre Research Institute is supported by a Centres grant from the Fonds de recherche du Québec-Santé.

CHAPTER V

HIGH DIETARY FOLATE IN MICE ALTERS IMMUNE RESPONSE AND REDUCES SURVIVAL AFTER MALARIAL INFECTION

Danielle N. Meadows, Renata H. Bahous, Rima Rozen

5.1.Summary

Malaria is a parasitic infection with nearly 200 million cases in 2013 alone. The parasite can obtain folate from its host or synthesize it *de novo*. In the previous chapters, we examined the impact of MTHFR expression on resistance to malaria. However, with increased folate intake due to food fortification and vitamin supplementation in many countries, in this chapter we questioned whether high dietary folate would affect response to malarial infection in a murine model of cerebral malaria. Mice were fed control diets (CD, recommended folate level) or folic acid-supplemented diets (FASD, 10-fold higher than recommended) for 5 weeks before infection with *Plasmodium berghei* ANKA. FASD mice had reduced survival ($p<0.01$, log-rank test) and higher parasitemia ($p<0.05$, t-test) compared with CD mice. FASD mice had lower numbers of splenocytes, total T cells, as well as lower numbers of specific T and NK cell sub-populations ($CD4^+$ T cells, $CD4^+$ $IFN\gamma^+$ T cells, $CD8^+$ T cells, $CCR4^+$ NK cells and $CCR4^+$ $IFN\gamma^+$ NK cells), compared with CD mice ($p<0.05$, ANOVA). Increased brain TNFα immunoreactive protein ($p<0.001$, t-test) and increased liver *Abca1* mRNA ($p<0.01$, t-test), a modulator of TNFα, were observed in FASD mice; these variables correlated positively ($r_s= 0.58$, $p<0.01$). *Bcl-xl/Bak* mRNA was increased in liver of FASD mice ($p<0.0001$, t-test), suggesting reduced apoptotic potential. We conclude that high dietary folate increases parasite replication, disturbs immune response and reduces resistance to malaria in mice. These findings have relevance for malaria-endemic regions, when considering anti-folate anti-malarials, food fortification or vitamin supplementation programs.

5.2.Introduction

In 2013, there were 198 million reported cases of malaria in 97 malaria-endemic countries, with 584,000 deaths (WHO, 2014). Malaria is caused by infection with the *Plasmodium* parasite and spreads through mosquitos. In humans, the majority of morbidity and mortality comes from *Plasmodium falciparum* infection. Children, pregnant women, and poverty-stricken individuals are at greatest risk for poor outcomes (Nzila, et al., 2014; WHO, 2014).

In mice, several strains of *Plasmodium* are used to study different complications of malaria. We have used *Plasmodium berghei* ANKA, a strain that induces experimental cerebral malaria in mice. We recently showed that a genetic deficiency in methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) protects against *P. berghei* ANKA infection (Meadows, et al., 2014). *Mthfr*$^{+/-}$ mice, heterozygous for a null allele, were more resistant to infection and mice that overexpressed MTHFR (through an additional copy of the human *MTHFR* gene (*MTHFR*^{Tg} mice)) were more susceptible to infection, compared with wild-type animals (*MTHFR*^{Tg} and *Mthfr*$^{+/+}$ mice) (Meadows, et al., 2014). We found that resistant *Mthfr*$^{+/-}$ mice had differences in immune cell populations and cytokine expression, and that these changes could contribute to the increased survival (Meadows, et al., 2014).

Folate derivatives are required for nucleotide and amino acid synthesis, as well as for methylation reactions. For many years, anti-folate drugs have been used to inhibit growth of pathogens, including the malarial parasite. Two folate-dependent enzymes, dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHSP) are popular targets in malaria (Muller and Hyde, 2013; Nzila, et al., 2005a). Blocking these enzymes inhibits *de novo* folate synthesis in the

parasite; mammalian hosts do not synthesize folate. Unfortunately, resistance has developed to many of these drugs (Muller and Hyde, 2013). To limit the impact of disease, one preventative strategy has been the prescription of folate-blocking anti- malarials to late-term pregnant women and to children during routine health care screenings (Nzila, et al., 2014; WHO, 2014). The latest reports show that these programs are not reaching the desired level of efficacy and dissemination.

Low folate intake is a risk factor for neural tube defects. Consequently many countries have introduced folate fortification of food to reduce incidence (Boulet, et al., 2008; Crider, et al., 2011; De Wals, et al., 2007). Some malaria-endemic countries such as Ghana and Cote d'Ivoire have already implemented dietary fortification programs (EUROCAT, 2015). However, with fortification and the general increased use of vitamin supplements among many population sub-groups, there are concerns that increased folate intake may have negative health consequences, including immune cell cytotoxicity (Troen, et al., 2006) and increased inflammation (Protiva, et al., 2011). Food fortification and vitamin supplementation use folic acid, a synthetic folate that may inhibit folate-dependent enzymes and transporters (Smith, et al., 2008). These issues and others have collectively led to questions regarding the risks and benefits of universal fortification (Crider, et al., 2011; Smith, et al., 2008).

Our laboratory has shown that high folate intake in mice, using a diet with 10-fold higher folate than the recommended level for rodents, negatively impacts embryonic development (Mikael, et al., 2013a) and leads to liver injury through alterations in lipid metabolism (Christensen, et al., 2015). The higher dietary folate in these studies resulted in a 3-fold increase in plasma folate (Mikael, et al., 2013a), the same degree of increase that has been reported in the United States

(Pfeiffer, et al., 2012). Considering our finding that a genetic disturbance in folate metabolism has an impact on resistance to malaria and the fact that anti-folate drugs are frequently used as anti-malarials, we sought to determine whether variation in dietary folate could affect the outcome of malaria infection in mice. We observed reduced resistance to malaria infection in mice with higher dietary folate, and identified some host changes in immune cell populations and immune modulators that could contribute to the altered resistance.

5.3.Results

5.3.1. Decreased survival of infected mice fed FASD

Murine diets that investigate the impact of folate intake, as used in previous work in our laboratory, often contain an antibiotic to prevent folate synthesis by intestinal flora (Sepehr, et al., 2003). To ensure that the antibiotic routinely used in our control diet, succinylsulfathiazole (SST), did not affect survival of mice infected with the malarial parasite, we compared survival of male *Mthfr*$^{+/+}$mice fed the control diet with SST (CD+ SST), the control diet without SST (CD) and standard mouse chow (MC) for 5 weeks before infection. Both control diets are amino acid defined and contain the recommended amount of folic acid for rodents (2 mg/kg diet). Mouse chow contains a mix of whole food products and is estimated to contain approximately 4 mg folic acid /kg diet. After 5 weeks on diet, mice were injected with 10^5 *P. berghei* ANKA parasites and survival was monitored. CD (Figure 5.1A; $p<0.0001$, log-rank test) and CD+SST (Fig 1a; $p<0.001$, log-rank test) mice survived significantly longer than MC mice, with 100% survival in both CD groups at two weeks post-infection. Survival between CD and CD+SST mice did not differ (Fig 1a; $p=1.0$, log-rank

test). Onset of mortality was observed in MC mice at 7 or 8 dpi, with 100% mortality before 10 dpi; this result is the typical outcome in mice fed standard chow and dying of cerebral malaria, as observed by our group (Meadows, et al., 2014) and others (Hood, et al., 1996; Roth, et al., 1988).

Mouse chow and amino acid-defined diets, such as CD and CD+SST, are very different in content, not just in the level of folic acid, but also in other amino acids and nutrients, including methionine, choline and glycine which can participate in one-carbon metabolism. To determine whether differences in survival were due only to differences in folate content, we used a folic acid- supplemented diet (FASD), in which the diet was identical to CD in all components except for a 10-fold increase in folate content (20 mg/kg diet). Since there were similar rates of survival between CD and CD+SST mice, we did not add SST to our CD or FASD diets. After five weeks on CD, FASD or MC, *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice were infected with 10^5 *P. berghei* ANKA parasites and survival was monitored. There were no significant differences in survival between *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice on these diets (CD *Mthfr*$^{+/+}$ compared with CD *Mthfr*$^{+/-}$, $p>0.05$, log-rank test, and FASD *Mthfr*$^{+/+}$ compared with FASD *Mthfr*$^{+/-}$, $p>0.05$, log-rank test; data not shown). Genotypes were therefore combined for further analyses. Figure 1B indicates that CD mice survived significantly longer than FASD mice ($p<0.01$, log-rank test); this experiment was repeated and the same conclusion was reached in the second experiment (data not shown; $p<0.001$, log-rank test). Symptoms of cerebral malaria such as tremors, lethargy, and impaired gait, were observed in FASD mice starting at 8-9 dpi. It is unclear whether the ultimate demise after 20 dpi in these mice was due to cerebral complications or complications of severe malaria-induced anemia.

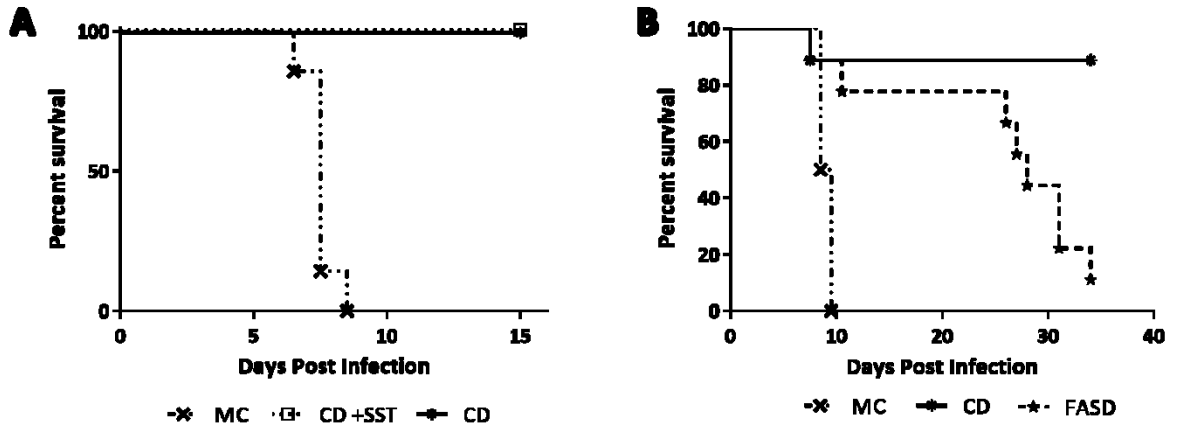


Figure 5.1. Survival of mice fed diets with variable folate content. (A) CD mice (n=8; p<0.0001, log-rank test) and CD+SST mice (n=7; p<0.001, log-rank test) survived longer than MC mice (n=7). There was no survival difference between CD and CD+SST mice at 15 dpi (p=1.0, log-rank test) (B) CD mice (n=9) survived longer than FASD mice (n=9; p<0.01, log-rank test). The MC mice (n=2) served as a positive control for a successful infection.

5.3.2. Increased parasite levels in infected FASD mice

Parasitemia was measured every 48h from 6-20 dpi (Figure 5.2). Early in infection, parasitemia was higher in FASD mice with 3.64 ± 0.62% at 6 dpi compared with 1.39 ± 0.53% in CD mice (Figure 5.2; $p<0.05$, t-test), and at 8 dpi (Figure 5.2; $p<0.05$, t-test) with 5.34 ± 0.68% parasitemia in FASD mice compared with 2.56 ± 0.90% in CD mice. As the infection progressed, parasite levels increased in both groups but remained significantly higher in FASD mice. At 18dpi, FASD mice had 49.88 ± 6.64% parasitemia compared with 18.71 ± 6.98% in CD mice (Figure 5.2; $p<0.01$, t-test), and at 20 dpi, 53.52 ± 6.28% in FASD mice and 20.36 ± 8.59% in CD mice (Figure 5.2; $p<0.01$, t-test).

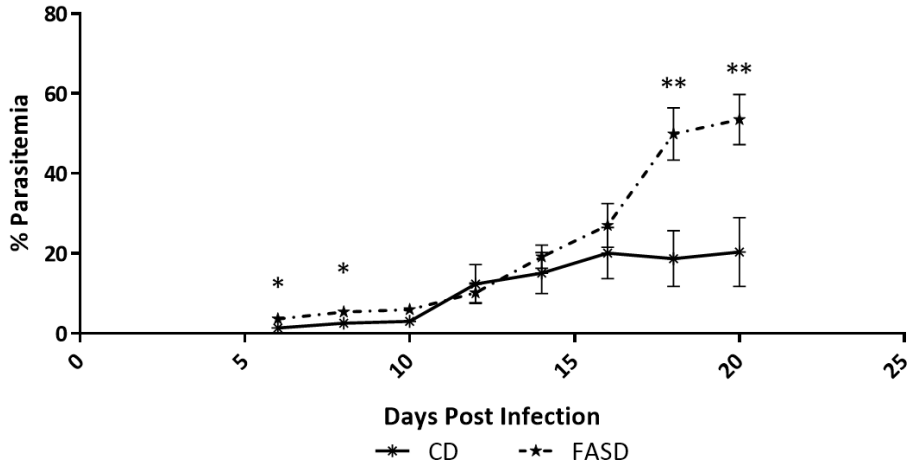


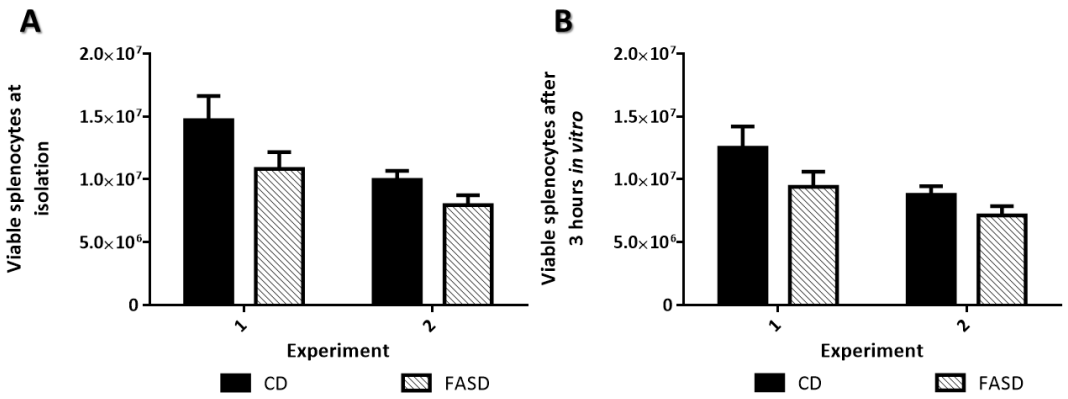
Figure 5.2. Parasitemia in CD and FASD mice. Values are means ± SEM. Parasitemia was measured every 48h from 6-20dpi. FASD mice (n=9) had significantly higher levels of parasitemia than CD mice (n=9) at 6, 8, 18 and 20 dpi. *p<0.05, **p<0.01, t-test.

5.3.3. Decreased immune cell populations in infected FASD mice

In earlier work (Meadows, et al., 2014), we found increases in some immune cell populations in the spleen of mice that were resistant to *P. berghei* ANKA. To examine the same cell populations in this study, we isolated splenocytes from CD and FASD mice at 7 dpi, counted viable cells and performed flow cytometry. Splenocytes were collected at this time point before the onset of cerebral symptoms or deaths in FASD mice at 8-9 dpi. FASD mice had significantly less viable splenocytes at isolation compared with CD mice (Figure 5.3A; $p<0.05$, ANOVA). Viable splenocytes were then plated at equal densities (5.0×10^6 cells) and incubated at 37°C with cell media enriched with the cytokine secretion inhibitor GolgiStop™. After 3 hours in culture, cells were re-stained for viability. Again, FASD mice had less viable cells than CD mice (Figure 5.3B; $p<0.05$, ANOVA), despite being plated at equal densities of viable cells.

Staining for surface cellular differentiation markers CD4, CD8, DX5 (CD49B), CCR4 (CD194) and intracellular staining for CD3 and IFNγ allowed us to identify and quantify various immune cell populations. Our staining panel specifically targeted natural killer (NK) and T cell populations, which are critical in fighting malarial infection (Hansen, et al., 2007; Spence and Langhorne, 2012). Shown are the cell populations that had significant differences between CD and FASD mice (Figure 4A-F).

FASD mice had decreased populations of T cells (Figure 5.4A; $p<0.05$, ANOVA). Within T cell subsets, they had decreased $CD8^+$ T cells (Figure 5.4B; $p<0.05$, ANOVA), as well as decreased populations of $CD4^+$ T cells (Figure 5.4C; $p<0.05$, ANOVA). Within the $CD4^+$ T cell population, FASD mice also had decreased $CCR4^+$ $CD4^+$ T cells (Figure 5.4D; $p<0.05$, ANOVA). There were no changes in the total number of NK cells, but FASD mice had fewer $CCR4^+$ NK cells (Figure 5.4E; $p<0.01$, ANOVA) than CD mice. They also had a borderline decrease in $IFN\gamma^+$ NK cells (Figure 5.4F; $p=0.061$, ANOVA). $CCR4^+$ NK cells have been reported to produce IFNγ and IL-17 (Pandya, et al., 2011), which are important responders in malarial infection.



Diet: p<0.05
Experiment: p<0.01

Diet: p<0.05
Experiment: p<0.05

Figure 5.3. Differences in viable immune cell populations at 7 dpi between CD and FASD mice. Values are means ± SEM. Two experiments are shown. (A) FASD mice (diagonal bars; n=5 (experiment 1) and n=8 (experiment 2)) had lower numbers of viable splenocytes compared with CD mice (black bars; n=7 (experiment 1) and n=9 (experiment 2)) ($p<0.05$, ANOVA). Experiment 1 had higher cell numbers than experiment 2 ($p<0.01$, ANOVA). (B) Viable splenocyte numbers were lower in FASD murine cultures after incubation with GolgiStop™ ($p<0.05$, ANOVA). Experiment 1 had higher cell numbers than experiment 2 ($p<0.05$, ANOVA).

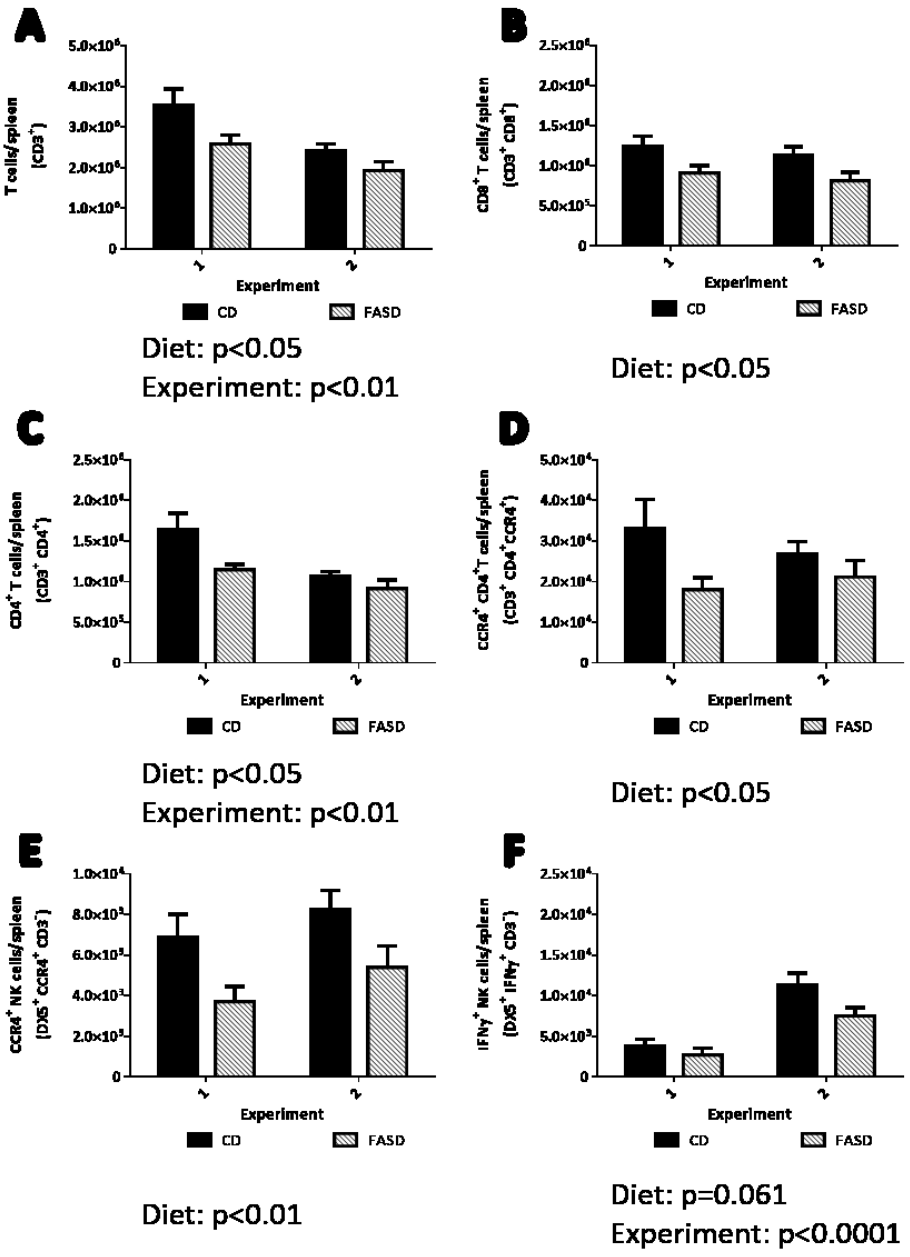


Figure 5.4. Differences in spleen immune cell populations at 7 dpi between CD and FASD mice. Values are means ± SEM. Two experiments are shown. FASD mice (diagonal bars; n=5 (experiment 1) and n=8 (experiment 2)) had significantly decreased populations of (A) T cells ($p<0.05$, ANOVA), (B) $CD8^+$ T cells ($p<0.05$, ANOVA), (C) $CD4^+$ T cells ($p<0.05$, ANOVA), (D) $CCR4^+$ $CD4^+$ T cells ($p<0.05$, ANOVA) and (E) $CCR4^+$ NK cells ($p<0.01$, ANOVA) compared with CD mice (black bars; n=7 (experiment 1) and n=9 (experiment 2)). There was also a borderline decrease in (F) $IFN\gamma^+$ NK cells ($p=0.061$, ANOVA) in these FASD mice. Experiment 1 had significantly increased (A) T cells ($p<0.01$, ANOVA) and (C) $CD4^+$ T cells ($p<0.01$, ANOVA), and significantly decreased numbers of (F) $IFN\gamma^+$ NK cells ($p<0.0001$, ANOVA).

5.3.4. Increased expression of TNFα in brain and increased *Abca1* mRNA

Cerebral symptoms are due to an exacerbated inflammatory response to fight the infection (Hunt and Grau, 2003). One of the primary mediators of this inflammatory response is TNFα; decreases in TNFα have been consistently shown to improve the outcome of cerebral malaria (Grau, et al., 1987; Grau, et al., 1989a; Rudin, et al., 1997a). In 3 separate experiments, we measured the expression in brain of TNFα (Figure 5.5A), by immunoblotting. FASD mice had an approximate 2-fold increase in TNFα protein, compared with CD mice (Figure 5.5A; $p<0.001$, t-test).

High levels of TNFα may relate, at least in part, to increased expression of ATP-binding cassette sub-family A, member 1, *Abca1*, a gene important in cholesterol transport. *Abca1*$^{-/-}$ mice have fewer pro-inflammatory microparticles in plasma and, decreased brain TNFα compared with wild-type littermates, and are protected from cerebral malaria. These observations in *Abca1*$^{-/-}$ mice suggested that brain TNFα expression may be regulated by *Abca1* expression (Combes, et al., 2005). We therefore elected to measure *Abca1* mRNA in liver, as *Abca1* expression is quite high in this tissue. We found that FASD mice had an approximate 2-fold higher level of *Abca1* expression compared with CD mice (Figure 5.5B; $p<0.01$, t-test). Brain TNFα expression levels positively correlated with liver *Abca1* expression levels in CD and FASD mice (Figure 5.5C; $r_s=0.58$, Spearman correlation, $p<0.01$).

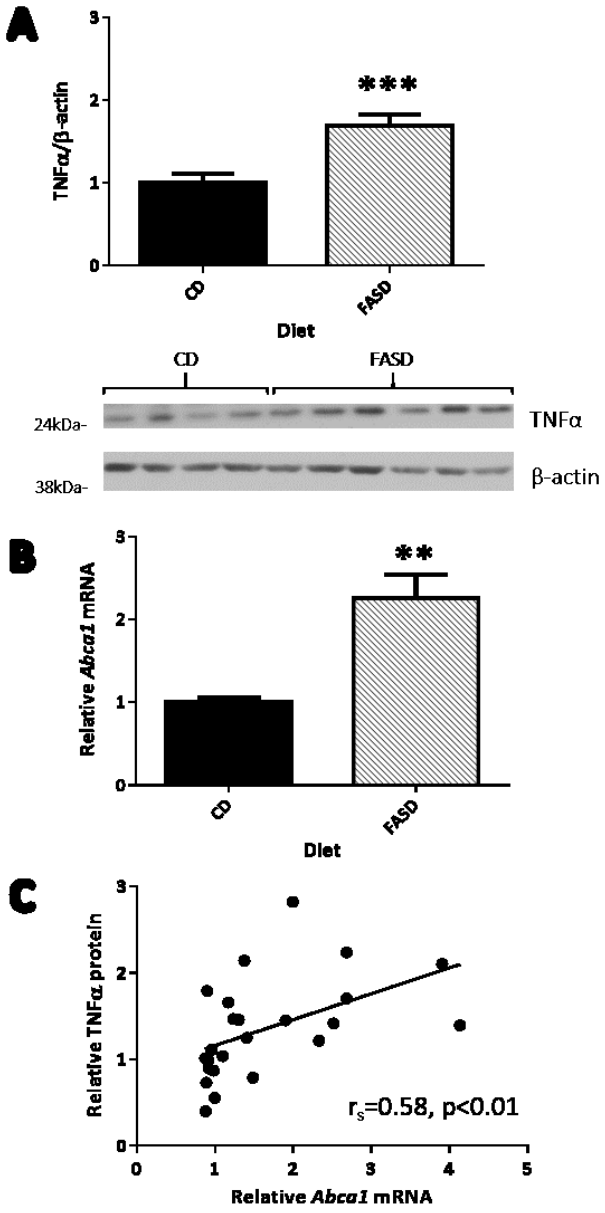


Figure 5.5. Differences in TNFα immunoreactive protein in brain and in relative *Abca1* mRNA in liver between CD and FASD mice at 7 dpi. Values are means ± SEM for A and B. (A) FASD mice (diagonal bars; 3 experiments combined; n=13) had significantly higher levels of TNFα protein in brain compared with CD mice (black bars; 3 experiments combined; n=12). Representative Western blot is shown below the graph. (B) Livers of FASD mice (diagonal bars; 3 experiments combined; n=12) had significantly higher relative *Abca1* mRNA levels than livers of CD mice (black bars; 3 experiments combined; n=14)). (C) Brain TNFα protein correlated with *Abca1* mRNA levels in liver of mice in both dietary groups (r_s=0.58, Spearman correlation, p<0.01; n=24). **p<0.01, ***p<0.001, t-test.

5.3.5. *Bcl-xl/Bak* ratio suggests reduced apoptotic environment in liver of FASD mice

Liver pathology, driven by resident parasites, often precedes cerebral pathology in *P. berghei* ANKA infection, and removal of parasites can reverse the liver pathology (Haque, et al., 2011). To explore liver apoptotic potential, we measured mRNA levels of B cell lymphoma-extra-large *(Bcl-xl)* and BCL2-agonist/killer 1 *(Bak),* and determined their ratio in CD and FASD mice (Table 5.1). *Bcl-xl* mRNA levels were increased in FASD mice ($p<0.01$, t-test), with no differences in *Bak* mRNA between dietary groups. In FASD mice, the *Bcl-xl/Bak* ratio was higher compared with that in CD mice ($p<0.0001$, t-test). This higher ratio is suggestive of a reduced apoptotic environment (Castilla, et al., 2006; Garcia-Crespo, et al., 2009) in FASD mice.

Table 5.1: Relative *Bcl-xl, Bak,* and *Bcl-xl/Bak* mRNA in liver of CD and FASD mice at 7 dpi.

Gene	mRNA expression		p-value (t-test)
	CD	FASD	
Bcl-xl	1.00 ± 0.06	1.56 ± 0.20	p<0.01
Bak	1.00 ± 0.06	0.95 ± 0.11	p>0.05
Bcl-xl/Bak	1.00 ± 0.05	1.66 ± 0.13	p<0.0001

Expression of *Bcl-xl*, *Bak*, and *Bcl-xl/Bak* in liver of CD mice (n=14; 3 combined experiments) and FASD mice (n=12; 3 combined experiments) indicates that FASD murine livers had a lower apoptotic environment (higher *Bcl-xl/Bak* ratio) compared with that in CD mice.

5.4.Discussion

Folate is required for synthesis of nucleotides and amino acids, and for methylation reactions. The malarial parasite also requires folate for these functions, and obtains folate derivatives from the host, or, unlike the host, can synthesize folate *de novo* (Muller and Hyde, 2013; Nzila, et al., 2005a; Nzila, et al., 2005b). Drugs that target folate metabolism in the parasite have been in use for many years, and reduce the availability of important building blocks for parasite replication.

Our work indicates that lower levels of dietary folate result in reduced parasitemia and increased survival, as observed in mice fed CD. Our control diet has the recommended amount of folate necessary for normal function of the host, but it has less folate than the standard laboratory mouse chow routinely used for experimental malarial studies, and less folate than FASD. In addition to higher folate content, mouse chow also has higher levels of other one-carbon donors, such as choline, methionine and glycine, which can be metabolized for one-carbon transfer reactions. Our findings are consistent with those of an earlier study where lack of p-aminobenzoic acid (pABA), a folate precursor, in the diet led to lack of growth of *Plasmodium yoelii* and increased survival of the host (Kicska, et al., 2003). In our experiments, it appears that the effect of lower dietary folate is much more profound than the effect of the genetic *Mthfr* deficiency (Meadows, et al., 2014). This observation, i.e. a greater effect of diet than genotype, is similar to that in our previous work on the effect of *Mthfr* deficiency and dietary folate on tumor development (Knock, et al., 2011; Lawrance, et al., 2009) or pregnancy outcomes (Mikael, et al., 2013a; Pickell, et al., 2009).

Increased blood folate has been observed following food fortification with folate in North America and other countries (Britto, et al., 2014; McDowell, et al., 2008). Folic acid, a synthetic form of the vitamin used in food fortification and vitamin supplements, is included in the total folate pool and appears at greater levels in the circulation, compared to pre-fortification values (Kalmbach, et al., 2008). Studies are emerging alluding to the potential negative consequences of high folate intake, although it is not clear whether the concerns relate to the increase in the total folate pool or to the unmetabolized folic acid. These concerns include immune dysfunction (Troen, et al., 2006) and lower performance in cognitive tests (Morris, et al., 2010; Valera-Gran, et al., 2014). In other work using murine models, we demonstrated increased heart defects in embryos of dams fed high folate during pregnancy (Mikael, et al., 2013a), and disturbances in lipid metabolism and liver injury in adult mice fed high folate diets for 6 months (Christensen, et al., 2015). Folic acid may inhibit folate-dependent enzymes or transporters. In the liver study, we showed that folic acid can inhibit MTHFR activity but it also decreased MTHFR immunoreactive protein in liver of mice fed FASD (Christensen, et al., 2015). In the current study, we have no evidence for a direct effect on MTHFR since our diets were administered for a short period of time.

The decreased resistance to malaria in mice fed FASD presumably relates to the increased availability of folate for growth of the parasite. Similar conclusions were reached in studies where therapeutic administration of folic acid tablets appeared to minimize the efficacy of anti-folate anti-malarial drugs (Carter, et al., 2005; Mulenga, et al., 2006). However, it is likely that changes in the host immune response also contribute to the outcome. In FASD mice, we observed lower levels of splenocytes, T cells and some sub-populations of T cells and NK cells, compared with levels in CD mice. These immune cells are usually up-regulated to fight malarial infection (Hansen,

et al., 2007; Spence and Langhorne, 2012). Our findings are consistent with our earlier work in which we demonstrated an increase in the same populations in *Mthfr*$^{+/-}$ mice which showed greater resistance compared with wild-type mice (Meadows, et al., 2014). Although the exact mechanisms responsible for the change in immune cells require identification, it is possible that folic acid inhibits other critical folate-dependent enzymes involved in proliferation, methylation or immune function. The decreases in some NK populations in FASD mice are also consistent with a report suggesting that high levels of unmetabolized folic acid may be toxic to human NK cells (Troen, et al., 2006).

Cerebral malaria results from an exacerbated inflammatory response (Hunt and Grau, 2003). In our experiments, the FASD mice exhibited cerebral symptoms including tremors and impaired motor function before death. Such symptoms were not observed in CD mice. We also observed increases in brain TNFα in FASD mice. Decreases in TNFα have been consistently shown to improve the outcome of cerebral malaria (Grau, et al., 1987; Grau, et al., 1989b; Rudin, et al., 1997a). High levels of TNFα are present in early stages of infection and *in vivo* neutralization of TNFα can prevent symptoms (Grau, et al., 1987). The increase in this immunomodulator in FASD mice is consistent with the poorer outcome and the cerebral symptoms. *Abca1*$^{-/-}$ mice survive cerebral malaria after *P. berghei* ANKA infection and have significantly lower levels of TNFα than wild-type mice. *Abca1* increases release of microparticles following malarial infection; these microparticles are involved in inflammation and result in an increase of TNFα (Combes, et al., 2005). Our finding of increased *Abca1* expression with increased TNFα expression in FASD mice, and the positive correlation between TNFα and *Abca1* in all mice, is consistent with a significant

disturbance in the immune response of the host and with the poor outcomes attributed to an increase in TNFα in FASD mice.

The higher *Bcl-xl/*Bak ratio in liver of FASD mice is suggestive of a reduced apoptotic environment compared with that in liver of CD mice. Parasite clearance in liver protects against cerebral symptoms (Haque, et al., 2011), and *P. berghei* ANKA typically blocks apoptosis in liver (van de Sand, et al., 2005). The altered hepatic environment with high dietary folate may represent another mechanism that enhances parasite expansion, in addition to a simple change in one-carbon availability for nucleotide and amino acid synthesis.

In the last few decades, there has been considerable effort to increase folate intake to minimize the incidence of neural tube defects and other disorders (Smith, et al., 2008). Food fortification with folate is routine in many countries worldwide, including some countries in malaria-endemic regions (EUROCAT, 2015). Pregnant women, children and the elderly often use vitamin supplements that contain folic acid. Fortification and supplementation has led to increased blood folate (Kalmbach, et al., 2008; Morris, et al., 2010; Troen, et al., 2006); increased blood folate has been suggested to limit the efficacy of anti-folate anti-malarial treatment (Carter, et al., 2005; Mulenga, et al., 2006). It is interesting to note that a study in Malawi found that children from a region with high vegetable intake had greater risk of malarial treatment failure than children from a region with a predominant fish or meat diet; the authors speculated that the higher folate in the vegetable diet could have contributed to this difference (Dzinjalamala, et al., 2005).

Although there is a body of literature suggesting the potential impact of folic acid on malarial infection, our work is novel in its direct demonstration of the impact of dietary folate on resistance to malarial infection and on the host response in relevant tissues. We found that increased intake of folate, prior to malarial infection, is associated with increased parasite levels in blood, decreased amounts of critical immune cells, and an increase in a major cytokine, TNFα, which leads to the cerebral symptoms. Increased dietary folate could lead to decreased resistance to malaria in human populations or to reduced efficacy of anti-folate drugs. Additional studies are warranted to examine the impact of variable dietary folate in human malaria, to ensure that excess folate, through fortification or vitamin supplementation, is not particularly harmful in malarial-endemic regions.

5.5.Acknowledgements

We are grateful to Liyuan Deng and Qing Wu for their technical assistance and handling of mice, to Dr. Philippe Gros for providing the *P. berghei* ANKA parasite, and to Ana Best for advice in statistical modelling. This work was supported by a Canadian Institutes of Health Research grant to RR (MOP 43232). The McGill University Health Centre Research Institute is supported by a Centres grant from the Fonds de recherche du Québec-Santé.

CHAPTER VI

GENERAL DISCUSSION

6.1.Genetic and nutritional deficiency

In this thesis we have shown that alteration of folate metabolism, whether through genetic modification of MTHFR or through dietary means, improves the outcome of malaria infection. Our data suggest that *Mthfr*$^{+/-}$ deficiency confers resistance to malaria, while over-expression in *MTHFR*^{Tg} mice increases susceptibility to malaria. Excess folic acid renders mice more susceptible to malaria, as observed in mice fed FASD.

The genetic differences observed between *Mthfr*$^{+/-}$ mice, *MTHFR*^{Tg} mice, and their wild type litter mates are not as dramatic as the differences seen between the CD and the FASD mice. The effect of dietary folate s much more profound than the effect of the genetic manipulation of MTHFR. The stronger effect of nutritional changes is likely due to dietary changes having a global effect on folate metabolism. Genetic changes in MTHFR alter only one reaction in the folate pathway, the reduction of 5,10-methyleneTHF to 5-methylTHF. Dramatically changing the size of the total folate pool by scaling it up 10-fold, is going to have a strong effect on all parts of folate metabolism because there will be more substrate available. This is something we have seen before in tumor studies (Knock, et al., 2011; Lawrance, et al., 2009) and in pregnancy studies (Mikael, et al., 2013a; Pickell, et al., 2009).

Additionally, there is also some degree of metabolic redundancy in the *Mthfr*$^{+/-}$ mice. The standard mouse chow used in these experiments had higher levels of folate than CD, and they also had higher levels of other one-carbon donors than CD or FASD. These carbon donors, such as choline, methionine and glycine, can be metabolized for one-carbon transfer reactions. *Mthfr*$^{+/-}$

mice aren't strictly reliant on 5-methylTHF to remethylate homocysteine, they can also make use of the choline in their diet.

It is also worth reiterating that in our genetic studies, deficiency and over-expression of MTHFR are mild. In *MTHFR*677TT individuals, deficiency is mild, which is why we used *Mthfr*$^{+/-}$ mice in these experiments. In many murine genetic studies of the outcome of *P. berghei* ANKA infection, the animals used were knockouts that had little residual protein function (Kossodo, et al., 1997; Min-Oo, et al., 2003; Roth, et al., 1988; Rudin, et al., 1997b). More similar to our model with a mild enzymatic deficiency, Hood et al, using a different cerebral malaria parasite, showed that even a modest increase in sickle cell hemoglobin resulted in longer survival and obtained a survival curve that looked similar to those in our genetic models (Hood, et al., 1996).

6.2.Changes in immune cell populations

In all of our infection models, and in the uninfected genetic models, we found changes in many of the same immune cell populations. We observed increased total splenocyte populations in infected *Mthfr*$^{+/-}$ mice, and we also observed increased populations of CD4$^+$ and CD8$^+$ T cells, and CCR4$^+$ NK cells. In the uninfected *Mthfr*$^{+/-}$ mice, we see changes not in the number of cells, but in the percentage of the immune cell population that is comprised of CCR4$^+$ NK cells and IFNγ^+ NK cells. In infected *MTHFR*^{Tg} mice, we did not find changes in total splenocytes or in lymphocyte numbers, but we did find decreased numbers of NK cells and CCR4$^+$ NK cells. In uninfected *MTHFR*^{Tg} mice we found decreased numbers of total splenocytes, total T cells and CD8$^+$ T cells. There was also a trend toward a decrease in CD4$^+$ T cells. In FASD mice, we observed lower levels

of splenocytes, T cells and some sub-populations of T cells and NK cells, compared with levels in CD mice.

Many of the immune cells populations we see changed in the infected animals, are cell types often up-regulated to fight malarial infection (Hansen, et al., 2007; Spence and Langhorne, 2012). More experiments are needed to definitively explain the exact mechanisms responsible for the change in immune cell populations, but we do have some hypotheses.

The availability of folate derivatives to produce the nucleotides needed to synthesize DNA likely plays a major role. In the *Mthfr*$^{+/-}$ mice, *Mthfr* deficiency would result in increased levels of the substrate, methyleneTHF, which is required for thymidine production. Enhanced thymidine synthesis could potentially enhance DNA synthesis and replication of lymphocytes (Bagley and Selhub, 1998; Celtikci, et al., 2009). Due to increased MTHFR activity, *MTHFR*^{Tg} mice have decreased levels of 10-formylTHF (Celtikci, et al., 2008). Less 10-formylTHF would indicate that purine synthesis is limited. There is also evidence that the conversion of dUMP and dTMP is compromised in *MTHFR*^{Tg} mice (Celtikci, et al., 2008), which would also limit the nucleotides available for proliferation and likely lead to the decreases we see in cell number. In the FASD it is possible that folic acid inhibits some other critical folate-dependent enzyme involved in proliferation. Methylation changes may also potentially alter immune function.

Of particular interest in many of these experiments are the changes observed in CCR4^{+} NK cells. There were *Mthfr*-dependent increases in *Mthfr*$^{+/-}$ mice regardless of infection and decreases in infected *MTHFR*^{Tg} mice. In infected FASD mice, there are also decreases in these cells. It is clear

that these cells are sensitive to folate metabolism changes and malaria infection. In humans, it is proposed that these cells are potent producers of IFNγ and IL-17 (Pandya, et al., 2011).

It would be interesting to see whether, like the uninfected *Mthfr*$^{+/-}$ mice, *MTHFR*677TT individuals also have more of these CCR4^{+} NK cells than *MTHFR*677CC individuals. Hyperhomocysteinemia has been associated with elevated inflammation and autoimmunity (Lazzerini, et al., 2007; Papatheodorou and Weiss, 2007). It is possible that these proinflammatory cells may be helping to drive inflammation and autoimmunity in individuals with hyperhomocystenemia. Functional studies would also be interesting given findings that high levels of unmetabolized folic acid may be toxic to human NK cells (Troen, et al., 2006).

6.3.Folate supplementation and malaria

Our findings that folic acid supplementation in the diet results in increased susceptibility to poor outcome during a malaria infection adds to a growing body of evidence that folic acid supplementation and dietary fortification may have potentially negative consequences (Barua, et al., 2014; Martinez-Vega, et al., 2015; Mikael, et al., 2013a; Morris, et al., 2010; Pickell, et al., 2011; Troen, et al., 2006; Valera-Gran, et al., 2014). There is also a growing body of clinical evidence that folic acid supplementation may cause issues for anti-folate antimalarial drugs (Carter, et al., 2005; Dzinjalamala, et al., 2005; Mulenga, et al., 2006; Nzila, et al., 2014). We are the first to show this experimentally in a murine model, and our findings suggest that not only is the folate itself potentially interfering with these drugs, but that there may also be unforeseen immune changes that could be interfering as well.

There are malaria-endemic countries where mandatory folic acid supplementation has been implemented, including Colombia, Ghana and Cote d'Ivoire (EUROCAT, 2015). It is also standard clinical practice to prescribe folic acid to pregnant women during pregnancy, especially in those who are malaria infected (WHO, 2014). There is growing evidence that these practices and dosage levels should be reconsidered. Though in our experiments we use a fairly high dose of folic acid (10x recommended daily intake), the animals only have a 3-fold increase in plasma folate (Mikael, et al., 2013a). A similar increase has been reported in the United States (Pfeiffer, et al., 2012) where mandatory fortification has been implemented (USFDA, 1996) and supplementation is widespread. In light of our experiments, it is clear that such high levels of supplementation during malaria infection are an issue, but lower levels may prove therapeutic without causing unintended consequences during malaria infection.

Others have suggested that other alternatives to folic acid could be useful to prevent NTDs and not to interfere with the efficacy of anti-folate anti-malarials. Nzila, et al. (2014) have suggested that instead of folic acid supplements, pregnant women should be treated with 5-methylTHF supplements. They propose that use of 5-methylTHF could still minimize the incidence of NTDs, while simultaneously not undermining the efficacy of anti-folate antimalarial drugs, no matter how high of a dose used (Nzila, et al., 2014). MTHFR is the enzyme responsible for the production of 5-methylTHF. Given our findings in the *Mthfr*$^{+/-}$ mice that *Mthfr*-deficiency is protective during malaria infection and that *MTHFR*-over-expression in *MTHFR*^{Tg} mice increases susceptibility, we would caution widespread implementation of 5-methylTHF without further studies. If the protection afforded by *Mthfr*-deficiency is related to nucleotide availability for cellular proliferation, treatment with 5-methylTHF would be relatively harmless. However, if the

protection is afforded because of unidentified methylation changes, treatment with 5-methylTHF would likely cause more harm than good.

6.4.*MTHFR* 677TT selection hypothesis

Our findings suggest that *Mthfr*$^{+/-}$ mice are protected against malaria and that *MTHFR*^{Tg} mice are more susceptible to malaria infection. These observations are consistent with earlier reports that demonstrate that severe *Mthfr*-deficiency is protective in mCMV (Fodil-Cornu, et al., 2009). They are also in line with clinical reports that West African MTHFR 677T carriers are protected from chronic hepatitis B infection (Bronowicki, et al., 2008). Our work is the first to demonstration that mild *Mthfr*-deficiency, as seen in *MTHFR* 677TT individuals, confers protection against a pathogen associated with selection. We suggest that a selective advantage against malaria may have helped to maintain the 677 polymorphism in human populations.

The highest frequencies of *MTHFR677TT* are found in areas of the Mediterranean, including Italy and Spain (Figure 1.2). There are also relatively high "founder effect" frequencies in the countries and regions that were settled by the Spanish conquistadors. In the United States, Hispanics have the highest frequencies of *MTHFR677TT*, and South American countries like Colombia also have very high frequencies of *MTHFR677TT*. The regions where malaria was historically endemic (Figure 1.3) have significant overlap with regions where *MTHFR677TT* frequencies are high. Other malaria-protective disorders, such as thalassemias (Flint, et al., 1986; Willcox, et al., 1983) and glucose-6-phosphate dehydrogenase deficiency (Ruwende and Hill, 1998), are also highly prevalent this historically malaria-endemic region.

6.5.Future project directions

All of the experiments in this thesis were performed using *P. berghei* ANKA parasites. Cerebral malaria only occurs in a small subset of humans infected with *P. falciparum.* The *Mthfr*-deficient mice have already been bred onto on a BALB/c background (Chen, et al., 2001; Schwahn, et al., 2004). BALB/c mice are susceptible to *P. chabaudi chabaudi* infection. Dietary and genetic experiments using *P. chabaudi chabaudi* parasites can help to formulate a more complete picture of the role *Mthfr*-deficiency and folic acid supplementation may play in malaria infection.

Our experiments use diets with fairly high doses of folic acid (10x recommended daily intake (Reeves, 1997)). Though these mice had plasma folate levels similar to humans (Mikael, et al., 2013a; Pfeiffer, et al., 2012), it is a dose much higher than the WHO's recommendation (WHO, 2012). It would be useful to repeat these experiments with a dose similar to the WHO's recommendation to determine whether that dose also has an effect on the outcome of malaria infection.

Several experiments have suggested that *Mthfr*-deficiency may be protective in pathogens. Earlier work in mCMV and the clinical report in hepatitis B suggest that *Mthfr*-deficiency is protective against viruses (Bronowicki, et al., 2008; Fodil-Cornu, et al., 2009). In our experiments, we have shown that *Mthfr*-deficiency is protective in a parasite. These experiments showed similar immune cell changes in the same types of immune cells. Further experiments using a bacterial pathogen to help narrow down some of the immune cell changes seen in viral and parasite infection and provide additional evidence to help determine mechanism.

Experiments to further characterize the $CCR4^+$ NK cells should also be performed. There is very little known about these cells, but what is known suggests that they may be pro-inflammatory (Pandya, et al., 2011). If these cells are increased in *MTHFR* 677TT individuals, it may help explain some of the elevated inflammation in individuals with hyperhomocysteinemia (Lazzerini, et al., 2007; Mikael, et al., 2013b; Papatheodorou and Weiss, 2007). Ideally these cell populations would be measured in *MTHFR* 677TT individuals, *MTHFR* 677CT individuals and *MTHFR* 677CC controls. From there, these cells can be isolated and functional studies performed.

There is a lot of evidence that the gut microbiome is affected by diet (reviewed by Bermon, et al. (2015)). It is likely that changing the folic acid content in the diet would also affect the composition of the gut microbiome. The gut microbiome has a huge impact on the immune response in these mice (Bermon, et al., 2015). Studies comparing the gut microbiomes of CD and FASD mice would help us to determine whether the immune changes seen in infected FASD mice may also be, in part, due to changes in the gut microbiome.

CLAIMS TO ORIGINALITY

1. *Mthfr*$^{+/-}$ mice infected with *P. berghei* ANKA survived infection longer than wild-type mice and had a trend toward lower parasite levels. They also had lower serum IFNγ than wild-type mice.
2. *Mthfr*$^{+/-}$ mice infected with *P. berghei* ANKA had higher levels of brain IFNγ and PPARγ, and lower levels of the active IL-10 dimer. They also had higher levels of spleen IFNγ.
3. *Mthfr*$^{+/-}$ mice infected with *P. berghei* ANKA had higher numbers of total splenocytes, total T cells, $CD4^+$ T cells, $CD8^+$ T cell and $CCR4^+$ NK cells.
4. Uninfected *Mthfr*$^{+/-}$ mice had lower levels of brain IFNγ and elevated levels of the inactive IL-10 monomer. They also had elevated numbers of $CCR4^+$ NK cells and $IFN\gamma^+$ NK cells.
5. *MTHFR*^{Tg} mice infected with *P. berghei* ANKA survived infection longer than wild-type mice and had a trend toward elevated parasite levels. They had lower numbers of NK cells and $CCR4^+$ NK cells.
6. Uninfected *MTHFR*^{Tg} mice higher numbers of total splenocytes, total T cells, and $CD8^+$ T cells. They also had a trend toward lower numbers of $CD4^+$ T cells.
7. Mice fed a CD diet were resistance to *P. berghei* ANKA infection than mice fed standard mouse chow.
8. *Mthfr*$^{+/-}$ mice and *Mthfr*$^{+/+}$ mice fed CD and FASD, showed no genotype differences. FASD mice were more susceptible to infection than CD mice. MC mice were more susceptible to infection than CD or FASD mice.
9. FASD mice infected with *P. berghei* ANKA had higher parasite levels during infection.

10. FASD mice infected with *P. berghei* ANKA had lower numbers of total splenocytes, total T cells, $CD4^+$ T cells, $CCR4^+$ $CD4^+$ T cells, and $CD8^+$ T cells. They also had decreases in $CCR4^+$ NK cells a trend toward lower numbers of $IFN\gamma^+$ NK cells.
11. FASD mice infected with *P. berghei* ANKA had elevated brain TNFα and liver *Abca1* expression. The levels of brain TNFα and liver *Abca1* expression positively correlated with one another.
12. FASD mice infected with *P. berghei* ANKA had an elevated liver *Bcl-xl/Bak* ratio, suggestive of a less apoptotic environment in their liver.

REFERENCES

Alfthan G, Laurinen MS, Valsta LM, Pastinen T, Aro A. 2003. Folate intake, plasma folate and homocysteine status in a random Finnish population. Eur J Clin Nutr 57(1):81-8.

Allison AC. 1954. Protection afforded by sickle-cell trait against subtertian malareal infection. Br Med J 1(4857):290-4.

Angius A, Simpore J, Persico I, Sassu A, Prodi DA, Musumeci S. 2007. Methylenetetrahydrofolate reductase gene polymorphisms in Burkina Faso: impact on plasma fasting homocysteine and after methionine loading test. Clin Lab 53(1-2):29-33.

Ayi K, Min-Oo G, Serghides L, Crockett M, Kirby-Allen M, Quirt I, Gros P, Kain KC. 2008. Pyruvate kinase deficiency and malaria. N Engl J Med 358(17):1805-10.

Bagley PJ, Selhub J. 1998. A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells. Proc Natl Acad Sci U S A 95(22):13217-20.

Barua S, Chadman KK, Kuizon S, Buenaventura D, Stapley NW, Ruocco F, Begum U, Guariglia SR, Brown WT, Junaid MA. 2014. Increasing maternal or post-weaning folic acid alters gene expression and moderately changes behavior in the offspring. PLoS One 9(7):e101674.

Berghout J, Higgins S, Loucoubar C, Sakuntabhai A, Kain KC, Gros P. 2012. Genetic diversity in human erythrocyte pyruvate kinase. Genes Immun 13(1):98-102.

Berghout J, Min-Oo G, Tam M, Gauthier S, Stevenson MM, Gros P. 2010. Identification of a novel cerebral malaria susceptibility locus (Berr5) on mouse chromosome 19. Genes Immun 11(4):310-8.

Bermon S, Petriz B, Kajeniene A, Prestes J, Castell L, Franco OL. 2015. The microbiota: an exercise immunology perspective. Exerc Immunol Rev 21:70-9.

Beutler E, Dern RJ, Flanagan CL. 1955. Effect of sickle-cell trait on resistance to malaria. Br Med J 1(4923):1189-91.

Bi XH, Zhao HL, Zhang ZX, Zhang JW. 2009. Association of RFC1 A80G and MTHFR C677T polymorphisms with Alzheimer's disease. Neurobiology of aging 30(10):1601-7.

Bjelland I, Tell GS, Vollset SE, Refsum H, Ueland PM. 2003. Folate, vitamin B12, homocysteine, and the MTHFR 677C->T polymorphism in anxiety and depression: the Hordaland Homocysteine Study. Arch Gen Psychiatry 60(6):618-26.

Bopp SE, Ramachandran V, Henson K, Luzader A, Lindstrom M, Spooner M, Steffy BM, Suzuki O, Janse C, Waters AP and others. 2010. Genome wide analysis of inbred mouse lines identifies a locus containing Ppar-gamma as contributing to enhanced malaria survival. PLoS One 5(5):e10903.

Boulet SL, Yang Q, Mai C, Kirby RS, Collins JS, Robbins JM, Meyer R, Canfield MA, Mulinare J, National Birth Defects Prevention N. 2008. Trends in the postfortification prevalence of spina bifida and anencephaly in the United States. Birth Defects Res A Clin Mol Teratol 82(7):527-32.

Bozovic IB, Vranekovic J, Cizmarevic NS, Mahulja-Stamenkovic V, Prpic I, Brajenovic-Milic B. 2011. MTHFR C677T and A1298C polymorphisms as a risk factor for congenital heart defects in

Down syndrome. Pediatrics international : official journal of the Japan Pediatric Society 53(4):546-50.
Britto JC, Cancado R, Guerra-Shinohara EM. 2014. Concentrations of blood folate in Brazilian studies prior to and after fortification of wheat and cornmeal (maize flour) with folic acid: a review. Rev Bras Hematol Hemoter 36(4):275-86.
Bronowicki JP, Abdelmouttaleb I, Peyrin-Biroulet L, Venard V, Khiri H, Chabi N, Amouzou EK, Barraud H, Halfon P, Sanni A and others. 2008. Methylenetetrahydrofolate reductase 677 T allele protects against persistent HBV infection in West Africa. Journal of hepatology 48(4):532-9.
Brunham LR, Kruit JK, Iqbal J, Fievet C, Timmins JM, Pape TD, Coburn BA, Bissada N, Staels B, Groen AK and others. 2006. Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. J Clin Invest 116(4):1052-62.
Campanella GS, Tager AM, El Khoury JK, Thomas SY, Abrazinski TA, Manice LA, Colvin RA, Luster AD. 2008. Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria. Proc Natl Acad Sci U S A 105(12):4814-9.
Canada Go. 1998. Regulations Amending the Food and Drug Regulations (1066). Canada Gazette. 24 p.
Carter JY, Loolpapit MP, Lema OE, Tome JL, Nagelkerke NJ, Watkins WM. 2005. Reduction of the efficacy of antifolate antimalarial therapy by folic acid supplementation. Am J Trop Med Hyg 73(1):166-70.
Castilla C, Congregado B, Chinchon D, Torrubia FJ, Japon MA, Saez C. 2006. Bcl-xL is overexpressed in hormone-resistant prostate cancer and promotes survival of LNCaP cells via interaction with proapoptotic Bak. Endocrinology 147(10):4960-7.
CDC. 1991. Use of folic acid for prevention of spina bifida and other neural tube defects--1983-1991. MMWR Morb Mortal Wkly Rep 40(30):513-516.
Celtikci B, Lawrance AK, Wu Q, Rozen R. 2009. Methotrexate-induced apoptosis is enhanced by altered expression of methylenetetrahydrofolate reductase. Anticancer Drugs 20(9):787-93.
Celtikci B, Leclerc D, Lawrance AK, Deng L, Friedman HC, Krupenko NI, Krupenko SA, Melnyk S, James SJ, Peterson AC and others. 2008. Altered expression of methylenetetrahydrofolate reductase modifies response to methotrexate in mice. Pharmacogenet Genomics 18(7):577-89.
Chan D, Cushnie DW, Neaga OR, Lawrance AK, Rozen R, Trasler JM. 2010a. Strain-specific defects in testicular development and sperm epigenetic patterns in 5,10-methylenetetrahydrofolate reductase-deficient mice. Endocrinology 151(7):3363-73.
Chan J, Deng L, Mikael LG, Yan J, Pickell L, Wu Q, Caudill MA, Rozen R. 2010b. Low dietary choline and low dietary riboflavin during pregnancy influence reproductive outcomes and heart development in mice. Am J Clin Nutr 91(4):1035-43.
Chen J, Giovannucci E, Kelsey K, Rimm EB, Stampfer MJ, Colditz GA, Spiegelman D, Willett WC, Hunter DJ. 1996. A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. Cancer Res 56(21):4862-4.
Chen Z, Karaplis AC, Ackerman SL, Pogribny IP, Melnyk S, Lussier-Cacan S, Chen MF, Pai A, John SW, Smith RS and others. 2001. Mice deficient in methylenetetrahydrofolate reductase

exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. Hum Mol Genet 10(5):433-43.
Choi BO, Kim NK, Kim SH, Kang MS, Lee S, Ahn JY, Kim OJ, Kim S, Oh D. 2003. Homozygous C677T mutation in the MTHFR gene as an independent risk factor for multiple small-artery occlusions. Thromb Res 111(1-2):39-44.
Christensen KE, Mikael LG, Leung KY, Levesque N, Deng L, Wu Q, Malysheva OV, Best A, Caudill MA, Greene ND and others. 2015. High folic acid consumption leads to pseudo-MTHFR deficiency, altered lipid metabolism, and liver injury in mice. Am J Clin Nutr 101(3):646-58.
Cohen V, Panet-Raymond V, Sabbaghian N, Morin I, Batist G, Rozen R. 2003. Methylenetetrahydrofolate reductase polymorphism in advanced colorectal cancer: a novel genomic predictor of clinical response to fluoropyrimidine-based chemotherapy. Clin Cancer Res 9(5):1611-5.
Combes V, Coltel N, Alibert M, van Eck M, Raymond C, Juhan-Vague I, Grau GE, Chimini G. 2005. ABCA1 gene deletion protects against cerebral malaria: potential pathogenic role of microparticles in neuropathology. Am J Pathol 166(1):295-302.
Crider KS, Bailey LB, Berry RJ. 2011. Folic acid food fortification-its history, effect, concerns, and future directions. Nutrients 3(3):370-84.
Culleton R, Martinelli A, Hunt P, Carter R. 2005. Linkage group selection: rapid gene discovery in malaria parasites. Genome Res 15(1):92-7.
de Miranda AS, Lacerda-Queiroz N, de Carvalho Vilela M, Rodrigues DH, Rachid MA, Quevedo J, Teixeira AL. 2011. Anxiety-like behavior and proinflammatory cytokine levels in the brain of C57BL/6 mice infected with Plasmodium berghei (strain ANKA). Neurosci Lett 491(3):202-6.
De Wals P, Tairou F, Van Allen MI, Uh SH, Lowry RB, Sibbald B, Evans JA, Van den Hof MC, Zimmer P, Crowley M and others. 2007. Reduction in neural-tube defects after folic acid fortification in Canada. N Engl J Med 357(2):135-42.
Devlin AM, Arning E, Bottiglieri T, Faraci FM, Rozen R, Lentz SR. 2004. Effect of Mthfr genotype on diet-induced hyperhomocysteinemia and vascular function in mice. Blood 103(7):2624-9.
Dzinjalamala FK, Macheso A, Kublin JG, Taylor TE, Barnes KI, Molyneux ME, Plowe CV, Smith PJ. 2005. Blood folate concentrations and in vivo sulfadoxine-pyrimethamine failure in Malawian children with uncomplicated Plasmodium falciparum malaria. Am J Trop Med Hyg 72(3):267-72.
Ergul E, Sazci A, Utkan Z, Canturk NZ. 2003. Polymorphisms in the MTHFR gene are associated with breast cancer. Tumour Biol 24(6):286-90.
EUROCAT. 2015. Folic Acid Mandatory Fortification. In: (EUROCAT) ESoCA, editor.
Ferreira A, Marguti I, Bechmann I, Jeney V, Chora A, Palha NR, Rebelo S, Henri A, Beuzard Y, Soares MP. 2011. Sickle hemoglobin confers tolerance to Plasmodium infection. Cell 145(3):398-409.
Flint J, Hill AV, Bowden DK, Oppenheimer SJ, Sill PR, Serjeantson SW, Bana-Koiri J, Bhatia K, Alpers MP, Boyce AJ and others. 1986. High frequencies of alpha-thalassaemia are the result of natural selection by malaria. Nature 321(6072):744-50.

Fodil-Cornu N, Kozij N, Wu Q, Rozen R, Vidal SM. 2009. Methylenetetrahydrofolate reductase (MTHFR) deficiency enhances resistance against cytomegalovirus infection. Genes Immun 10(7):662-6.

Fortin A, Stevenson MM, Gros P. 2002a. Complex genetic control of susceptibility to malaria in mice. Genes and immunity 3(4):177-86.

Fortin A, Stevenson MM, Gros P. 2002b. Susceptibility to malaria as a complex trait: big pressure from a tiny creature. Human molecular genetics 11(20):2469-78.

Franke-Fayard B, Fonager J, Braks A, Khan SM, Janse CJ. 2010. Sequestration and tissue accumulation of human malaria parasites: can we learn anything from rodent models of malaria? PLoS Pathog 6(9):e1001032.

Friso S, Udali S, Guarini P, Pellegrini C, Pattini P, Moruzzi S, Girelli D, Pizzolo F, Martinelli N, Corrocher R and others. 2013. Global DNA hypomethylation in peripheral blood mononuclear cells as a biomarker of cancer risk. Cancer Epidemiol Biomarkers Prev 22(3):348-55.

Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP and others. 1995. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10(1):111-3.

Frosst P, Zhang Z, Pai A, Rozen R. 1996. The methylenetetrahydrofolate reductase (Mthfr) gene maps to distal mouse chromosome 4. Mammalian genome : official journal of the International Mammalian Genome Society 7(11):864-5.

Gao CM, Tang JH, Cao HX, Ding JH, Wu JZ, Wang J, Liu YT, Li SP, Su P, Matsuo K and others. 2009. MTHFR polymorphisms, dietary folate intake and breast cancer risk in Chinese women. Journal of human genetics 54(7):414-8.

Garcia-Crespo D, Knock E, Jabado N, Rozen R. 2009. Intestinal neoplasia induced by low dietary folate is associated with altered tumor expression profiles and decreased apoptosis in mouse normal intestine. J Nutr 139(3):488-94.

Goodwin LG. 1956. Sulphonamides and folic acid antagonists in malaria and toxoplasmosis. Proceedings of the Royal Society of Medicine 49(11):871-3.

Goyette P, Christensen B, Rosenblatt DS, Rozen R. 1996. Severe and mild mutations in cis for the methylenetetrahydrofolate reductase (MTHFR) gene, and description of five novel mutations in MTHFR. American journal of human genetics 59(6):1268-75.

Goyette P, Frosst P, Rosenblatt DS, Rozen R. 1995. Seven novel mutations in the methylenetetrahydrofolate reductase gene and genotype/phenotype correlations in severe methylenetetrahydrofolate reductase deficiency. Am J Hum Genet 56(5):1052-9.

Goyette P, Pai A, Milos R, Frosst P, Tran P, Chen Z, Chan M, Rozen R. 1999. Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR) (vol 9, pg 652, 1998). Mammalian Genome 10(2):204-204.

Goyette P, Sumner JS, Milos R, Duncan AM, Rosenblatt DS, Matthews RG, Rozen R. 1994. Human methylenetetrahydrofolate reductase: isolation of cDNA mapping and mutation identification. Nat Genet 7(4):551.

Grau GE, Fajardo LF, Piguet PF, Allet B, Lambert PH, Vassalli P. 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. Science 237(4819):1210-2.

Grau GE, Heremans H, Piguet PF, Pointaire P, Lambert PH, Billiau A, Vassalli P. 1989a. Monoclonal antibody against interferon gamma can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor. Proc Natl Acad Sci U S A 86(14):5572-4.

Grau GE, Piguet PF, Vassalli P, Lambert PH. 1989b. Tumor-necrosis factor and other cytokines in cerebral malaria: experimental and clinical data. Immunol Rev 112:49-70.

Green R, Miller JW. 1999. Folate deficiency beyond megaloblastic anemia: hyperhomocysteinemia and other manifestations of dysfunctional folate status. Semin Hematol 36(1):47-64.

Griffiths MJ, Shafi MJ, Popper SJ, Hemingway CA, Kortok MM, Wathen A, Rockett KA, Mott R, Levin M, Newton CR and others. 2005. Genomewide analysis of the host response to malaria in Kenyan children. J Infect Dis 191(10):1599-611.

Guan LX, Du XY, Wang JX, Gao L, Wang RL, Li HB, Wang SX. 2005. [Association of genetic polymorphisms in plasminogen activator inhibitor-1 gene and 5,10-methylenetetrahydrofolate reductase gene with recurrent early spontaneous abortion]. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 22(3):330-3.

Gueant-Rodriguez RM, Gueant JL, Debard R, Thirion S, Hong LX, Bronowicki JP, Namour F, Chabi NW, Sanni A, Anello G and others. 2006. Prevalence of methylenetetrahydrofolate reductase 677T and 1298C alleles and folate status: a comparative study in Mexican, West African, and European populations. The American journal of clinical nutrition 83(3):701-7.

Hafalla JC, Cockburn IA, Zavala F. 2006. Protective and pathogenic roles of CD8+ T cells during malaria infection. Parasite Immunol 28(1-2):15-24.

Hansen DS, D'Ombrain MC, Schofield L. 2007. The role of leukocytes bearing Natural Killer Complex receptors and Killer Immunoglobulin-like Receptors in the immunology of malaria. Curr Opin Immunol 19(4):416-23.

Haque A, Best SE, Amante FH, Ammerdorffer A, de Labastida F, Pereira T, Ramm GA, Engwerda CR. 2011. High parasite burdens cause liver damage in mice following Plasmodium berghei ANKA infection independently of CD8(+) T cell-mediated immune pathology. Infect Immun 79(5):1882-8.

Haworth JC, Dilling LA, Surtees RA, Seargeant LE, Lue-Shing H, Cooper BA, Rosenblatt DS. 1993. Symptomatic and asymptomatic methylenetetrahydrofolate reductase deficiency in two adult brothers. Am J Med Genet 45(5):572-6.

Hill AV, Allsopp CE, Kwiatkowski D, Anstey NM, Twumasi P, Rowe PA, Bennett S, Brewster D, McMichael AJ, Greenwood BM. 1991. Common west African HLA antigens are associated with protection from severe malaria. Nature 352(6336):595-600.

Hobbs CA, Sherman SL, Yi P, Hopkins SE, Torfs CP, Hine RJ, Pogribna M, Rozen R, James SJ. 2000. Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome. Am J Hum Genet 67(3):623-30.

Hood AT, Fabry ME, Costantini F, Nagel RL, Shear HL. 1996. Protection from lethal malaria in transgenic mice expressing sickle hemoglobin. Blood 87(4):1600-3.
Hosseini M, Houshmand M, Ebrahimi A. 2011. MTHFR polymorphisms and breast cancer risk. Archives of medical science : AMS 7(1):134-7.
Houcher B, Houcher Z, Touabti A, Begag S, Torun D, Egin Y, Akar N, Kadour F. 2010. Association of methylenetetrahydrofolate reductase C677T and cystathionine beta-synthase polymorphisms in cardiovascular disease in the algerian population. Genet Test Mol Biomarkers 14(6):775-80.
Huang Y, He Y, Sun X, He Y, Li Y, Sun C. 2014. Maternal high folic acid supplement promotes glucose intolerance and insulin resistance in male mouse offspring fed a high-fat diet. Int J Mol Sci 15(4):6298-313.
Hunt NH, Grau GE. 2003. Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. Trends Immunol 24(9):491-9.
Ishih A, Nagata T, Kobayashi F, Muregi FW, Ohori K, Miyase T. 2008. Possible involvement of IFN-gamma in early mortality of Plasmodium berghei NK65-infected BALB/c mice after febrifugine treatment. The Southeast Asian journal of tropical medicine and public health 39(6):949-58.
Jacques P, Choumenkovitch S. 2005. Mild MTHFR Deficiency and Folate Status. In: Ueland PM, Rozen R, editor. MTHFR Polymorphisms and Disease. Georgetown, Texas USA: Eurekah.com. p 54-70.
Jadavji NM, Deng L, Leclerc D, Malysheva O, Bedell BJ, Caudill MA, Rozen R. 2012. Severe methylenetetrahydrofolate reductase deficiency in mice results in behavioral anomalies with morphological and biochemical changes in hippocampus. Mol Genet Metab 106(2):149-59.
Joober R, Benkelfat C, Lal S, Bloom D, Labelle A, Lalonde P, Turecki G, Rozen R, Rouleau GA. 2000. Association between the methylenetetrahydrofolate reductase 677C-->T missense mutation and schizophrenia. Mol Psychiatry 5(3):323-6.
Kadaveru K, Protiva P, Greenspan EJ, Kim YI, Rosenberg DW. 2012. Dietary methyl donor depletion protects against intestinal tumorigenesis in Apc(Min/+) mice. Cancer Prev Res (Phila) 5(7):911-20.
Kalmbach RD, Choumenkovitch SF, Troen AM, D'Agostino R, Jacques PF, Selhub J. 2008. Circulating folic acid in plasma: relation to folic acid fortification. Am J Clin Nutr 88(3):763-8.
Kang SS, Zhou J, Wong PW, Kowalisyn J, Strokosch G. 1988. Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase. Am J Hum Genet 43(4):414-21.
Kelly TL, Neaga OR, Schwahn BC, Rozen R, Trasler JM. 2005. Infertility in 5,10-methylenetetrahydrofolate reductase (MTHFR)-deficient male mice is partially alleviated by lifetime dietary betaine supplementation. Biol Reprod 72(3):667-77.
Khor CC, Hibberd ML. 2011. Revealing the molecular signatures of host-pathogen interactions. Genome Biol 12(10):229.
Kicska GA, Ting LM, Schramm VL, Kim K. 2003. Effect of dietary p-aminobenzoic acid on murine Plasmodium yoelii infection. J Infect Dis 188(11):1776-81.

Knock E, Deng L, Krupenko N, Mohan RD, Wu Q, Leclerc D, Gupta S, Elmore CL, Kruger W, Tini M and others. 2011. Susceptibility to intestinal tumorigenesis in folate-deficient mice may be influenced by variation in one-carbon metabolism and DNA repair. J Nutr Biochem 22(11):1022-9.

Knock E, Deng L, Wu Q, Lawrance AK, Wang XL, Rozen R. 2008. Strain differences in mice highlight the role of DNA damage in neoplasia induced by low dietary folate. J Nutr 138(4):653-8.

Knock E, Deng L, Wu Q, Leclerc D, Wang XL, Rozen R. 2006. Low dietary folate initiates intestinal tumors in mice, with altered expression of G2-M checkpoint regulators polo-like kinase 1 and cell division cycle 25c. Cancer Res 66(21):10349-56.

Kossodo S, Monso C, Juillard P, Velu T, Goldman M, Grau GE. 1997. Interleukin-10 modulates susceptibility in experimental cerebral malaria. Immunology 91(4):536-40.

Krawinkel MB, Strohm D, Weissenborn A, Watzl B, Eichholzer M, Barlocher K, Elmadfa I, Leschik-Bonnet E, Heseker H. 2014. Revised D-A-CH intake recommendations for folate: how much is needed? Eur J Clin Nutr 68(6):719-23.

Kwiatkowski DP. 2005. How malaria has affected the human genome and what human genetics can teach us about malaria. Am J Hum Genet 77(2):171-92.

Lamb TJ, Brown DE, Potocnik AJ, Langhorne J. 2006. Insights into the immunopathogenesis of malaria using mouse models. Expert Rev Mol Med 8(6):1-22.

Lambrot R, Xu C, Saint-Phar S, Chountalos G, Cohen T, Paquet M, Suderman M, Hallett M, Kimmins S. 2013. Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. Nat Commun 4:2889.

Langevin SM, Lin D, Matsuo K, Gao CM, Takezaki T, Stolzenberg-Solomon RZ, Vasavi M, Hasan Q, Taioli E. 2009. Review and pooled analysis of studies on MTHFR C677T polymorphism and esophageal cancer. Toxicology letters 184(2):73-80.

Lawrance AK, Deng L, Rozen R. 2009. Methylenetetrahydrofolate reductase deficiency and low dietary folate reduce tumorigenesis in Apc min/+ mice. Gut 58(6):805-11.

Lawrance AK, Racine J, Deng L, Wang X, Lachapelle P, Rozen R. 2011. Complete deficiency of methylenetetrahydrofolate reductase in mice is associated with impaired retinal function and variable mortality, hematological profiles, and reproductive outcomes. J Inherit Metab Dis 34(1):147-57.

Lazzerini PE, Capecchi PL, Selvi E, Lorenzini S, Bisogno S, Galeazzi M, Laghi Pasini F. 2007. Hyperhomocysteinemia, inflammation and autoimmunity. Autoimmun Rev 6(7):503-9.

Leclerc D, Cao Y, Deng L, Mikael LG, Wu Q, Rozen R. 2013. Differential gene expression and methylation in the retinoid/PPARA pathway and of tumor suppressors may modify intestinal tumorigenesis induced by low folate in mice. Mol Nutr Food Res 57(4):686-97.

Leclerc D, Sibani S, Rozen R. 2005. *Molecular Biology of Methylenetetrahydrofolate Reductase (MTHFR) and Overview of Mutations/Polymorphisms*. In: Ueland PM, Rozen R, editors. MTHFR Polymorphism and Disease. Georgetown, TX: Landes Bioscience. p 1-20.

Li D, Ahmed M, Li Y, Jiao L, Chou TH, Wolff RA, Lenzi R, Evans DB, Bondy ML, Pisters PW and others. 2005a. 5,10-Methylenetetrahydrofolate reductase polymorphisms and the risk of pancreatic cancer. Cancer Epidemiol Biomarkers Prev 14(6):1470-6.

Li D, Pickell L, Liu Y, Wu Q, Cohn JS, Rozen R. 2005b. Maternal methylenetetrahydrofolate reductase deficiency and low dietary folate lead to adverse reproductive outcomes and congenital heart defects in mice. Am J Clin Nutr 82(1):188-95.

Longley R, Smith C, Fortin A, Berghout J, McMorran B, Burgio G, Foote S, Gros P. 2011. Host resistance to malaria: using mouse models to explore the host response. Mammalian genome : official journal of the International Mammalian Genome Society 22(1-2):32-42.

Lu Z, Serghides L, Patel SN, Degousee N, Rubin BB, Krishnegowda G, Gowda DC, Karin M, Kain KC. 2006. Disruption of JNK2 decreases the cytokine response to Plasmodium falciparum glycosylphosphatidylinositol in vitro and confers protection in a cerebral malaria model. Journal of immunology 177(9):6344-52.

Ly A, Lee H, Chen J, Sie KK, Renlund R, Medline A, Sohn KJ, Croxford R, Thompson LU, Kim YI. 2011. Effect of maternal and postweaning folic acid supplementation on mammary tumor risk in the offspring. Cancer Res 71(3):988-97.

MacFarlane AJ, Behan NA, Matias FM, Green J, Caldwell D, Brooks SP. 2013. Dietary folate does not significantly affect the intestinal microbiome, inflammation or tumorigenesis in azoxymethane-dextran sodium sulphate-treated mice. Br J Nutr 109(4):630-8.

Martinez-Vega R, Garrido F, Partearroyo T, Cediel R, Zeisel SH, Martinez-Alvarez C, Varela-Moreiras G, Varela-Nieto I, Pajares MA. 2015. Folic acid deficiency induces premature hearing loss through mechanisms involving cochlear oxidative stress and impairment of homocysteine metabolism. FASEB J 29(2):418-32.

McDowell MA, Lacher DA, Pfeiffer CM, Mulinare J, Picciano MF, Rader JI, Yetley EA, Kennedy-Stephenson J, Johnson CL. 2008. Blood folate levels: the latest NHANES results. NCHS Data Brief(6):1-8.

Meadows DN, Pyzik M, Wu Q, Torre S, Gros P, Vidal SM, Rozen R. 2014. Increased Resistance to Malaria in Mice with Methylenetetrahydrofolate Reductase (Mthfr) Deficiency Suggests a Mechanism for Selection of the MTHFR 677C>T (c.665C>T) Variant. Hum Mutat 35(5):594-600.

Mendis K, Rietveld A, Warsame M, Bosman A, Greenwood B, Wernsdorfer WH. 2009. From malaria control to eradication: The WHO perspective. Trop Med Int Health 14(7):802-9.

Mikael LG, Deng L, Paul L, Selhub J, Rozen R. 2013a. Moderately high intake of folic acid has a negative impact on mouse embryonic development. Birth Defects Res A Clin Mol Teratol 97(1):47-52.

Mikael LG, Genest J, Jr., Rozen R. 2006. Elevated homocysteine reduces apolipoprotein A-I expression in hyperhomocysteinemic mice and in males with coronary artery disease. Circ Res 98(4):564-71.

Mikael LG, Pancer J, Jiang X, Wu Q, Caudill M, Rozen R. 2013b. Low dietary folate and methylenetetrahydrofolate reductase deficiency may lead to pregnancy complications through modulation of ApoAI and IFN-gamma in spleen and placenta, and through reduction of methylation potential. Mol Nutr Food Res 57(4):661-70.

Mikael LG, Pancer J, Wu Q, Rozen R. 2012. Disturbed one-carbon metabolism causing adverse reproductive outcomes in mice is associated with altered expression of apolipoprotein AI and inflammatory mediators PPARalpha, interferon-gamma, and interleukin-10. J Nutr 142(3):411-8.

Mikael LG, Rozen R. 2008. Homocysteine modulates the effect of simvastatin on expression of ApoA-I and NF-kappaB/iNOS. Cardiovasc Res 80(1):151-8.
Miller JW, Garrod MG, Allen LH, Haan MN, Green R. 2009. Metabolic evidence of vitamin B-12 deficiency, including high homocysteine and methylmalonic acid and low holotranscobalamin, is more pronounced in older adults with elevated plasma folate. Am J Clin Nutr 90(6):1586-92.
Miller LH, Mason SJ, Clyde DF, McGinniss MH. 1976. The resistance factor to Plasmodium vivax in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med 295(6):302-4.
Min-Oo G, Fortin A, Tam MF, Gros P, Stevenson MM. 2004. Phenotypic expression of pyruvate kinase deficiency and protection against malaria in a mouse model. Genes Immun 5(3):168-75.
Min-Oo G, Fortin A, Tam MF, Nantel A, Stevenson MM, Gros P. 2003. Pyruvate kinase deficiency in mice protects against malaria. Nat Genet 35(4):357-62.
Morris MS, Jacques PF, Rosenberg IH, Selhub J. 2007. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. Am J Clin Nutr 85(1):193-200.
Morris MS, Jacques PF, Rosenberg IH, Selhub J. 2010. Circulating unmetabolized folic acid and 5-methyltetrahydrofolate in relation to anemia, macrocytosis, and cognitive test performance in American seniors. Am J Clin Nutr 91(6):1733-44.
Mulenga M, Malunga P, Bennett S, Thuma P, Shulman C, Fielding K, Greenwood B. 2006. Folic acid treatment of Zambian children with moderate to severe malaria anemia. Am J Trop Med Hyg 74(6):986-90.
Muller IB, Hyde JE. 2013. Folate metabolism in human malaria parasites--75 years on. Mol Biochem Parasitol 188(1):63-77.
Naidu CA, Moodley J, Pegoraro R, Rom L. 2007. Methylenetetrahydrofolate (MTHFR) reductase gene polymorphism in African women with abruptio placentae. European journal of obstetrics, gynecology, and reproductive biology 132(2):241-3.
NIH OoDS. 2012. Folate: Dietary Supplement Fact Sheet. http://ods.od.nih.gov/factsheets/Folate-HealthProfessional/: NIH.
Nzila A, Okombo J, Molloy AM. 2014. Impact of folate supplementation on the efficacy of sulfadoxine/pyrimethamine in preventing malaria in pregnancy: the potential of 5-methyl-tetrahydrofolate. J Antimicrob Chemother 69(2):323-30.
Nzila A, Ward SA, Marsh K, Sims PF, Hyde JE. 2005a. Comparative folate metabolism in humans and malaria parasites (part I): pointers for malaria treatment from cancer chemotherapy. Trends Parasitol 21(6):292-8.
Nzila A, Ward SA, Marsh K, Sims PF, Hyde JE. 2005b. Comparative folate metabolism in humans and malaria parasites (part II): activities as yet untargeted or specific to Plasmodium. Trends Parasitol 21(7):334-9.
Pandya AD, Al-Jaderi Z, Hoglund RA, Holmoy T, Harbo HF, Norgauer J, Maghazachi AA. 2011. Identification of human NK17/NK1 cells. PLoS One 6(10):e26780.
Papatheodorou L, Weiss N. 2007. Vascular oxidant stress and inflammation in hyperhomocysteinemia. Antioxid Redox Signal 9(11):1941-58.

Patel SN, Berghout J, Lovegrove FE, Ayi K, Conroy A, Serghides L, Min-oo G, Gowda DC, Sarma JV, Rittirsch D and others. 2008. C5 deficiency and C5a or C5aR blockade protects against cerebral malaria. The Journal of experimental medicine 205(5):1133-43.

Pfeiffer CM, Hughes JP, Lacher DA, Bailey RL, Berry RJ, Zhang M, Yetley EA, Rader JI, Sempos CT, Johnson CL. 2012. Estimation of trends in serum and RBC folate in the U.S. population from pre- to postfortification using assay-adjusted data from the NHANES 1988-2010. J Nutr 142(5):886-93.

Pickell L, Brown K, Li D, Wang XL, Deng L, Wu Q, Selhub J, Luo L, Jerome-Majewska L, Rozen R. 2011. High intake of folic acid disrupts embryonic development in mice. Birth Defects Res A Clin Mol Teratol 91(1):8-19.

Pickell L, Li D, Brown K, Mikael LG, Wang XL, Wu Q, Luo L, Jerome-Majewska L, Rozen R. 2009. Methylenetetrahydrofolate reductase deficiency and low dietary folate increase embryonic delay and placental abnormalities in mice. Birth Defects Res A Clin Mol Teratol 85(6):531-41.

Protiva P, Mason JB, Liu Z, Hopkins ME, Nelson C, Marshall JR, Lambrecht RW, Pendyala S, Kopelovich L, Kim M and others. 2011. Altered folate availability modifies the molecular environment of the human colorectum: implications for colorectal carcinogenesis. Cancer Prev Res (Phila) 4(4):530-43.

Rah H, Jeon YJ, Choi Y, Shim SH, Yoon TK, Choi DH, Cha SH, Kim NK. 2012. Association of methylenetetrahydrofolate reductase (MTHFR 677C>T) and thymidylate synthase (TSER and TS 1494del6) polymorphisms with premature ovarian failure in Korean women. Menopause 19(11):1260-6.

Rajkovic A, Mahomed K, Rozen R, Malinow MR, King IB, Williams MA. 2000. Methylenetetrahydrofolate reductase 677 C --> T polymorphism, plasma folate, vitamin B(12) concentrations, and risk of preeclampsia among black African women from Zimbabwe. Molecular genetics and metabolism 69(1):33-9.

Reeves PG. 1997. Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr 127(5 Suppl):838S-841S.

Rosenberg N, Murata M, Ikeda Y, Opare-Sem O, Zivelin A, Geffen E, Seligsohn U. 2002. The frequent 5,10-methylenetetrahydrofolate reductase C677T polymorphism is associated with a common haplotype in whites, Japanese, and Africans. American journal of human genetics 70(3):758-62.

Rosenblatt DS, Fenton WA. 2001. Inherited disorders of folate and cobalamin transport and metabolism. In: Scriver CR, Beaudet AL, Sly WS, editors. The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill. p 3897-3933.

Rosenblatt DS, Lue-Shing H, Arzoumanian A, Low-Nang L, Matiaszuk N. 1992. Methylenetetrahydrofolate reductase (MR) deficiency: thermolability of residual MR activity, methionine synthase activity, and methylcobalamin levels in cultured fibroblasts. Biochem Med Metab Biol 47(3):221-5.

Roth EF, Jr., Shear HL, Costantini F, Tanowitz HB, Nagel RL. 1988. Malaria in beta-thalassemic mice and the effects of the transgenic human beta-globin gene and splenectomy. J Lab Clin Med 111(1):35-41.

Rouissi K, Ouerhani S, Oliveira E, Marrakchi R, Cherni L, Ben Othman F, Ben Slama MR, Sfaxi M, Ayed M, Chebil M and others. 2009. Polymorphisms in one-carbon metabolism pathway genes and risk for bladder cancer in a Tunisian population. Cancer genetics and cytogenetics 195(1):43-53.

Rudin W, Eugster HP, Bordmann G, Bonato J, Muller M, Yamage M, Ryffel B. 1997a. Resistance to cerebral malaria in tumor necrosis factor-alpha/beta-deficient mice is associated with a reduction of intercellular adhesion molecule-1 up-regulation and T helper type 1 response. Am J Pathol 150(1):257-66.

Rudin W, Favre N, Bordmann G, Ryffel B. 1997b. Interferon-gamma is essential for the development of cerebral malaria. Eur J Immunol 27(4):810-5.

Ruwende C, Hill A. 1998. Glucose-6-phosphate dehydrogenase deficiency and malaria. J Mol Med (Berl) 76(8):581-8.

Sachs J, Malaney P. 2002. The economic and social burden of malaria. Nature 415(6872):680-5.

Schmidt KE, Schumak B, Specht S, Dubben B, Limmer A, Hoerauf A. 2011. Induction of pro-inflammatory mediators in Plasmodium berghei infected BALB/c mice breaks blood-brain-barrier and leads to cerebral malaria in an IL-12 dependent manner. Microbes Infect 13(10):828-36.

Schwahn BC, Chen Z, Laryea MD, Wendel U, Lussier-Cacan S, Genest J, Jr., Mar MH, Zeisel SH, Castro C, Garrow T and others. 2003. Homocysteine-betaine interactions in a murine model of 5,10-methylenetetrahydrofolate reductase deficiency. FASEB J 17(3):512-4.

Schwahn BC, Laryea MD, Chen Z, Melnyk S, Pogribny I, Garrow T, James SJ, Rozen R. 2004. Betaine rescue of an animal model with methylenetetrahydrofolate reductase deficiency. Biochem J 382(Pt 3):831-40.

Selhub J, Morris MS, Jacques PF, Rosenberg IH. 2009. Folate-vitamin B-12 interaction in relation to cognitive impairment, anemia, and biochemical indicators of vitamin B-12 deficiency. Am J Clin Nutr 89(2):702S-6S.

Sepehr E, Peace RW, Storey KB, Jee P, Lampi BJ, Brooks SP. 2003. Folate derived from cecal bacterial fermentation does not increase liver folate stores in 28-d folate-depleted male Sprague-Dawley rats. J Nutr 133(5):1347-54.

Serghides L, Patel SN, Ayi K, Lu Z, Gowda DC, Liles WC, Kain KC. 2009. Rosiglitazone modulates the innate immune response to Plasmodium falciparum infection and improves outcome in experimental cerebral malaria. J Infect Dis 199(10):1536-45.

Shotelersuk V, Ittiwut C, Siriwan P, Angspatt A. 2003. Maternal 677CT/1298AC genotype of the MTHFR gene as a risk factor for cleft lip. J Med Genet 40(5):e64.

Sie KK, Medline A, van Weel J, Sohn KJ, Choi SW, Croxford R, Kim YI. 2011. Effect of maternal and postweaning folic acid supplementation on colorectal cancer risk in the offspring. Gut 60(12):1687-94.

Skibola CF, Smith MT, Kane E, Roman E, Rollinson S, Cartwright RA, Morgan G. 1999. Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. Proc Natl Acad Sci U S A 96(22):12810-5.

Smith AD, Kim YI, Refsum H. 2008. Is folic acid good for everyone? Am J Clin Nutr 87(3):517-33.

Spence PJ, Langhorne J. 2012. T cell control of malaria pathogenesis. Curr Opin Immunol 24(4):444-8.

Thomas M, Rosenblatt D. 2005. Severe Methylenetetrahydrofolate Reductase Deficiency. In: PM U, R R, editors. MTHFR Polymorphisms and Disease. Georgetown, Texas: Eurekah.com. p 41-53.

Timmann C, Evans JA, Konig IR, Kleensang A, Ruschendorf F, Lenzen J, Sievertsen J, Becker C, Enuameh Y, Kwakye KO and others. 2007. Genome-wide linkage analysis of malaria infection intensity and mild disease. PLoS Genet 3(3):e48.

Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. 2012. Into the eye of the cytokine storm. Microbiol Mol Biol Rev 76(1):16-32.

Troen AM, Mitchell B, Sorensen B, Wener MH, Johnston A, Wood B, Selhub J, McTiernan A, Yasui Y, Oral E and others. 2006. Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. J Nutr 136(1):189-94.

Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. 2001. Biological and clinical implications of the MTHFR C677T polymorphism. Trends Pharmacol Sci 22(4):195-201.

Ueland PM, Rozen R. 2005. MTHFR polymorphisms and disease. Georgetown, Tex.: Landes Bioscience : Eurekah.com.

USFDA. 1996. Food standards: amendment of standards of identify for enriched grain products to require addition of folic acid. Federal Register. 44 p.

Valera-Gran D, Garcia de la Hera M, Navarrete-Munoz EM, Fernandez-Somoano A, Tardon A, Julvez J, Forns J, Lertxundi N, Ibarluzea JM, Murcia M and others. 2014. Folic acid supplements during pregnancy and child psychomotor development after the first year of life. JAMA Pediatr 168(11):e142611.

van de Sand C, Horstmann S, Schmidt A, Sturm A, Bolte S, Krueger A, Lutgehetmann M, Pollok JM, Libert C, Heussler VT. 2005. The liver stage of Plasmodium berghei inhibits host cell apoptosis. Mol Microbiol 58(3):731-42.

van der Put NM, Steegers-Theunissen RP, Frosst P, Trijbels FJ, Eskes TK, van den Heuvel LP, Mariman EC, den Heyer M, Rozen R, Blom HJ. 1995. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet 346(8982):1070-1.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7):RESEARCH0034.

Villegas-Mendez A, de Souza JB, Murungi L, Hafalla JC, Shaw TN, Greig R, Riley EM, Couper KN. 2011. Heterogeneous and tissue-specific regulation of effector T cell responses by IFN-gamma during Plasmodium berghei ANKA infection. Journal of immunology 187(6):2885-97.

WHO. 2012. Guideline: Daily iron and folic acid supplementation in pregnant women. Geneva: WHO.

WHO. 2014. World Malaria Report 2014. Geneva, Switzerland: World Health Organization. 242 p.

WHO. 2015. Malaria: Information for travellers. In: Organization GMP-WH, editor. Geneva, Switzerland.

Willcox M, Bjorkman A, Brohult J, Pehrson PO, Rombo L, Bengtsson E. 1983. A case-control study in northern Liberia of Plasmodium falciparum malaria in haemoglobin S and beta-thalassaemia traits. Ann Trop Med Parasitol 77(3):239-46.

Wilson RD, Johnson JA, Wyatt P, Allen V, Gagnon A, Langlois S, Blight C, Audibert F, Desilets V, Brock JA and others. 2007. Pre-conceptional vitamin/folic acid supplementation 2007: the use of folic acid in combination with a multivitamin supplement for the prevention of neural tube defects and other congenital anomalies. J Obstet Gynaecol Can 29(12):1003-26.

Yajnik CS, Deshpande SS, Jackson AA, Refsum H, Rao S, Fisher DJ, Bhat DS, Naik SS, Coyaji KJ, Joglekar CV and others. 2008. Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. Diabetologia 51(1):29-38.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13:134.