Oxidative damage and transmethylation micronutrient effects on the T cell epigenome in systemic lupus erythematosus

by

Donna Ray

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Toxicology)
in The University of Michigan
2014

Doctoral Committee:

      Professor Bruce C. Richardson, Co-Chair
      Professor Craig Harris, Co-Chair
      Assistant Professor Dana Dolinoy
      Professor Jonathan Maybaum

To my family and friends

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5azaC	5-azacytidine
Ag	antigen
AP-1	activating protein 1
APCs	antigen-presenting cells
ART	assisted reproductive technology
ATP	adenosine triphosphate
B_2	riboflavin
B_6	pyridoxine
B_{12}	cobalamin
BAFF/BlyS	B cell activating factor/B lymphocyte stimulator
BCR	B cell receptor
BHMT	betaine-homocysteine-S-methyltransferase
CG	cytosine-guanine
CH_3	methyl group
CpG	cytosine-phosphate-guanine
dC	deoxycytosine
d^mC	deoxymethylcytosine
Dnmt	DNA methyltransferase
DOHaD	developmental origins of health and disease
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FMN	flavin mononucleotide
GSH	reduced glutathione
GWAS	genome-wide association studies
Hyd	hydralazine
ICAM-1	intercellular adhesion molecule-1
IL-2	interleukin-2
IL-17	interleukin-17
KIR	killer cell Ig-like receptor
5,10-methyleneTHF	5,10-methylenetetrahydrofolate
5-methylTHF	5-methyltetrahydrofolate
MAT	methionine adenosyltransferase
mls	milliliters

NK	natural killer
iNOS	inducible nitric oxide synthase
NO	nitric oxide
3NT	3-nitrotyrosine
$O_2^{\cdot-}$	superoxide anion
$ONOO^-$	peroxynitrite
PBMCs	peripheral blood mononuclear cells
Pca	procainamide
PEMT	phosphatidylethanolamine N-methyltransferase
PHA	phytohemagglutinin
PKC	protein kinase C
PKCα	protein kinase C alpha
PKCδ	protein kinase C delta
PKCθ	protein kinase C theta
PLP	pyridoxal 5'-phosphate
PMA	phorbol myristate acetate
RNS	reactive nitrogen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SLE	systemic lupus erythematosus
SLEDAI	systemic lupus erythematosus disease activity index
SNP	single nucleotide polymorphism
SEM	standard error of the mean
TCR	T cell receptor
THF	tetrahydrofolate
Treg	regulatory T cell

ABSTRACT

Systemic lupus erythematosus is a potentially fatal autoimmune disease that predominantly affects women. Genetic and environmental factors both contribute to disease development, but how environmental factors cause epigenetic changes leading to pathogenesis is unclear. CD4+ T cell DNA demethylation results in overexpression of several immune-relevant methylation-sensitive genes, leading to functional consequences in lupus. Multiple environmental factors affect the methylation cycle, including micronutrients and oxidation of cellular components. This dissertation aims to address the relationship between micronutrients, redox changes, and the expression of methylation-sensitive T cell genes in lupus.

The first objective of this dissertation assessed levels of 8 micronutrients that are involved in methylation, in the serum of lupus patients. We found zinc, vitamin B6, and methionine to be decreased and homocysteine to be increased in our cohort of lupus patients compared to the normal population. In the second objective, we investigated whether altered levels of the same 8 micronutrients could alter expression of methylation-sensitive T cell genes *in vitro*. We did this by developing a custom media in which the transmethylation micronutrient levels were adjusted to physiologic levels. Our findings suggest that micronutrient

levels alter the expression of methylation-sensitive T cell genes and may have implications in lupus.

We then examined the relationship between micronutrients and peroxynitrite, a potent pro-oxidant, in the third objective. By treating T cells with peroxynitrite and culturing them in media with altered concentrations of folate and methionine, we determined that oxidant and micronutrient effects are additive in promoting increased gene expression. Finally, we compared the effects of altered methionine on CD4+ T cells from lupus patients and age- and gender-matched controls, in the fourth and final objective of this dissertation. The results suggest that T cells from lupus patients may be more sensitive to micronutrient alterations potentially due to increased levels of pro-oxidants.

Overall, these studies strongly suggest a potential role for micronutrient modulation of T cells in lupus and establish a link between redox status and micronutrient effects on methylation-sensitive gene expression. These findings provide a basis for future research on therapeutic benefits of dietary modification in autoimmunity.

CHAPTER I: INTRODUCTION

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic relapsing autoimmune disease affecting several organs of the body. The most common organs involved are the skin, joints, and kidney, though other organs such as the heart, lungs, and brain may also be affected. Clinical manifestations of lupus include discoid rash and the classic malar rash across the nose and cheeks, as well as oral ulcers, arthritis, pericarditis, pleurisy, glomerulonephritis, neuropsychiatric involvement and hematologic manifestations.

Lupus affects women in their childbearing years 9 times more often than men [1]. The exact prevalence of the disease is unknown, but is estimated at 14.6-100 per 100,000 [2, 3]. Currently, there is no cure for lupus. Due to improvements in treatment over the last several decades, the morbidity and mortality of SLE has significantly decreased, with the 10-year survival now at about 70% [4]. However, chronic complications associated with prolonged immune dysregulation and pharmaceutical use are significant.

The exact etiology of SLE is unknown, however a genetic predisposition is necessary. The concordance rate of lupus in familial lupus is 2% [5] and 25-40% [6] in identical twins. Further evidence for genetic involvement comes from the higher incidence of lupus in women compared to men. Estrogen is partially

responsible since women in their childbearing years have the highest incidence of lupus and estrogen supplementation is associated with lupus flares (SELENA trial). However, the presence of 2X chromosomes also contributes to the higher incidence. Women with Turner's syndrome (XO) have a lower risk of lupus, similar to that of men while men with Klinefelter's syndrome (XXY) have an approximately 14-fold higher risk than normal men, and a lupus risk similar to women [7]. Genome wide association studies (GWAS) have recently identified single nucleotide polymorphisms (SNPs) associated with lupus in at least 51 genes (see Table 1). However, despite the association of several genes with lupus, the estimated heritability of the disease according to GWAS reports is only 15% [8]. This, along with incomplete concordance in identical twins, indicates a role for environmental factors in disease development.

Environmental factors that have been associated with lupus include drugs, UV light, silica exposure and cigarette smoking, as well as infections [9-16]. Increased oxidative stress is common to these and may be a mechanism by which environment contributes to autoimmunity.   In the case of drugs, structurally unrelated drugs have been implicated in lupus suggesting that more than one mechanism may be involved [17]. It is unclear how environmental factors contribute to lupus in a genetically predisposed individual, but multiple mechanisms are likely involved. To date, few studies have examined the role of diet in lupus, another environmental factor likely to impact the disease.

Pathogenesis

Several immune abnormalities characterize SLE, including autoantibody production, B and T cell defects, aberrant cytokine release and persistent activation of the complement system, all of which ultimately lead to tissue damage. The presence of autoantibodies precedes clinical symptoms of disease by an average of 2-3 years [18]. Cells that are undergoing apoptosis provide antigenic stimulation. Antigen-presenting-cells (APCs) such as dendritic cells, monocytes/macrophages and B cells present autoantigens resulting in activation of effector T cells that help B cells produce autoantibodies. Autoantibodies can aggregate with antigens to form immune complexes which can become trapped in basement membranes of the kidney. Autoantibodies can also bind antigens in target tissues and activate complement.

While autoantibodies are key players in the onset of SLE, they are not sufficient; genes are also required. Only a small subset of individuals with autoantibodies go on to develop lupus. Complement activation as well as cytokine, chemokine, and proteolytic enzyme release, and oxidative damage are necessary for organ inflammation and damage to occur. Complement is needed for the removal of pathogens, immune complexes, apoptotic cells and debris. Indeed, hereditary deficiencies of the complement proteins C1q and C4 are associated with the monogenic forms of SLE. However, dysregulated persistent activation of the complement system by immune complexes in lupus results in chronic inflammation.

B and T cell abnormalities are abundant and contribute to disease pathogenesis in SLE. Certain subsets of B cells including memory B cells as well as activated B cells and plasma cells, are increased in SLE, and require lesser stimuli to be activated [19, 20]. Normally B cells go through several selection checkpoints to remove autoreactive B cells. However these checkpoints are defective in SLE leading to survival of autoreactive B cell subsets [21]. B-cell activating factor (BAFF/BLyS), a cytokine involved in promoting proliferation and survival of B cells, is increased in SLE patients [22]. Several genetic polymorphisms that predispose to SLE include those that affect B cell receptor (BCR) signaling, such as protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22) and BLK proto-oncogene, Src family tyrosine kinase (BLK) [23, 24] .

T cells also respond to stimulation with an exaggerated response in lupus [25]. Assembly of the signaling apparatus after T cell receptor (TCR) activation is abnormal in SLE CD4+ T cells. This results in suppression of IL-2 production, a cytokine important in the development and survival of regulatory T cells (Tregs) that function to limit inflammatory responses, and increased IL-17 production, which promotes inflammation [26]. Other T cell defects include possible defects in CD8+ cytotoxic T cells, decreased mitogen-activated protein (MAP) kinase activity, and altered cell surface receptors [27]. Proper cell surface receptor function is essential to the immune system. Epigenetic changes in T cells have also been implicated in SLE pathogenesis.

DNA Methylation

The intersection of gene and environmental interactions lies in the field of epigenetics. Epigenetics is defined as heritable changes in gene expression that occur without a change in DNA sequence. Epigenetic modifications include histone modifications such as acetylation, deacetylation, methylation and phosphorylation, and DNA methylation.

DNA methylation refers to the postsynthetic modification of deoxycytosine (dC) bases where a methyl group is added to carbon 5 to form deoxymethylcytosine (dmC) [28]. A 'methyl mark' is thus created on the DNA, resulting in a stable epigenetic modification. Cytosines that are methylated are almost always found in cytosine-guanine (CG) pairs. Most (approximately 70-80%) CG pairs are methylated in the mammalian genome [29]. The exceptions occur in regions of DNA rich in CG pairs, referred to as CpG islands (cytosine and guanine nucleosides linked by a phosphate), which are found in or near promoters of active genes [30, 31]. Methylation of regulatory regions usually corresponds to transcriptionally silent DNA while transcriptionally active DNA is typically unmethylated. DNA methylation results in a closed, transcriptionally inactive chromatin configuration. This occurs when methylcytosine binding proteins such as MECP2, MBD1, and MBD2 bind to dmC and recruit chromatin inactivation complexes to the methylated sequences. This promotes chromatin condensation into a compressed configuration which is inaccessible to transcription initiation complexes [32].

DNA methylation is mediated by a group of enzymes called the DNA methyltransferases (Dnmts). The importance of DNA methylation is demonstrated by the fact that homozygous deletion of any one of the Dnmts is embryonic lethal in mice [33]. During development, DNA methylation patterns are established by the 'de novo' methyltransferases DNA methyltransferase 3a and 3b (Dnmt3a and Dnmt3b). This serves to suppress expression of genes that are unnecessary or potentially detrimental to the function of the mature cells. Following differentiation, methylation patterns are maintained by DNA methyltransferase I (Dnmt1) which replicates the methylation patterns during mitosis [34-37]. Dnmt1 is upregulated by extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) pathway signaling during mitosis [38]. Dnmt1 maintains methylation by binding the replication fork, then recognizing methylated CG pairs on the parent strand and methylating the corresponding CG pair on the daughter strand. During this process, Dnmt1 transfers a methyl group from S-adenosylmethionine (SAM) to the unmethylated cytosine, forming 5-methylcytosine and S-adenosylhomocysteine (SAH). The transmethylation forward velocity is directly proportional to SAM concentration and Dnmt1 activity, and inversely proportional to SAH concentration (Figure 1.1). Environmental factors causing a decrease in SAM concentration or Dnmt activity, or an increase in SAH concentration will effectively decrease DNA methylation.

T cells in lupus

The first evidence of a role for DNA methylation in lupus came from studies done where normal, antigen specific CD4+ T cells treated with 5-azacytidine (5azaC), a DNA methylation inhibitor, became autoreactive, losing restriction for nominal antigen (Ag) and responding to self class II MHC molecules presenting inappropriate antigens [39]. 5azaC is a nucleoside analog developed 50 years ago [40] which incorporates into DNA causing rapid passive loss of DNA methylation when Dnmt1 becomes covalently bound to 5-azacytosine residues in the DNA [41]. CD4+ T cells treated with 5azaC also induced autologous B cell differentiation in the absence of exogenous antigen [42]. Further, adoptive transfer of murine CD4+ T cells treated with DNA methylation inhibitors into syngeneic hosts caused a lupus-like disease [43]. The autoreactivity was subsequently attributed in part to overexpression of lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18) on the demethylated cells [44], due to demethylation of the *CD11a* promoter [45]. LFA-1 is an adhesion molecule that binds to intercellular adhesion molecule-1 (ICAM-1) on APCs to stabilize T cell receptor-Ag binding to form the "immunologic synapse" resulting in T cell activation [46]. LFA-1 also acts as a costimulatory molecule for T cell activation [47]. Overexpression of LFA-1 contributes to T cell responsiveness in the absence of antigen in the binding cleft likely due to overstabilization of the T cell receptor-MHC interaction and/or increased costimulatory signals [48].

The fact that 5azaC induced autoreactivity suggested that other environmental agents may induce autoreactivity through similar epigenetic mechanisms. Interestingly, procainamide (Pca) and hydralazine (Hyd), two drugs strongly associated with lupus [17], were found to be DNA methylation inhibitors. Procainamide is an antiarrhythmic drug and hydralazine an antihypertensive drug. Treating human CD4+ T cells with procainamide, hydralazine, or 5azaC resulted in a decrease of total T cell genomic d^mC content, as measured by reverse-phase HPLC [49], demonstrating that both procainamide and hydralazine are DNA methylation inhibitors. Subsequently, human or murine CD4+ T cells treated with Pca or Hyd overexpressed LFA-1 and became autoreactive , and adoptive transfer of the autoreactive murine cells into syngeneic recipients caused a lupus-like disease identical to 5azaC treated T cells [50]. The observation that these drugs have distinct chemical structures suggests that multiple mechanisms may be involved in inducing autoimmunity [17]. Procainamide reversibly inhibits Dnmt1 enzymatic activity and hydralazine prevents Dnmt1 upregulation by inhibiting ERK pathway signaling through PKCδ [17]. These studies suggest that other environmental agents may be involved in triggering lupus through similar mechanisms.

A hallmark of lupus is increased antibody production. T cells made autoreactive by DNA methylation inhibitors recognize self class II MHC molecules on B cells and overstimulate antibody production [51]. The experimentally demethylated cells also induce macrophage apoptosis by responding to self class II MHC molecules on macrophages. The resultant

release of apoptotic material is highly antigenic and since macrophages normally remove apoptotic material, apoptotic debris accumulates. Decreased clearance of apoptotic material or inducing macrophage apoptosis with clodronate vesicles in mice [52, 53] resulted in production of autoantibodies to nuclear antigens, thus the macrophage apoptosis likely stimulates autoantibody formation.

In lupus patients, T cell DNA methylation levels were decreased compared to controls, and patients with active lupus had lower total methylcytosine content compared to patients with inactive lupus [42]. Dnmt1 levels were decreased in lupus patients due to decreased ERK pathway signaling [54] caused by defective PKCδ phosphorylation [55]. Importantly, the defect in PKCδ activation was subsequently attributed to oxidative stress [56].

As mentioned above, LFA-1 was overexpressed in T cells treated with DNA methylation inhibitors. Patients with active lupus also overexpress LFA-1 on a CD4+ T cell subset [44, 57]. The overexpression was due to demethylation of CD11a regulatory regions, identical to those demethylated in 5azaC-treated T cells [45]. Later studies found several other immune genes to be demethylated and overexpressed in CD4+ T cells treated with DNA methylation inhibitors and in CD4+ T cells from lupus patients. These include CD70, perforin and the KIR genes [51, 58-60]. CD70 is a B cell costimulatory molecule that stimulates antibody production. The overexpression of CD70 on experimentally demethylated cells and lupus T cells caused B cell overstimulation and antibody production that was abrogated by anti-CD70 [51]. The perforin and KIR genes are noteworthy since they are not normally expressed on CD4+ T cells, but

aberrantly expressed when demethylated as in the case of 5azaC-treated CD4+ T cells or lupus CD4+ T cells. The KIR genes are normally expressed on natural killer (NK) cells and either stimulate or suppress various effector functions depending on the cytoplasmic domain [61]. KIR expression on demethylated CD4+ T cells and lupus T cells resulted in IFN-γ overexpression and macrophage killing [62]. IFN-γ stimulates antibody production [63] and as noted above, macrophage killing leads to autoantibody formation. Perforin is a pore-forming cytotoxic molecule also expressed in NK cells and aberrantly expressed in demethylated and lupus CD4+ T cells. Perforin lyses target cells by disrupting their cytoplasmic membranes [64] and promotes autoreactive macrophage killing by lupus CD4+ T cells [65], which can be inhibited by concanamycin A, a perforin inhibitor [66]. The functional consequences of these aberrantly expressed methylation-sensitive genes may contribute to the autoimmunity and pathogenesis seen in lupus through effects such as antibody production and macrophage killing.

Micronutrients and DNA Methylation

Several micronutrients and metabolites are involved in the transmethylation cycle (Figure 1.2). SAM serves as the universal methyl donor for a number of methyl acceptors including DNA, RNA and protein [67]. SAH is the by-product of methylation and strongly inhibits the transmethylation reaction by binding the catalytic domain of SAM-dependent methyltransferases [68]. The SAM/SAH ratio is reflective of the flow of methyl groups from SAM to methyl

acceptors [69], with a decrease in the ratio indicative of lower methylation capacity [70]. Transmethylation micronutrients and their complex interactions affect DNA methylation by potentially mediating the SAM/SAH ratio. Some of the key micronutrients/metabolites are outlined below.

Methionine

Methionine is an essential amino acid that cannot be synthesized *de novo* in humans. Methionine combines with ATP to form SAM by the enzyme methionine adenosyltransferase (MAT), and is therefore critical for maintaining the methylation cycle. The importance of methionine in the methylation cycle is demonstrated by MAT1A knockout mice. Methionine levels are increased 7-fold in MAT1A knockout mice and SAM is severely reduced by 74%, as might be expected from the inability to produce SAM. The reactions to produce reduced glutathione (GSH) from methionine are reversible and it has been argued that GSH depletion leads to DNA hypomethylation by drawing methionine away from the methylation cycle [71].   However in the MAT1A knockout mice, GSH is surprisingly reduced, considering the presence of excess methionine. This suggests that GSH is redirected towards the SAM cycle as a methionine source to drive methylation in the absence of MAT1A [72], highlighting the necessity for methionine in the methylation cycle.

Homocysteine

Homocysteine is not a dietary micronutrient, but an important metabolite in the methylation cycle. Following DNA methylation by SAM, SAH can be hydrolyzed to homocysteine and adenosine. In this reversible reaction, SAH synthesis is strongly favored over hydrolysis. Therefore, efficient homocysteine removal prevents SAH accumulation which inhibits Dnmt1. Homocysteine can be remethylated to form methionine or catabolized to sulfate and pyruvate. Plasma homocysteine is a very sensitive biomarker of folate adequacy [73] in deficient but also normal and above-normal levels of red blood cell folate [74]. Elevations in plasma homocysteine have been associated with cardiovascular disease [75, 76] and stroke [77]. The increased risk is present at modest homocysteine elevations at greater than 11μmol/l [75] which is within the generally accepted normal range of 5-15μmol/l [78]. Interestingly, homocysteine levels are elevated in lupus patients [79], and SAH and homocysteine accumulation causes lymphocyte DNA demethylation [70].

Folate

Folate is a water-soluble B vitamin (vitamin B9) that is essential to the DNA biosynthesis and methylation cycles. During insufficient times of dietary intake of folate or its synthetic form folic acid, DNA biosynthesis and methylation decreases. A decrease in DNA biosynthesis results in reduced cell division causing anemia, leukopenia, and thrombocytopenia. A decrease in the methylation cycle is observed by an elevation in plasma homocysteine [73]. The

primary form of folate to enter the bloodstream from intestinal cells is 5-methylTHF [73]. In the methylation cycle, 5-methylTHF transfers a methyl group to homocysteine catalyzed by methionine synthase to synthesize methionine [80, 81], and subsequently SAM. Folate modulates DNA methylation through its effects on SAM [82]. Low folate status that is still considered clinically normal has been associated with increased risk of colorectal cancer [83, 84]. This is further supported by the observation that genetic polymorphisms such as MTHFR C677T that affect the folate pathway, modify colorectal cancer risk [85-87]. DNA methylation in lymphocytes may be affected by another polymorphism in the same gene, MTHFR A1298C [88, 89]. Furthermore, a polymorphic variant of the gene encoding methionine synthase, MTR 2756A>G, has been associated with an increased risk of SLE in the Polish population [90]. It is clear from numerous studies that folate modulates DNA methylation, however the degree to which this occurs and direction of the change is still unclear.

Choline

Choline is critical for membrane structural integrity, lipid and cholesterol metabolism, and is a precursor for the neurotransmitter acetylcholine. Choline is also important in transmethylation pathways. It is unclear whether normal dietary intake meets the nutrient needs for most people [91]. The US Institute of Medicine's Food and Nutrition Board established intake recommendations for choline for the first time in 1998 [92]. Choline is a major dietary source of methyl groups, though endogenous choline is present in the body from the *de novo*

biosynthesis of phosphatidylcholine catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT) in the liver. PEMT utilizes SAM as a methyl donor to form a new choline moiety, subsequently producing homocysteine [93]. Interestingly, estrogen upregulates PEMT, thereby affecting endogenous choline synthesis [94] and consequently resistance to choline deficiency: premenopausal women fed a choline-deficient diet were more resistant to developing organ dysfunction compared to males and postmenopausal women [95]. In the transmethylation cycle, choline is important for one of the two parallel pathways by which homocysteine is remethylated to form methionine [96] thus lowering homocysteine concentrations [97]. The first pathway involves folate and vitamin B12 (see section on folate). The second pathway utilizes betaine, a metabolite of choline, as the methyl group donor for the conversion of homocysteine to methionine. Oral betaine supplementation has been shown to lower plasma homocysteine concentrations [98, 99]. Individuals with choline deficiency have elevated homocysteine levels and a decreased capacity to methylate homocysteine after a methionine load [100]. Additionally, dietary choline deficiency causes hypomethylation of DNA [101, 102] by decreasing SAM concentrations in tissues [103, 104]. Given the elevated homocysteine levels [79] and hypomethylation of T cell DNA [105] in SLE, choline may have implications in disease pathogenesis.

Vitamin B12

Vitamin B12, also known as cobalamin, is used by two enzymes: methylmalonyl coenzyme (CoA) mutase and methionine synthase. Methylmalonyl coenzyme (CoA) mutase functions in the citric acid cycle to metabolize amino acids and is compromised during vitamin B12 deficiency [73], however it only appears to have a critical function during embryonic life and in children, but not in adults. Methionine synthase, on the other hand, is vital to the methylation cycle. Vitamin B12 is utilized by methionine synthase to convert homocysteine to methionine. In this process, 5-methyltetrahydrofolate (5-methylTHF) is converted to tetrahydrofolate. Any interruption in the vitamin B12-dependent methionine synthase results in accumulation of homocysteine and decrease in SAM, thereby affecting methylation. This is seen in the neuropathy that occurs with vitamin B12 deficiency, characterized by demyelination of the peripheral nerves and spinal column [106, 107]. Additionally, folate becomes 'trapped' as 5-methylTHF and cannot be reversed to its precursor, therefore affecting DNA biosynthesis and cell division. This is seen in the anemia that presents with vitamin B12 deficiency [73]. Vitamin B12 enters the human food chain from foods of animal origin. Absorption of vitamin B12 is dependent on the production of intrinsic factor and hydrochloric acid by the parietal cells of the stomach. The production of intrinsic factor and hydrochloric acid may be compromised in elderly populations in the fairly rare form of pernicious anemia [108] or more common atrophic gastritis [109], respectively. The methylation disturbances caused by vitamin B12 deficiency may have implications for other

diseases in this population, particularly autoimmune diseases and cancer which both increase with age and are affected by methylation [110].

Zinc

Zinc affects several aspects of the immune system. These effects are numerous and include susceptibility to infection [111-120], fetal immunologic development [121-123], and effects on immunosuppressive conditions [124-128]. Zinc also affects specific cells of the immune system including T and B lymphocytes. Lymphocytes, particularly T lymphocytes, are reduced in number in the blood and peripheral lymphoid organs of zinc-deficient children with acrodermatitis enteropathica and patients receiving total parenteral nutrition [129, 130].

Zinc may also affect methylation. The first study examining the relationship between zinc and methylation patterns was conducted in 1985. Rats that were fed a zinc deficient diet had reduced utilization of methyl groups from SAM and this resulted in DNA and histone hypomethylation [131, 132]. Zinc affects two enzymes in the methylation pathway: betaine-homocysteine-S-methyltransferase (BHMT) and methionine synthase. BHMT is a zinc metalloenzyme that synthesizes methionine by catalyzing the transfer of methyl groups from betaine to homocysteine [133]. Methionine synthase utilizes zinc for active site conformational changes needed to catalyze the transfer of a methyl group to homocysteine to produce methionine [134, 135]. Both of these pathways are essential for remethylating homocysteine to form methionine and

subsequently the SAM needed for methylation, therefore depletion of zinc may lead to impaired enzyme activity and consequently hypomethylation.

Vitamin B6

Though overt B-vitamin deficiencies have decreased, there is some evidence of widespread subclinical deficiency, particularly pyridoxine and riboflavin [73]. Subclinical deficiencies are not as obvious in their manifestations, but can have detrimental metabolic effects. Vitamin B6, also known as pyridoxine, is important in the synthesis of enzymes of amino acid metabolism. Deficiency of vitamin B6 has been associated with immune system impairment [73]. Circulating lymphocytes are decreased and anemia may be present as well [136]. In the methylation cycle, vitamin B6 functions in the conversion of homocysteine to cystathionine, a precursor of GSH. A decrease in vitamin B6 results in elevated homocysteine levels. The best biochemical marker of vitamin B6 deficiency is decreased plasma levels of the coenzyme pyridoxal 5'-phosphate (PLP) [73]. Plasma PLP levels decrease with age, therefore separate guidelines for adequate intake of vitamin B6 have been established for the elderly population [137].

Riboflavin

Riboflavin, also known as vitamin B2, participates in numerous oxidation-reduction reactions by conversion to coenzymes flavin mononucleotide (FMN) and to flavin adenine dinucleotide (FAD). These coenzymes function in

metabolic pathways and energy production through the respiratory chain [138, 139]. Riboflavin deficiency occurs through inadequate dietary intake or through malabsorption in certain conditions such as lactose intolerance and celiac disease. Riboflavin is necessary for the production of the coenzyme PLP (see section on vitamin B6), therefore riboflavin deficiency often occurs with vitamin B6 deficiency [140] and can affect the methylation cycle through interactions with vitamin B6.

Oxidative stress and DNA methylation in lupus

Oxidative stress is traditionally defined as an imbalance between pro-oxidants and antioxidants [141] and more recently to include a disruption of redox signaling and control and/or molecular damage [142] . Oxidative stress has been associated with aging [143] and several pathological conditions including cardiovascular disease [144], diabetes [145], pulmonary diseases [146], neurodegenerative diseases [147], and cancer [148] . Significant evidence indicates that oxidative stress may also contribute to SLE.

Lupus is characterized by chronic inflammation and a prooxidative state [149, 150]. Several markers of oxidative stress are increased in lupus patients. Urinary F2 isoprostane has been associated with patient-reported symptoms in SLE, suggesting that oxidative stress may contribute to the debilitating symptoms associated with the disease [150]. Multiple markers of protein oxidation are associated with lupus. Protein carbonyls and 3-nitrotyrosine levels were increased in sera from SLE patients and correlated with disease activity [149,

151]. Furthermore, protein carbonyl levels were increased in patients with arthritis or arthralgia, cardiac involvement, or renal disease compared to those without, suggestive of a role for protein oxidation in organ damage [152].

Reactive nitrogen species (RNS) have been implicated in lupus pathogenesis. Nitric oxide (NO), in particular, has several immunoregulatory functions including regulation of T lymphocytes. NO affects T cell function by mediating mitochondrial hyperpolarization following T cell activation [153]. Additionally, T cell receptor signaling to antigen at the immunological synapse is selectively potentiated by NO derived from endothelial nitric oxide synthase (eNOS) [154]. The CD4+ T cell proliferation and differentiation into T helper 1 (Th1) and Th2 cells that occurs following activation is influenced by NO whereby NO preferentially enhances Th1 cell differentiation [155, 156]. In SLE, NO production is increased. One study measuring serum nitrite and nitrate levels found an increase in lupus patients that correlated with disease activity and damage [157]. Several studies demonstrate a role for NO in SLE T cell dysregulation [158-160]. Lupus T cells have permanently high mitochondrial membrane potential [161, 162]. Interestingly, lupus and normal T cells generate similar amounts of NO, however monocytes from lupus patients produce higher levels of NO compared to normal monocytes. Since NO is a diffusible gas, the NO produced by monocytes may be affecting neighboring T cells. NO produced by monocytes in SLE has been shown to contribute to T lymphocyte mitochondrial dysfunction [163]. Other NO-induced T cell effects in SLE include

increased mTOR activity [164] and TCR-induced rapid and sustained Ca2+ signal [163], and decreased TCRζ expression [165] and ATP level [166].

NO is produced by the enzyme inducible nitric oxide synthase (iNOS) and can combine with superoxide anion ($O_2^{\cdot-}$) to generate the highly reactive metabolite, peroxynitrite ($ONOO^-$). $ONOO^-$ can modify proteins in several ways including oxidation and nitration. Nitration of tyrosine residues by $ONOO^-$ results in the formation of 3-nitrotyrosine (3NT), a stable by-product of peroxynitrite formation [151, 167] . While other measures of NO synthesis can be falsely elevated by medications and dietary nitrates, 3NT is a reliable marker of NO production that is unaffected by exogenous sources [167]. Importantly, 3NT levels are increased in lupus patients, and the increase correlated with disease activity [167]. Furthermore, 3NT was increased in patients with lupus nephritis compared to those without renal disease [167]. These findings suggest a role for nitrated proteins in the pathogenesis of SLE. Recently, nitration of proteins has been implicated in epigenetic mechanisms of lupus pathogenesis.

As discussed above, DNA methylation is mediated by DNA methyltransferases. The Ras-MAPK signaling pathway regulates Dnmt1 levels by activating the transcription factors activating protein 1 (AP-1). DNA methylation was inhibited in actively proliferating cells by inhibiting this signaling pathway [168, 169]. Furthermore, human T cell Dnmt levels were decreased when RAS-MAPK pathway signaling was inhibited [170]. SLE patients have an impaired T cell extracellular signal-regulated kinase (ERK) 1/2 phosphorylation that is proportional to disease activity [54]. CD4+ T cells become autoreactive

when treated with DNA methylation inhibitors [171] and MEK inhibitors, which are ERK signaling pathway inhibitors [172]. An inducible transgenic mouse model that expresses a dominant negative MEK in T cells had decreases in T cell ERK pathway signaling when the transgene was turned on. Additionally, the mice developed anti-ds-DNA antibodies and an "interferon signature" characteristic of autoimmunity [173]. Purified CD4+ T cells from patients also have an impaired ERK pathway [42]. These observations together suggest that aberrant ERK pathway signaling may decrease T cell DNA methylation and contribute to lupus.

Subsequent studies have shown that the defect in ERK pathway signaling is due to impaired PKCδ phosphorylation. T cells from lupus patients with active disease and T cells treated with hydralazine, and ERK pathway inhibitor, were stimulated with phorbol myristate acetate (PMA), a molecule that induces activation of the ERK pathway by directly activating protein kinase C (PKC). The PKCδ isoform but not PKCα or PKCθ showed impaired phosphorylation in response to stimulation. Furthermore, human CD4+ T cells treated with Rottlerin, a selective PKCδ inhibitor, had similar decreases in ERK phosphorylation and overexpression of CD70 with demethylation of the TNFSF7 (CD70) promoter as lupus and hydralazine-treated T cells. CD4+ T cells transfected with a dominant negative PKCδ also showed the same results [55]. Mice lacking PKCδ develop a lupus-like disease [174], supporting the hypothesis that defective PKCδ phosphorylation may contribute to lupus through impaired ERK pathway signaling and subsequent T cell DNA demethylation.

The defect in PKCδ phosphorylation has recently been attributed to oxidative stress, specifically the nitration of PKCδ [56]. Treating CD4+ T cells from healthy subjects with increasing concentrations of ONOO$^-$ and stimulating with PMA resulted in PKCδ nitration. This altered the phosphorylation pattern by decreasing threonine (T^{505}) phosphorylation and increasing tyrosine (Y^{311}) phosphorylation. The PKCδ nitration induced by ONOO$^-$ also correlated with PMA-stimulated ERK phosphorylation, and since PKCδ T^{505} phosphorylation is upstream of ERK, this suggests a role for PKCδ nitration in the impaired ERK pathway signaling [55, 56]. Additionally, the study found increased amounts of nitrated PKCδ in T cells of patients with active lupus compared to controls. Oxidative stress may be contributing to SLE by altering signaling through the effects of ONOO$^-$ on PKCδ. This in turn may affect Dnmt1 regulation and subsequently methylation-sensitive autoimmune-relevant gene expression.

Summary

SLE is a multi-factorial disease in which genetic and environmental factors play a role. Epigenetic changes, particularly DNA methylation in T cells, contribute to disease pathogenesis and may in part account for the environmental impact on the disease. Which environmental factors and how they trigger disease in genetically predisposed individuals is unclear. Micronutrients from diet impact the transmethylation reaction, and may play a part. Additionally, oxidative stress is increased in lupus patients, affects DNA methylation, and is common to several environmental triggers associated with lupus.

We hypothesize that micronutrients affect methylation-sensitive gene expression in SLE and interact with oxidant effects to further promote aberrant gene expression. This is examined in the following four specific aims:

I. To determine if transmethylation micronutrient levels are decreased in lupus patients.

II. To examine whether altered micronutrient levels affect methylation-sensitive T cell gene expression.

III. To investigate the interaction between oxidants and altered micronutrient levels on methylation-sensitive T cell gene expression.

IV. To compare methylation-sensitive gene expression in lupus versus control T cells exposed to altered micronutrients.

$$V= \frac{k[Dnmt1][SAM]}{[SAH]}$$

Figure 1.1: Transmethylation reaction forward velocity.
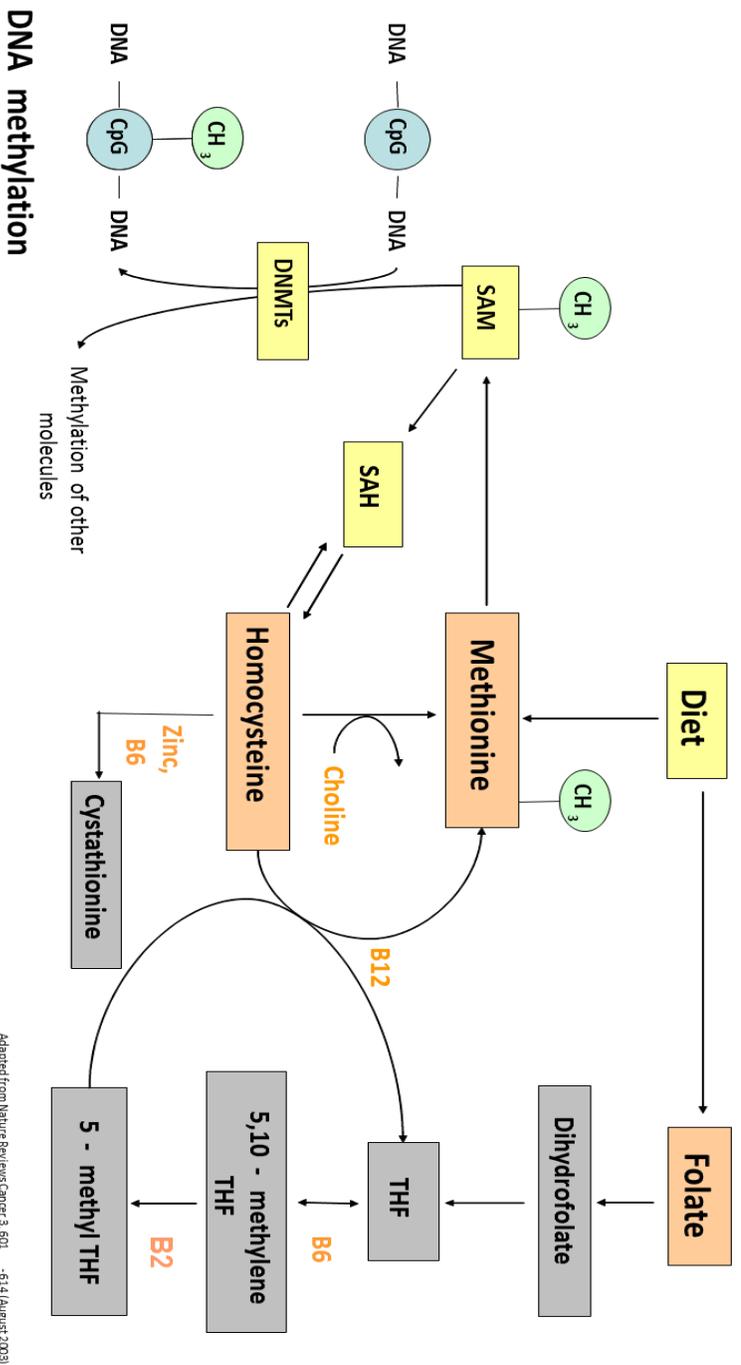
DNA methylation

Figure 1.2: Interaction between micronutrients and DNA methylation. Diagram adapted from Nature Reviews Cancer 3, 601-614 (August 2003) Abbreviations: methyl group (CH₃); DNA methyltransferases (DNMTs); cytosine-phosphate-guanine (CpG); S-adenosylmethionine (SAM); S-adenosylhomocysteine (SAH); riboflavin (B2); pyridoxine (B6); cobalamin (B12); tetrahydrofolate (THF); 5,10-methylenetetrahydrofolate (5,10-methyleneTHF); 5-methyltetrahydrofolate (5-methylTHF).

Table 1.1 Single nucleotide polymorphisms associated with lupus

Gene/region	Chromosome	Associated SNP	Risk allele	Odds ratio*	European American	African American	Hispanic	Asian	Selected reference
AFF1	4q21	rs340630	A	1.21				x	Okada et al, 2012
ARID5B	10q21	rs4948496	C	1.18	x			x	Yang W et al, 2013
ATG5	6p21	rs2245214	G	1.15	x				Gateva V et al, 2009
BANK1	4q24	rs10516487	G	1.38	x	x		x	Kozyrev SV et al, 2008
C8orf13-BLK	8p22-23	rs13277113	A	1.39	x	x		x	Hom G et al, 2008
CD80	3q13	rs6804441	A	1.27		x		x	Yang W et al, 2013
CDKN1B	12p13	rs34330	C	1.19				x	Yang W et al, 2013
CREBL2	12p13	rs12822507	A	1.16				x	Yang W et al, 2013
CTLA4	2q33	rs231775	G	1.23	x	x		x	Barreto M et al, 2004
DDX6	11q23.3	rs503425	C	1.16	x				Gateva V et al, 2009
DRAM1	12q23	rs4622329	A	1.19				x	Yang W et al, 2013
ETS1	11q24.3	rs6590330	A	1.37	x			x	Han JW et al, 2009
FCGR2A	1q23	rs1801274	C	1.35	x	x		x	Harley JB et al, 2008
FCGR3A	1q23	rs396991	T	1.3	x	x	x		Magnusson V et al, 2004
GPR19	12p13	rs10845606	C	1.19			x	x	Yang W et al, 2013
HLA region1	6p21.33	rs3131379	A	2.36	x	x	x	x	Harley JB et al, 2008
HLA region2	6p21.32	rs1270942	G	2.35	x	x	x	x	Harley JB et al, 2008
ICAM1-ICAM4-ICAM5	19p13.3	rs3093030	A	1.16	x	x	x	x	Kim K et al, 2012
IFIH1	2q24	rs1990760	T	1.11	x			x	Cunninghame Graham et al, 2011
IKZF1	7p12.2	rs4917014	A	1.39	x	x		x	Han JW et al, 2009
IKZF3-ZPBP2	17q21	rs14453560	C	1.37	x	x			Lessard et al, 2012
IL10	1q31-32	rs3024505	A	1.19	x	x			Gateva V et al, 2009
IL-21	4q26	rs907715	G	1.29	x	x			Sawalha AH et al, 2007
IRF5	7q32	rs2004640	T	1.47	x	x	x	x	Graham RR et al, 2006
IRF8	16q24.1	rs11644034	G	1.28	x				Lessard et al, 2012
IRF8	16q24.1	rs2280381	A	1.11	x				Cunninghame Graham et al, 2011
ITGAM	16p11.2	rs11143679	A	1.78	x	x	x	x	Nath SK et al, 2008
JAZF1	7p15	rs849142	T	1.19	x				Gateva V et al, 2009
KIAA1542	11p15.5	rs4963128	C	1.28	x	x		x	Harley JB et al, 2008
LRRC18-WDFY4	10q11.22	rs1913517	A	1.24	x	x		x	Han JW et al, 2009

Table 1.1 Single nucleotide polymorphisms associated with lupus continued

Gene	Locus	SNP	Allele	OR				Reference
MECP2/IRAK1	Xq28	rs17435	T	1.39	x		x	x	Sawalha AH et al, 2008
NCF2	1q25	rs10911363	T	1.23	x			x	Cunninghame Graham et al, 2011
PDCD1	2q37.3	rs11568821	A	2.85	x		x	x	Prokunina L et al, 2002
PDHX-CD44	11p13	rs2732552	C	1.22	x	x		x	Lessard et al, 2011
PRDM1	6q21-22.1	rs6568431	A	1.2	x				Gateva V et al, 2009
PTPN22	1p13	rs2476601	A	1.53	x			x	Harley JB et al, 2008
PTPN22	1p13.2	rs34933034	A	1.32	x			x	Manjarrez-Orduño N et al, 2012
PTTG1	5q33.3	rs2431099	G	1.15	x			x	Gateva V et al, 2009
PXK	3p14.3	rs6445975	C	1.25	x			x	Harley JB et al, 2008
RASGRP3	2p22.3	rs13385731	A	1.43	x			x	Han JW et al, 2009
SLC15A4	12q24.32	rs1385374	A	1.26	x			x	Han JW et al, 2009
STAT4	2q32.2	rs7574865	T	1.55	x	x		x	Remmers EF et al, 2007
TET3	2p13	rs6705628	C	1.33	x			x	Yang W et al, 2013
TET3	2q13	rs48523324	T	1.27	x			x	Yang W et al, 2013
TMEM39A	3q13.33	rs1132200	G	1.2	x			x	Lessard et al, 2012
TNFAIP3	6q23	rs5029939	G	2.09	x			x	Bates JS et al, 2009
TNFSF4	1q25	rs2205960	T	1.28	x		x	x	Graham DS et al, 2008
TNIP1	5q32-33.1	rs7708392	C	1.27	x			x	Gateva V et al, 2009
TYK2	19p13.2	rs280519	A	1.2	x				Cunninghame Graham et al, 2011
UBE2L3	22q11.2	rs5754217	T	1.2	x			x	Gateva V et al, 2009
UHRF1BP1	6p21	rs11755393	G	1.17	x			x	Gateva V et al, 2009

27

CHAPTER II: TRANSMETHYLATION MICRONUTRIENT LEVELS IN LUPUS PATIENTS

Introduction

SLE is a chronic relapsing autoimmune disease characterized by multiple immune abnormalities that lead to multi-system involvement and organ damage. Genetic and environmental factors play a role in disease development and age may also contribute. Epigenetic changes in T cells, specifically DNA methylation, have been implicated as a mechanism by which environment may promote disease progression.

DNA methylation is a post-synthetic modification where a methyl group is transferred from SAM to cytosine nucleotides in the DNA by the Dnmt enzymes. DNA methylation is dependent on the enzymatic activity of Dnmt and the availability of SAM which is generated through the methylation cycle. Multiple metabolites affect the methylation cycle including several micronutrients from diet. After transferring a methyl group from SAM to the DNA, Dnmt1 produces the by-product SAH, a potent inhibitor of Dnmt1. Any interruption of the methylation cycle affecting production of SAM will decrease DNA methylation. Furthermore, an increase in the metabolite homocysteine favors SAH synthesis thereby decreasing DNA methylation through Dnmt1 inhibition.

Most CG pairs in DNA are methylated except in and around active promoter regions. Methylation of promoter regions generally corresponds to transcriptionally inactive genes whereas unmethylated promoters are associated with active gene transcription. In lupus, several methylation-sensitive immune genes, such as *CD11a*, *CD70*, *KIR*, and *perforin*, are overexpressed due to hypomethylation of CD4+ T cell DNA [175]. The overexpression pattern is identical to that seen in normal CD4+ T cells treated with DNA methylation inhibitors. Furthermore, the genes that are overexpressed are demethylated at their promoters in both the lupus T cells and the experimentally demethylated T cells, compared to normal T cells. The overexpression of these genes in CD4+ T cells has functional consequences such as macrophage apoptosis and autoantibody production caused by the altered T cells, which may in part be responsible for disease pathogenesis.

We hypothesized that the T cell hypomethylation seen in lupus patients may be due in part to changes in serum/plasma levels of key micronutrients involved in the methylation cycle. We therefore obtained blood from lupus patients and compared the serum or plasma levels of seven micronutrients and homocysteine to the normal population values. The results suggest that subclinical deficiencies in certain micronutrients may play a role in lupus due to potential alterations of the methylation cycle.

Materials and Methods

Human Subjects. The University of Michigan Institutional Review Board for Human Subject Research reviewed and approved this study. Patient samples were collected from the University of Michigan outpatient clinic with informed consent according to the Declaration of Helsinki. Patient characteristics are listed in Table 2.1. Subjects were questioned regarding vitamin supplements, fasting status, and alcohol consumption for the 24 hour period preceeding sample collection since these may affect test results. In most cases we were not able to collect fasting samples, however we did take note of any patients that may have already been fasting.

Sample processing. A total of 37 milliliters (mls) of blood was collected in green top (sodium heparin) Vacutainer tubes for methionine, choline, riboflavin, and vitamin B6 testing, SST™ Vacutainer tubes for folate and vitamin B12 testing, lavender top (EDTA) Vacutainer tubes for homocysteine testing, and royal blue (no additive) tubes for zinc testing. Plasma or serum was extracted within 30 minutes of blood collection by centrifugation. Samples were then aliquoted and frozen at -20°C until analysis.

Sample testing. Serum folate, zinc, and vitamin B12 were measured by the University of Michigan Chemical Pathology Laboratories. Plasma homocysteine, riboflavin, and vitamin B6 were also measured by the University of Michigan

Chemical Pathology Laboratories. Plasma methionine was measured by the Mayo Medical Laboratories. Plasma choline was measured by Ralston Analytical Laboratories. Test methodology is listed in Table 2.2.

SLEDAI score. To compare disease activity, the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used [176] (Table 2.3). A score was assigned to each patient based on clinical lab findings and physician's findings within the past 10 days of sample collection. The portion of the score based on the doctor's note may be subjective, therefore scores that were not definitive were confirmed by the physician in charge of the lupus cohort who independently assigned a score.

Statistical Analysis. To compare the levels of different micronutrients in the lupus patients to the normal population values, the mean of the lupus patients' value was compared to the midpoint of the normal range, using a one-sample t-test. For non-normally distributed variables, the median of the micronutrient value was compared to the midpoint of the normal range using a non-parametric Wilcoxon sign rank test.

The percentage of patients who were out of the normal range (either above or below the normal range) was assessed using a one-sample binomial proportion, and a 95% confidence interval was computed.

Statistical analyses were conducted using IBM SPSS Statistics, version 21 for Windows, copyright IBM corporation 1989, 2012.

Results

Serum/plasma nutrient concentrations. Serum folate, zinc, and vitamin B12, and plasma homocysteine, riboflavin, vitamin B6, and methionine were tested in 28-35 lupus patients and compared to the mean population reference values given by each laboratory. Mean population reference values have been established from historical data spanning large population groups. Plasma choline was tested in 10 lupus patients and compared to 10 age- and gender-matched controls (Table 2.4). Serum zinc levels were significantly decreased in lupus patients compared to the normal female population mean value for zinc concentration (0.71 vs 0.86; p=0.0001). Vitamin B6 was also decreased in the lupus patients tested compared to the mean population value (16.48 vs. 22.5; p=0.021). Homocysteine was significantly increased in lupus patients compared to the mean population value (11.37 vs. 9.5; p=0.013). Methionine was also significantly decreased in lupus patients compared to the mean population value (23.09 vs. 26.0; p-0.012). There were no significant differences in serum folate and vitamin B12, and plasma riboflavin in lupus patients compared to the mean population value. There was also no significant difference in plasma choline levels in the ten lupus patients tested compared to ten age- and gender- matched controls. These results were independent of age and disease activity.

Discussion

These studies indicate lower than average levels of zinc, vitamin B6, and methionine, and higher homocysteine levels in lupus patients compared to the normal population. While the changes seen here are within the normal ranges established, the possibility of subclinical changes in dietary micronutrients having metabolic effects has been raised [73].

The change in zinc was small but significant compared to the normal population. Considering plasma zinc is under close homeostatic control [73], the decrease may have implications for pathogenesis. The role of zinc has been well established in numerous immune functions including effects on T lymphocytes [177]. Responses involving T lymphocytes such as delayed hypersensitivity and cytotoxic activity are impaired during zinc deficiency and restored by zinc supplementation [178-181]. T cell proliferative responses to mitogens such as PHA are reduced during zinc deficiency, as in the case of patients receiving total parenteral nutrition devoid of zinc [130]. Importantly, zinc may alter T cell surface molecules. Pretreatment of T lymphocytes with zinc *in vitro* enhanced rosetting by sheep erythrocytes [182]. Furthermore, zinc was shown to increase transcription and expression of ICAM-1, an adhesion molecule, on the surface of lymphocytes [183]. Other lymphocyte surface molecules have been reported to be altered in SLE due to hypomethylation [184]. Zinc may contribute to these changes due to its role in the methylation cycle. Zinc is important in the regeneration of methionine in the methylation cycle. Two key enzymes utilize zinc to remethylate homocysteine to form methionine. Methionine is necessary

33

for the formation of the methyl donor SAM, therefore a decrease in zinc has potential for interrupting methylation.

We also found vitamin B6 and methionine to be decreased in lupus patients compared to the means of the normal population. As mentioned above, methionine is essential for maintaining the methylation cycle through formation of SAM. Vitamin B6 is necessary for the conversion of homocysteine to cystathione and a decrease in vitamin B6 results in elevated homocysteine levels. Vitamin B6 levels decrease with age and evidence of potential widespread subclinical deficiency has been reported [73]. Interestingly, a study by Mohan et al. has recently also reported decreased vitamin B6 and methionine levels in lupus patients [185]. In the study, the authors used an LC/MS and GC/MS based platform to perform a comprehensive metabolic scan of sera from 20 SLE patients and 9 healthy controls. They found a decrease in methionine levels, and a decrease in vitamin B6 levels that correlated with disease activity. They further validated the vitamin B6 decrease using an independent cohort of 38 SLE patients and 14 healthy controls. These results are corroborated by our findings that vitamin B6 and methionine are decreased in lupus patients.

Homocysteine levels were increased in lupus patients compared to the normal population in our study. This is in accordance with a previous study also demonstrating increased homocysteine levels in lupus patients [79]. Homocysteine is a key metabolite in the methylation cycle, where it is remethylated by methionine synthase and 5-methylTHF as the methyl donor to form methionine. An increase in homocysteine results in increased synthesis of

SAH, a potent inhibitor of methylation [68]. Vitamin B6 is involved in the metabolism of homocysteine and as stated above, was decreased in our study. Interestingly, the decrease in vitamin B6 along with an increase in homocysteine is similar to the results of a prospective study of lupus patients [79]. In the prospective study, elevated homocysteine levels correlated with risk of stroke and arterial thrombotic events in patients with SLE after adjusting for established risk factors. Elevated homocysteine concentrations have also been associated with atherosclerosis and thrombotic events in the elderly [186-189]. In the Framingham Heart Study, homocysteine concentrations above 14.1 µmol/L were found in 29.3% of the study population with 67% of the high homocysteine cases attributable to low plasma concentrtions of folate, vitamin B12, and PLP (vitamin B6) [190]. Since vascular thrombotic events are a major contributor to morbidity and mortality in SLE and homocysteine is a modifiable risk factor that plays a role in methylation, our findings reiterate the potential importance that vitamin intervention may have in SLE.

Folate was not decreased in our study, contrary to what we might have expected. Folate is an important methyl donor In the methylation cycle and is well known to affect methylation. Considering lupus patients have hypomethylated T cell DNA, we hypothesized that they may have lower than average folate levels. Furthermore, homocysteine is a sensitive indicator of folate status [73], and since homocysteine was elevated in our cohort of patients, we expected folate levels to be decreased. Importantly, in a study by Petri et al., decreases in folate levels did correlate with the elevated homocysteine levels

seen in lupus patients [79]. The difference may be due to the patient population studied. In the study by Petri et al., a large number of patients was studied (337), allowing capture of several patients with high homocysteine levels: 15% (51) of the patients had homocysteine concentrations above 14.1 µmol/L. Furthermore, in our study, the average SLEDAI score, an index used to assess disease activity, was 3, which is considered inactive disease. Studying patients with more active disease and higher SLEDAI scores may yield higher homocysteine levels and corresponding decreases in folate levels. Additionally, we questioned our patients regarding vitamin/folate supplementation and alcohol consumption in the 24 hours preceeding the test, since these affect test results however due to the widespread fortification of foods with folic acid in the US, it is difficult to control for dietary intake. We used plasma folate as a measure of folate levels in our study based on guidelines from the University of Michigan Chemical Pathology Laboratories, however it has been suggested that red blood cell folate may be a more accurate measure of folate since plasma folate is subject to greater fluctuation [74].

We didn't observe any difference in the vitamin B12 levels in lupus patients compared to the normal population. Vitamin B12 is involved in the metabolism of homocysteine and levels of vitamin B12 correlated with increased homocysteine levels in the Framingham Heart Study [190]. However, the Framingham population is elderly, and vitamin B12 deficiency can occur in the elderly because of malabsorption due to atrophic gastritis, whereas our study

population was an average age of 33 years. This, along with a low average SLEDAI score may explain the lack of change seen in our study.

There was no difference in choline levels, though this may be due to our small sample number of 10 patients and 10 controls. Mohan et al., did observe a reduction in choline levels in lupus patients which was accompanied by decreased phosphocholine levels [185].

Our study and the study by Mohan et al., did not demonstrate any change in the riboflavin levels of lupus patients compared with controls.

In conclusion, we found decreases in zinc, methionine, and vitamin B6, and an increase in homocysteine levels in lupus patients compared to the normal population. The decreases in methionine and vitamin B6 and the increase in homocysteine that we observed corroborate findings from other studies, in a different cohort of patients [79, 185]. This is however the first study to note a change in zinc levels in lupus patients compared to the normal population. The major limitations of our study were the low SLEDAI scores of the patients and possibly the sample size. The micronutrients studied here are key players in the methylation cycle and are potentially modifiable through dietary intervention, therefore deserve further study into potential benefits for autoimmunity.

No.	37
Female, no.	35
Age, mean, years (range)	40 (21-79)
Race	
Caucasian	30
African-American	6
Hispanic	1
SLEDAI, mean (range)	3 (0-12)

Table 2.1: Patient characteristics

Methionine	HPLC
Homocysteine	Chemiluminescent Assay
Folate	Chemiluminescent Immunoassay
Choline	Enzymatic assay based on oxidation by choline oxidase
Vitamin B12	Chemiluminescent Immunoassay
Zinc	Atomic Absorption
Vitamin B6	Liquid Chromatography-Tandem Mass Spectrometry
Riboflavin	Liquid Chromatography-Tandem Mass Spectrometry

Table 2.2: Test methodology

Systemic Lupus Erythematosus Disease Activity Index SELENA Modification

Physicians Global Assessment _____

0123
NoneMildMedSevere

SLEDAI Score

Check box: If descriptor is present at the time of visit or in the proceeding 10 days.

Wt	Present	Descriptor	Definition
8	☐	Seizure	Recent onset. Exclude metabolic, infectious or drug cause.
8	☐	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganized, or catatonic behavior. Excluded uremia and drug causes.
8	☐	Organic Brain Syndrome	Altered mental function with impaired orientation, memory or other intelligent function, with rapid onset fluctuating clinical features. Include clouding of consciousness with reduced capacity to focus, and inability to sustain attention to environment, plus at least two of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious or drug causes.
8	☐	Visual Disturbance	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serious exodate or hemorrhages in the choroids, or optic neuritis. Exclude hypertension, infection, or drug causes.
8	☐	Cranial Nerve Disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8	☐	Lupus Headache	Severe persistent headache: may be migrainous, but must be nonresponsive to narcotic analgesia.
8	☐	CVA	New onset of cerebrovascular accident(s). Exclude arteriosclerosis.
8	☐	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual, infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis.
4	☐	Arthritis	More than 2 joints with pain and signs of inflammation (i.e. tenderness, swelling, or effusion).
4	☐	Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/adolase or electromyogram changes or a biopsy showing myositis.
4	☐	Urinary Casts	Heme-granular or red blood cell casts.
4	☐	Hematuria	>5 red blood cells/high power field. Exclude stone, infection or other cause.

Table 2.3: SLEDAI criteria

https://www.rheumatology.org/Practice/Clinical/Indexes/Systemic_Lupus_Erythematosus_Disease_Activity_Index_SELENA_Modification/

4	☐	Proteinuria	>0.5 gm/24 hours. New onset or recent increase of more than 0.5 gm/24 hours.
4	☐	Pyuria	>5 white blood cells/high power field. Exclude infection.
2	☐	New Rash	New onset or recurrence of inflammatory type rash.
2	☐	Alopecia	New onset or recurrence of abnormal, patchy or diffuse loss of hair.
2	☐	Mucosal Ulcers	New onset or recurrence of oral or nasal ulcerations.
2	☐	Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2	☐	Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram confirmation.
2	☐	Low Complement	Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory.
2	☐	Increased DNA binding	>25% binding by Farr assay or above normal range for testing laboratory.
1	☐	Fever	>38Â°C. Exclude infectious cause.
1	☐	Thrombocytopenia	<100,000 platelets/mm3.
1	☐	Leukopenia	<3,000 White blood cell/mm3. Exclude drug causes.

_____ TOTAL SCORE (Sum of weights next to descriptors marked present)

Table 2.3: SLEDAI criteria continued
https://www.rheumatology.org/Practice/Clinical/Indexes/Systemic_Lupus_Erythematosus_Disease_Activity_Index_SELENA_Modification/

Nutrient	Reference Mean/range	Lupus* Mean/range	*p*-value
Zinc (µg/ml)	0.86 (0.55-1.50)	0.71 (0.5-0.9)	0.0001
B6 (µg/L)	22.5 (5-50)	16.48 (2-57)	0.021
Homocysteine (µM)	9.5 (5-15)	11.37 (6-25)	0.013
Methionine (µM)	26 (10-42)	23.09 (13-47)	0.012
Folate (ng/ml)	7.1 (1.5-22)	18.27 (6.4->24)	N.S.
B12 (pg/ml)	500 (211-911)	569.80 (220-1770)	N.S.
B2 (µg/L)	6.0 (3-15)	9.0 (1-78)	N.S.
Choline (ppm)	225.4 (194-261)	225 (201-260)	N.S.

Table 2.4: Serum/plasma nutrient concentrations in lupus petients vs. population. *N=28-35 patients; choline=10 patients

CHAPTER III: MICRONUTRIENT LEVELS MODULATE METHYLATION-SENSITIVE GENE EXPRESSION IN T CELLS

<u>Introduction</u>

The methylation cycle is dependent on several dietary micronutrients and metabolites. Micronutrient levels affecting methylation have been studied in various aspects of health and disease. Recently, the importance of maternal nutrition affecting epigenetic patterns established *in utero* and the consequent lifelong impacts has garnered significant interest in the field of developmental origins of health and disease (DOHaD).   Epigenetic changes due to micronutrients have been studied in the field of cancer as well [191-195]. Autoimmunity is also affected by DNA methylation, however epigenetic changes in immune cells due to micronutrient alterations is poorly understood.

It has been well established that CD4+ T cell hypomethylation occurs in SLE, a prototypic autoimmune disease [184]. This has been shown in terms of global methylation [42] as well as gene-specific hypomethylation [45, 58-60]. The hypomethylation causes overexpression of immune-relevant methylation-sensitive genes with functional consequences. CD70 is a B cell costimulatory molecule whose overexpression results in increased antibody production [51]. KIR and perforin are not normally expressed in T cells, but are aberrantly expressed due to hypomethylation of their gene promoter regions, resulting in effects including macrophage killing [58, 62]. The functional consequences of

these overexpressed genes likely contributes to lupus pathogenesis. We

therefore sought to determine whether changes in micronutrient levels could

affect methylation-sensitive gene expression in T cells.

When studying methylation *in vitro*, effects may be masked by the large

quantities of pro-methylation micronutrients present in the tissue culture medium.

We developed a custom media containing transmethylation micronutrient levels

that were similar to physiologic levels. We then compared T cell methylation-

sensitive gene expression in custom media and the standard media. The results

suggest that micronutrient levels do in fact modify T cell gene expression and

that conventional tissue culture medium may conceal some of the effects.

Materials and Methods

Reagents. RPMI 1640 was purchased from Invitrogen Life Technologies. RPMI

1640 without vitamin B6, methionine, folic acid, vitamin B12, riboflavin, and

choline was custom ordered from Invitrogen Life Technologies. Zinc, vitamin B6,

methionine, folate, vitamin B12 and riboflavin were also purchased from

Invitrogen Life Technologies. Homocysteine was purchased from Sigma. Ficoll

Paque Plus was purchased from GE Healthcare, phytohemagglutinin was

purchased from Remel, and recombinant human IL-2 was purchased from

Peprotech. Antibodies were purchased from BD Pharmingen, Beckman Coulter,

and R & D Systems. Cytofix/Cytoperm Kit was purchased from BD Biosciences.

Standardization particles were purchased from Bangs Laboratories. Magnetic

beads were purchased from Miltenyi Biotech, SV Total RNA Isolation System

was purchased from Promega Corporation, and QuantiTect SYBR Green RT-PCR kit was purchased from Qiagen.

Human subjects.   The University of Michigan Institutional Review Board for Human Subject Research reviewed and approved this study. Healthy donors were recruited by advertising. Both male and female donors were utilized, ranging in age from 18-45.

Custom media. Custom media was created using RPMI 1640 without vitamin B6, methionine, folate, vitamin B12, riboflavin, and choline as the base. Vitamin B6, methionine, folate, vitamin B12, riboflavin, and choline were added back at concentrations adjusted to physiologic levels, listed in Table 3.1. Homocysteine and zinc were also added at physiologic concentrations (Table 3.1). Fetal bovine serum was dialyzed against Hank's Balanced Salt Solution (HBSS) using SpectraPor dialysis tubing with a molecular weight cutoff of 2,000 to exclude additional micronutrients.

Cell culture. Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus density gradient centrifugation. Cells were cultured at 1×10^6/mL in RPMI 1640 with 10% fetal bovine serum (FBS) or custom media with 10% dialyzed FBS. Phytohemagglutin (PHA) was added at a concentration of 1μg/mL for 18-24hrs to stimulate T cells. Cells were washed and cultured for an additional 3 days in their respective culture media without PHA. Cells were then harvested for flow cytometric or real-time RT-PCR analysis.

Flow cytometry. Extracellular cell surface staining was performed using human antibodies for CD11a, CD70 and KIR. Since the KIR molecules belong to a family of polymorphic genes, a cocktail of KIR antibodies reactive to various KIR receptors was used: CD158b1/b2,j; CD158i; NKB1; CD158b; CD158a,h; KIR2DL4/CD158d. Intracellular staining was performed using a human perforin antibody and the Cytofix/Cytoperm Kit. Cells were stained and fixed, then analyzed on a FACSCalibur flow cytometer at least 24 hours later to allow for cell equilibration after contraction due to fixing. Isotype and single-color positive controls were included in each condition. Standardization particles for fluorescein isothiocyanate (FITC), phycoerythrin (PE) and cychrome (CYC) were run during each experiment for instrument calibration. Ten thousand events in the gated lymphocyte population were captured. Percent-positive cells were assessed for CD70, KIR and perforin. Mean fluorescence intensity was assessed for CD11a and CD70. Flow cyometric results were analyzed using FCS Express Software, version 3 (De Novo™ Software, Los Angeles, CA).

Real-time RT-PCR. Total RNA was isolated using the Promega SV Total RNA Isolation System. CD4+ and CD8+ T cells were isolated using magnetic bead purification. CD70, perforin, and β-actin transcripts were then measured using a Rotor-Gene 3000 (Corbett Robotics, San Francisco, CA) and the QuantiTect SYBR Green RT-PCR kit according to the manufacturers' instructions. CD70, perforin, and β-actin primer sequences were as previously published [51, 58].

Statistical analysis. A linear mixed model (LMM) was used to estimate the linear relationship between micronutrient concentration and gene expression. A

random effect was included for each subject, to take into account that there were multiple concentrations measured for each subject. Statistical analyses were conducted using IBM SPSS Statistics, version 21 for Windows, copyright IBM corporation 1989, 2012.

Results

RPMI 1640 is associated with decreased protein expression of methylation-sensitive T cell genes. RPMI 1640 contains high levels of pro-methylation micronutrients, therefore we developed a custom media with physiologic levels of transmethylation micronutrients (Table 3.1) to study the expression of methylation-sensitive T cell genes. PBMCs were isolated from 9 different healthy donors and cultured as described in "Materials and Methods" section. Cells were then harvested and stained with antibodies for CD11a, CD70, KIR, and perforin along with either CD4 or CD8, and analyzed by flow cytometry. Multiple repeats were performed on several donors; in those cases, an average value for expression was calculated for each donor. In CD4+ T cells, KIR and perforin had significantly higher expression (p=0.016; p=0.001, respectively) in custom media relative to RPMI 1640. In CD8+ T cells, CD70, KIR, and perforin had significantly higher expression (p=0.004; p=0.001; p=0.004, respectively) in custom media relative to RPMI 1640 (Figure 3.1). No change was observed in CD11a expression (data not shown).

RPMI 1640 is associated with decreased CD70 and perforin mRNA expression. We examined mRNA expression of two methylation-sensitive genes in CD4+ T cells cultured in RPMI 1640 compared to custom media. PBMCs were isolated from 5 different healthy donors and cultured as described in "Materials and Methods" section. Cells were then harvested and separated into CD4+ and CD8+ T cell subsets by positive selection using magnetic bead purification. Total RNA was extracted and transcripts were measured using Real-time RT-PCR. CD70 and perforin mRNA expression in CD4+ T cells was significantly increased (p=0.02; p-0.04, respectively) in custom media compared to RPMI 1640. Perforin mRNA expression was significantly increased (p=0.03) in CD8+ T cells compared to RPMI 1640 (Figure 3.2).

Increasing folate suppresses perforin expression. To investigate individual micronutrient effects on methylation-sensitive gene expression, custom media was used, varying the concentration of one micronutrient at a time. PBMCs were isolated from 3 different healthy donors and cultured in RPMI 1640 or custom media with varying concentrations of folic acid, as described in "Materials and Methods" section. Cells were then harvested and intracellularly stained with the perforin antibody, and analyzed by flow cytometry for protein expression. Folate levels that were increased above the normal physiologic range caused a significant suppression of perforin expression in CD4+ (p=0.006) and CD8+ (p=0.006) T cells (Figure 3.3).

Increasing vitamin B6 decreases perforin expression. The effect of vitamin B6 on T cell methylation-sensitive gene expression was also studied. PBMCs were isolated from 4 different healthy donors and cultured in RPMI 1640 or custom media with varying concentrations of vitamin B6, as described in "Materials and Methods" secton. Cells were then harvested and intracellularly stained with the perforin antibody, and analyzed by flow cytometry for protein expression. CD4+ T cells had a significant decrease (p=0.032) in perforin expression when exposed to higher concentrations of vitamin B6. There was no significant effect in CD8+ T cells (Figure 3.4).

Increasing homocysteine causes CD70 overexpression. Since homocysteine levels can interrupt the methylation cycle by favoring synthesis of SAH, an inhibitor of Dnmt1 [68], we examined the effects of increasing homocysteine on methylation-sensitive gene expression in T cells. PBMCs were isolated from 4 different healthy donors and cultured in RPMI 1640 or custom media with varying concentrations of homocysteine, as described in "Materials and Methods" section. Cells were then harvested and stained with anti-CD70 and analyzed by flow cytometry for protein expression. CD4+ and CD8+ T cells had a significant increase (p=0.005; p=0.045, respectively) in CD70 expression when exposed to increasing homocysteine concentrations (Figure 3.5).

Discussion

These studies demonstrate that altered levels of micronutrients can modulate immune- relevant methylation-sensitive gene expression in T cells. Conventional tissue culture media such as RPMI 1640 contain high levels of transmethylation micronutrients that may favor methylation, thus masking methylation effects (Table 3.1). For example, the level of methionine found in RPMI 1640 is approximately 3 times the level present in human serum, while folate in RPMI 1640 is approximately 56 times the level found in human serum. We developed a custom media where key micronutrients involved in the methylation cycle are adjusted to physiologic levels. Zinc and homocysteine are two important metabolites involved in the methylation cycle but not present in RPMI 1640. We therefore added zinc and homocysteine to our custom media at physiologic levels to study their effects as well.

Our results show significant increases in CD70, KIR, and perforin protein expression in the custom media (Figure 3.1), supporting the hypothesis that micronutrient levels alter methylation-sensitive gene expression in T cells. Furthermore, the results suggest that RPMI 1640 may conceal methylation-related effects in T cells studied *in vitro*. We also examined mRNA expression of CD70 and perforin in custom media versus RPMI 1640. CD70 and perforin mRNA expression was significantly increased in custom media compared to RPMI 1640 (Figure 3.2), corroborating our protein data.

Based on the results using custom media, we investigated which particular micronutrients in the media may be affecting expression of methylation-sensitive genes in T cells. To do this, we used the custom media with all transmethylation micronutrients of interest at physiologic levels, with one being varied at a time, spanning concentrations below and above the physiologic range. We varied each of the 8 micronutrients of interest and examined the protein expression of the 4 immune-relevant methylation-sensitive genes. We found significant effects with folate, vitamin B6, and homocysteine. Perforin expression was decreased in CD4+ and CD8+ T cells when folate concentration was increased. Perforin expression was also decreased in CD4+ T cells when vitamin B6 levels were increased. Increasing homocysteine concentration in the custom media caused overexpression of CD70. The other micronutrient and gene combinations had no significant effects in this system (data not shown).

These data suggest an important role of micronutrients in the expression of methylation-sensitive genes in T cells. As noted earlier, the genes studied are overexpressed in SLE, therefore micronutrient modulation of their expression is of particular interest since dietary modification may have potential benefits. In the first portion of this study, custom media altered the expression of CD70, KIR and perforin compared to RPMI 1640, demonstrating modulation by micronutrients. Additionally, the study suggests limitations of conventional tissue culture media when studying methylation-related changes. Indeed, methylation changes due to tissue culture media composition have been implicated in assisted reproductive technology (ART). Beckwith-Wiedemann syndrome, a

disorder characterized by overgrowth and neoplasia due to aberrant methylation and imprinting of a group of genes, has been linked to ART. The changes may be due to culture conditions, including media, and potentially other aspects of the ART [196].

In the second portion of this study where individual micronutrients were varied one at a time, three micronutrients emerged as having clear effects on protein expression: folate, vitamin B6, and homocysteine. Folate and vitamin B6 are well known to be involved in the metabolism of homocysteine. Interestingly, in Chapter 2 of this dissertation, we studied serum levels of micronutrients in lupus patients and found vitamin B6 to be decreased and homocysteine to be increased compared to the normal population. We did not observe a change in folate levels likely due to the limitations of our study, however Petri et al., did find high homocysteine levels to be associated with folate and vitamin B6 levels in a cohort of lupus patients [79]. We also observed decreased zinc and methionine in the serum of lupus patients (Chapter 2), but did not observe any significant effects on T cell methylation-sensitive gene expression in the present study. This may be due to the limitations of a cell culture system where cell-cell or tissue-tissue interactions are missing. Methionine is an essential amino acid and an essential component of tissue culture media; it is possible that decreasing methionine in the presence of other decreased transmethylation micronutrients may cause suboptimal culture conditions. Zinc is not normally added to tissue culture media and can be detrimental in too high concentrations in tissue culture. This may also result in suboptimal tissue culture conditions. Additionally, zinc is

maintained under very close homeostatic control in the body which is not replicated in an *in vitro* system. Nevertheless, these studies suggest an important role for folate, vitamin B6, and homocysteine in methylation-sensitive gene expression in T cells with potential implications for lupus pathogenesis. Several mechanisms may be involved in the altered micronutrient effects on methylation-sensitive gene expression. For example, the altered micronutrients may be altering the SAM/SAH ratio, thus affecting methylation. Measurements of SAM and SAH levels will help to determine this. Further, it is possible that changes in transport and metabolism of the micronutrients and not the quantities themselves are resulting in the methylation-sensitive gene expression changes seen. Studies in which additional information from donors such as the presence of SNPs involved in micronutrient metabolism and hypermethylation of genes involved in folate biosynthesis pathways can be correlated with altered micronutrient-induced methylation-sensitive gene expression *in vitro*, will aid in establishing the mechanisms involved.

Component	RPMI 1640	Normal Serum Levels	Custom Media
Methionine (µM)	101	10-42	33
Choline (µM)	21.4	7-20	10
Folic acid (ng/ml)	1000	3-17.5	7
B6 (µg/L)	1000	5-50	22
B2 (µg/L)	200	3-15	6
B12 (pg/ml)	5000	211-911	350
Zn (µg/ml)	0	0.55-1.5	0.5
Homocysteine (µM)	0	5-15	9

Table 3.1: Comparison of transmethylation micronutrient levels in RPMI 1640, human serum, and custom media.
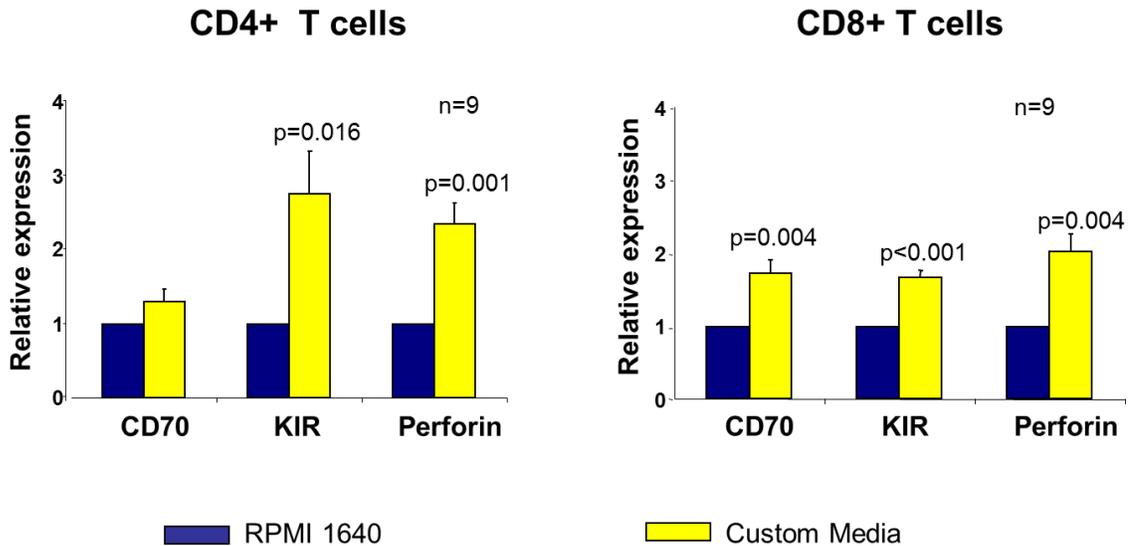
Figure 3.1 RPMI 1640 is associated with decreased protein expression of methylation-sensitive T cell genes. PBMCs were isolated from 9 different healthy donors and cultured in either RPMI 1640 or custom media, as described in "Materials and Methods" section. After culture, cells were stained with antibodies and analyzed by flow cytometry. Results are shown relative to expression in RPMI 1640. KIR and perforin expression in CD4+ T cells were significantly increased in custom media compared to RPMI 1640. CD70, KIR and perforin expression in CD8+ T cells were significantly increased in custom media compared to RPMI 1640.
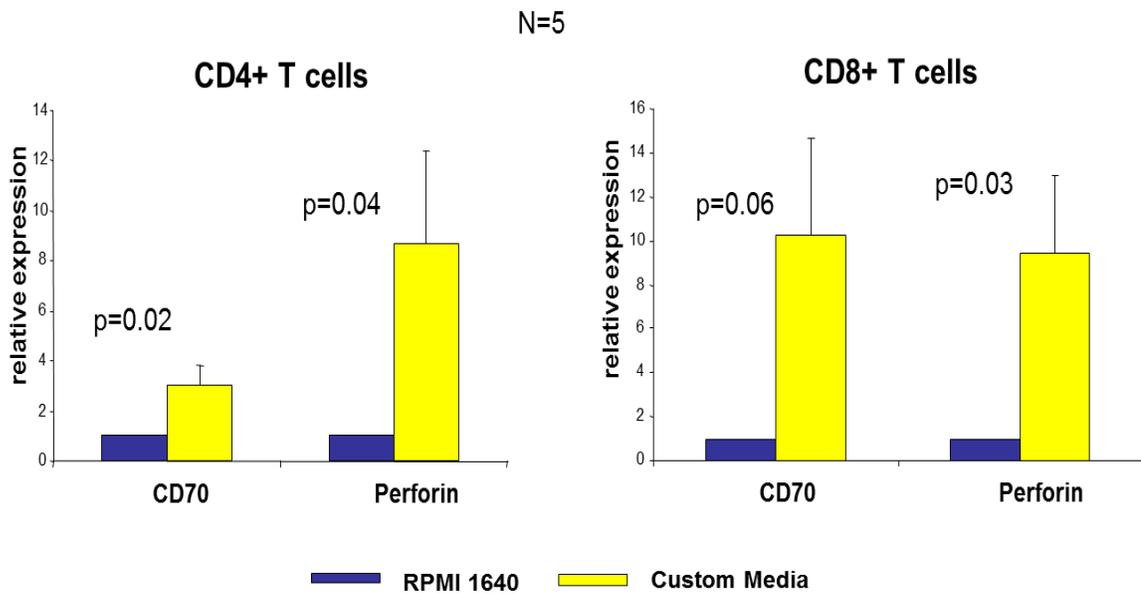
Figure 3.2 RPMI 1640 is associated with decreased CD70 and perforin mRNA expression. PBMCs were isolated from 5 healthy donors and cultured in either RPMI 1640 or custom media as described in "Materials and Methods" section. After culture, cells were separated into CD4+ and CD8+ subsets, total RNA was extracted and analyzed by Real-time RT-PCR. Results are shown relative to expression in RPMI 1640. CD70 and perforin mRNA expression was significantly increased in both CD4+ and CD8+ T cells cultured in custom media compared to RPMI 1640.

CD4+ T cells

CD8+ T cells
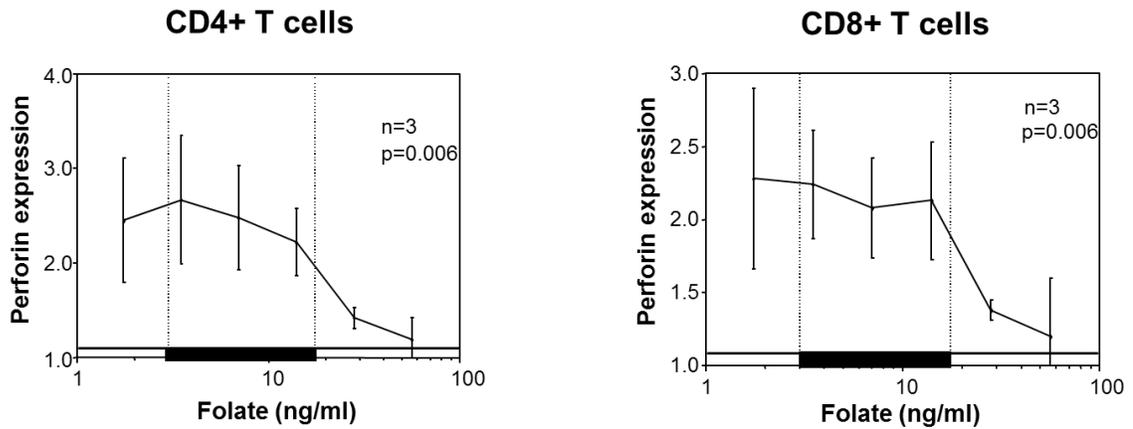
Figure 3.3 Increasing folate suppresses perforin protein expression. PBMCs were isolated from 3 healthy donors and cultured in RPMI 1640, or custom media with varying concentrations of folic acid, as described in "Materials and Methods" section. After culture, cells were stained with antibodies for CD4 or CD8, and perforin. Black horizontal shaded bar represents physiologic range of folate. Results are standardized to RPMI 1640 and are shown as mean ± SEM. Higher concentrations of folate significantly decreased perforin expression in both CD4+ and CD8+ T cells.

CD4+ T cells

Perforin expression

n=4
p=0.032

B6 (μg/L)

CD8+ T cells

Perforin expression

n=4
n.s.

B6 (μg/L)
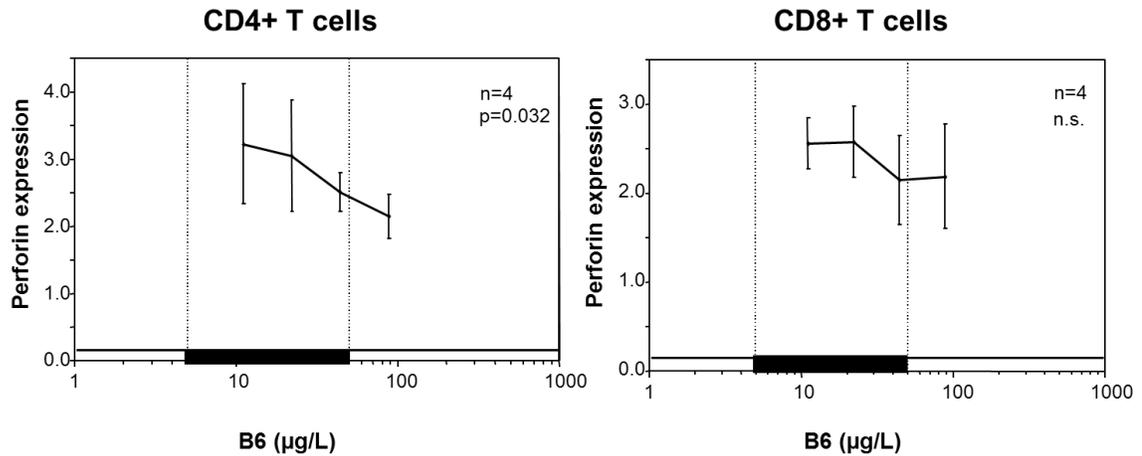
Figure 3.4 Increasing vitamin B6 decreases perforin protein expression. PBMCs were isolated from 4 healthy donors and cultured in RPMI 1640, or custom media with varying concentrations of vitamin B6, as described in "Materials and Methods" section. After culture, cells were stained with antibodies for CD4 or CD8, and perforin. Black horizontal shaded bar represents physiologic range of vitamin B6. Results are standardized to RPMI 1640 and are shown as mean ± SEM. Higher concentrations of vitamin B6 significantly decreased perforin expression in CD4+ T cells.
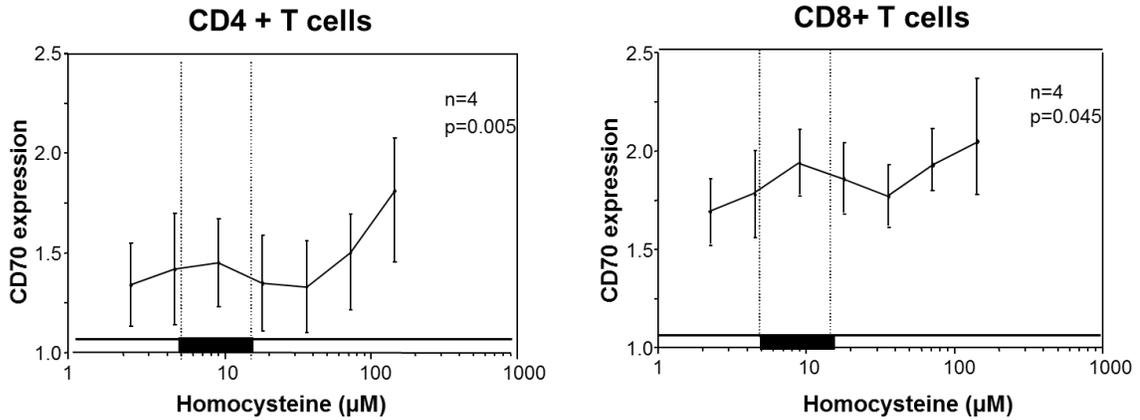
Figure 3.5 Increasing homocysteine leads to CD70 protein overexpression. PBMCs were isolated from 4 healthy donors and cultured in RPMI 1640, or custom media with varying concentrations of homocysteine, as described in "Materials and Methods" section. After culture, cells were stained with antibodies for CD4 or CD8, and CD70. Black horizontal shaded bar represents physiologic range of homocysteine. Results are standardized to RPMI 1640 and are shown as mean ± SEM. Higher concentrations of homocysteine significantly increased CD70 expression in CD4+ and CD8+ T cells.

CHAPTER IV: OXIDANTS AND ALTERED MICRONUTRIENT LEVELS SYNERGIZE TO ALTER METHYLATION-SENSITIVE GENE EXPRESSION IN T CELLS.

Introduction

Oxidative stress is defined as an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage [142]. Oxidative stress has been implicated in lupus pathogenesis. Several markers of oxidative stress are elevated in lupus patients including urinary F_2 isoprostane [150], protein carbonyls [149], and 3-nitrotyrosine [151]. Interestingly, protein carbonyl and 3NT levels correlated with disease activity, and protein carbonyl levels were increased in patients with organ damage compared to those without [152].

3-nitrotyrosine is formed when tyrosine residues are nitrated by peroxynitrite ($ONOO^-$). Nitration of proteins is one method by which oxidative stress may be contributing to lupus. The highly reactive $ONOO^-$ is formed when nitric oxide combines with superoxide anion. The increased NO levels present in lupus patients [151, 157] may be causing increased nitration of proteins [149, 151] through formation of $ONOO^-$ [197]. Recent evidence has suggested that cytotoxicity caused by NO may in fact be due to the effects of $ONOO^-$[197].

Nitration of proteins has recently been linked to epigenetic mechanisms contributing to lupus. Decreased DNA methylation resulting in overexpression of several immune-related genes has been associated with SLE [105]. DNA methylation is regulated in part by the Dnmt enzymes. In lupus, Dnmt1 levels are decreased due to defective ERK pathway signaling [170]. The defect has been traced to impaired phosphorylation of the PKCδ isoform [55] which is upstream of ERK. Subsequent studies revealed that nitration of PKCδ is responsible for the aberrant PKCδ phosphorylation [56] and likely the ERK pathway signaling abnormalities associated with decreased Dnmt1 levels.

DNA methylation is also affected by dietary nutrients involved in the transmethylation pathway. Two metabolites of particular interest are folate and methionine. The main methyl group donor for most biological methylation reactions is S-adenosylmethionine (SAM). SAM is formed from methionine and ATP by the enzyme methionine adenosyltransferase (MAT). Methionine, along with N-methyltetrahydrofolate and choline are the primary dietary sources of methyl groups (~10, 5, and 30 mmol methyl/d, respectively) [198]. Since methionine is an essential amino acid and cannot be synthesized *de novo* in humans, dietary intake is likely indispensable for maintaining SAM levels [199]. S-adenosylhomocysteine (SAH) is the by-product of DNA methylation by SAM and an inhibitor of Dnmt [68]. SAH can then be hydrolyzed to adenosine and homocysteine. Folate is important in the remethylation of homocysteine to methionine [110]. Elevated homocysteine has been associated with disease

states such as atherosclerosis [200] and uremia [201], and is a strong indicator of folate status [73].

Based on the knowledge that lupus patients have increased oxidative stress and altered T cell DNA methylation patterns, and that folate and methionine play a role in the methylation cycle, we hypothesize that oxidative stress and decreases in folate or methionine may be synergistic in causing overexpression of methylation-sensitive genes. We therefore cultured T cells from normal healthy donors in media containing altered folate or methionine, treated them with variable concentrations of $ONOO^-$ and examined expression of methylation-sensitive genes. The results suggest a potential additive effect of oxidative stress and decreased methyl donors, which may have implications for autoimmunity.

Materials and Methods

Reagents. RPMI 1640, RPMI1640 without folate and RPMI 1640 without methionine,and folate and methionine were all purchased from Invitrogen Life Technologies; Ficoll-Paque Plus was purchased from GE Healthcare; phytohemagglutinin was purchased from Remel; recombinant human IL-2 was purchased from Peprotech; and peroxynitrite was purchased from Calbiochem. Antibodies were purchased from BD Pharmingen, Beckman Coulter and R & D Systems. Cytofix/Cytoperm Kit was purchased from BD Biosciences. Standardization particles were purchased from Bangs Laboratories.

Human subjects. The University of Michigan Institutional Review Board for Human Subject Research reviewed and approved this study. Healthy donors were recruited by advertising. Average age of donors for folate studies was 31, ranging from 22-37 (n=6; 2 males, 4 females). Average age of donors for methionine studies was 33, ranging from 29-37 (n=6; 1 male, 5 female).

Cell culture and treatment. Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus density gradient centrifugation. Cells were cultured at $1x10^6$/ml in RPMI 1640 with 10% fetal bovine serum (FBS), and 1µg/ml phytohemagglutinin, for 18-24hrs. Cells were then washed and incubated with RPMI 1640 and either RPMI 1640 without folate or RPMI 1640 without methionine, regular or dialyzed FBS, 10ng/ml recombinant human IL-2, and 0, 20 or 25µM peroxynitrite (Appendix A) for 3 days. Cells were then harvested and washed for flow cytometric analysis.

Flow cytometry. Extracellular cell surface staining was performed using human antibodies for CD11a, CD70 and KIR. Since the KIR molecules belong to a family of polymorphic genes, a cocktail of KIR antibodies reactive to various KIR receptors was used: CD158b1/b2,j; CD158i; NKB1; CD158b; CD158a,h; KIR2DL4/CD158d. Intracellular staining was performed using a human perforin antibody and the Cytofix/Cytoperm Kit. Cells were stained and fixed, then analyzed on a FACSCalibur flow cytometer at least 24 hours later to allow for cell

equilibration after contraction due to fixing. Isotype and single-color positive controls were included in each condition. Standardization particles for fluorescein isothiocyanate (FITC), phycoerythrin (PE) and cychrome (CYC) were run during each experiment for instrument calibration. Ten thousand events in the gated lymphocyte population were captured. Percent-positive cells were assessed for CD70, KIR and perforin. Mean fluorescence intensity was assessed for CD11a and CD70. Flow cyometric results were analyzed using FCS Express Software, version 3 (De Novo™ Software, Los Angeles, CA).

Statistical analysis. A repeated measures ANOVA was used to analyze the relationship between the three folate concentrations (2270nM, 40nM, 10nM) or three methionine concentrations (101µM, 30µM, 5µM), and the three peroxynitrite concentrations (0µM, 20µM, 25µM) on gene expression among normal subjects. Because cells from each subject received all combinations of folate or methionine and peroxynitrite, we included folate or methionine concentration and peroxynitrite concentration plus their interaction as the within-subjects effects. Post-hoc tests, with a Bonferroni correction for multiple comparisons, were used to examine the specific effects of peroxynitrite at each given concentration of folate or methionine and the specific effects of folate or methionine at each level of peroxynitrite. Statistical analyses were conducted using IBM SPSS Statistics, version 21 for Windows, copyright IBM corporation 1989, 2012.

Results

Peroxynitrite and folate treatment. CD4+ T cells cultured in 40nM and 10nM folate concentrations increased expression of CD70, as compared to the standard media, RPMI 1640. Simultaneous treatment with 20µM and 25µM peroxynitrite further increased the expression of CD70 (Figure 4.1A; $p<0.001$ for overall folate effect; $p<0.001$ for overall peroxynitrite effect). CD4+ T cells cultured in 40nM and 10nM folate concentrations also increased KIR expression, as compared to RPMI 1640. Simultaneous treatment with 20µM and 25µM peroxynitrite further increased expression of KIR (Figure 4.2B; $p<0.001$ for overall folate effect; $p<0.05$ for overall peroxynitrite effect). No significant effects were observed in CD11a or perforin expression (data not shown).

Peroxynitrite and methionine treatment. CD4+ T cells cultured in 30µM and 5µM methionine concentrations increased expression of CD70, as compared to the standard media, RPMI 1640. Simultaneous treatment with 20µM and 25µM peroxynitrite further increased the expression of CD70 (Figure 4.2A; $p<0.001$ for overall methionine effect; $p<0.001$ for overall peroxynitrite effect). CD4+ T cells cultured in 30µM and 5µM methionine concentrations also increased KIR expression, as compared to RPMI 1640. Simultaneous treatment with 20µM and 25µM peroxynitrite further increased expression of KIR (Figure 4.2B; $p<0.001$ for overall methionine effect; $p<0.01$ for overall peroxynitrite effect). No significant effects were observed in CD11a or perforin expression (data not shown).

Discussion

The goal of this study was to examine how the combined effects of oxidative stress and altered nutrient status may affect expression of immune-relevant methylation-sensitive genes. The findings demonstrate an overexpression of methylation-sensitive genes when T cells are cultured in media with lower folate or methionine concentrations, and that $ONOO^-$ potentiates the effect. Considering folate and methionine are key components of the transmethylation cycle, and $ONOO^-$ affects Dnmt through PKCδ, DNA methylation is a likely mechanism for the overexpression observed.

Decreasing folate concentrations on untreated CD4+ T cells had a small effect on CD70 and KIR expression that increased significantly with $ONOO^-$ treatment. The effect was more pronounced with KIR. Similarly, decreasing methionine concentrations on untreated CD4+ T cells also had a small effect on CD70, a larger effect on KIR, and this was increased with $ONOO^-$ treatment. The overexpression of CD70 and KIR was further increased when the cells were exposed to both lower nutrient and prooxidant conditions compared to just one of the conditions, suggesting that multiple 'insults' may be additive. Furthermore, the results support our hypothesis that T cells exposed to suboptimal nutrient conditions may be more sensitive to oxidative stress and vice versa.

We chose to examine gene expression of CD70 and KIR since it has previously been demonstrated that these genes are demethylated and

overexpressed in SLE. CD70 is a B cell costimulatory molecule that is involved in antibody production. The CD70 promoter (TNFSF7) is demethylated and CD70 is overexpressed in patients with active lupus . CD4+ T cells treated with DNA methylation inhibitors and CD4+ lupus T cells also overstimulate B cell IgG production which is abrogated by anti-CD70 [51]. Importantly, CD70 expression was also increased in CD4+ T cells treated with Rottlerin, a selective PKCδ inhibitor [55]. KIR is normally expressed on NK cells but suppressed by DNA methylation on T cells [61]. Experimentally demethylated CD4+ T cells and T cells from lupus patients aberrantly express KIR, and in lupus patients, the expression correlates with disease activity. Aberrant KIR expression may contribute to lupus by promoting macrophage killing and release of interferon-γ [62]. We also examined expression of CD11a and perforin but found no significant effect (data not shown).

Folate and methionine are highly relevant to the transmethylation cycle and therefore were the micronutrients chosen for this study. Folate is known to modify DNA methylation patterns [110]. 5-methyltetrahydrofolate (5-methylTHF), an intermediate in folate metabolism, provides the methyl donor to remethylate homocysteine to methionine [80, 81]. Hyperhomocysteinaemia in patients with chronic renal failure contributes to DNA demethylation, which can be reversed with folate supplementation [201]. Importantly, homocysteine levels are also elevated in lupus patients [79]. Since elevated homocysteine is a marker of possible folate deficiency [73], folate affects methylation, and T cells are hypomethylated in lupus, further investigation into the possible benefits of folate

supplementation in SLE may be indicated. Methionine is an essential amino acid that maintains SAM levels [199]. Interestingly, reduced glutathione (GSH), a potent antioxidant, is depleted in SLE lymphocytes [185, 202], contributing to oxidative stress and DNA hypomethylation. It has been hypothesized that methionine may be diverted from SAM synthesis in order to regenerate GSH [71], further highlighting the potential importance of dietary methionine in SLE.

Together, these studies suggest that an interruption in the methylation cycle from altered nutrient levels combined with decreased Dnmt1 enzymatic activity due to oxidative stress results in overexpression of CD70 and KIR. The results demonstrate that blockade of the methylation cycle via multiple mechanisms is additive. Future studies such as those examining the levels of SAM and SAH will help determine the route by which altered micronutrients interrupt the methylation cycle. Additional methylation studies of the CD70 and KIR promoter regions will help confirm the mechanism of action. Further examination of other nutrient interactions with prooxidants will shed light on the complex interactions potentially contributing to autoimmunity.

A

B

Fol 2270nM   Fol 40nM   Fol10nM

■ ONOO⁻0   ■ ONOO⁻20   ■ ONOO⁻25
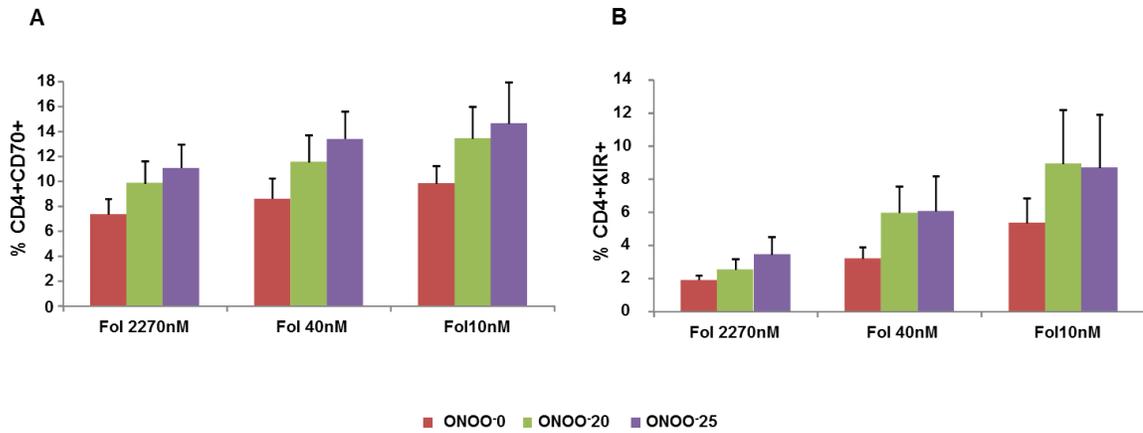
Figure 4.1 Peroxynitrite and lower folate concentrations increase CD70 and KIR expression. Culturing peroxynitrite-treated CD4+ T cells in media containing lower folate concentrations increases **(A)** CD70 ($p<0.001$ for overall folate effect; $p<0.001$ for overall peroxynitrite effect) and **(B)** KIR ($p<0.001$ for overall folate effect; $p<0.05$ for overall peroxynitrite effect) expression. n=6.
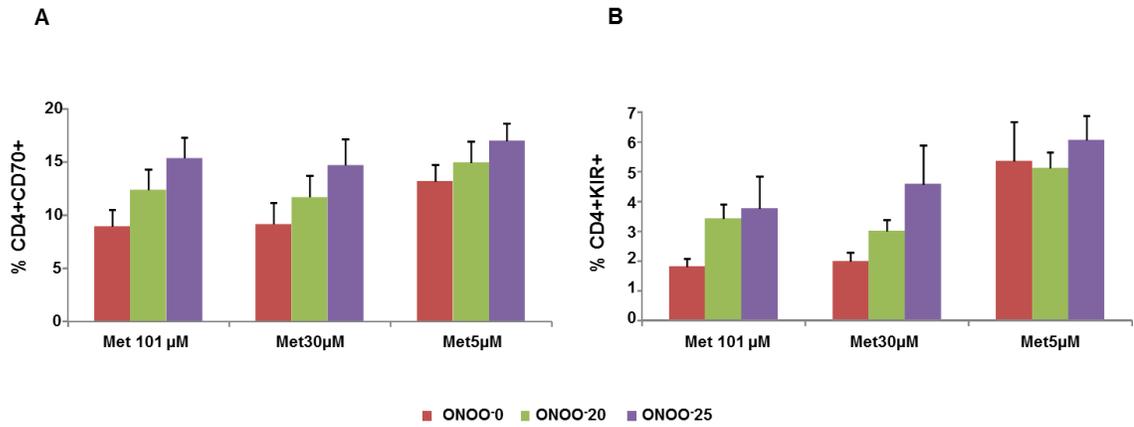
**Figure 4.2 Peroxynitrite and lower methionine concentrations increase CD70 and KIR expression.** Culturing peroxynitrite-treated CD4+ T cells in media containing lower methionine concentrations increases **(A)** CD70 (p<0.001 for overall folate effect; p<0.001 for overall peroxynitrite effect; n=6) and **(B)** KIR (p<0.001 for overall folate effect; p<0.01 for overall peroxynitrite effect; n=5) expression.

APPENDIX A: DETERMINATION OF PEROXYNITRITE CONCENTRATIONS AND OXIDATIVE DAMAGE ENDPOINT

Materials and Methods

To determine the optimal concentrations of peroxynitrite at which protein expression of methylation-sensitive genes is affected while likely causing oxidative damage, we performed several dose-response experiments to assess protein expression and used flow cytometry to assess cell integrity. Blood was obtained from 4 different healthy donors. PBMCs were isolated using Ficoll-Hypaque Plus and stimulated with PHA for 24hours. Cells were then washed and cultured in RPMI 1640 with 10% FBS at 37°C at 5% CO_2 in a humidified environment, and treated with various concentrations of peroxynitrite ($ONOO^-$) (ranging from 0-35µM) for 3 days. After culture, cells were stained with antibodies for CD70, KIR, and perforin and analyzed by flow cytometry.

Results

Cell Integrity. Forward and side scatter was observed for PBMCs, gating on the lymphocyte population (Figure A.1). In the untreated (0µM $ONOO^-$) sample, the majority of the cells can be seen in the gated lymphocyte (gate 1) population (Figure A.1.A). In the 15µM, 20µM, and 25µM peroxynitrite concentrations, progressively fewer cells are seen in the gated lymphocyte population and more

cells can be seen shifted into the region of dead or dying cells (Figures A.1.B-A.1.D). At concentrations of 30μM and 35μM ONOO⁻, few cells remain in the gated lymphocyte population and most are present in the dead or dying population (Figures A.1.E and A.1.F). Figure A.1 shows an overall progression of loss of cell integrity with increasing doses of ONOO⁻.

Protein expression. Cells were assessed for CD70, KIR, and perforin expression following peroxynitrite treatment to determine concentrations at which a change in expression occurs. PBMCs from 3 healthy donors were treated with 0μM, 2.5μM, 5μM, 10μM or 20μM ONOO⁻ and then analyzed by flow cytometry for CD70, KIR and perforin expression (Figure A.2). An increase in CD70, KIR and perforin expression was observed at concentrations of 10μM and 20μM ONOO⁻. It was unclear whether higher than 20μM concentrations would have a similar increase, therefore cells from a 4th donor were used to assess protein expression at ONOO⁻ concentrations of 0-30μM (Figure A.3). Increasing protein expression of CD70 and KIR was observed at ONOO⁻ concentrations of 15μM-25μM.

Summary

These results confirm that 20μM and 25μM peroxynitrite are appropriate concentrations for our studies. We chose these concentrations based on the forward and side scatter plots where there were still sufficient cells remaining in the lymphocyte population and at which there were observable effects on protein

expression. Higher than 25µM concentrations (i.e.30µM) did show an increase in

KIR expression on CD4+ T cells (Figure A.3), however this effect is likely only on

very few cells as the scatter plots show very few cells remain in that population

(Figure A.1E). We did not directly measure oxidative damage in our studies,

however it is likely that the concentrations used resulted in increased levels of

oxidative stress since they are the highest concentrations at which cells were still

viable and peroxynitrite is a known inducer of oxidative stress. Furthermore, the

concentrations used are well within the physiologic range, which has been

estimated to be around 50µM [203] and reported to be as high as 500µM in

phagolysosomes of activated macrophages [204].
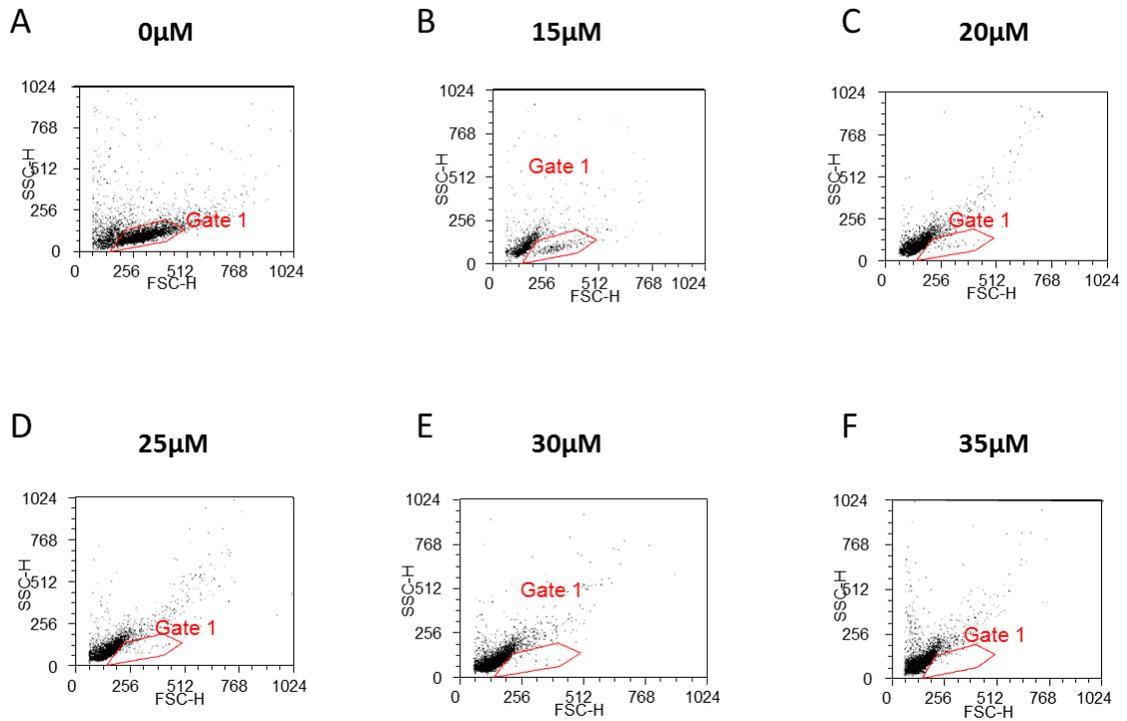
Figure A.1: Forward and Side Scatter of PBMCs treated with various concentrations of ONOO⁻. Cells were treated with 0µM (A), 15µM (B), 20µM (C), 25µM (D), 30µM (E), or 35µM (F) ONOO⁻ and forward and side scatter was observed by flow cytometry. Cells are gated on the lymphocyte population (Gate 1).
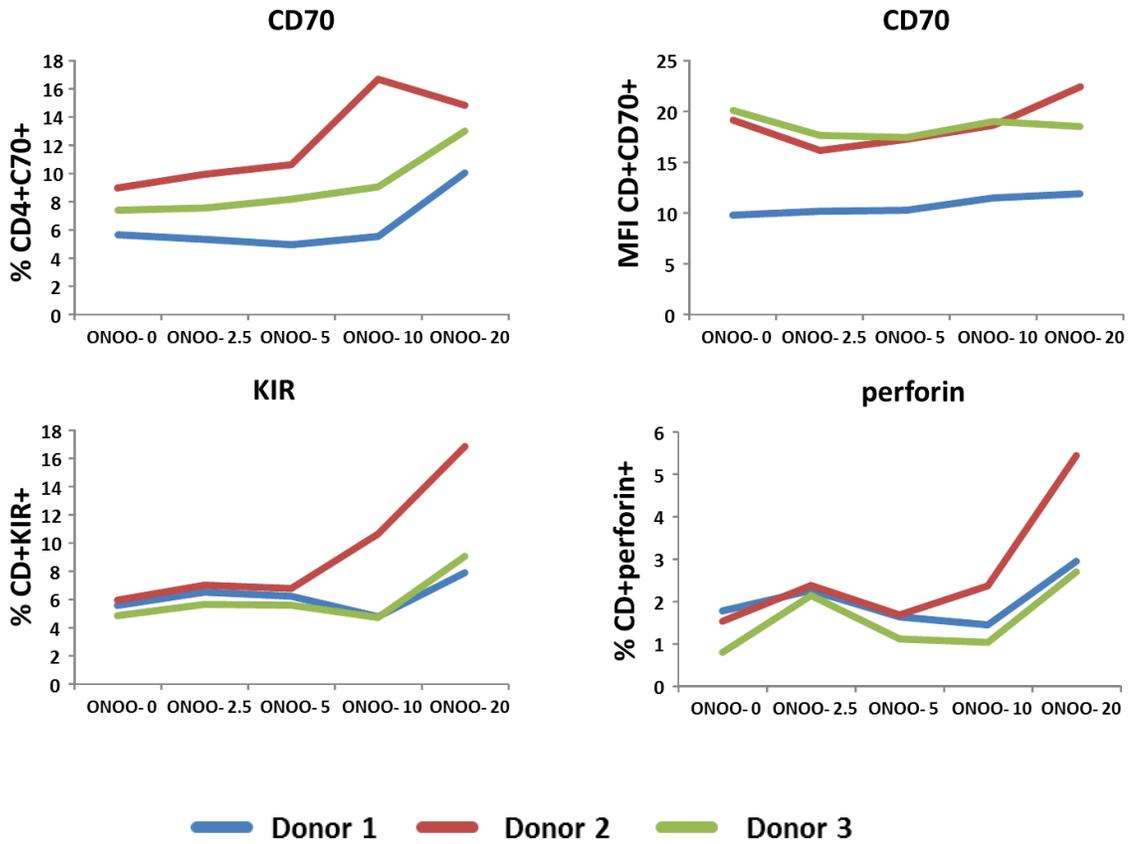
Figure A.2: CD70, KIR, and perforin expression on CD4+ Tcells treated with 0-20µM ONOO⁻ from 3 donors.
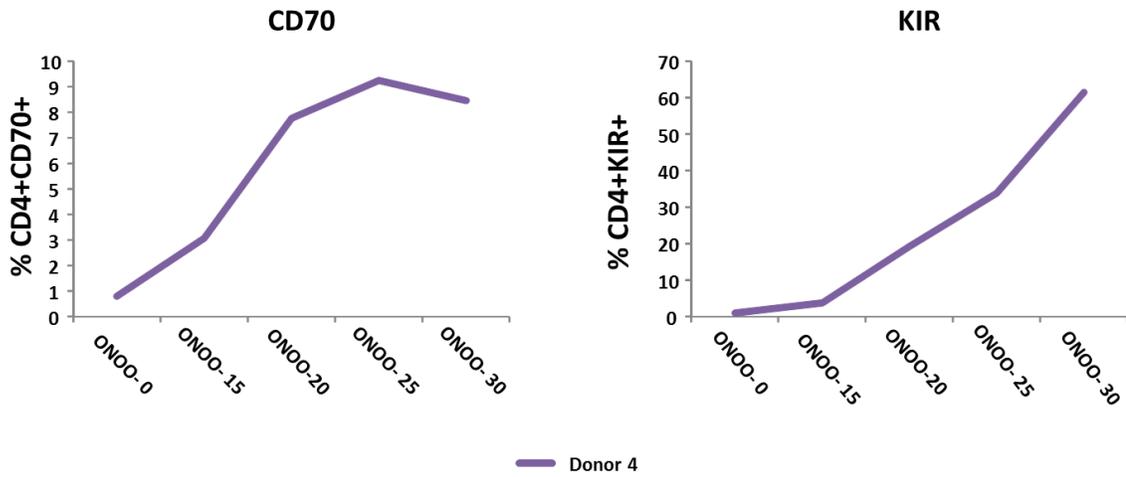
Figure A.3: CD70 and KIR expression on CD4+ T cells treated with 0-30μM ONOO⁻ from a 4th donor.

CHAPTER V: METHYLATION-SENSITIVE GENES ARE DIFFERENTIALLY EXPRESSED IN LUPUS VERSUS CONTROL CD4+ T CELLS EXPOSED TO ALTERED MICRONUTRIENTS

Introduction

Systemic lupus erythematosus is an autoimmune disease characterized by CD4+ T cell demethylation. DNA methylation is a postsynthetic modification where a methyl group is transferred to a cytosine nucleotide in the DNA. The methylation reaction is mediated by the Dnmt group of enzymes and is also dependent on intracellular stores of the methyl donor SAM. SAH, a by-product of methylation, inversely affects the methylation reaction by inhibiting Dnmt. Methylation of promoter regions of genes is typically associated with transcriptional suppression while demethylation results in gene expression.

In lupus, CD4+ T cells are demethylated at several promoter regions including CD11a, CD70, KIR, and perforin [184]. The demethylation causes overexpression of the genes, resulting in excess antibody production and macrophage killing. Lupus patients have decreased Dnmt1 levels [170] that may be due in part to oxidative stress. Dnmt1 is regulated by ERK pathway signaling. In lupus, defective PKCδ phosphorylation is at least in part responsible for defective ERK pathway signaling, resulting in failure to upregulate Dnmt1 [55]. The defect has recently been attributed to the nitration of PKC δ [56].

Micronutrients from the diet also affect DNA methylation. Methionine is necessary for the synthesis of SAM, the universal methyl donor [67]. Methionine combines with ATP to produce SAM, therefore a decrease in methionine is likely to interrupt the methylation cycle. Folate is an important component of the methylation cycle as well. Folate serves as the methyl donor for homocysteine in the form of 5-methylTHF, to re-synthesize methionine. A decrease in folate results in an accumulation of homocysteine, which participates in a reversible reaction strongly favoring SAH production. Since SAH is an inhibitor of DNA methylation, a reduction in folate results in decreased DNA methylation.

Based on the knowledge that Dnmt1 levels are decreased in lupus patients and that micronutrients may alter DNA methylation, we hypothesized that lupus CD4+ T cells may be more sensitive to lower concentrations of methionine than cells from unaffected individuals. We cultured CD4+ T cells from lupus patients and age- and gender-matched controls in media with varying amounts of methionine and examined protein expression of methylation-sensitive genes. The results suggest that lupus patients may be less resistant to micronutrient deficiencies due to their already decreased levels of Dnmt1.

Materials and Methods

Reagents. RPMI 1640, RPMI1640 without folate and RPMI 1640 without methionine, and folate and methionine were all purchased from Invitrogen Life Technologies; Ficoll-Paque Plus was purchased from GE Healthcare;

phytohemagglutinin was purchased from Remel; and recombinant human IL-2 was purchased from Peprotech. Antibodies were purchased from BD Pharmingen, Beckman Coulter and R & D Systems. Cytofix/Cytoperm Kit was purchased from BD Biosciences. Standardization particles were purchased from Bangs Laboratories.

Human subjects. The University of Michigan Institutional Review Board for Human Subject Research reviewed and approved this study. Patient samples were collected from the University of Michigan outpatient clinic with informed consent according to the Declaration of Helsinki. Age- and gender-matched controls were recruited by advertising.

Cell culture and treatment. Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus density gradient centrifugation. Cells were cultured at 1×10^6/ml in RPMI 1640 with 10% fetal bovine serum (FBS), and 1µg/ml phytohemagglutinin, for 18-24hrs. Cells were then washed and incubated with RPMI 1640 and either RPMI 1640 without folate or RPMI 1640 without methionine, conventional or dialyzed FBS, and 10ng/ml recombinant human IL-2 for 3 days. Cells were then harvested and washed for flow cytometric analysis.

Flow cytometry. Extracellular cell surface staining was performed using human antibodies for CD11a, CD70 and KIR. Since the KIR molecules belong to a

family of polymorphic genes, a cocktail of KIR antibodies reactive to various KIR receptors was used: CD158b1/b2,j; CD158i; NKB1; CD158b; CD158a,h; KIR2DL4/CD158d. Intracellular staining was performed using a human perforin antibody and the Cytofix/Cytoperm Kit. Cells were stained and fixed, then analyzed on a FACSCalibur flow cytometer at least 24 hours later to allow for cell equilibration after contraction due to fixing. Isotype and single-color positive controls were included in each condition. Standardization particles for fluorescein isothiocyanate (FITC), phycoerythrin (PE) and cychrome (CYC) were run during each experiment for instrument calibration. Ten thousand events in the gated lymphocyte population were captured. Percent-positive cells were assessed for CD70, KIR and perforin. Mean fluorescence intensity was assessed for CD11a and CD70. Flow cyometric results were analyzed using FCS Express Software, version 3 (De Novo™ Software, Los Angeles, CA).

Statistical analysis. The gene expression in lupus patients was compared to age- and gender-matched controls at three different concentrations of methionine for each patient using a linear mixed model. This model included a random effect for subject and a random effect for pair. The fixed factors in the model were group (lupus or control), concentration (methionine 101μM, 30μM, and 5μM), and their interaction. Post-hoc tests were used to compare the lupus patients to normal controls at each level of methionine concentration, and to compare the methionine concentrations within a group (lupus or control).

Statistical analyses were conducted using IBM SPSS Statistics, version 21 for Windows, copyright IBM corporation 1989, 2012.

Results

Low methionine causes overexpression of CD70 in lupus CD4+ T cells. To investigate whether lupus patients may be more sensitive to decreases in methionine, we cultured CD4+ T cells from patients and controls in various concentrations of methionine and examined CD70 protein expression. PBMCs from 9 lupus patients and 9 age- and gender-matched controls were cultured in RPMI 1640 with 101μM, 30μM, or 5μM methionine for 4 days. Cells were then stained with antibodies for CD4 and CD70 and analyzed by flow cytometry. Patients had a significant increase in CD70 expression (p=0.028 for overall methionine effect) compared to controls, when cultured in lower concentrations of methionine. Patients had a significant increase between the highest and lowest methionine concentrations (p<0.026) (Figure 5.1).

Low methionine causes overexpression of KIR in lupus CD4+ T cells. To investigate whether lupus patients may be more sensitive to decreases in methionine, we cultured CD4+ T cells from patients and controls in various concentrations of methionine and examined KIR protein expression. PBMCs from 9 lupus patients and 9 age- and gender-matched controls were cultured in RPMI 1640 with 101μM, 30μM, or 5μM methionine for 4 days. Cells were then stained with antibodies for CD4 and KIR and analyzed by flow cytometry.

Patients and controls both had a significant increase in KIR expression (p=0.001 and p=0.024 for overall methionine effect, respectively), when cultured in decreasing concentrations of methionine. Patients and controls had a significant increase in KIR expression between 101µM and 5µM methionine concentrations (p=0.006; p=0.021, respectively). Patients had a significant increase in KIR expression between 101µM and 30µM (p=0.003), but controls had no significant effect (Figure 5.2).

Low methionine causes overexpression of perforin in lupus CD4+ T cells.

To investigate whether lupus patients may be more sensitive to decreases in methionine, we cultured CD4+ T cells from patients and controls in various concentrations of methionine and examined perforin protein expression. PBMCs from 9 lupus patients and 9 age- and gender-matched controls were cultured in RPMI 1640 with 101µM, 30µM, or 5µM methionine for 4 days. Cells were then intracellularly stained with antibodies for CD4 and perforin and analyzed by flow cytometry. Patients had a significant increase in perforin expression (p=0.008 for overall methionine effect), when cultured in decreasing concentrations of methionine. Controls had no significant effect. Patients had a significant increase in expression when comparing methionine 101µM and 5µM (p=0.007). (Figure 5.3).

Folate modifies expression of CD11a and CD70 in lupus T cells. PBMCs from 11 lupus patients were cultured in RPMI 1640 with 2270nM, 40nM, or 10nM folic acid for 4 days. Cells were then stained with antibodies for CD4 or CD8, and CD11a or CD70, and analyzed by flow cytometry. Patients had a significant

increase in CD11a expression in both CD4+ and CD8+ T cells (p<0.001) when

cultured in decreasing concentrations of folate (Figure 5.4). Patients also had a

significant increase in CD70 expression in both CD4+ and CD8+ T cells (p<0.001)

when cultured in decreasing concentrations of folate (Figure 5.5).

Discussion

Lupus patients have increased oxidative stress and decreased Dnmt1

levels [170, 199]. We have shown in Chapter II of this dissertation that lupus

patients have decreased serum methionine levels. Since methionine is a

necessary micronutrient in the methylation cycle, we examined whether lupus

CD4+ T cells would be more sensitive to alterations in methionine levels due to

their already low Dnmt1 levels in comparison to healthy controls.

We used 2 concentrations of methionine (30µM, 5µM) that are in the

physiologic range, with one being at the high end and one in the lower end. We

also used standard RPMI 1640 for comparison, which contains 101µM

methionine. CD70 expression was unaffected in control CD4+ T cells in the

lower concentrations of methionine. Patient CD4+ T cells, however, had

significant differential expression in the 3 concentrations of methionine (Figure

5.1). This supports our hypothesis that lupus patients are more sensitive to

transmethylation micronutrient alterations. We also assessed KIR expression. In

the controls, there was a significant increase in KIR expression in the lowest

methionine concentration, resulting in an overall methioinine effect, however

there was no difference in expression in the 2 higher concentrations. The patients also had a significant overall effect of methionine concentration and interestingly did have a significant increase in expression between the 2 higher concentrations (101µM and 30µM) (Figure 5.2). Considering 30µM is considered 'high physiologic' concentration, this suggests that in lupus patients, higher than 30µM concentrations of methionine may be needed to prevent increasing KIR expression. Finally, we examined perforin expression in the presence of the 3 methioinine concentrations. The controls once again had no significant increase in perforin expression across the concentrations of methionine, however the patients had a significant increase overall, and in the lowest concentration compared to standard RPMI 1640 (Figure 5.3). Together, these results suggest that lupus CD4+ T cells are more susceptible to overexpressing CD70, KIR, and perforin when exposed to a low methionine environment.

Additionally, we examined the effects of altered folate levels on T cell gene expression in lupus T cells. We used 2 concentrations of folic acid, 40nM and 10nM, which are considered to be the higher and lower physiologic levels. Standard RPMI 1640, which contains 2270nM folic acid, was used as a control. Expression of CD11a and CD70 both significantly increased when cultured in decreasing levels of folate, in both CD4+ and CD8+ T cells. We do not have data comparing this to control CD4+ T cells at this time, however considering folate is decreased in some lupus patients [79], and that in Chapter IV of this dissertation we show a synergistic effect of lower folate concentrations and oxidants on methylation-sensitive gene expression, we hypothesize that the

lupus CD4+ T cells will be more sensitive to lower folate concentrations compared to controls as well. Studies are ongoing to investigate this.
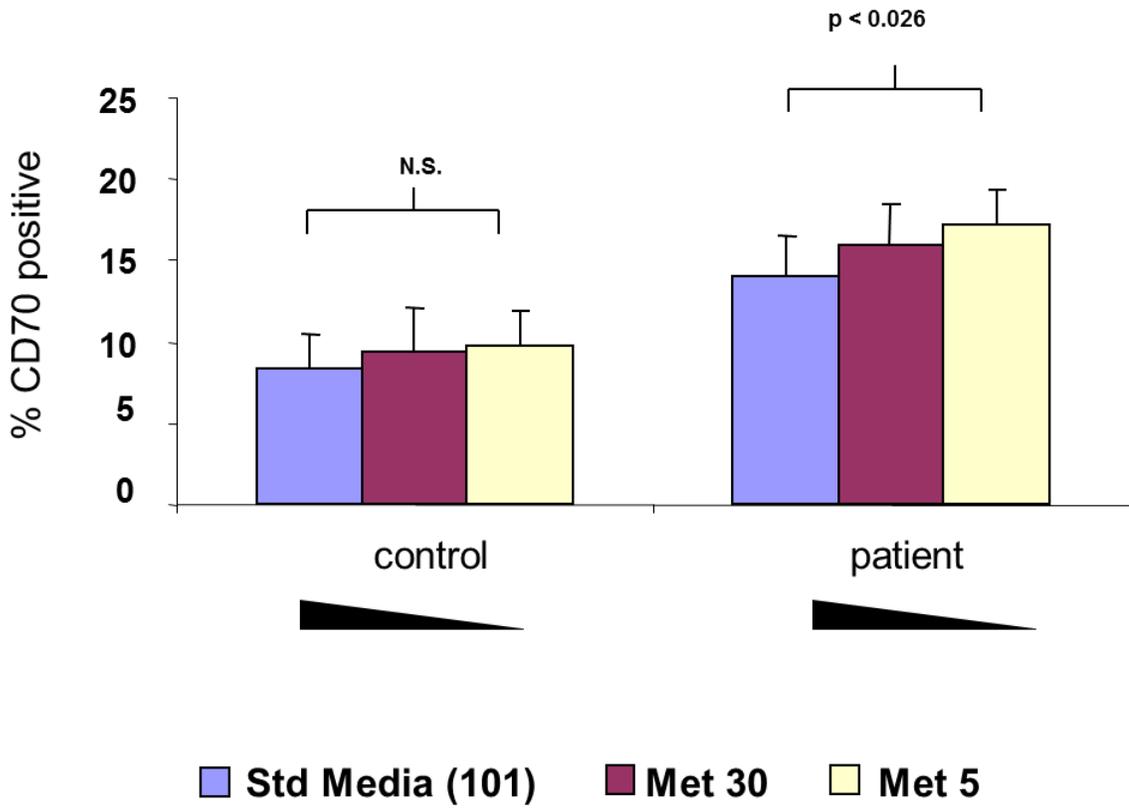
Figure 5.1 Low methionine causes overexpression of CD70 in lupus CD4+ T cells. PBMCs from 9 lupus patients and 9 age- and gender-matched controls were cultured in RPMI 1640 with 101μM, 30μM or 5μM methionine as described in "Materials and Methods" section. Cells were than stained with antibodies for CD4 or CD70 and analyzed by flow cytometry. There was a significant overall methionine effect (p=0.028) in patients but not controls.
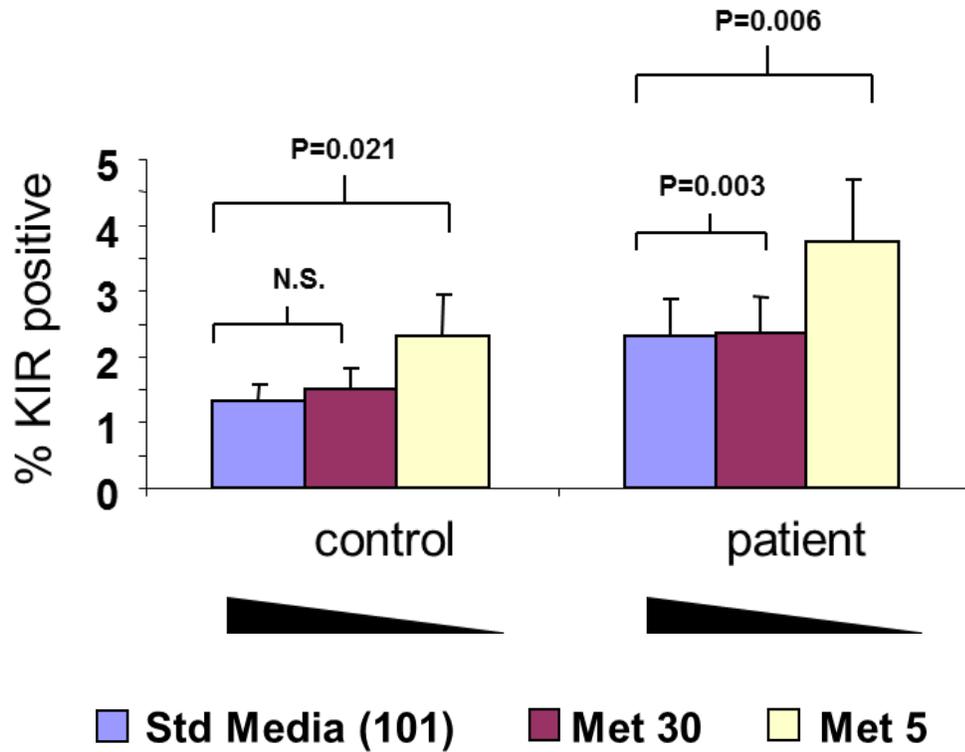
Figure 5.2 Low methionine causes overexpression of KIR in lupus CD4+ T cells. PBMCs from 9 lupus patients and 9 age- and gender- matched controls were cultured in RPMI 1640 with 101µM, 30µM, or 5µM methionine as described in " Materials and Methods" section. Cells were than stained with antibodies for CD4 or KIR and analyzed by flow cytometry. There was a significant overall methionine effect in controls (p=0.024) and in patients (p=0.001).
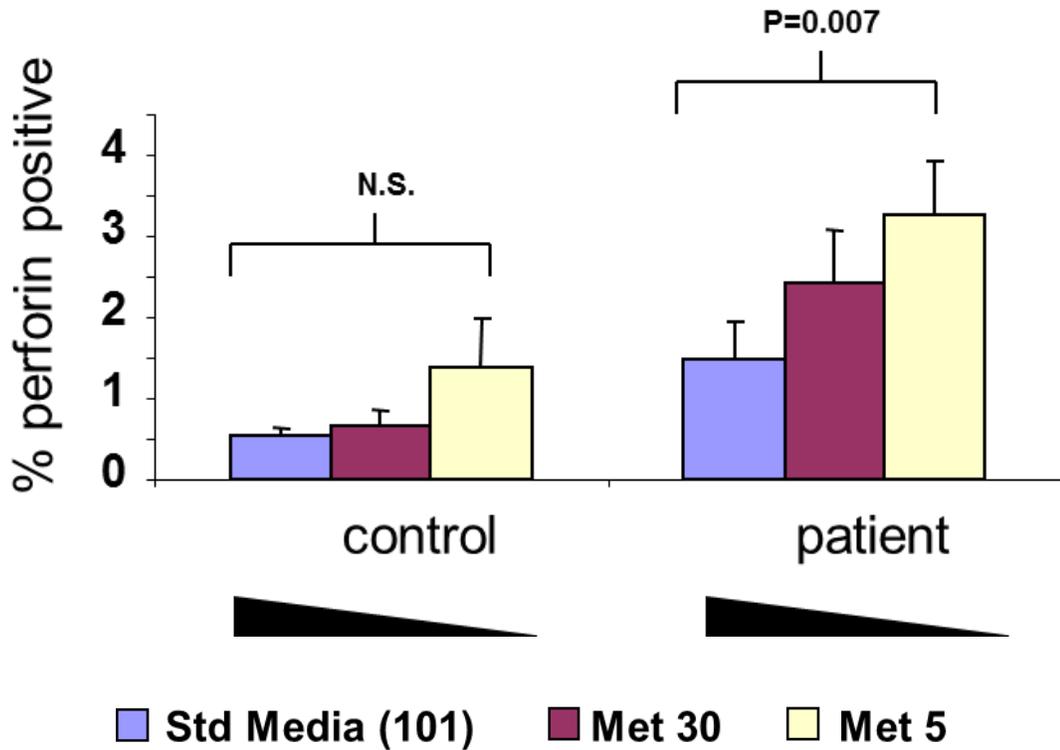
Figure 5.3 Low methionine causes overexpression of perforin in lupus CD4+ T cells. PBMCs from 9 lupus patients and 9 age- and gender-matched controls were cultured in RPMI 1640 with 101µM, 30µM, or 5µM methionine as described in "Materials and Methods" section. Cells were then stained with antibodies for CD4 or perforin and analyzed by flow cytometry. There was a significant overall methionine effect in patients (p=0.008) but not in controls.
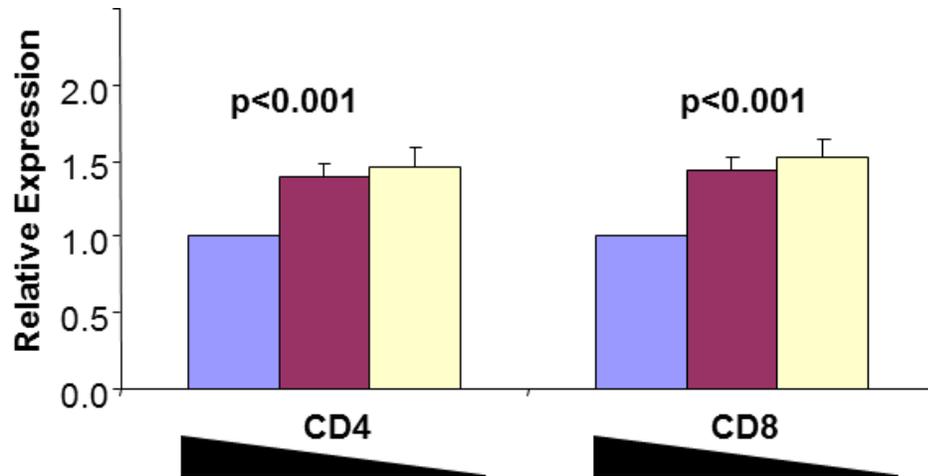
Figure 5.4 Folate modifies expression of CD11a in lupus CD4+ T cells. PBMCs from 11 lupus patients were cultured in RPMI 1640 with 2270nM, 40nM, or 10nM folic acid as described in "Materials and Methods" section. Cells were then stained with antibodies for CD4 or CD8, and CD11a and analyzed by flow cytometry. The effect of folate on CD11a expression was significant in both CD4+ (p<0.001) and CD8+ (p<0.001) T cells.
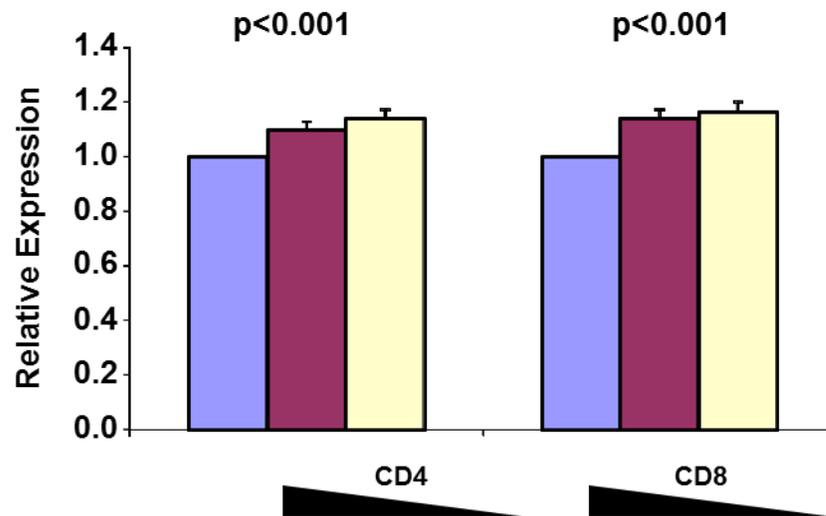
Figure 5.5 Folate modifies expression of CD70 in lupus T cells.
PBMCs from 11 lupus patients were cultured in RPMI 1640 with 2270nM, 40nM, or 10nM folic acid as described in "Materials and Methods" section. Cells were then stained with antibodies for CD4 or CD8, and CD70. The effect of folate on CD11a expression was significant in both CD4+ (p<0.001) and CD8+ (p<0.001) T cells.

CHAPTER VI: CONCLUSION

<u>Introduction</u>

T cell DNA demethylation is associated with autoimmunity in SLE. The mechanisms linking environmental factors and dysregulation of methylation in the disease, however still remain unclear. Through the research presented in this dissertation, we have demonstrated that transmethylation micronutrient alterations affect methylation-sensitive gene expression in T cells in lupus. We have shown that lupus patients have serum/plasma levels of transmethylation micronutrients that differ from the normal population. *In vitro*, transmethylation mmicronutrient levels in tissue culture media modulate expression of methylation-sensitive genes in T cells. Furthermore, we demonstrate the synergistic effects of oxidative stress and micronutrient alterations in promoting increased protein expression of methylation-sensitive genes in T cells. CD4+ T cells from lupus patients display an increased sensitivity to altered methionine levels by aberrantly expressing methylation-sensitive genes compared to CD4+ T cells from healthy controls. This is likely due to the decreased Dnmt1 levels and increased oxidative stress in lupus. To our knowledge, this is the first report demonstrating a relationship between oxidative stress and micronutrient effects on T cell methylation-sensitive gene expression relating to SLE.

Further work is needed to establish the mechanisms involved. Currently, methylation studies are underway to confirm the methylation changes that are likely occurring. Additional studies including the measurement of SAM/SAH ratios would help to establish a mechanism by which altered micronutrients are exerting their effects. Several factors may be involved. The metabolism of micronutrients in addition to quantities available are likely to be a factor. A polymorphism of the methionine synthase enzyme involved in folate and methionine metabolism has been associated with increased risk of SLE [90]. Furthermore, a recent study has shown several genes involved in the folate biosynthesis pathway to be hypermethylated in lupus CD4+ T cells compared to normal controls [205]. Studies in which these changes can be correlated with micronutrient effects *in vitro* may shed light on mechanisms by which altered micronutrients may be differentially affecting lupus patients. Oxidants such as peroxynitrite may be affecting Dnmt1 enzymatic activity through nitration of tyrosine residues of PKCδ [56] as discussed earlier in this dissertation, thus having a concurrent effect on methylation. Peroxynitrite can also react with methionine and inactivate methioinine-containing proteins by oxidizing methionine residues [206]. This may represent another mechanism by which altered micronutrients and oxidative stress interact to cause altered methylation-sensitive gene expression, and merits further study.

This work is significant because the overexpression of CD4+ T cell methylation-sensitive genes in lupus has functional consequences, including increased antibody production and macrophage killing [51, 62]. These effects

contribute to disease pathogenesis and subsequent morbidity (Figure 6.1). By demonstrating that transmethylation micronutrients alter methylation-sensitive gene expression in lupus, this work identifies a potentially modifiable mechanism which may have therapeutic applications.

Public Health Implications

The burden of autoimmune disease worldwide is significant. In the United States, it is estimated that 5-8% of the population has at least one autoimmune disease, which corresponds to between 15 and 24 million people [207]. The majority of autoimmune diseases disproportionately affect women for reasons that are still unclear. Furthermore, all autoimmune diseases have a genetic and environmental component that combine to cause pathogenesis.

SLE is one of many autoimmune diseases, however it is a prototypic autoimmune disease to study. The features of SLE are common to many other autoimmune diseases, and findings from lupus research can be extended to some of the other diseases. During the past 50 years, only one new drug has been developed and FDA approved for the treatment of lupus. The drug, Benlysta, is a human monoclonal antibody that targets B-lymphocyte stimulator, thereby reducing autoantibody production. However, there are significant costs and side effects associated with this new drug. Side effects include infections, cancers, depression, and suicide. Moreover, the drug is modestly effective, offering benefit to about 30% of patients, and not those with the most severe

disease involving organ damage [208]. This highlights the need for further research into novel treatments for SLE and other autoimmune diseases.

SLE has a genetic component, evidenced by the monogenic forms of the disease, though generally lupus is a polygenic disease. Lupus also has an environmental component, and this is best seen in cases of drug-induced lupus where certain drugs directly cause lupus and the disease remits when the inciting agent is removed [17]. Age may also play a role in disease development since the incidence of lupus increases with age [209], and this may be due to an accumulation of environmental 'insults' over time. These components make SLE a model autoimmune disease in which to study environmental effects. Several environmental agents in addition to drugs have been associated with SLE, including silica dust [14], cigarette smoking [15], and infectious agents [16]. Oxidative stress is a common mechanism to these three environmental triggers and may be occurring with others. We explored the role of dietary micronutrients as a possible environmental factor in affecting lupus as well as oxidative stress. In Chapter II of this dissertation, we show decreased levels of zinc, vitamin B6, and methionine and increased levels of homocysteine in lupus patients compared to the normal population. In Chapter IV, we establish a relationship between oxidative stress and micronutrient level effects on T cell methylation-sensitive gene expression, and show that lupus CD4+ T cells are more sensitive to micronutrient alterations in Chapter V. Given the fact that dietary micronutrients are relatively easily modified, these results warrant further

investigation into the role of micronutrients as a potential benefit for therapy and/or prevention.

<u>Summary</u>

In summary, through the research presented in this thesis, we have demonstrated a role for micronutrients in modulating T cell methylation-sensitive gene expression in lupus. We have shown a decrease in zinc, vitamin B6, and methionine, and an increase in homocysteine in the serum of lupus patients. Alterations in micronutrient levels modulate T cell methylation-sensitive gene expression *in vitro*. Furthermore, oxidative stress and altered micronutrient levels are additive in affecting immune-relevant methylation-sensitive gene expression in T cells. Given that lupus patients have decreased Dnmt1 levels likely resulting from oxidative stress, and we show that CD4+ T cells from lupus patients are more sensitive to decreases in methionine, dietary modification may have therapeutic implications in SLE and potentially other autoimmune diseases. Further studies are needed to elucidate the complex interactions of transmethylation micronutrients, oxidative stress, and DNA methylation in lupus. The work presented in this dissertation will hopefully serve as a basis for future investigation into potential therapies in the field of autoimmunity.

Genes

Environment
Diet
Oxidative Stress

Overexpression of methylation-sensitive genes

CD70   **KIR, perforin**

Antibody prodution   **Macrophage killing**

Pathogenesis & Morbidity in Lupus
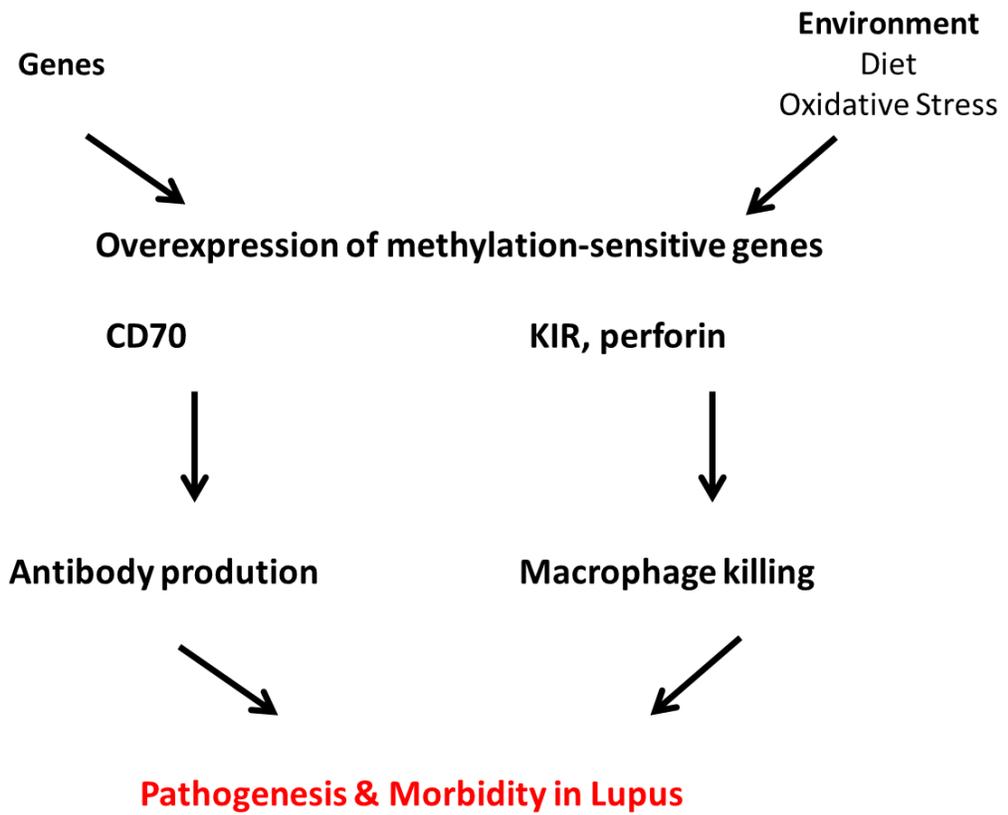
Figure 6.1: Model

REFERENCES

1.   Wallace, D.J., B.H. Hahn, and E.L. Dubois, *Dubois' lupus erythematosus.* 2007, Philadelphia: Lippincott Williams & Wilkin.

2.   Jacobson, D.L., et al., *Epidemiology and estimated population burden of selected autoimmune diseases in the United States.* Clinical immunology and immunopathology, 1997. **84**(3): p. 223-43.

3.   Rus, V., E.E. Maury, and M.C. Hochberg, *The Epidemiology of systemic lupus erythematosus.*, in *Dubois; lupus erythematosus.* 2007, Lippincott Williams & Wilkin: Philadelphia. p. 34-44.

4.   Pons-Estel, G.J., et al., *Understanding the epidemiology and progression of systemic lupus erythematosus.* Seminars in arthritis and rheumatism, 2010. **39**(4): p. 257-68.

5.   Kyttaris, V. and G. Tsokos, *Uncovering the genetics of systemic lupus erythematosus: implications for therapy.* Am J Pharmacogenomics, 2003. **3**(3): p. 193-202.

6.   Deapen, D., et al., *A revised estimate of twin concordance in systemic lupus erythematosus.* Arthritis Rheum, 1992. **35**(3): p. 311-8.

7.   Scofield, R.H., et al., *Klinefelter's syndrome (47,XXY) in male systemic lupus erythematosus patients: support for the notion of a gene-dose effect from the X chromosome.* Arthritis Rheum, 2008. **58**(8): p. 2511-7.

8.   Manolio, T.A., et al., *Finding the missing heritability of complex diseases.* Nature, 2009. **461**(7265): p. 747-53.

9.   Rubin, R.L., *Drug-Induced Lupus*, in *DuBois' Lupus Erythematosus*, Wallace, Editor. 2007, Lippincott Williams & Wilkins: Philadelphia. p. 870-900.

10.   Lehmann, P., et al., *Experimental reproduction of skin lesions in lupus erythematosus by UVA and UVB radiation.* J Am Acad Dermatol, 1990. **22**(2 Pt 1): p. 181-7.

11.   Herzinger, T., G. Plewig, and M. Rocken, *Use of sunscreens to protect against ultraviolet-induced lupus erythematosus.* Arthritis Rheum, 2004. **50**(9): p. 3045-6.

12.   Fruchter, O. and Y. Edoute, *First presentation of systemic lupus erythematosus following ultraviolet radiation exposure in an artificial tanning device.* Rheumatology (Oxford), 2005. **44**(4): p. 558-9.

13.   Haga, H.J., et al., *Seasonal variations in activity of systemic lupus erythematosus in a subarctic region.* Lupus, 1999. **8**(4): p. 269-73.

14.   Parks, C.G., et al., *Occupational exposure to crystalline silica and risk of systemic lupus erythematosus: a population-based, case-control study in the southeastern United States.* Arthritis Rheum, 2002. **46**(7): p. 1840-50.

15.   Harel-Meir, M., Y. Sherer, and Y. Shoenfeld, *Tobacco smoking and autoimmune rheumatic diseases.* Nat Clin Pract Rheumatol, 2007. **3**(12): p. 707-15.

16.   Cooper, G.S., et al., *Recent advances and opportunities in research on lupus: environmental influences and mechanisms of disease.* Environ Health Perspect, 2008. **116**(6): p. 695-702.

17.   Yung, R. and B. Richardson, *Drug-Induced Lupus Mechanisms*, in *Systemic Lupus Erythematosus*, R. Lahita, et al., Editors. 2011, Elsevier. p. 385-404.

18.   Arbuckle, M.R., et al., *Development of autoantibodies before the clinical onset of systemic lupus erythematosus.* N Engl J Med, 2003. **349**(16): p. 1526-33.

19.   Jacobi, A.M., et al., *Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus.* Arthritis Rheum, 2003. **48**(5): p. 1332-42.

20.   Jacobi, A.M., et al., *Activated memory B cell subsets correlate with disease activity in systemic lupus erythematosus: delineation by expression of CD27, IgD, and CD95.* Arthritis Rheum, 2008. **58**(6): p. 1762-73.

21.   Bensimon, C., P. Chastagner, and M. Zouali, *Human lupus anti-DNA autoantibodies undergo essentially primary V kappa gene rearrangements.* EMBO J, 1994. **13**(13): p. 2951-62.

22.   Davidson, A., *The rationale for BAFF inhibition in systemic lupus erythematosus.* Current rheumatology reports, 2012. **14**(4): p. 295-302.

23.   Kariuki, S.N., M.K. Crow, and T.B. Niewold, *The PTPN22 C1858T polymorphism is associated with skewing of cytokine profiles toward high interferon-alpha activity and low tumor necrosis factor alpha levels in patients with lupus.* Arthritis Rheum, 2008. **58**(9): p. 2818-23.

24.   Hom, G., et al., *Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX.* N Engl J Med, 2008. **358**(9): p. 900-9.

25. Vassilopoulos, D., B. Kovacs, and G.C. Tsokos, *TCR/CD3 complex-mediated signal transduction pathway in T cells and T cell lines from patients with systemic lupus erythematosus.* J Immunol, 1995. **155**(4): p. 2269-81.

26. Alunno, A., et al., *Balance between regulatory T and Th17 cells in systemic lupus erythematosus: the old and the new.* Clinical & developmental immunology, 2012. **2012**: p. 823085.

27. Hahn, B.H., *The Pathogenesis of SLE*, in *Dubois' Lupus Erythematosus and Related Syndromes*, D.J. Wallace and B.H. Hahn, Editors. 2013, Elsevier Saunders: Philadelphia, PA. p. 25-34.

28. Adams, R.L. and R.H. Burdon, *DNA methylation in the cell.*, in *Molecular Biology of DNA Methylation.*, A. Rich, Editor. 1985, Springer-Verlag: New York. p. 9-18.

29. Ehrlich, M. and R.Y. Wang, *5-Methylcytosine in eukaryotic DNA.* Science, 1981. **212**(4501): p. 1350-7.

30. Razin, A. and H. Cedar, *Distribution of 5-methylcytosine in chromatin.* Proc Natl Acad Sci U S A, 1977. **74**(7): p. 2725-8.

31. McClelland, M. and R. Ivarie, *Asymmetrical distribution of CpG in an 'average' mammalian gene.* Nucleic Acids Res, 1982. **10**(23): p. 7865-77.

32. Bird, A.P. and A.P. Wolffe, *Methylation-induced repression--belts, braces, and chromatin.* Cell, 1999. **99**(5): p. 451-4.

33. Li, E., T.H. Bestor, and R. Jaenisch, *Targeted mutation of the DNA methyltransferase gene results in embryonic lethality.* Cell, 1992. **69**(6): p. 915-26.

34. Holliday, R. and J.E. Pugh, *DNA modification mechanisms and gene activity during development.* Science, 1975. **187**(4173): p. 226-32.

35. Riggs, A.D., *X inactivation, differentiation, and DNA methylation.* Cytogenet Cell Genet, 1975. **14**(1): p. 9-25.

36. Attwood, J.T., R.L. Yung, and B.C. Richardson, *DNA methylation and the regulation of gene transcription.* Cell Mol Life Sci, 2002. **59**(2): p. 241-57.

37. Ray, D. and B. Richardson, *Failure to maintain T cell DNA methylation and chromatin structure contributes to human lupus*, in *Molecular Autoimmunity*, M. Zouali, Editor. 2005, Springer: New York. p. 69-84.

38. Rouleau, J., A.R. MacLeod, and M. Szyf, *Regulation of the DNA methyltransferase by the Ras-AP-1 signaling pathway.* The Journal of biological chemistry, 1995. **270**(4): p. 1595-601.

39. Richardson, B., *Effect of an inhibitor of DNA methylation on T cells. II. 5-Azacytidine induces self-reactivity in antigen-specific T4+ cells.* Hum Immunol, 1986. **17**(4): p. 456-70.

40. Sorm, F., et al., *5-Azacytidine, a new, highly effective cancerostatic.* Experientia, 1964. **20**(4): p. 202-3.

41. Jones, P.A., S.M. Taylor, and V.L. Wilson, *Inhibition of DNA methylation by 5-azacytidine.* Recent Results Cancer Res, 1983. **84**: p. 202-11.

42. Richardson, B., et al., *Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis.* Arthritis Rheum, 1990. **33**(11): p. 1665-73.

43. Quddus, J., et al., *Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-azacytidine or procainamide, is sufficient to cause a lupus-like disease in syngeneic mice.* J Clin Invest, 1993. **92**(1): p. 38-53.

44. Richardson, B.C., et al., *Phenotypic and functional similarities between 5-azacytidine-treated T cells and a T cell subset in patients with active systemic lupus erythematosus.* Arthritis Rheum, 1992. **35**(6): p. 647-62.

45. Lu, Q., et al., *Demethylation of ITGAL (CD11a) regulatory sequences in systemic lupus erythematosus.* Arthritis Rheum, 2002. **46**(5): p. 1282-91.

46. Grakoui, A., et al., *The immunological synapse: a molecular machine controlling T cell activation.* Science, 1999. **285**(5425): p. 221-7.

47. Wulfing, C., M.D. Sjaastad, and M.M. Davis, *Visualizing the dynamics of T cell activation: intracellular adhesion molecule 1 migrates rapidly to the T cell/B cell interface and acts to sustain calcium levels.* Proc Natl Acad Sci U S A, 1998. **95**(11): p. 6302-7.

48. Richardson, B., et al., *Lymphocyte function-associated antigen 1 overexpression and T cell autoreactivity.* Arthritis Rheum, 1994. **37**(9): p. 1363-72.

49. Cornacchia, E., et al., *Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity.* J Immunol, 1988. **140**(7): p. 2197-200.

50. Yung, R., et al., *Mechanisms of drug-induced lupus. IV. Comparison of procainamide and hydralazine with analogs in vitro and in vivo.* Arthritis Rheum, 1997. **40**(8): p. 1436-43.

51. Oelke, K., et al., *Overexpression of CD70 and overstimulation of IgG synthesis by lupus T cells and T cells treated with DNA methylation inhibitors.* Arthritis Rheum, 2004. **50**(6): p. 1850-60.

52.   Scott, R.S., et al., *Phagocytosis and clearance of apoptotic cells is mediated by MER.* Nature, 2001. **411**(6834): p. 207-11.

53.   Denny, M.F., et al., *Accelerated macrophage apoptosis induces autoantibody formation and organ damage in systemic lupus erythematosus.* J Immunol, 2006. **176**(4): p. 2095-104.

54.   Deng, C., et al., *Decreased Ras-mitogen-activated protein kinase signaling may cause DNA hypomethylation in T lymphocytes from lupus patients.* Arthritis Rheum, 2001. **44**(2): p. 397-407.

55.   Gorelik, G., et al., *Impaired T cell protein kinase C delta activation decreases ERK pathway signaling in idiopathic and hydralazine-induced lupus.* J Immunol, 2007. **179**(8): p. 5553-63.

56.   Gorelik, G.J., S. Yarlagadda, and B.C. Richardson, *Protein kinase Cdelta oxidation contributes to ERK inactivation in lupus T cells.* Arthritis and rheumatism, 2012. **64**(9): p. 2964-74.

57.   Yung, R., et al., *Mechanisms of drug-induced lupus. II. T cells overexpressing lymphocyte function-associated antigen 1 become autoreactive and cause a lupuslike disease in syngeneic mice.* J Clin Invest, 1996. **97**(12): p. 2866-71.

58.   Lu, Q., et al., *DNA methylation and chromatin structure regulate T cell perforin gene expression.* J Immunol, 2003. **170**(10): p. 5124-32.

59.   Lu, Q., A. Wu, and B.C. Richardson, *Demethylation of the same promoter sequence increases CD70 expression in lupus T cells and T cells treated with lupus-inducing drugs.* J Immunol, 2005. **174**(10): p. 6212-9.

60.   Liu, Y., et al., *DNA methylation inhibition increases T cell KIR expression through effects on both promoter methylation and transcription factors.* Clin Immunol, 2009. **130**(2): p. 213-24.

61.   Vilches, C. and P. Parham, *KIR: diverse, rapidly evolving receptors of innate and adaptive immunity.* Annu Rev Immunol, 2002. **20**: p. 217-51.

62.   Basu, D., et al., *Stimulatory and inhibitory killer Ig-like receptor molecules are expressed and functional on lupus T cells.* J Immunol, 2009. **183**(5): p. 3481-7.

63.   Seery, J.P., *IFN-gamma transgenic mice: clues to the pathogenesis of systemic lupus erythematosus?* Arthritis Res, 2000. **2**(6): p. 437-440.

64.   Podack, E.R., H. Hengartner, and M.G. Lichtenheld, *A central role of perforin in cytolysis?* Annu Rev Immunol, 1991. **9**: p. 129-57.

65.   Kaplan, M.J., et al., *The apoptotic ligands TRAIL, TWEAK, and Fas ligand mediate monocyte death induced by autologous lupus T cells.* J Immunol, 2002. **169**(10): p. 6020-9.

66.   Kaplan, M.J., et al., *Demethylation of promoter regulatory elements contributes to perforin overexpression in CD4+ lupus T cells.* J Immunol, 2004. **172**(6): p. 3652-61.

67.   Finkelstein, J.D., *Methionine metabolism in mammals.* J Nutr Biochem, 1990. **1**(5): p. 228-37.

68.   James, S.J., et al., *Elevation in S-adenosylhomocysteine and DNA hypomethylation: potential epigenetic mechanism for homocysteine-related pathology.* J Nutr, 2002. **132**(8 Suppl): p. 2361S-2366S.

69.   Kramer, D.L., et al., *Combined modulation of S-adenosylmethionine biosynthesis and S-adenosylhomocysteine metabolism enhances inhibition of nucleic acid methylation and L1210 cell growth.* Cancer Res, 1990. **50**(13): p. 3838-42.

70.   Yi, P., et al., *Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation.* J Biol Chem, 2000. **275**(38): p. 29318-23.

71.   Lertratanangkoon, K., et al., *Alterations of DNA methylation by glutathione depletion.* Cancer Lett, 1997. **120**(2): p. 149-56.

72.   Lu, S.C., et al., *Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation.* Proc Natl Acad Sci U S A, 2001. **98**(10): p. 5560-5.

73.   WHO, *Vitamin and mineral requirements in human nutrition.* 2004: Bangkok, Thailand.

74.   Sauberlich, H., *Folate status in the US population groups.*, in *Folate in health and disease*, L.B. Bailey, Editor. 1995, Marcel Dekker: New York, NY. p. 171-194.

75.   Wald, N.J., et al., *Homocysteine and ischemic heart disease: results of a prospective study with implications regarding prevention.* Arch Intern Med, 1998. **158**(8): p. 862-7.

76.   Boushey, C.J., et al., *A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes.* JAMA, 1995. **274**(13): p. 1049-57.

77.   Perry, I.J., et al., *Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men.* Lancet, 1995. **346**(8987): p. 1395-8.

78.   Refsum, H., et al., *Homocysteine and cardiovascular disease.* Annu Rev Med, 1998. **49**: p. 31-62.

79.   Petri, M., et al., *Plasma homocysteine as a risk factor for atherothrombotic events in systemic lupus erythematosus.* Lancet, 1996. **348**(9035): p. 1120-4.

80.   Chen, L.H., et al., *Human methionine synthase. cDNA cloning, gene localization, and expression.* J Biol Chem, 1997. **272**(6): p. 3628-34.

81.   Leclerc, D., et al., *Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria.* Proc Natl Acad Sci U S A, 1998. **95**(6): p. 3059-64.

82.   Kim, Y.I., *Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility.* J Nutr, 2005. **135**(11): p. 2703-9.

83.   Kim, Y. and J.B. Mason, *Folate, epithelial dysplasia and colon cancer.* Proc Assoc Am Physicians, 1995. **107**(2): p. 218-27.

84.   Kim, Y.I., et al., *Colonic mucosal concentrations of folate correlate well with blood measurements of folate status in persons with colorectal polyps.* Am J Clin Nutr, 1998. **68**(4): p. 866-72.

85.   Bailey, L.B., *Folate, methyl-related nutrients, alcohol, and the MTHFR 677C-->T polymorphism affect cancer risk: intake recommendations.* J Nutr, 2003. **133**(11 Suppl 1): p. 3748S-3753S.

86.   Kim, Y.I., *Role of the MTHFR polymorphisms in cancer risk modification and treatment.* Future Oncol, 2009. **5**(4): p. 523-42.

87.   Potter, J.D., *Methyl supply, methyl metabolizing enzymes and colorectal neoplasia.* J Nutr, 2002. **132**(8 Suppl): p. 2410S-2412S.

88.   Castro, R., et al., *5,10-methylenetetrahydrofolate reductase (MTHFR) 677C-->T and 1298A-->C mutations are associated with DNA hypomethylation.* J Med Genet, 2004. **41**(6): p. 454-8.

89.   Friso, S., et al., *The MTHFR 1298A>C polymorphism and genomic DNA methylation in human lymphocytes.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(4): p. 938-43.

90.   Burzynski, M., et al., *MTR 2756 A > G polymorphism is associated with the risk of systemic lupus erythematosus in the Polish population.* Lupus, 2007. **16**(6): p. 450-4.

91.   Zeisel, S.H., *Gene response elements, genetic polymorphisms and epigenetics influence the human dietary requirement for choline.* IUBMB Life, 2007. **59**(6): p. 380-7.

92. Medicine, I.o. and N.A.o.S. USA, *Choline*, in *Dietary reference intakes for folate, thiamin, riboflavin, niacin, vitamin B12, panthothenic acid, biotin, and choline*. 1998, National Academy Press: Washington, DC. p. 3909-422.

93. Blusztajn, J.K., S.H. Zeisel, and R.J. Wurtman, *Developmental changes in the activity of phosphatidylethanolamine N-methyltransferases in rat brain.* Biochem J, 1985. **232**(2): p. 505-11.

94. Resseguie, M., et al., *Phosphatidylethanolamine N-methyltransferase (PEMT) gene expression is induced by estrogen in human and mouse primary hepatocytes.* FASEB J, 2007. **21**(10): p. 2622-32.

95. Fischer, L.M., et al., *Sex and menopausal status influence human dietary requirements for the nutrient choline.* Am J Clin Nutr, 2007. **85**(5): p. 1275-85.

96. Finkelstein, J.D., *Pathways and regulation of homocysteine metabolism in mammals.* Semin Thromb Hemost, 2000. **26**(3): p. 219-25.

97. Olthof, M.R., et al., *Low dose betaine supplementation leads to immediate and long term lowering of plasma homocysteine in healthy men and women.* J Nutr, 2003. **133**(12): p. 4135-8.

98. Steenge, G.R., P. Verhoef, and M.B. Katan, *Betaine supplementation lowers plasma homocysteine in healthy men and women.* J Nutr, 2003. **133**(5): p. 1291-5.

99. Wendel, U. and H.J. Bremer, *Betaine in the treatment of homocystinuria due to 5,10-methylenetetrahydrofolate reductase deficiency.* Eur J Pediatr, 1984. **142**(2): p. 147-50.

100. da Costa, K.A., et al., *Choline deficiency in mice and humans is associated with increased plasma homocysteine concentration after a methionine load.* Am J Clin Nutr, 2005. **81**(2): p. 440-4.

101. Locker, J., T.V. Reddy, and B. Lombardi, *DNA methylation and hepatocarcinogenesis in rats fed a choline-devoid diet.* Carcinogenesis, 1986. **7**(8): p. 1309-12.

102. Tsujiuchi, T., et al., *Hypomethylation of CpG sites and c-myc gene overexpression in hepatocellular carcinomas, but not hyperplastic nodules, induced by a choline-deficient L-amino acid-defined diet in rats.* Jpn J Cancer Res, 1999. **90**(9): p. 909-13.

103. Zeisel, S.H., et al., *Effect of choline deficiency on S-adenosylmethionine and methionine concentrations in rat liver.* Biochem J, 1989. **259**(3): p. 725-9.

104.   Shivapurkar, N. and L.A. Poirier, *Tissue levels of S-adenosylmethionine and S-adenosylhomocysteine in rats fed methyl-deficient, amino acid-defined diets for one to five weeks.* Carcinogenesis, 1983. **4**(8): p. 1051-7.

105.   Ray, D. and B. Richardson, *Toxicoepigenomics in lupus*, in *Toxicology and Epigenetics*, S. Sahu, Editor. 2012, John Wiley & Sons, Ltd.

106.   Weir, D.G. and J.M. Scott, *Cobalamins physiology, dietary sources and requirements.*, in *Encyclopedia of human nutrition*, M. Sadler, J.J. Strain, and B. Caballero, Editors. 1998, Academic Press: San Diego, CA. p. 394-401.

107.   Weir, D.G. and J.M. Scott, *Vitamin B12*, in *Modern nutrition in health and disease*, M.E. Shils, Editor. 1999, Williams & Wilkins: Baltimore, MA. p. 447-458.

108.   Chanarin, I., *The megaloblastic anaemias.* 2nd ed. 1979, Oxford: Blackwell Scientific Publications.

109.   Carmel, R., *Prevalence of undiagnosed pernicious anemia in the elderly.* Arch Intern Med, 1996. **156**(10): p. 1097-100.

110.   Ly, A., et al., *Folate and DNA methylation.* Antioxid Redox Signal, 2012. **17**(2): p. 302-26.

111.   Sazawal, S., et al., *Zinc supplementation reduces the incidence of persistent diarrhea and dysentery among low socioeconomic children in India.* J Nutr, 1996. **126**(2): p. 443-50.

112.   Ruel, M.T., et al., *Impact of zinc supplementation on morbidity from diarrhea and respiratory infections among rural Guatemalan children.* Pediatrics, 1997. **99**(6): p. 808-13.

113.   Sazawal, S., et al., *Zinc supplementation reduces the incidence of acute lower respiratory infections in infants and preschool children: a double-blind, controlled trial.* Pediatrics, 1998. **102**(1 Pt 1): p. 1-5.

114.   Gibson, R.S., et al., *Growth in children from the Wosera subdistrict, Papua New Guinea, in relation to energy and protein intakes and zinc status.* Am J Clin Nutr, 1991. **53**(3): p. 782-9.

115.   Bates, C.J., et al., *A trial of zinc supplementation in young rural Gambian children.* Br J Nutr, 1993. **69**(1): p. 243-55.

116.   Friis, H., et al., *The impact of zinc supplementation on Schistosoma mansoni reinfection rate and intensities: a randomized, controlled trial among rural Zimbabwean schoolchildren.* Eur J Clin Nutr, 1997. **51**(1): p. 33-7.

117. Hambidge, K.M., P.A. Walravens, and K.H. Neldner, *The role of zinc in the pathogenesis and treatment of acrodermatitis enteropathica.* Prog Clin Biol Res, 1977. **14**: p. 329-42.

118. Mathur, N.K., et al., *Serum zinc levels in subtypes of leprosy.* Int J Lepr Other Mycobact Dis, 1984. **52**(3): p. 327-30.

119. Godfrey, J.C., N.J. Godfrey, and S.G. Novick, *Zinc for treating the common cold: review of all clinical trials since 1984.* Altern Ther Health Med, 1996. **2**(6): p. 63-72.

120. Tang, A.M., N.M. Graham, and A.J. Saah, *Effects of micronutrient intake on survival in human immunodeficiency virus type 1 infection.* Am J Epidemiol, 1996. **143**(12): p. 1244-56.

121. Meadows, N.J., et al., *Zinc and small babies.* Lancet, 1981. **2**(8256): p. 1135-7.

122. Dutz, W., et al., *Persistent cell mediated immune-deficiency following infantile stress during the first 6 months of life.* Eur J Pediatr, 1976. **122**(2): p. 117-30.

123. Ferguson, A.C., *Prolonged impairment of cellular immunity in children with intrauterine growth retardation.* J Pediatr, 1978. **93**(1): p. 52-6.

124. Sandstead, H.H., *Understanding zinc: recent observations and interpretations.* J Lab Clin Med, 1994. **124**(3): p. 322-7.

125. Prasad, A.S., et al., *Zinc deficiency in elderly patients.* Nutrition, 1993. **9**(3): p. 218-24.

126. Garfinkel, D., *Is aging inevitable? The intracellular zinc deficiency hypothesis of aging.* Med Hypotheses, 1986. **19**(2): p. 117-37.

127. Dardenne, M., et al., *Restoration of the thymus in aging mice by in vivo zinc supplementation.* Clin Immunol Immunopathol, 1993. **66**(2): p. 127-35.

128. Congy, F., et al., *[Plasma zinc levels in elderly hospitalized subjects. Correlation with other nutritional and immunological markers and survival].* Sem Hop, 1983. **59**(45): p. 3105-8.

129. Moynahan, E.J., *Letter: Acrodermatitis enteropathica: a lethal inherited human zinc-deficiency disorder.* Lancet, 1974. **2**(7877): p. 399-400.

130. Allen, J.I., N.E. Kay, and C.J. McClain, *Severe zinc deficiency in humans: association with a reversible T-lymphocyte dysfunction.* Ann Intern Med, 1981. **95**(2): p. 154-7.

131. Wallwork, J.C. and J.A. Duerre, *Effect of zinc deficiency on methionine metabolism, methylation reactions and protein synthesis in isolated perfused rat liver.* J Nutr, 1985. **115**(2): p. 252-62.

132. Duerre, J.A. and J.C. Wallwork, *Methionine metabolism in isolated perfused livers from rats fed on zinc-deficient and restricted diets.* Br J Nutr, 1986. **56**(2): p. 395-405.

133. Breksa, A.P., 3rd and T.A. Garrow, *Recombinant human liver betaine-homocysteine S-methyltransferase: identification of three cysteine residues critical for zinc binding.* Biochemistry, 1999. **38**(42): p. 13991-8.

134. Koutmos, M., et al., *Metal active site elasticity linked to activation of homocysteine in methionine synthases.* Proc Natl Acad Sci U S A, 2008. **105**(9): p. 3286-91.

135. Matthews, R.G. and C.W. Goulding, *Enzyme-catalyzed methyl transfers to thiols: the role of zinc.* Curr Opin Chem Biol, 1997. **1**(3): p. 332-9.

136. McCormick, D.B. and H.L. Greene, *Vitamins*, in *Tietz textbook of clinical chemistry*, V.A. Burtis and E.R. Ashwood, Editors. 1994, WB Saunders: Philadelphia, PA. p. 1275-1316.

137. Board, F.a.N., *Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline.* 1998: Washington, DC.

138. McCormick, D.B., *Coenzymes, Biochemistry of*, in *Encyclopedia of molecular biology and molecular medicine*, R.A. Meyers, Editor. 1996, Verlag Chemie: Weinheim. p. 396-406.

139. McCormick, D.B., *Coenzymes, Biochemistry*, in *Encyclopedia of human biology*, R. Dulbecco, Editor. 1997, Academic Press: San Diego, CA. p. 847-864.

140. McCormick, D.B., *Vitamin B$_6$*, in *Modern nutrition in health and disease*, M.E. Shils and V.R. Young, Editors. 1988, Lea & Febiiger: Philadelphia, PA. p. 376-382.

141. Sies, H., *Oxidative Stress: Introductory Remarks.*, in *Oxidative Stress*, H. Sies, Editor. 1985, Academic Press: London: London. p. 1-8.

142. Sies, H. and D.P. Jones, *Oxidative Stress*, in *Encyclopedia of Stress*, G. Fink, Editor. 2007, Academic Press. p. 45-48.

143. Jones, D.P., et al., *Redox analysis of human plasma allows separation of pro-oxidant events of aging from decline in antioxidant defenses.* Free radical biology & medicine, 2002. **33**(9): p. 1290-300.

144.   Stocker, R. and J.F. Keaney, Jr., *Role of oxidative modifications in atherosclerosis.* Physiological reviews, 2004. **84**(4): p. 1381-478.

145.   Bonnefont-Rousselot, D., *The role of antioxidant micronutrients in the prevention of diabetic complications.* Treatments in endocrinology, 2004. **3**(1): p. 41-52.

146.   Cantin, A.M., *Potential for antioxidant therapy of cystic fibrosis.* Current opinion in pulmonary medicine, 2004. **10**(6): p. 531-6.

147.   Vina, J., et al., *Molecular bases of the treatment of Alzheimer's disease with antioxidants: prevention of oxidative stress.* Molecular aspects of medicine, 2004. **25**(1-2): p. 117-23.

148.   Berger, M.M., *Can oxidative damage be treated nutritionally?* Clinical nutrition, 2005. **24**(2): p. 172-83.

149.   Morgan, P.E., A.D. Sturgess, and M.J. Davies, *Increased levels of serum protein oxidation and correlation with disease activity in systemic lupus erythematosus.* Arthritis and rheumatism, 2005. **52**(7): p. 2069-79.

150.   Avalos, I., et al., *Oxidative stress in systemic lupus erythematosus: relationship to disease activity and symptoms.* Lupus, 2007. **16**(3): p. 195-200.

151.   Wang, G., et al., *Markers of oxidative and nitrosative stress in systemic lupus erythematosus: correlation with disease activity.* Arthritis and rheumatism, 2010. **62**(7): p. 2064-72.

152.   Zhang, Q., et al., *Oxidative protein damage and antioxidant status in systemic lupus erythematosus.* Clinical and experimental dermatology, 2010. **35**(3): p. 287-94.

153.   Nagy, G., A. Koncz, and A. Perl, *T cell activation-induced mitochondrial hyperpolarization is mediated by Ca2+- and redox-dependent production of nitric oxide.* J Immunol, 2003. **171**(10): p. 5188-97.

154.   Ibiza, S., et al., *Endothelial nitric oxide synthase regulates T cell receptor signaling at the immunological synapse.* Immunity, 2006. **24**(6): p. 753-65.

155.   Niedbala, W., et al., *Nitric oxide preferentially induces type 1 T cell differentiation by selectively up-regulating IL-12 receptor beta 2 expression via cGMP.* Proc Natl Acad Sci U S A, 2002. **99**(25): p. 16186-91.

156.   Koncz, A., et al., *Nitric oxide mediates T cell cytokine production and signal transduction in histidine decarboxylase knockout mice.* J Immunol, 2007. **179**(10): p. 6613-9.

157.   Oates, J.C., et al., *Association of serum nitrate and nitrite levels with longitudinal assessments of disease activity and damage in systemic lupus erythematosus and lupus nephritis.* Arthritis Rheum, 2008. **58**(1): p. 263-72.

158.   Nagy, G., et al., *Central role of nitric oxide in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus.* Arthritis Res Ther, 2010. **12**(3): p. 210.

159.   Perl, A., *Emerging new pathways of pathogenesis and targets for treatment in systemic lupus erythematosus and Sjogren's syndrome.* Curr Opin Rheumatol, 2009. **21**(5): p. 443-7.

160.   Perl, A., et al., *T-cell and B-cell signaling biomarkers and treatment targets in lupus.* Curr Opin Rheumatol, 2009. **21**(5): p. 454-64.

161.   Perl, A., et al., *Mitochondrial hyperpolarization: a checkpoint of T-cell life, death and autoimmunity.* Trends Immunol, 2004. **25**(7): p. 360-7.

162.   Perl, A., et al., *Apoptosis and mitochondrial dysfunction in lymphocytes of patients with systemic lupus erythematosus.* Methods Mol Med, 2004. **102**: p. 87-114.

163.   Nagy, G., et al., *Nitric oxide-dependent mitochondrial biogenesis generates Ca2+ signaling profile of lupus T cells.* J Immunol, 2004. **173**(6): p. 3676-83.

164.   Fernandez, D.R., et al., *Activation of mammalian target of rapamycin controls the loss of TCRzeta in lupus T cells through HRES-1/Rab4-regulated lysosomal degradation.* J Immunol, 2009. **182**(4): p. 2063-73.

165.   Kirchgessner, H., et al., *The transmembrane adaptor protein TRIM regulates T cell receptor (TCR) expression and TCR-mediated signaling via an association with the TCR zeta chain.* J Exp Med, 2001. **193**(11): p. 1269-84.

166.   Gergely, P., Jr., et al., *Mitochondrial hyperpolarization and ATP depletion in patients with systemic lupus erythematosus.* Arthritis Rheum, 2002. **46**(1): p. 175-90.

167.   Oates, J.C., et al., *Prospective measure of serum 3-nitrotyrosine levels in systemic lupus erythematosus: correlation with disease activity.* Proceedings of the Association of American Physicians, 1999. **111**(6): p. 611-21.

168.   MacLeod, A.R., J. Rouleau, and M. Szyf, *Regulation of DNA methylation by the Ras signaling pathway.* The Journal of biological chemistry, 1995. **270**(19): p. 11327-37.

169. Yang, J., et al., *Effect of mitogenic stimulation and DNA methylation on human T cell DNA methyltransferase expression and activity.* J Immunol, 1997. **159**(3): p. 1303-9.

170. Deng, C., et al., *Role of the ras-MAPK signaling pathway in the DNA methyltransferase response to DNA hypomethylation.* Biological chemistry, 1998. **379**(8-9): p. 1113-20.

171. Richardson, B.C., M.R. Liebling, and J.L. Hudson, *CD4+ cells treated with DNA methylation inhibitors induce autologous B cell differentiation.* Clin Immunol Immunopathol, 1990. **55**(3): p. 368-81.

172. Deng, C., et al., *Hydralazine may induce autoimmunity by inhibiting extracellular signal-regulated kinase pathway signaling.* Arthritis Rheum, 2003. **48**(3): p. 746-56.

173. Sawalha, A.H., et al., *Defective T-cell ERK signaling induces interferon-regulated gene expression and overexpression of methylation-sensitive genes similar to lupus patients.* Genes Immun, 2008. **9**(4): p. 368-78.

174. Miyamoto, A., et al., *Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cdelta.* Nature, 2002. **416**(6883): p. 865-9.

175. Richardson, B., *Primer: epigenetics of autoimmunity.* Nat Clin Pract Rheumatol, 2007. **3**(9): p. 521-7.

176. Bombardier, C., et al., *Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE.* Arthritis Rheum, 1992. **35**(6): p. 630-40.

177. Shankar, A.H. and A.S. Prasad, *Zinc and immune function: the biological basis of altered resistance to infection.* Am J Clin Nutr, 1998. **68**(2 Suppl): p. 447S-463S.

178. Fernandes, G., et al., *Impairment of cell-mediated immunity functions by dietary zinc deficiency in mice.* Proc Natl Acad Sci U S A, 1979. **76**(1): p. 457-61.

179. Pekarek, R.S., et al., *Abnormal cellular immune responses during acquired zinc deficiency.* Am J Clin Nutr, 1979. **32**(7): p. 1466-71.

180. Sazawal, S., et al., *Effect of zinc supplementation on cell-mediated immunity and lymphocyte subsets in preschool children.* Indian Pediatr, 1997. **34**(7): p. 589-97.

181. Kramer, T.R., et al., *Lymphocyte responsiveness of children supplemented with vitamin A and zinc.* Am J Clin Nutr, 1993. **58**(4): p. 566-70.

182.   McMahon, L.J., et al., *In vitro effects of zinc chloride on spontaneous sheep red blood cell (e) rosette formation by lymphocytes from cancer patients and normal subjects.* Immunol Commun, 1976. **5**(1-2): p. 53-67.

183.   Martinotti, S., et al., *Heavy-metal modulation of the human intercellular adhesion molecule (ICAM-1) gene expression.* Biochim Biophys Acta, 1995. **1261**(1): p. 107-14.

184.   Richardson, B.C., *Epigenetics and autoimmunity. Overview.* Autoimmunity, 2008. **41**(4): p. 243-4.

185.   Wu, T., et al., *Metabolic disturbances associated with systemic lupus erythematosus.* PloS one, 2012. **7**(6): p. e37210.

186.   Stampfer, M.J., et al., *A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians.* JAMA, 1992. **268**(7): p. 877-81.

187.   Selhub, J., et al., *Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis.* N Engl J Med, 1995. **332**(5): p. 286-91.

188.   Clarke, R., et al., *Hyperhomocysteinemia: an independent risk factor for vascular disease.* N Engl J Med, 1991. **324**(17): p. 1149-55.

189.   Verhoef, P., et al., *A prospective study of plasma homocyst(e)ine and risk of ischemic stroke.* Stroke, 1994. **25**(10): p. 1924-30.

190.   Selhub, J., et al., *Vitamin status and intake as primary determinants of homocysteinemia in an elderly population.* JAMA, 1993. **270**(22): p. 2693-8.

191.   Kim, Y.I., *Folate and carcinogenesis: evidence, mechanisms, and implications.* J Nutr Biochem, 1999. **10**(2): p. 66-88.

192.   Kim, Y.I., *Role of folate in colon cancer development and progression.* J Nutr, 2003. **133**(11 Suppl 1): p. 3731S-3739S.

193.   Kim, Y.I., *Folate and colorectal cancer: an evidence-based critical review.* Mol Nutr Food Res, 2007. **51**(3): p. 267-92.

194.   Kim, Y.I., *Folic acid supplementation and cancer risk: point.* Cancer Epidemiol Biomarkers Prev, 2008. **17**(9): p. 2220-5.

195.   Arthur, A.E., et al., *Diet and proinflammatory cytokine levels in head and neck squamous cell carcinoma.* Cancer, 2014.

196.   DeBaun, M.R., E.L. Niemitz, and A.P. Feinberg, *Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19.* Am J Hum Genet, 2003. **72**(1): p. 156-60.

197. Pacher, P., J.S. Beckman, and L. Liaudet, *Nitric oxide and peroxynitrite in health and disease.* Physiol Rev, 2007. **87**(1): p. 315-424.

198. Fukagawa, N.K., *Sparing of methionine requirements: evaluation of human data takes sulfur amino acids beyond protein.* J Nutr, 2006. **136**(6 Suppl): p. 1676S-1681S.

199. Oaks, Z. and A. Perl, *Metabolic control of the epigenome in systemic Lupus erythematosus.* Autoimmunity, 2013.

200. Welch, G.N. and J. Loscalzo, *Homocysteine and atherothrombosis.* N Engl J Med, 1998. **338**(15): p. 1042-50.

201. Ingrosso, D., et al., *Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia.* Lancet, 2003. **361**(9370): p. 1693-9.

202. Perl, A., R. Hanczko, and E. Doherty, *Assessment of mitochondrial dysfunction in lymphocytes of patients with systemic lupus erythematosus.* Methods Mol Biol, 2012. **900**: p. 61-89.

203. Mihm, M.J. and J.A. Bauer, *Peroxynitrite-induced inhibition and nitration of cardiac myofibrillar creatine kinase.* Biochimie, 2002. **84**(10): p. 1013-9.

204. Denicola, A., et al., *Peroxynitrite-mediated cytotoxicity to Trypanosoma cruzi.* Arch Biochem Biophys, 1993. **304**(1): p. 279-86.

205. Jeffries, M.A., et al., *Genome-wide DNA methylation patterns in CD4+ T cells from patients with systemic lupus erythematosus.* Epigenetics, 2011. **6**(5): p. 593-601.

206. Moreno, J.J. and W.A. Pryor, *Inactivation of alpha 1-proteinase inhibitor by peroxynitrite.* Chem Res Toxicol, 1992. **5**(3): p. 425-31.

207. *National Institutes of Health Autoimmune Disease Coordinating Committee Report, 2005.* 2005: Bethesda (MD).

208. Boyce, E.G. and B.E. Fusco, *Belimumab: review of use in systemic lupus erythematosus.* Clin Ther, 2012. **34**(5): p. 1006-22.

209. Somers, E.C., et al., *Incidence of systemic lupus erythematosus in the United Kingdom, 1990-1999.* Arthritis Rheum, 2007. **57**(4): p. 612-8.