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Age-dependent decreases in DNA methyltransferase levels and low transmethylation micronutrient levels synergize to promote overexpression of genes implicated in autoimmunity and acute coronary syndromes

YePeng Li^a, Ying Liu^a, Faith M. Strickland^a, and Bruce Richardson^{a,b,*}

^a Dept. of Medicine, University of Michigan, Ann Arbor MI 48109, USA

^b Ann Arbor VA Medical Center, Ann Arbor MI 48105, USA

Abstract

T cell DNA methylation levels decline with age, activating genes such as KIR and TNFSF7 (CD70), implicated in lupus-like autoimmunity and acute coronary syndromes. The mechanisms causing age-dependent DNA demethylation are unclear. Maintenance of DNA methylation depends on DNA methyltransferase 1 (Dnmt1) and intracellular S-adenosylmethionine levels, and is inhibited by S-adenosylhomocysteine (SAH). SAM levels depend on dietary micronutrients including folate and methionine. SAH levels depend on serum homocysteine concentrations. T cell Dnmt1 levels also decline with age. We hypothesized that age-dependent Dnmt1 decreases synergize with low folate, low methionine or high homocysteine levels to demethylate and activate methylation-sensitive genes. T cells from healthy adults ages 22–81, stimulated and cultured with low folate, low methionine, or high homocysteine concentrations showed demethylation and overexpression of KIR and CD70 beginning at age ~50 and increased further with age. The effects were reproduced by Dnmt1 knockdowns in T cells from young subjects. These results indicate that maintenance of T cell DNA methylation patterns is more sensitive to low folate and methionine levels in older than younger individuals, due to low Dnmt1 levels, and that homocysteine further increases aberrant gene expression. Thus, attention to proper nutrition may be particularly important in the elderly.

Keywords

Aging; Epigenetics; DNA methylation; Senescence

1. Introduction

DNA methylation is a post-synthetic modification that silences gene expression. DNA methylation patterns are established during differentiation, and serve to suppress genes

* Corresponding Author: Tel. 734 647-6383, Fax 734 936-9220; brichard@umich.edu. (B Richardson).

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unnecessary or detrimental to the function of any given cell, but for which the cell expresses activating transcription factors. DNA methylation is particularly important in T lymphocytes, which differentiate throughout life into multiple subsets with distinct functions but express a partially overlapping repertoire of transcription factors. Consequently, some subset-specific genes are silenced primarily by DNA methylation. For example, inhibiting T lymphocyte DNA methylation activates the Th1 cytokine IFN- γ in Th2 cells, the Th2 cytokine IL-4 in Th1 cells, the cytotoxic molecule perforin in CD4 cells, and the KIR gene family, normally confined to natural killer (NK) lymphocytes, on CD4 and CD8 cells (Basu et al., 2009; Liu et al., 2009a; Lu et al., 2003; Richardson, 2007). These studies support the importance of DNA methylation in maintaining subset-specific T lymphocyte gene expression.

DNA methylation patterns must be replicated each time a cell divides. However, the maintenance of DNA methylation patterns deteriorates with age in most cells, causing aberrant methylation of some CpG islands that can lead to malignant transformation, as well as demethylation of other regions that promotes gene overexpression (Yung and Julius, 2008). In T cells a small number of CpG islands methylate with age, suppressing gene expression (Tra et al., 2002). However, the predominant effect is a decline in total deoxymethylcytosine (d^mC) content, causing aberrant overexpression of genes normally silenced by methylation (Richardson, 2003). The mechanisms causing T cell DNA demethylation are important to understand, because the demethylation may contribute to the development of lupus-like autoimmunity as well as acute coronary syndromes, by causing aberrant overexpression of genes including the KIR gene family, perforin, CD70, IFN- γ , LFA-1 and others (Jones and Chen, 2006; Kaplan et al., 2004; Liu et al., 2009b; Liu et al., 2009a; Lu et al., 2002; Lu et al., 2005; Nakajima et al., 2003).

DNA methylation patterns are replicated during mitosis by the maintenance DNA methyltransferase Dnmt1. Dnmt1 binds proliferating cell nuclear antigen (PCNA) in the replication fork and recognizes CpG pairs (Iida et al., 2002). If the parent DNA strand is methylated, Dnmt1 catalyzes the transfer of the methyl group from S-adenosylmethionine to the corresponding dC in the daughter strand, producing 5-methylcytosine (mC) and S-adenosylhomocysteine (SAH) (Ross and Poirier, 2002):

The forward velocity of this reaction is directly proportional to SAM concentrations and Dnmt1 activity, and inversely proportional to SAH:

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

T cell Dnmt1 levels decrease with age, which contributes to the decline in overall d^mC content (Zhang et al., 2002). However, diet is also important in replicating DNA methylation patterns, and is a mechanism by which the environment can modify the epigenome. SAM levels depend on micronutrients such as folate and methionine (Met) (Ross and Poirier, 2002), and restricting dietary methyl donors can demethylate DNA in tissues like the liver (Pogribny et al., 1995) and peripheral blood leukocytes (Rampersaud et al., 2000). SAH levels are also affected by diet.

SAH concentrations are dependent on homocysteine (Hcy) levels, and folate is required for the metabolism of Hcy to Met (Ross and Poirier, 2002). Elevated serum Hcy levels contribute to DNA demethylation in conditions such as chronic renal failure, and folate supplementation

can reverse this effect (Ingrosso et al., 2003). How dietary transmethylation micronutrients, Hcy and decreased Dnmt1 levels interact to cause decreases in DNA methylation and aberrant gene overexpression in aging is unknown.

We hypothesized that decreases in folate or Met, increases in Hcy, and age-dependent decreases in Dnmt1 levels may be synergistic in inhibiting T cell DNA methylation. We therefore cultured T cells from healthy young and older individuals, as well as T cell Dnmt1 “knockdowns”, in media containing variable amounts of folate, Met, and/or Hcy, and examined the effects on the expression and methylation of genes normally suppressed by DNA methylation. The results suggest a potentially important mechanism increasing sensitivity of older people, and perhaps people with some forms of autoimmunity, to adverse effects from a diet poor in methyl donors.

2. Materials and Methods

2.1. Subjects

Healthy men and women ages 22-81 were recruited from the Arthritis clinic of the University of Michigan, the Human Subjects Core of the University of Michigan Claude D. Pepper Older Americans Independence Center, and by advertising. The older cohort (age > 50) was 50% women, 50% men and 90% Caucasian, 10% African-American. The younger cohort (age < 50) was 44% male, 56% female and 88% Caucasian, 12% Asian. Subjects with autoimmune or other inflammatory conditions were specifically excluded, as were subjects receiving medications known to affect DNA methylation such as procainamide and hydralazine. The protocols were approved by the University of Michigan IRB.

2.2. Cells and cell culture

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation then cultured in complete tissue culture medium (RPMI 1640/10% FBS) and stimulated for 18-24 h with 1 µ/ml phytohemagglutinin (PHA) (Murex, Norcross, GA) as previously described (Lu and Richardson, 2004). The cells were then washed with PBS X3 and cultured in custom media containing 40 nM or 10 nM folate, or 30 µM or 5 µM Met and supplemented with 10% FBS dialyzed against Hanks balanced salt solution (HBSS), 1% Pen/Strep (Gibco, Grand Island, New York) and 10 ng/ml recombinant IL-2 (Peprotech, Rocky Hill, NJ) for another 72 hours. RPMI 1640 contains no Hcy (Table 1), so where indicated Hcy (mixed D and L enantiomers, Sigma Chemical Co, St. Louis MO) was added at 15 µM together with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (Sigma). Controls included cells cultured with EHNA and no Hcy (Kredich and Martin, 1977). Total T cells, CD4⁺ T cells or CD8⁺ T cells were then purified using the pan T cell isolation kit II or the CD4⁺ T cell isolation kit II from Miltenyi (Auburn, CA).

2.3. Flow cytometric analysis

The following monoclonal antibodies were used: CY5-anti-CD3, FITC-anti-CD8, CY5-anti-CD8, CY5-anti-CD4, and FITC-anti-CD28 (all from BD PharMingen, San Diego, CA). Anti-CD158b1/b2,j-PE (GL183, reactive with KIR 2DL2/2DL3/2DS2) was obtained from Beckman Coulter Immunotech (Fullerton, CA). Cell staining and fixation were performed as previously described (Liu et al., 2009b), and cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes NJ).

2.4. Real-time RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA). KIR2DL2, CD70, Dnmt1, β -actin, and histone H4 transcripts were measured in magnetic bead purified T cells using a Rotor-Gene 3000 (Corbett Robotics, San Francisco, CA) and the QuantiTect

SYBR Green RT-PCR kit (QIAGEN) according to the manufacturer's instructions. CD70, Dnmt1, Dnmt3a, β -actin and histone H4 primers and amplification protocols were as published (Liu et al., 2009b; Liu et al., 2009a; Lu et al., 2005). Primers designed to match polymorphic positions unique to KIR2DL2 genes were as described by Uhrberg et al (Liu et al., 2009a; Uhrberg et al., 1997).

2.5. T cell siRNA transfection and culture

PBMC from healthy subjects were isolated by density gradient centrifugation and total T cells purified using a Pan T cell isolation kit II (Miltenyi). T cells were typically > 94% CD3⁺. The T cells were then cultured in hTC Culture medium (Amaxa, Gaithersburg MD) containing 10% fetal bovine serum and 1% penicillin/streptomycin. and transfected with 50nM siRNA-DNMT1 (QIAGEN) using the Amaxa human T cell nucleofector kit and program U-14. Controls included a nonspecific siRNA provided by the manufacturer. 6 hours later the cells were cultured in the custom Met or folate media and stimulated with PHA. Dnmt1 transcripts were measured relative to histone H4 24 hours following stimulation, while methylation and expression of CD70 and KIR2DL2 were measured relative to β -actin 72 hours after PHA stimulation.

2.6. Bisulfite Sequencing

Genomic DNA was isolated from T cells using FlexiGene DNA Kits (QIAGEN) then treated with sodium bisulfite using KIR2DL2 primers and amplification as published, interrogating 18 CG pairs (Liu et al., 2009b; Liu et al., 2009a). PCR products were purified using QIAEXII Gel Extraction Kits (QIAGEN), and cloned using the pGEM-T Easy Vector System (Promega, Madison, WI). 10 cloned fragments were sequenced for each sample by the University of Michigan Sequencing Core.

2.7. Statistical analysis

Differences in gene expression between cells cultured in media with differing concentrations of folate or Met were determined using Student's t-test, and the relationship between age and gene expression by ANOVA, using Systat software (Chicago IL).

3. Results

3.1. T cells from older people demethylate and express KIR genes when folate or Met concentrations are limiting

KIR genes are normally expressed by NK cells and a senescent CD28⁻ T cell subset, but not on normal T cells (Liu et al., 2009b). However, inhibiting DNA methylation in vitro with the irreversible DNA methyltransferase inhibitor 5-azacytidine induces KIR gene expression in CD4⁺ and CD8⁺ T cells from healthy young people (Liu et al., 2009a). We used a similar protocol to compare the effects of folate and Met restriction on KIR gene expression in T cells from healthy subjects with a wide age range. PBMC from subjects ages 25-76 were stimulated with PHA, cultured briefly (72 hours) in media containing dialyzed FCS and variable concentrations of Met (30 μ M or 5 μ M) or folate (40 nM or 10 nM), corresponding to normal or low human serum levels (Table 1), then stained with mAb to CD4, CD8, and a mAb crossreactive with KIR2DL2/2DL3/2DS2 (KIR) and analyzed by multicolor flow cytometry. KIR2DL3 is found in approximately 90% of Caucasians, which were the majority of the subjects studied, as well in ~90% of individuals from other ethnic groups, while KIR2DL2 and 2DS2 are found in approximately 50% of these ethnic groups (Denis et al., 2005; Momot et al., 2006). Figure 1A shows that varying Met concentrations has little effect on KIR expression in CD4⁺ T cells from younger (26-36 years old) individuals ($p > 0.05$ by paired t-test, low vs high Met concentrations). In contrast CD4⁺ T cells from the older (57-76 years) subjects

expressed higher levels of KIR when cultured in low Met concentrations relative to high concentrations ($p=0.012$). In addition more CD4+ T cells from the older individuals expressed KIR genes when cultured in either 30 μM Met ($0.23\pm0.07\%$ vs $1.65\pm0.43\%$, young vs old, mean $\pm$ SEM, $p=0.017$ by 2 sample t-test) or 5 μM Met ($0.36\pm0.08\%$ vs $3.08\pm0.80\%$, $p=0.013$).

Figs. 1 A-D show that varying folate or Met concentrations has little effect on KIR expression in CD4+ or CD8+ T cells from young (25-45 years) subjects. In contrast, a subset of CD4+ and CD8+ T cells from older (> 50 years) subjects express KIR when cultured in media containing 30 μM Met or 40 nM folate, and decreasing the Met or folate concentrations causes a further increase in the number of cells affected ($p\leq0.022$ high vs low folate/Met concentrations in the older group). Overall the percentage of cells expressing KIR increases significantly with increasing age ($p\leq0.016$ in all 4 experiments by ANOVA). Controls included culturing the same cells in complete media. There was no significant difference in KIR expression on CD8+ T cells from young and old individuals or on CD4+ T cells from older individuals relative to T cells cultured in complete media or media containing 30 μM Met or 40 nM folate (not shown). However, there was a small but statistically significant increase in KIR expression when CD4+ T cells from young individuals were cultured in media containing 40 nM folate relative to complete media ($0.63\pm0.11\%$ vs $0.34\pm0.13\%$, 40 nM folate vs complete media, mean $\pm$ SEM, $p=0.018$). The significance of this small increase is uncertain.

These results were confirmed at the mRNA level using RT-PCR. CD4+ T cells from 3 younger (mean age 30 years) and 3-4 older subjects (mean ages 67-72 years) were stimulated and cultured in media containing similar concentrations of Met (30 μM or 5 μM) or folate (40 nM or 10 nM), then KIR2DL2 transcripts were measured relative to β -actin. Figures 1E and 1F show that restricting Met or folate increases KIR2DL2 mRNA levels to a greater extent in the older subjects. Also, KIR2DL2 levels were higher in CD4+ T cells from the older vs younger donors cultured in media containing 30 μM Met ($p=0.026$), 5 μM Met ($p=0.009$), 40 nM folate ($p=0.02$) or 10 nM folate ($p=0.02$).

The effect of Met and folate restriction on KIR2DL2 promoter (bp -257 - +95) methylation was measured. CD4+ T cell DNA from three younger (31 ± 13 years) and three older (69 ± 10.6 years) healthy subjects from Fig 1 was analyzed by bisulfite sequencing, cloning and sequencing 10 fragments from each subject. Fig 2A shows the average methylation of the 10 determinations for each of the CG pairs in CD4+ T cells from a representative young subject following culture in 40 nM folate media. Fig 2B shows the methylation pattern in cells from the same subject cultured with 10 nM folate. In the young subject, the promoter remains almost completely methylated regardless of the folate concentration. In contrast, many of the CG sites in the KIR2DL2 promoter were partially demethylated in an older subject following culture in 40 nM folate (Fig. 2C). Low (10 nM) folate caused even more demethylation in cells from the older subject (Fig. 2D), particularly in the region between -36 and -215, compared to either higher (40 nM) folate concentrations or CD4+ T cells from the young subject cultured in either folate concentration (Fig. 2D vs Fig 2A, 2B). The effect of folate and Met concentrations on the average methylation across the region between -36 and -215 in CD4+ T cells from all 3 younger and all 3 older subjects is summarized in Figs 2E and Fig 2F. Low folate levels caused a statistically significant ($p=0.0045$ by Student's t-test) decrease in overall promoter methylation only in T cells from older subjects compared to T cells from the young (Fig 2E). Similar results were observed for Met (Fig 2F). Decreases in KIR2DL2 promoter methylation were also observed in T cells from the older individuals when cultured in the higher concentrations of folate or Met, but these did not achieve statistical significance.

3.2. Limiting Met/folate increases CD70 expression on T cells from older people

The effects of age, Met and folate were confirmed for TNFSF7 (CD70), another T cell gene regulated in part by DNA methylation. Fig 3A compares the effects of 30 and 5 M Met on CD4

+ T cells. In contrast to KIR, there was no significant increase in CD70 protein expression with age on CD4+ T cells cultured in media containing 30 μ M Met ($p=0.217$ by ANOVA). However, an age-dependent increase was observed when the same cells were cultured in media containing 5 μ M Met ($p=0.03$). Fig 3B shows similar studies comparing CD70 expression on CD8+ T cells. There was an age-dependent increase in CD70 expression on CD8+ T cells cultured in 30 μ M ($p=0.001$) and 5 μ M Met ($p<0.001$), and CD70 expression was also higher on CD4+ T cells from the older subjects when cultured in 5 μ M Met than in 30 μ M Met ($p=0.012$ by paired t-test).

Fig 3C compares the effect of age on CD70 expression by CD4+ T cells cultured in 40 or 10 nM folate. While there was a small increase in CD70 protein with age in media containing 40 nM folate this increase was not significant ($p=0.266$). However, a significant ($p=0.007$) increase was seen in media containing 10 nM folate. Fig 3D shows similar studies using CD8+ T cells. An age dependent increase in CD70 expression was seen using 40 nM ($p=0.002$) and 10 nM ($p<0.001$) folate, and more CD8+ T cells from the older individuals expressed CD70 when cultured in 10nM vs 40 nM folate ($p=0.0006$ by paired t-test). Controls again included similarly culturing cells from the younger and older subjects in complete RPMI 1640 medium. Compared to T cells cultured in media containing 30 μ M Met or 40 nM folate, there was no difference in CD70 expression on CD4+ or CD8+ T cells from young or old individuals (not shown).

These results were confirmed using RT-PCR. CD4+ T cells from 3 younger and 3-4 older subjects were stimulated and cultured in media containing variable amounts of Met (30 μ M or 5 μ M) or folate (40 nM or 10 nM) as before, then CD70 transcripts were measured relative to β -actin. There was a significant increase in CD70 mRNA in T cells from older people cultured in media contain 5 μ M vs 30 μ M Met (Fig. 3E). A similar increase in was observed in T cells from the older subjects when cultured in media containing 10 vs 40 nM folate (Fig 3F). While smaller increases were observed in T cells from the younger donors, these were not statistically significant. CD70 mRNA levels were higher in CD4+ T cells from the older vs younger donors when cultured in media containing 30 μ M Met ($p=0.002$), 5 μ M Met ($p=0.04$), 40 nM folate ($p=0.009$) or 10 nM folate ($p=0.003$).

3.3. Hcy increases KIR expression only in older people and only when folate levels are low

Since elevated serum Hcy levels contribute to DNA demethylation and folate is required for the metabolism of Hcy to Met (Ross and Poirier, 2002), we examined the interaction of Hcy and folate on the expression of methylation-sensitive genes. PBMC from healthy subjects ages 21-75 were stimulated with PHA and cultured in media containing 40 or 10 nM folate as in Fig 1, but this time with or without 15 μ M Hcy. Fig 4A shows the effects of age, 40 nM folate and 0 and 15 μ M Hcy on KIR expression by CD4+ T cells. An age-dependent increase in KIR expression was seen as in Fig 1, but Hcy had no further effect. In contrast, Hcy caused a significant ($p=0.018$ by paired t-test) increase in KIR expression when T cells from the older subjects were cultured in media containing 10 nM folate (Fig 4B). Figs 4C and 4D show the same experiments using CD8+ T cells from the same subjects. Again, an interaction between age and KIR expression is seen, and Hcy has no effect in the presence of 40 nM folate (4C), but a KIR increase is seen in T cells from older but not younger subjects when cultured with Hcy and 10 nM folate (4D, $p=0.005$ by paired t-test).

3.4. Age, but not low Met or folate concentrations, decreases T cell Dnmt1 levels

We have previously reported that T cell Dnmt1 levels decline with age (Zhang et al., 2002). However, the effects of folate and Met combined with aging on Dnmt1 levels are unknown. We therefore, considered the possibility that low folate or Met levels may alter T cell gene expression through effects on Dnmt1 levels. PBMC from five healthy younger (28.4 ± 10.0

years) and five healthy older (63.2 ± 7.2 years) subjects were stimulated and cultured in media containing 30 or 5 μM Met or 40 or 10 nM folate as before and 24 hours later Dnmt1 transcripts were measured in CD4⁺ T cells. Fig 5A compares the effects of 30 and 5 μM Met on Dnmt1 levels in CD4⁺ T cells from the young and old subjects. While Dnmt1 levels were lower in the older group as expected ($p < 0.01$), varying the Met concentration had no effect on Dnmt1 levels within each age group (1.61 ± 0.23 vs 1.78 ± 0.29 , mean $\pm$ SEM, 30 vs 5 μM Met in the young and 0.71 ± 0.07 vs 0.63 ± 0.06 , mean $\pm$ SEM, 30 vs 5 μM Met in the older). Fig 5B similarly shows an age-dependent decrease in Dnmt1, but again there was no difference in Dnmt1 levels in T cells cultured in 40 or 10 nM folate within each age group (1.57 ± 0.19 vs 1.78 ± 0.22 40 vs 10 nM folate in the young, and 0.85 ± 0.06 vs 0.77 ± 0.05 , 40 vs 10 nM folate in the older). Fig 5C shows the relationship between age and Dnmt1 levels in the CD4⁺ T cells from the 10 subjects cultured in media containing 40 nM folate, and confirms a progressive decrease with age.

3.5. Decreasing Dnmt1 is sufficient to make T cells sensitive to low Met/folate levels

The relationship between low Dnmt1 levels, low Met and folate concentrations and increased KIR expression was confirmed using siRNA “knock downs”. T cells were isolated from four healthy young subjects (average age 23.3 ± 0.96 years), transfected with control or Dnmt1 siRNA, stimulated with PHA, then cultured in media containing 30 or 5 μM Met (Fig 6A) or 40 or 10 nM folate (Fig 6B) for another 72 hours. Dnmt1 transcript levels in cells transfected with the Dnmt1 siRNA were $50 \pm 3.39\%$ of controls, similar in magnitude to the suppression previously reported by our group (Liu et al., 2009b) and to those shown in Figs 5A and 5B. Our group has previously reported a good correlation between T cell Dnmt1 mRNA and protein levels across a wide range of mRNA levels (Chen et al., 2009; Zhang et al., 2002). KIR2DL2 transcripts were measured relative to β -actin in these cells using RT-PCR. Met or folate restriction had no significant effect on KIR2DL2 expression by T cells transfected with the control siRNA. In contrast, both Met and folate restriction caused a significant ($p < 0.04$) increase in KIR2DL2 levels in T cells transfected with the Dnmt1 siRNA, confirming an interaction between decreased Dnmt1 levels, Met/folate restriction and methylation sensitive gene overexpression.

3.6. Effect of Met/folate restriction on KIR expression in CD28⁺ and CD28[−] T cells

The results presented above demonstrate that Met/folate restriction causes KIR expression on a relatively small number of CD4⁺ and CD8⁺ T cells. We considered the possibility that Met/folate restriction preferentially affects a subset of T cells. “Senescent” T cells, characterized by the absence of CD28, develop with aging, in chronic inflammatory/autoimmune diseases like rheumatoid arthritis, and with replicative senescence (Vallejo, 2005). These cells have decreased Dnmt1 levels and are the only T cell subset known to demethylate and overexpress KIR genes (Liu et al., 2009b) with the exception of a pathogenic CD28⁺ subset in patients with active lupus (Basu et al., 2009). We therefore compared the effects of micronutrient restriction on CD28⁺ and CD28[−] T cells from young and older healthy subjects. Fig 7A compares the effects of 30 and 5 μM Met on KIR expression on CD4⁺CD28⁺ T cells, and Fig 7B their effects on CD4⁺CD28[−] T cells. As before, Met restriction had no significant effect on T cells from the younger donors, while 5 μM Met caused a greater increase in KIR expression than 30 μM on both CD28⁺ ($p = 0.04$) and CD28[−] ($p = 0.05$) T cells from older people. However, 5 μM Met induced KIR expression on a greater number of CD4⁺CD28[−] than CD4⁺CD28⁺ T cells from the older group ($9.1 \pm 2.4\%$ vs $2.7 \pm 0.3\%$, mean $\pm$ SEM, $p = 0.04$). Fig 7C similarly compares the effects of 40 and 10 nM folate on KIR expression on CD4⁺CD28⁺ T cells from the same subjects, while Fig 7D shows the effects on the CD4⁺CD28[−] subset. Again, folate restriction increases KIR expression on both CD4⁺CD28⁺ and CD4⁺CD28[−] T cells, but more CD28[−] cells express KIR than CD28⁺ cells particularly when cultured in 10 nM folate ($8.1 \pm 2.5\%$ vs $3.1 \pm 0.5\%$, $p < 0.001$).

The same experiments were performed on CD8⁺ T cells from the same subjects. Fig 7E compares the effect of 30 and 5 μ M Met on KIR expression on CD8⁺CD28⁺ T cells, while Fig 7F compares their effects on KIR expression on CD8⁺CD28⁻ T cells. Again, Met restriction had no significant effect on T cells from the younger donors, while in the older subjects 5 μ M Met caused a greater increase in KIR expression than 30 μ M on both CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells, and 5 μ M Met induced KIR expression on a greater number of CD8⁺CD28⁻ T cells than on CD8⁺CD28⁺ T cells from the older group ($10.5 \pm 3.3\%$ vs. $3.0 \pm 0.8\%$, mean $\pm$ SEM, $p=0.05$). Similarly, Fig 7G compares the effects of 40 and 10 nM folate on KIR expression on CD8⁺CD28⁺ T cells from the same subjects, while Fig 7H shows the effects on the CD8⁺CD28⁻ subset. Folate restriction increased KIR expression on both CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells. However, the effect of folate restriction on CD8⁺CD28⁺ T cells was not significant ($p=0.06$), while the effect on the CD28⁻ subset was somewhat more significant ($p=0.04$). There was no significant difference in KIR⁺ cells between the CD8⁺CD28⁺ and CD8⁺CD28⁻ subsets when cultured in 10 nM folate. Together these results demonstrate that Met/folate restriction can affect gene expression in both CD28⁺ and CD28⁻ T cells from the older group, but that the effect tends to be greater in CD28⁻ T cells, consistent with their lower Dnmt1 levels (Liu et al., 2009b).

4.0. Discussion

These studies demonstrate an age-dependent demethylation and overexpression of genes normally suppressed by DNA methylation when T cells are cultured in media with low Met or folate concentrations, and that Hcy potentiates the effect. While multiple age-dependent changes in transmethylation biochemistry may contribute, age-dependent decreases in Dnmt1 are likely important, since Dnmt1 siRNA knockdowns in T cells from young people reproduced the effect. T cells also express DNA methyltransferase 3a, and Dnmt3a levels decrease with aging (Liu et al., 2009b). However, selective Dnmt1 and Dnmt3a knockdowns demonstrate that Dnmt3a has only a minor role in maintenance T cell DNA methylation (Liu et al., 2009b) and so is unlikely to have a significant role in the age-dependent effects reported here. T cells express very little Dnmt3b (Zhang et al., 2002) and its role in T cell function is uncertain.

There was little effect of varying folate or Met concentrations on T cells from subjects up to ~50 years of age, after which methylation sensitive gene expression increased steadily with age, particularly when dietary transmethylation factors were limiting. Since Dnmt1 levels decrease throughout life (Zhang et al., 2002) the change may reflect a threshold effect of Dnmt1, such that Dnmt1 levels must drop below a critical level before alterations in the micronutrient concentrations have an effect.

Hcy also contributed to gene overexpression in T cells from older people, but only when folate levels were low. Hcy is converted intracellularly to SAH, and SAH binds the catalytic domain of most SAM-dependent methyltransferases with high affinity, making it a potent inhibitor of transmethylation reactions (James et al., 2002). Folate is required for the conversion of Hcy to Met (James et al., 2002), and the lower folate levels used in these studies may thus decrease the efficiency of this clearance mechanism.

In these studies Met/folate restriction had a smaller effect on KIR expression in CD4⁺ than CD8⁺ T cells from older people. Similarly, greater numbers of CD8⁺ T cells overexpressed CD70 relative to CD4⁺ cells. We and others have reported that greater numbers of CD8⁺ cells overexpress methylation sensitive genes in lupus, aging and following treatment with DNA methylation inhibitors (Li et al., 2008; Liu et al., 2009a; Lu et al., 2003). The reason is unknown, but may reflect a differential stringency in the maintenance of methylation patterns in these subsets. However, the partial effects on KIR and CD70 in both populations suggest the existence of subsets with different Dnmt1 levels. This possibility prompted studies comparing

micronutrient restriction in CD28⁺ and CD28⁻ T cells, and demonstrated a greater effect on CD28⁻ T cells. We recently reported that this subset expresses lower Dnmt1 levels than CD28⁺ cells from the same individuals (Liu et al., 2009b), supporting the contention that decreased Dnmt1 levels make CD28⁻ T cells more sensitive to nutrient restriction. However, the observation that some CD28⁺ cells can overexpress KIR suggests that Dnmt1 levels may vary within this subset as well. Since CD4⁺CD28⁺ cells do not aberrantly express methylation-sensitive markers like KIR though, the subset affected is not immediately obvious.

The aberrant overexpression of methylation sensitive genes can have pathologic consequences. The “senescent” CD4⁺CD28⁻ T cell subset, which infiltrates atherosclerotic plaques and is implicated in plaque rupture and myocardial infarctions (Nakajima et al., 2003), also overexpresses genes normally suppressed by DNA methylation in CD4⁺CD28⁺ T cells. The genes include the KIR gene family, CD70, IFN- γ and perforin (Liu et al., 2009b; Nakajima et al., 2003). This subset increases with aging as well as with proliferative stress *in vitro* and in chronic inflammatory diseases like rheumatoid arthritis (Vallejo, 2005). Interestingly, serum Hcy levels increase with aging (Refsum et al., 2006) and in patients with lupus (Roman et al., 2007), and Hcy is implicated in atherosclerotic vascular disease (Selhub et al., 1995) which accelerates in aging and lupus (Refsum et al., 2006; Roman et al., 2007). The present studies raise the possibility that increased Hcy levels may contribute to atherosclerosis and myocardial infarctions by inhibiting DNA methylation in T lymphocytes or other cell types, and raise the possibility that diets poor in Met or folate, together with effects of replicative stress on T cell Dnmt1 levels (Liu et al., 2009b), may contribute to these conditions.

Overexpression of methylation sensitive genes may also contribute to some forms of autoimmunity. Murine T cells treated with 5-azacytidine demethylate and overexpress genes like LFA-1, making them autoreactive, and these cells cause a lupus-like disease *in vivo*. T cells overexpressing LFA-1 by transfection also become autoreactive and cause a lupus-like disease *in vivo* (Quddus et al., 1993; Yung et al., 1996), suggesting that demethylation of LFA-1 and likely other genes contribute to the development of lupus-like autoimmunity. The lupus inducing drugs procainamide and hydralazine are also DNA methylation inhibitors (Cornacchia et al., 1988), and murine T cells treated with these drugs and other DNA methylation inhibitors cause a lupus-like disease in adoptive transfer models (Quddus et al., 1993; Yung et al., 1997). Finally, CD4⁺ T cells from patients with active lupus demethylate and overexpress the same genes and demonstrate the same functional changes as those caused by DNA methylation inhibitors *in vitro*, indicating a role for T cell DNA demethylation in lupus-like human autoimmunity as well (Richardson, 2007). Interestingly, a recent study of more than 1600 men and women with lupus demonstrated an age dependent increase in disease onset, with a linear increase in incidence through age 74 in men, and a steady increase in women up to menopause (Somers et al., 2007), suggesting an age contribution to lupus.

In conclusion, our results suggest that aging, a poor diet, and increased serum Hcy levels can have functionally significant effects on the expression of genes normally suppressed by DNA methylation in T lymphocytes and perhaps other cells. Further, the methylation errors may accumulate over time. This mechanism could potentially contribute to a number of poorly understood diseases of aging, and clinical trials of folate/Met supplementation may be indicated.

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Abbreviations

d ^m C	deoxymethylcytosine
Dnmt1	DNA methyltransferase 1
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
Hcy	homocysteine
HBSS	Hanks balanced salt solution
^m C	5-methylcytosine
Met	methionine
NK	natural killer
PBMC	peripheral blood mononuclear cells
PHA	phytohemagglutinin
PCNA	Proliferating cell nuclear antigen
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine

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FIGURE 1. Age and Met/folate restriction increases KIR expression on CD4+ and CD8+ T cells
 PBMC from subjects of the indicated ages were stimulated with PHA for 18-24 h, washed and cultured in media containing 10% dialyzed FBS and 30 μ M or 5 μ M Met (panels **A** and **B**), or 40 or 10 nM folate (panels **C** and **D**) for an additional 72 hours. The cells were then stained with anti-KIR2DL2/2DL3/2DS2-PE, anti-CD4-APC and anti-CD8-FITC and analyzed by flow cytometry. Results are presented as the percent CD4+KIR+ (panels **A** and **C**) or CD8+KIR+ (panels **B** and **D**) relative to total CD4+ or CD8+ cells, with the results from each subject displayed above their age. P values refer to the difference in KIR expression between cells from the subjects ages ≥ 50 cultured in media with higher vs lower Met concentrations determined by paired t-test. There was no significant difference in KIR expression on T cells

from the younger subjects when cultured in media containing higher vs lower Met concentrations ($p>0.05$). **E.** CD4⁺ T cells from 3 young people (average age 30 ± 13.9 years, mean $\pm$ SD) and 3 older (71.6 ± 10.7 years) subjects were stimulated and cultured in media containing 30 μ M (dark bars) or 5 μ M Met (light bars) as in panel A then KIR2DL2 transcripts were measured relative to β -actin (* $p=0.05$ 30 μ M Met, young vs old, ** $p=0.02$ 5 μ M Met, young vs old). **F.** CD4⁺ T cells from the same 3 young subjects and 4 older subjects (67 ± 9.7 years) were similarly stimulated and cultured in media containing 40 nM (dark bars) or 10 nM folate (light bars) (* $p=0.03$ 40nM folate, young vs old, ** $p=0.04$ 10nM folate young vs old) then KIR transcripts were measured. Results are presented as the mean $\pm$ SEM.

FIGURE 2. Effect of age and Met/folate restriction on CD4+ T cell KIR2DL2 promoter methylation

A. PBMC from a young subject were stimulated with PHA and cultured with media containing 40 nM folate as in Fig 1. DNA was then isolated from CD4+ T cells, bisulfite treated, then the region shown on the X axis was amplified and 10 fragments were cloned and sequenced. The location of each CG pair is shown on the X axis, and the average methylation of the 10 determinations for each CG pair is shown on the Y axis. **B.** PBMC from the same young subject were similarly stimulated, cultured in 10 nM folate, then the average methylation for each CG pair determined in CD4+ cells as in panel A. **C.** PBMC from an older subject were similarly stimulated, cultured in 40 nM folate and KIR2DL2 promoter methylation determined in CD4+ cells as in panel A. **D.** PBMC from the same older subject were stimulated and cultured in

10 nM folate and promoter methylation measured as in panel B. **E.** PBMC from three younger (31 ± 13 years) and three older (69 ± 10.6 years) subjects were stimulated and cultured in media containing 40 or 10 nM folate then CD4⁺ T cell DNA was isolated and KIR2DL2 promoter methylation analyzed by sequencing of bisulfite treated DNA as in panels A-D. The results represent the mean $\pm$ SEM of the overall methylation of the KIR2DL2 promoter in the 3 younger (light bars) or older (dark bars) subjects (n.s. = not significant). **F.** PBMC from the same subjects were cultured in media containing 30 or 5 μ M Met then KIR2DL2 promoter methylation determined as in panel E. Results again represent the mean $\pm$ SEM of the overall methylation, determined by cloning and sequencing 10 fragments per subject under each condition. (n.s. = not significant).

FIGURE 3. Age and Met/folate restriction increases CD70 expression in CD4+ and CD8+ T cells PBMC from subjects of the indicated ages were stimulated, cultured in custom media, then CD70 expression was compared on CD4+ and CD8+ T cells as in Fig. 1. **A.** Effects of 30 μ M and 5 μ M Met on CD70 expression in CD4+ T cells. **B.** Effects of 30 μ M and 5 μ M Met on CD70 expression in CD8+ T cells. **C.** Effects of 40 nM and 10 nM folate on CD70 expression in CD4+ T cells. **D.** Effects of 40 nM and 10 nM folate on CD70 expression in CD8+ T cells. P values refer to the difference in CD70 expression between T cells from the subjects ages ≥ 60 cultured in media with higher vs lower nutrient concentrations. **E.** CD4+ T cells from 3 healthy younger (age 30 ± 13.9) and 3 older (age 71.6 ± 10.7) subjects were stimulated and cultured in media containing 30 μ M (dark bars) or 5 μ M Met (light bars) as in Fig. 2 then CD70 transcripts were measured relative β -actin. *p =0.01 Met 30 μ M vs Met 5 μ M for the older subjects. **F.** CD4+ T cells from the same subjects were similarly stimulated and cultured in

media containing 40 nM (dark bars) or 10 nM folate (light bars) then CD70 transcripts were similarly measured (**p =0.03 folate 40 nM vs 10 nM for the older subjects). Results are presented as the mean $\pm$ SEM.

FIGURE 4. Age, folate restriction and Hcy are synergistic in increasing KIR expression on T cells
PBMC from people of the ages indicated on the x-axis were stimulated with PHA then cultured in media containing 40 nM folate (panels **A** and **C**) or 10 nM folate (panels **B** and **D**) as in Fig 1, but with (round symbols) or without (diamonds) 15 μ M Hcy. KIR expression was measured on CD4+ T cells (panels **A** and **B**) or CD8+ T cells (panels **C** and **D**) 72 hours later by flow cytometry also as in Fig 1.

FIGURE 5. Dnmt 1 mRNA expression decreases with age but not Met/folate restriction

A. CD4⁺ T cells from five healthy younger people (average age 28.4 ± 10.0) and five healthy older people (average age 63.2 ± 7.2) were stimulated for 18-24 hours then cultured in media containing 30 μ M or 5 μ M Met for 24 hours then Dnmt1 transcripts measured relative to histone H4 by real-time RT-PCR. Results are presented as the mean $\pm$ SEM ($p = 0.005$ Met 5 μ M young vs old, $p = 0.006$, Met 30 μ M young vs old). **B.** CD4⁺ T cells from the same subjects were similarly stimulated, cultured in media containing 40 nM or 10 nM folate, and Dnmt1 transcripts measured. ($p = 0.002$ folate 10 nM young vs old, $p = 0.003$ folate 40 nM young vs old). **C.** Regression analysis of age vs Dnmt1 levels for CD4⁺ T cells cultured in media containing 40 nM folate. (* = $p < 0.01$, old vs young).

FIGURE 6. siRNA Dnmt1 suppression increases sensitivity to low Met/folate levels

T cells from four healthy young people (average age 23.3 ± 0.96) were isolated, transfected with control or Dnmt1 siRNA, cultured in normal media for 6 hours then stimulated with PHA for an additional 18 hours. The cells were then washed, cultured in the indicated media for another 72 hours, then KIR2DL2 transcripts were measured relative to β -actin. Results are presented as the mean $\pm$ SEM. **A.** $p = 0.001$ (Dnmt1 siRNA, 5 μ M vs 30 μ M Met). **B.** $p = 0.036$ (Dnmt1 siRNA, 10 nM vs 40 nM folate).

FIGURE 7. Effects of nutrient restriction on CD28+ and CD28- T cells

PBMC from healthy subjects of the indicated ages were stimulated with PHA and cultured in media containing 30 or 5 μ M Met or 40 or 10 nM folate as indicated in each panel and as in Fig 1, then stained with mAb to KIR, CD4 and CD28 or KIR, CD8 and CD28, and analyzed by flow cytometry. In all cases the square symbols represent the lower concentrations, and the triangles the higher concentrations. **A.** Percent CD4+KIR+CD28+ in cells cultured in 30 or 5 μ M Met. **B.** Percent CD4+KIR+CD28- in cells cultured in 30 or 5 μ M Met. **C.** Percent CD4+KIR+CD28+ in cells cultured in 40 or 10 nM folate. **D.** Percent CD4+KIR+CD28- in cells cultured in 40 or 10 nM folate. **E.** Percent CD8+KIR+CD28+ in cells cultured in 30 or 5 μ M Met. **F.** Percent CD8+KIR+CD28- in cells cultured in 30 or 5 μ M Met. **G.** Percent CD8+KIR+

+CD28+ in cells cultured in 40 or 10 nM folate. **H.** Percent CD8+KIR+CD28– in cells cultured in 40 or 10 nM folate. P values represent the significance of the differences in KIR expression on T cells from the older subjects following culture in media containing high or low concentrations of the indicated nutrients, determined by paired t-test.

Table 1

Transmethylation Micronutrients Levels

Component	RPMI 1640	Normal Serum Levels
Methionine	101 μ M	10-42 μ M
Folic acid	2268 nM	18-40 nM
Homocysteine	0	5-15 μ M