Correction

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B vitamins attenuate the epigenetic effects of ambient fine particles in a pilot human intervention trial

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Acute exposure to fine particle (PM2.5) induces DNA methylation changes implicated in inflammation and oxidative stress. We conducted a crossover trial to determine whether B-vitamin supplementation averts such changes. Ten healthy adults blindly received a 2-h, controlled-exposure experiment to sham under placebo, PM2.5 (250 μg/m³) under placebo, and PM2.5 (250 μg/m³) under B-vitamin supplementation (2.5 mg/d folic acid, 50 mg/d vitamin B12 and 1 mg/d vitamin B6), respectively. We profiled epigenome-wide methylation before and after each experiment using the Infinium HumanMethylation450 BeadChip in peripheral CD4+ T-helper cells. PM2.5 induced methylation changes in genes involved in mitochondrial oxidative energy metabolism. B-vitamin supplementation prevented these changes. Likewise, PM2.5 depleted 11.1% [95% confidence interval (CI) 0.4%, 21.7%; P = 0.04] of mitochondrial DNA content compared with sham, and B-vitamin supplementation attenuated the PM2.5 effect by 102% (Pinteraction = 0.01). Our study indicates that individual-level prevention may be used to complement regulations and control potential mechanistic pathways underlying the adverse PM2.5 effects, with possible significant public health benefit in areas with frequent PM2.5 peaks.

Air pollution | B vitamins | DNA methylation | mitochondria

Historical episodes of air pollution peaks were shown to be associated with up to >10 times increased death rates (1). According to the WHO, 92% of the world’s population currently lives in places where air quality levels exceed the WHO limits (2). Ambient PM2.5 particles (with an aerodynamic diameter of <2.5 μm) pollution is one of the most prominent air pollutants (3), because they deposit in the respiratory bronchioles and the alveoli and stimulate local and systemic inflammation and oxidative stress (4). Over the past few decades, substantial lowering of ambient PM2.5 levels has been achieved through large-scale emissions control policies (5). However, exposure peaks with adverse health consequences are still frequently recorded (6, 7), even in areas typically exhibiting low levels (5). The molecular mechanisms underlying PM2.5’s health effects are not fully understood, and the lack of preventative options at the individual level adds complexity to tackling this major public health challenge.

Recent studies in environmental epigenetics provide opportunities to understand the mechanistic underpinnings of exposure-related health effects and to develop novel individual-level interventions. DNA methylation, a potentially modifiable epigenetic mechanism, can regulate gene expression and chromosome integrity via addition of methyl groups to cytosine residues (8). The dynamic DNA landscape can be rapidly altered in peripheral leukocytes following PM2.5 exposure; indeed, such changes are postulated to underlie PM2.5-induced systemic inflammation and oxidative stress (9, 10). Most evidence of this phenomenon in humans is based on a heterogeneous mixture of leukocytes (9–12), but a loss of methylation in inflammatory genes and subsequent inflammatory responses, specifically in circulating Th cells, are observed in vivo after environmental challenge (13, 14). Notably, DNA methylation is dependent on a biochemical cycle that supplies methyl groups (CH3) while relying on methyl nutrients (i.e., B vitamins including folic acid, vitamin B12, and amino acids including methionine, betaine, and choline) (8, 15, 16). In animal studies, a methyl-nutrient-deficient diet leads to aberrant DNA methylation patterns (17), and administration of methyl nutrients enables restoration of epigenetic status (15, 18–20). Likewise, human studies show that dietary methyl nutrient intervention influences the plasticity of DNA methylation (21). The potential for epigenetic modulation has also been demonstrated in the presence of environmental stressors in animal models—Doliny et al. successfully used methyl nutrients to avert the DNA hypomethylation induced by bisphenol A exposure (22). These findings

Significance

Air pollution is a major public health concern worldwide. The molecular mechanistic underpinnings of the health effects of air pollution are not fully understood, and the lack of individual-level preventative options represent a critical knowledge gap. Our study demonstrated the epigenetic effects of air pollution and suggested that B vitamins might be used as prevention to complement regulations to attenuate the impact of air pollution on the epigenome. Our study inaugurated a line of research for the development of preventive interventions to minimize the adverse effects of air pollution on potential mechanistic markers. Because of the central role of epigenetic modifications in mediating environmental effects, our findings might be extended to other toxicants and environmental diseases.


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opened new avenues for the application of epigenetic intervention to reduce the health effects of air pollution. However, to date, epigenetic intervention in humans in the context of air pollution has not been described.

The present study is a placebo-controlled crossover pilot intervention trial with controlled human exposure experiments to concentrated ambient fine particles (PM₁₀). We hypothesized that acute PM₁₀ exposure can rapidly modify the DNA methylation profile in peripheral CD4⁺ Th cells—the most prolific cytokine producer mediating PM₁₀-induced inflammatory responses—and that PM₁₀-induced DNA methylation changes can be reduced by B vitamins (i.e., folic acid, vitamin B₁₂, and B₁₉), the primary source of methyl groups (Fig. 1).

**Results**

**Study Population, Plasma B-Vitamin Concentrations, and Exposure Levels.** In the present crossover placebo-controlled trial, 10 volunteers completed 30 controlled exposure experiments following the same order (Fig. S1): seven volunteers aged 19–29 y and three aged 30–49 y. Four volunteers were white, three were Asian, and three were other races. Six volunteers were female and three volunteers had a body mass index (BMI) ≥25. All volunteers maintained consistent dietary patterns throughout the trial.

The targeted concentration of PM₁₀ exposures was 250 μg/m³. However, the actual PM₁₀ concentrations varied among controlled exposure experiments to PM₁₀ (100.6–287.5 μg/m³; median: 234.0 μg/m³). There was no significant difference (P = 0.38) in PM₁₀ concentration between PM₁₀ experiment under placebo [median: 219.1 μg/m³; interquartile range (IQR): 33.1 μg/m³] and PM₁₀ experiment under B vitamins (median: 237.2 μg/m³; IQR: 48.7 μg/m³).

To confirm that supplementation affecting circulating nutrient levels, we measured plasma B vitamins before and after placebo and supplementation. The median plasma concentrations of folic acid and vitamins B₁₂ and B₁₉ were 35 nmol/L (IQR: 14 nmol/L), 41 nmol/L (IQR: 16 nmol/L), and 292 pmol/L (IQR: 72 pmol/L) before sham experiment, respectively. After volunteers took placebos for 4 wk, their median plasma concentrations were similar: 39 nmol/L (IQR: 24 nmol/L) for folic acid (P = 0.82), 37 nmol/L (IQR: 18 nmol/L) for vitamin B₁₂ (P = 0.75), and 262 pmol/L (IQR: 214 pmol/L) for vitamin B₁₉ (P = 0.42). B-vitamin supplementation significantly increased the median plasma concentrations of folic acid (56 nmol/L; IQR: 13; P = 0.02), vitamin B₁₂ (428 nmol/L; IQR: 321; P = 0.004), and vitamin B₁₉ (511 pmol/L; IQR: 85; P = 0.001). The abovementioned P values were based on Wilcoxon signed-rank test, a nonparametric paired difference test.

**CD4⁺ Th Cell Purity.** All CD4⁺ Th cell samples’ purity were over 80% (Table S1), with only minor contamination from CD8 T cells, B cells, granulocytes, and natural killer cells. The median purity of samples collected at sham experiment, PM₁₀ experiment under placebo, and PM₁₀ experiment under B vitamins was 96.9% (IQR: 5.1), 94.8% (IQR: 7.1), and 96.1% (IQR: 4.7), respectively.

**Effect of PM₁₀ and B-Vitamin Supplementation on DNA Methylation.** Two-hour PM₁₀ exposure substantially modified DNA methylation in CD4⁺ Th cells, and these changes were prevented by B-vitamin supplementation (Fig. 2). Because our study is limited in power, we present only the top 10 loci, selected following the method proposed by Maccani et al. (23)—first based on effect size and then P value (Table S2 and Fig. 2A). In the absence of B-vitamin supplementation, PM₁₀ exposure either increased or decreased DNA methylation levels, compared with sham, at these loci (Fig. 2B). Quantile-Quantile plots for expected vs. observed distribution of P values showed minimal genomic inflation with a lambda of 1.03 (Fig. 2B).

**Table S2** presents the genomic position, relation to CpG islands, and gene symbol for the 10 loci. The top two loci that are associated with known genes were cg06194186 and cg17157498. Locus cg06194186 is located in the promoter region (TSS1500) of the carboxypeptidase O (CPO) gene, and locus cg17157498 is located in the promoter region (TSS1500) of the NADH dehydrogenase (ubiquinone) Fe-S protein 7 (NDUF7) gene (Figs. S2 and S3). Fig. 3 presents the shift in methylation level distributions and corresponding IORs pre and post each exposure experiment for these two loci.

Four-week B-vitamin supplementation attenuated the PM₁₀ effect by 28–76% at the top 10 loci. Supplementing B vitamins resulted in a reduction in effect size by 57% for cg06194186, 49% for cg07689821, 73% for cg00068102, 31% for cg00647528, 45% for cg15426626, 28% for cg10719920, 76% for cg2198027, 74% for cg17157498, 63% for cg08075528, and 71% for cg26995744, respectively (Table S3 and Fig. 2C).

**Effect of PM₁₀ and B-Vitamin Supplementation on Mitochondrial DNA Content.** CPO and NDUF7 are both involved in mitochondrial oxidative energy metabolism—a pivotal function with substantial impact on mitochondrial biogenesis and clearance (24, 25). In the secondary exploratory analysis, we further tested the associations of PM₁₀ with mitochondrial DNA content, as well as the potential protective effect of B-vitamin supplementation. In the absence of B-vitamin supplementation, compared with sham, 2-h exposure to PM₁₀ was estimated to be nonsignificantly associated with a −0.3% change [95% confidence interval (CI): −10.1%, 9.5%; P = 0.94] in mitochondrial DNA content. However, 24 h after exposure experiments, we observed substantial reduction in mitochondrial DNA content associated with PM₁₀: 2-h exposure to PM₁₀ significantly depleted mitochondrial DNA content by 11.1% (95% CI: 0.4%,...
B vitamins have a long biological half-life (26), and the reduction of PM$_{2.5}$ effect by B vitamins. Bar height indicates PM$_{2.5}$ effect for one CpG. The vertical lines indicate suggestive threshold based on effect size, whereas the horizontal line reflects the suggestive threshold based on statistical significance. B is the quantile-quantile plot for associations of PM$_{2.5}$ with DNA methylation in circulating CD4$^+$ Th cells. C represents the top 10 loci associated with PM$_{2.5}$ and the reduction of PM$_{2.5}$ effect by B vitamins. Bar height indicates PM$_{2.5}$ effect, whereas the gray part indicates the magnitude of effect attenuation by B vitamins. D is the Manhattan plot representing the chromosome location of each loci. The dashed horizontal line reflects the suggestive threshold for statistical significance. Analyses were adjusted for season, chamber humidity, and temperature.

21.7%; $P = 0.04$) (Table S3). B-vitamin supplementation completely attenuated such effect of PM$_{2.5}$ by 102% ($P_{\text{interaction}} = 0.01$). With B-vitamin supplementation, 2-h exposure to PM$_{2.5}$ was not associated with mitochondrial DNA content (0.2%; 95% CI: −8.3%, 8.8%; $P = 0.96$) (Table S3).

**Exploratory Mediation Analysis and External Supporting Data.** We further deconstructed total PM$_{2.5}$ effects on mitochondrial DNA content into direct and indirect (i.e., mediated) effects to investigate whether DNA methylation levels at loci cg06194186 and cg17157498 mediate PM$_{2.5}$-mitochondrial DNA content relationship. Our result indicated that 16.0% (95% CI: 4.1%, 27.9%) and 18.4% (95% CI: 9.9%, 26.9%) of the PM effect on mitochondrial DNA content was mediated by cg06194186 and cg17157498 methylation, respectively. Consistent with our hypothesis, we observed correlation between mitochondrial DNA content and the methylation levels of cg06194186 ($r = -0.45; P = 0.06$) and cg17157498 ($r = 0.63; P = 0.01$) in CD4$^+$ Th cells, in an independent external dataset.

**Sensitivity Analysis.** B vitamins have a long biological half-life (26), therefore requires a washout period longer than four months. We designed the trial without randomizing the treatment order (placebo vs. B vitamins) to avoid long washout periods, which would have made exposure experiments on the same volunteer less comparable. Lifestyle factors may vary over several months, particularly in relation to seasonality, which also may directly affect DNA methylation (8, 10). To rule out the potential impact of temporal trend on our results, we adjusted for date-since-entry, and this adjustment did not affect our conclusion. Furthermore, we conducted permutation test on the two top loci to ensure the robustness of our analysis ($P_{\text{permutation}} < 0.001$). In the analysis involving the mitochondrial DNA content, we additionally adjusted for age (continuous), BMI (continuous), and race (categorical) to examine if our results are...
sensitive to covariates specification. Our results were robust and consistent (Table S3).

Discussion

This crossover intervention trial with controlled exposure experiments demonstrated that 2-h exposure to concentrated ambient PM$_{2.5}$ (250 μg/m$^3$) affects the dynamic epigenetic landscape in circulating CD$^+$ Th cells among healthy adults. We showed that these effects can be prevented with B-vitamin supplementation (i.e., folic acid and vitamins B$_6$ and B$_{12}$). Furthermore—as the top loci suggested potential modulation of mitochondrial metabolism—we followed up these findings by showing that exposure to PM$_{2.5}$ significantly altered mitochondrial DNA content in circulating CD$^+$ Th cells, and B-vitamin supplementation nearly completely prevented these effects.

Air pollution has been consistently associated with adverse health outcomes in epidemiological studies (1, 3, 4, 9). Although the biological mechanism underlying the health effects of PM$_{2.5}$ remains not fully understood, systemic inflammation and oxidative stress have been proposed as essential pathological pathways (4, 27). Furthermore, PM$_{2.5}$ can disturb DNA methylation profiles (9, 10, 13), which might exacerbate oxidative and inflammatory responses following exposure. A previous human exposure study demonstrated that toll-like receptor 4 (TLR4) gene hypomethylation in leukocytes mediates a part of PM effects on blood pressure (28). Recently, an epigenome-wide association study (EWAS) showed that low-concentration air pollution alters DNA methylation profiles in whole blood (9). However, the interpretation of those findings is limited, considering potential bias due to cell-type heterogeneity within whole blood, and by the correlational nature of observational studies (11, 12). In addition, these findings might not be generalizable to areas with frequent air pollution peaks. The present study—a cell type-specific EWAS using controlled exposure experiments—has the unique advantage of providing unbiased insight on the novel epigenetic underpinnings of the proinflammatory and prooxidative effects of PM$_{2.5}$ exposure peaks.

In line with our hypothesis, we demonstrated acute effects of PM$_{2.5}$ inhalation on DNA methylation in the promoter region of genes related to mitochondrial function and oxidative metabolism (24, 25): CPO, a member of the metalloenzyme family (25), is involved in metal ion binding, metallopeptidase, and metallocarboxylase activities—which are essential in regulation of the steady-state concentration of O$_2^-\textsuperscript{−}$ in the intracellular space of mitochondria (29); NDUF57 encodes one of the subunits of the mitochondrial respiratory chain complex I that transfers electrons from NADH to coenzyme Q, and NDUF57 mutations were of etiological significance in mitochondrial complex I deficiency (30). Although mitochondria have their own genetic material distinct from the nuclear DNA, the majority of mitochondrial proteins are encoded by the nuclear genome (31). The observation that PM$_{2.5}$ exposure substantially altered DNA methylation of nuclear genes in mitochondrial pathways indicate that mitochondria—the specialized organelles that regulate cellular-redox-balance and supplies energy—are a primary target of PM-induced cellular responses (32).

Our results on mitochondrial DNA content supplemented the findings from the epigenome-wide DNA methylation scan: exposure to PM$_{2.5}$ for 2 h was followed by reduced mitochondrial DNA content 24-h postexposure. The cellular mitochondrial genomic content is stringently regulated by biogenesis/degradation machinery (33), which is vital in the determination of cell survival and function. Compensatory mitochondria biogenesis can buffer an intracellular reactive oxygen species (ROS) challenge, as an adaptive stress response to eliminate cellular oxidative damage (34). However, persistent oxidative stress may eventually overwhelm the adaptive response system and lead to mitochondrial DNA depletion via mitophagy (35). Our results support this hypothesis by demonstrating that exposure to high-concentration PM$_{2.5}$ can reduce the mitochondrial DNA contents in circulating CD$^+$ Th cells. Consistent with our results, a recent study reported that a 10 μg/m$^3$ increase in coarse PM (PM$_{10-2.5}$) exposure during pregnancy was associated with a 16.1% decrease in placental mitochondrial DNA content (36). Our exploratory mediation analysis indicates that short-term exposure to high-concentration PM$_{2.5}$ depletes mitochondrial DNA content, likely via—at least in part—modulating DNA methylation levels of genes in mitochondrial pathways. Future studies are warranted to investigate the potential for targeted epigenetic interventions.

DNA methylation is a modifiable biochemical process relying on methyl-group supplying nutrients such as B vitamins, which is postulated to increase DNA methylation levels (8, 15, 16). This feature renders B-vitamin supplementation an attractive pharmacological intervention to counteract the PM effects, which has been associated with loss of DNA methylation on inflammatory genes (28). Landmark experiments on the Agouti A$^+$ mice and other models have shown that dietary methyl nutrients, added during gestation (15–18) or later even in adult life (20, 21), can be used to modulate DNA methylation status. In human studies, intake of a folic acid-depleted diet for several weeks promotes hypomethylation of lymphocyte genomic DNA among postmenopausal women, and this hypomethylation is reversible with folic acid replacement (21). Among patients with colorectal adenomatous polyps, folic acid supplementation led to a 31% increase in leukocyte DNA methylation and a 25% increase in DNA from the colonic mucosa (37). Potential for human translation is also demonstrated in animal models, as methyl group-supplying nutrients can be used to prevent the loss of DNA methylation induced by environmental pollutants in rodents (22). However, to the best of our knowledge, whether B vitamins can be used to limit adverse effects from PM pollution has not previously been tested in humans. Our research provided the experimental evidence showing that the epigenetic effects of PM$_{2.5}$ can be reduced using 4-wk B-vitamin supplementation. Remarkably, in our data, the B-vitamin supplementation not only prevented decreased DNA methylation but also increased DNA methylation following acute exposure to high-concentration PM. These findings suggest that B vitamins might protect against DNA hypomethylation as methyl group-supplying nutrients. On the other hand, we might also hypothesize that DNA methylation through interactions with regulatory pathways mediated by essential enzymes (such as DNA methyltransferases and methyltetrahydrofolate reductase).

A major innovation of the present study over previous human epigenome-wide studies is the use of isolated CD$^+$ Th cells—an essential cell type modulating human immunity through both its own immune activities and regulation of other leukocytes’ proliferation, apoptosis, migration, and other functions via cytokine signaling (31). Therefore, the epigenetic effects of PM$_{2.5}$ and protective effects of B vitamins observed in CD$^+$ Th genome might indicate subsequent modulation of essential cellular functions of other blood cell types. The Houseman cell proportion estimates indicated high purity of the analyzed samples. Although our study is subject to residual influence from differential CD$^+$ Th subsets, the observed effect of PM$_{2.5}$ or B vitamins on DNA methylation is unlikely to be surrogate for leukocyte composition variation. Our plating scheme for DNA methylation analysis is independent of the exposure and treatment status, and was designed to minimize potential bias due to technical variables. Thus, the measurement error of DNA methylation can be assumed to be nondifferential and, therefore, likely to bias the results toward null. We conducted sensitivity analysis to robustly evaluate the impact of PM$_{2.5}$ on top loci, and further supported the EWAS results with a widely accepted mitochondrial marker—mitochondrial DNA content—with a highly reproducible quantitative real-time PCR method.
crossover design controlled for time-invariant factors such as sex, race, BMI, SNP, etc. In addition, all exposure experiments were conducted at the same time of the day to eliminate any impact due to diurnal variation.

We acknowledge several limitations in the present pilot study. Although our EWAS is limited in power to meet the stringent Bonferroni threshold for significance with only 10 volunteers (30 exposure experiments), our sample size is comparable to previous controlled exposure studies that succeeded in demonstrating health effects of PM exposure (28, 38–40). As suggested by previous study (23), we selected the top loci based on both effect size and statistical significance, because those loci are more likely to infer biological significance. The short study duration was implemented to reduce the impact of within-volunteer seasonal variations. Therefore, we could not randomize on the treatment (placebo vs. B vitamins) order due to long biological half-life of body stores of B vitamins (26), which might create potential confounding due to a temporal trend or learning effect (i.e., the volunteers might be more tolerant with the PM$_{2.5}$ effects at the second PM$_{2.5}$ exposure). In the sensitivity analysis, we adjusted for the amount of time passing because the study entry, and our results were consistent. Although residual confounding is possible, considering the magnitude of our effect estimates and the consistency of our findings, it is unlikely that the observed association reflected bias resulting from confounding. In additional, actual PM$_{2.5}$ concentration in PM$_{2.5}$ experiments under placebo was nonsignificantly lower than PM$_{2.5}$ experiments under B-vitamin supplementation, which might bias our results on B vitamins’ protective effects toward the null. Finally, future validation studies are warranted because our unique study design using CD4$^+$ Th cells created major challenge to identify a suitable replication cohort, and our findings might not be generalizable to other cell types due to cell-type specificity of DNA methylation and mitochondrial DNA content.

The unclear molecular mechanistic underpinning of PM$_{2.5}$ health effects remains the major gap in current knowledge—therby creating challenges in developing preventative strategies. The present study is a pilot intervention trial in the investigation of mechanistic pathways underlying the adverse health effects of air pollution, and potential targeted preventive approaches. We demonstrated that ambient PM$_{2.5}$ exposure peak has unfavorable effects on epigenetic and pro-oxidative markers that can be neutralized by B-vitamin supplementation. Our findings suggest promising opportunities to aid the development of novel intervention strategies—which is particularly important for pathologies related to ubiquitous exposures such as PM$_{2.5}$ pollution. Future trials with larger sample sizes are warranted to shed light on the precise pathophysiological processes of PM-induced inflammatory and oxidative responses, the mechanism underlying the protective effect of B vitamins, and potential clinical application.

Methods

Study Population. We recruited 10 healthy, 18- to 60-y-old, nonsmoking volunteers who were not taking any medicines or vitamin supplements, from the University of Toronto campus and surrounding area. The trial protocols were approved by all participating institutional review board (University of Toronto, St. Michael’s Hospital, and Harvard T.H. Chan School of Public Health) and registered (ClinicalTrials.gov identifier NCT01864824; date of registration: May 8, 2013). All methods were performed in accordance with the relevant guidelines and regulations. We obtained written informed consent from every volunteer before enrollment.

Study Design. We conducted a single-blind, crossover intervention trial with controlled exposure experiments to concentrated ambient PM$_{2.5}$ (July 2013 to February 2014). The design (Fig. 1) started with a 2-wk run-in period with placebo, followed by the baseline sham experiment (2 h, particle-free medical air, exposure once). After sham experiment, each volunteer took placebo for 4 wk and then was exposed to PM$_{2.5}$ (2 h, target concentration: 250 μg/m$^3$, exposure two). Volunteers started the 4-wk B-vitamin supplementation after exposure two, and then were exposed again to PM$_{2.5}$ (2 h, target concentration: 250 μg/m$^3$, exposure three). All volunteers received three exposure experiments following the same order.

Exposure Facility. Ambient particles were drawn in from an inlet 1.5 m high, beside a busy (~1,000 vehicles per hour) street in downtown Toronto. We used the Harvard ambient particle concentrator to generate concentrated ambient PM$_{2.5}$ (41), and delivered PM$_{2.5}$ air stream to the volunteer seated inside a 4.9-m$^2$ (1.1 × 1.9 × 2.0 m) lexan enclosure via an “oxygen type” facemask. The sham exposure with medical air were generated as previously described (38). During each exposure experiment, PM$_{2.5}$ mass was collected on a 47-mm, 2-μm Teflon filter (Teflo, R2P947; Pall Corp.) and was monitored using the gravimetric determination of PM$_{2.5}$ exposure mass concentration (micrograms per cubic meter).

Folic Acid, Vitamin B$_6$, and Vitamin B$_12$ Supplement. We administered one placebo or B-vitamin supplement (2.5 mg of folic acid, 50 mg of vitamin B$_6$, and 1 mg of vitamin B$_12$) daily to each volunteer. Previous human trials showed that these doses rapidly increased plasma B-vitamin levels, modified methyl-cycle metabolite levels, and ameliorated cardiovascular measurements (42, 43). Preparation, packaging, and coding of the placebo and supplement were done by an external laboratory (Jameson Laboratories) and was blinded to the volunteers. Before each exposure experiment, we measured volunteers’ plasma folic acid and vitamin B$_12$ levels using competitive-binding immunoenzymatic assay (A98032 and 33000; Beckman Coulter), and vitamin B$_12$ levels using HPLC with fluorescence detection. At the first and the last visit, we assessed typical daily B-vitamin intake with a self-administered validated (44), semiquantitative food-frequency questionnaire used in the Nurses’ Health Study.

CD4$^+$ Th Cell Isolation and DNA Extraction. We collected blood samples via venous phlebotomy (preexposure, immediately postexposure, and 24 h postexposure), and within 4 h, isolated CD4$^+$ Th cells by removing unwanted cells using RosetteSep Human CD4$^+$ T Cell Enrichment Mixture (no. 15062; Stem Cell Technologies). DNA was then extracted using a Promega Maxwell 16 instrument with tissue DNA purification kit (Promega). We monitored the concentration and quality of extracted DNA using NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies). Unsatisfactory DNA samples were discarded and DNA was extracted again. We estimated the proportions of major leukocyte types (CD4 T cells, CD8 T cells, B cells, granulocytes, monocytes, and natural killer cells) to assess the purity of isolated CD4$^+$ Th cells using the Houseman method, a statistical deconvolution technique based on the 450K data (11).

Epigenome-Wide DNA Methylation Scan. We measured the epigenome-wide DNA methylation profile using the Infinium Human Methylation 450K BeadChip (Illumina) (45), which allows the assessment of approximately half million CpG sites across 99% of RefSeq genes within the genome (46). Because of the within-volunteer, cross-over design, we plated all samples from one volunteer in one chip, with pre- and postexposure samples randomly loaded onto each column on the same row. All samples were processed by one technician and analyzed in one batch to minimize batch effect. Sample preparation and quality control details are explained in SI Methods.

Mitochondrial DNA Content in CD4$^+$ Th Cells. We measured mitochondrial DNA content in CD4$^+$ Th cells through the mtDNA/nDNA ratio, a widely used biomarker representing the mitochondrial DNA copy number versus the nuclear DNA copy number (34). Mitochondrial DNA copy number was analyzed pre, immediately after, and 24 h after each exposure experiment using multiplex quantitative real-time PCR, as previously reported (34). The mtDNA/nDNA is used in the statistical analysis—a ratio value of 1 indicates that the mtDNA/nDNA of the test sample is equal to the mtDNA/nDNA in the reference DNA pool used in the assay.

CD4$^+$ Th Mitochondrial DNA Content and DNA Methylation in External Dataset. To strengthen our findings, we identified an external dataset based on 15 de-identified volunteers’ CD4$^+$ T cells—which were purified from fresh blood samples through magnetic-activated cell sorting using anti-CD4 antibody coupled paramagnetic microbeads (Miltenyi Biotech). Epigenome-wide DNA methylation profiles and mitochondrial DNA content were measured using the same methods of the present study.
Statistical Methods. We used linear mixed-effects models (Sj Methods) with random intercepts assigned to each volunteer to account for correlation among within-person measurements. The cross-over design minimized the influence from time-invariant factors. In all models, we adjusted for time-varying covariates with potential influences on DNA methylation, selected based on prior knowledge and the existing literature [i.e., season (fall/winter/spring/summer), chamber temperature, and chamber relative humidity (38)]. Rank-based normal transformation was performed on all DNA methylation measurements to improve normality and stabilize the variance. We further performed permutation tests on the observed top two loci to ensure the robustness of our results, and conducted exploratory mediation analysis to evaluate whether DNA methylation mediates the effect of PM$_{2.5}$ on mitochondrial DNA content (Sj Methods). Analyses were performed using SAS 9.4 (SAS Institute) and R statistical computing software (R Foundation for Statistical Computing).

Data Availability. Data are available on request due to privacy or other restrictions.

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