

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

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Edited by Kirk R. Smith, University of California, Berkeley, CA, and approved February 13, 2017 (received for review November 8, 2016)

Acute exposure to fine particle (PM_{2.5}) 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, PM_{2.5} (250 µg/m³) under placebo, and PM_{2.5} (250 µg/m³) under B-vitamin supplementation (2.5 mg/d folic acid, 50 mg/d vitamin B₆, and 1 mg/d vitamin B₁₂), respectively. We profiled epigenome-wide methylation before and after each experiment using the Infinium HumanMethylation450 BeadChip in peripheral CD4⁺ T-helper cells. PM_{2.5} induced methylation changes in genes involved in mitochondrial oxidative energy metabolism. B-vitamin supplementation prevented these changes. Likewise, PM_{2.5} 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 PM_{2.5} effect by 102% (*P*_{interaction} = 0.01). Our study indicates that individual-level prevention may be used to complement regulations and control potential mechanistic pathways underlying the adverse PM_{2.5} effects, with possible significant public health benefit in areas with frequent PM_{2.5} 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 PM_{2.5} (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 PM_{2.5} 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 PM_{2.5}'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 PM_{2.5}

exposure; indeed, such changes are postulated to underlie PM-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 B₆ and 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—Dolinoy 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.

Author contributions: J.Z., P.K., F.S., D.R.G., and A.A.B. designed research; J.Z., O.K., G.W., B.U., and M.S. performed research; J.Z., O.K., G.W., J.L., X.L., M.Z., M.S.-G., L.T., M.S., L.L., B.A.C., and T.W. contributed new reagents/analytic tools; J.L. and T.W. provided the replication dataset for revision; X.L. advised epigenome-wide analysis; L.T. served as data manager; L.L. and B.A.C. advised biostatistics; P.K. designed the exposure devices; J.Z. and Y.G. analyzed data; and J.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1618545114/-DCSupplemental.

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 ($PM_{2.5}$). We hypothesized that acute $PM_{2.5}$ exposure can rapidly modify the DNA methylation profile in peripheral $CD4^+$ Th cells—the most prolific cytokine producer mediating $PM_{2.5}$ -induced inflammatory responses—and that $PM_{2.5}$ -induced DNA methylation changes can be reduced by B vitamins (i.e., folic acid, vitamin B₆, and B₁₂), 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 $PM_{2.5}$ exposures was 250 $\mu\text{g}/\text{m}^3$. However, the actual $PM_{2.5}$ concentrations varied among controlled exposure experiments to $PM_{2.5}$ (100.6–287.5 $\mu\text{g}/\text{m}^3$; median: 234.0 $\mu\text{g}/\text{m}^3$). There was no significant difference ($P = 0.38$) in $PM_{2.5}$ concentration between $PM_{2.5}$ experiment under placebo [median: 219.1 $\mu\text{g}/\text{m}^3$; interquartile range (IQR): 33.1 $\mu\text{g}/\text{m}^3$] and $PM_{2.5}$ experiment under B vitamins (median: 237.2 $\mu\text{g}/\text{m}^3$; IQR: 48.7 $\mu\text{g}/\text{m}^3$).

To confirm that supplementation affected circulating nutrient levels, we measured plasma B vitamins before and after placebo and supplementation. The median plasma concentrations of folic acid and vitamins B₆ and B₁₂ 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 B₆ ($P = 0.75$), and 262 pmol/L (IQR: 214 pmol/L) for vitamin B₁₂ ($P = 0.42$). B-vitamin supplementation significantly increased the median plasma concentrations of folic acid (56 nmol/L; IQR: 13; $P = 0.02$), vitamin B₆ (428 nmol/L; IQR: 321; $P = 0.004$), and vitamin B₁₂ (511 pmol/L; IQR: 85; $P = 0.01$). 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, $PM_{2.5}$ experiment under placebo, and $PM_{2.5}$ experiment under B vitamins was 96.9% (IQR: 5.1), 94.8% (IQR: 7.1), and 96.1% (IQR: 4.7), respectively.

Effect of $PM_{2.5}$ and B-Vitamin Supplementation on DNA Methylation.

Two-hour $PM_{2.5}$ 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, $PM_{2.5}$ exposure either increased or decreased DNA methylation levels, compared with sham, at these loci (Fig. 2A). 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 (*NDUFS7*) gene (Figs. S2 and S3). Fig. 3 presents the shift in methylation level distributions and corresponding IQRs pre and post each exposure experiment for these two loci.

Four-week B-vitamin supplementation attenuated the $PM_{2.5}$ 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 cg21986027, 74% for cg17157498, 63% for cg08075528, and 71% for cg26995744, respectively (Table S3 and Fig. 2C).

Effect of $PM_{2.5}$ and B-Vitamin Supplementation on Mitochondrial DNA Content.

CPO and *NDUFS7* 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 $PM_{2.5}$ 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 $PM_{2.5}$ 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 $PM_{2.5}$: 2-h exposure to $PM_{2.5}$ significantly depleted mitochondrial DNA content by 11.1% (95% CI: 0.4%,

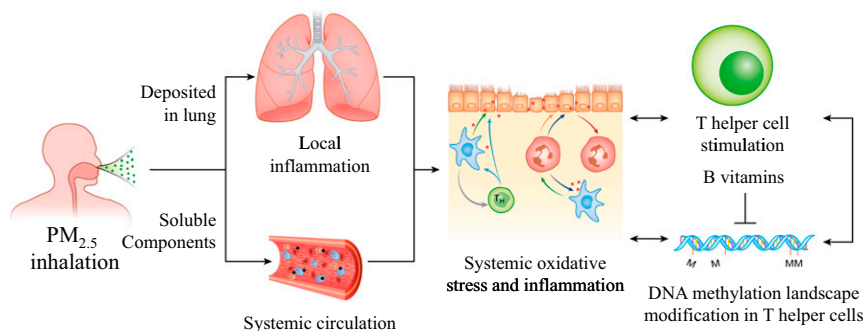


Fig. 1. Proposed conceptual model linking fine particulate matter ($PM_{2.5}$), systemic oxidative stress and inflammation, and altered DNA methylation landscape in Th cells. We hypothesized that $PM_{2.5}$ inhalation triggers local and systemic inflammation and oxidative stress, which alters the DNA methylation landscape in circulating $CD4^+$ Th cells and further stimulates $CD4^+$ Th cells. In return, stimulated $CD4^+$ Th cells undergo more epigenetic remodeling—possibly due to modulated methyl group availability—therefore creating a vicious circle which amplifies the inflammatory and oxidative effects of $PM_{2.5}$.

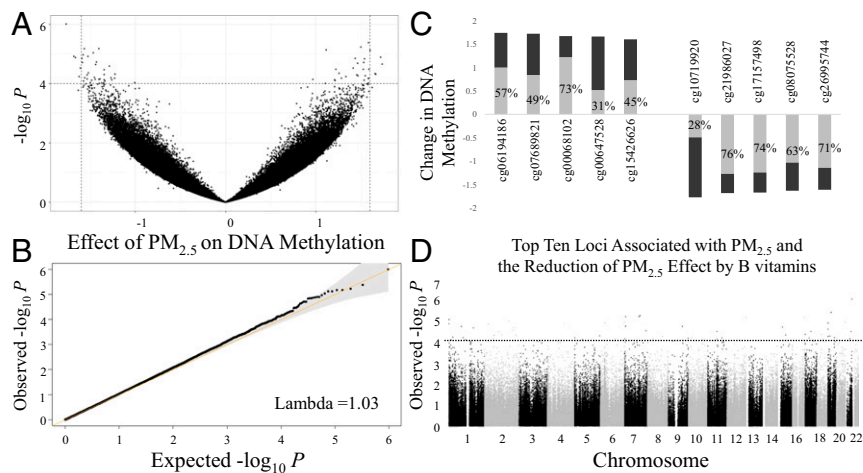


Fig. 2. The effect of 2-h exposure to PM_{2.5} on DNA methylation landscape and the reduction of PM_{2.5} effect by B-vitamin supplementation. *A* is the Volcano plot depicting the distribution of estimated effect of PM_{2.5} exposure on the epigenome. Each dot represents the estimated PM_{2.5} effect 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

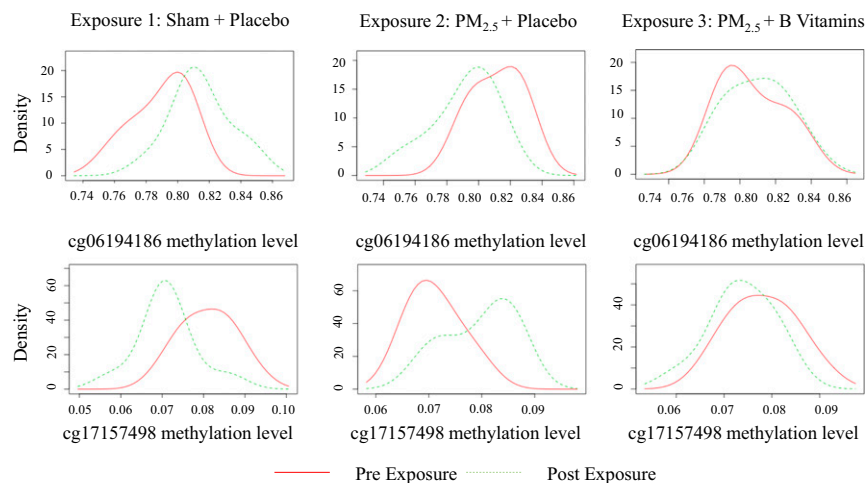


Fig. 3. Distribution of DNA methylation levels of cg06194186 and cg17157498 before and after each exposure experiment. Red solid lines and green dashed lines indicate the distribution of methylation levels measured before and after exposure, respectively.

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/m³) affects the dynamic epigenetic landscape in circulating CD4⁺ Th cells among healthy adults. We showed that these effects can be prevented with B-vitamin supplementation (i.e., folic acid and vitamins B₆ and B₁₂). 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 CD4⁺ 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 biological 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 effect 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 metal-carboxypeptidase activities—which are essential in regulation of the steady-state concentration of O₂⁻ in the intermembrane space of mitochondria (29); *NDUFS7* encodes one of the subunits of the mitochondrial respiratory chain complex I that transfers electrons from NADH to coenzyme Q, and *NDUFS7* 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 mitochondrial 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 CD4⁺ Th cells. Consistent with our results, a recent study reported that a 10 µg/m³ 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 pharmaceutical 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^{vy} 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 human. 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, B vitamins might also minimize DNA hypermethylation through interactions with regulatory pathways mediated by essential enzymes (such as DNA methyltransferases and methylenetetrahydrofolate reductase).

A major innovation of the present study over previous human epigenome-wide studies is the use of isolated CD4⁺ 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 CD4⁺ 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 CD4⁺ 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. Our

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 with-in 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 addition, 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}'s health effects remains the major gap in current knowledge—thereby 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 one). After sham experiment, each volunteer took placebo for 4 wk and was then exposed to PM_{2.5} (2 h, target concentration: 250 µg/m³, 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/m³, 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-m³ (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, R2PJ047; Pall Corp.) and was monitored using the gravimetric determination of PM_{2.5} exposure mass concentration (micrograms per cubic meter).

Folic Acid, Vitamin B₆, and Vitamin B₁₂ Supplement. We administered one placebo or B-vitamin supplement (2.5 mg of folic acid, 50 mg of vitamin B₆, and 1 mg of vitamin B₁₂) 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 (Jamieson Laboratories) and was blinded to the volunteers. Before each exposure experiment, we measured volunteers' plasma folic acid and vitamin B₁₂ levels using competitive-binding immunoassay (A98032 and 33000; Beckman Coulter), and vitamin B₁₂ 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 a 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 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 Biotec). 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 (*SI Methods*) with random intercepts assigned to each volunteer to account for correlation among within-volunteer measurements. The crossover 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 measures 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 (*SI 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.

ACKNOWLEDGMENTS. This study was supported by NIH Grants R21ES021895, R01ES015172, R01ES021733, R01ES020836, R01ES021357, P30ES009089, and P30ES000002; US Environmental Protection Agency funding (Grant RD-83479801); Environment Canada; AllerGen Networks of Centres of Excellence; and National Natural Science Foundation of China Grant 91643202.

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