

Correction

MEDICAL SCIENCES

Correction for “B vitamins attenuate the epigenetic effects of ambient fine particles in a pilot human intervention trial,” by Jia Zhong, Oskar Karlsson, Guan Wang, Jun Li, Yichen Guo, Xinyi Lin, Michele Zemplyeni, Marco Sanchez-Guerra, Letizia Trevisi, Bruce Urch, Mary Speck, Liming Liang, Brent A. Coull, Petros Koutrakis, Frances Silverman, Diane R. Gold, Tangchun Wu, and Andrea A. Baccarelli, which appeared in issue 13, March 28, 2017, of *Proc Natl Acad Sci USA* (114:3503–3508; first published March 13, 2017; 10.1073/pnas.1618545114).

The authors note that the following statement should be added to the Acknowledgments: “This publication was supported in part by United States Environmental Protection Agency (USEPA) Grant RD-83479801. Its contents are solely the responsibility of the grantee and do not necessarily represent the official views of the USEPA. Further, USEPA does not endorse the purchase of any commercial products or services mentioned in the paper.”

www.pnas.org/cgi/doi/10.1073/pnas.1704438114

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

Jia Zhong^{a,1}, Oskar Karlsson^{b,c}, Guan Wang^d, Jun Li^{e,f}, Yichen Guo^g, Xinyi Lin^h, Michele Zemplyni^g, Marco Sanchez-Guerraⁱ, Letizia Trevisi^j, Bruce Urch^{k,l,m,n}, Mary Speck^k, Liming Liang^g, Brent A. Coull^g, Petros Koutrakis^j, Frances Silverman^{k,l,m,n}, Diane R. Gold^{j,o}, Tangchun Wu^{e,f}, and Andrea A. Baccarelli^a

^aMailman School of Public Health, Columbia University, New York, NY 10032; ^bCenter for Molecular Medicine, Department of Clinical Neuroscience, Karolinska Institutet, 171 77 Stockholm, Sweden; ^cDepartment of Pharmaceutical Biosciences, Uppsala University, 752 37 Uppsala, Sweden; ^dInstitute for Genomic Medicine, Columbia University, New York, NY 10032; ^eDepartment of Occupational and Environmental Health, Key Laboratory of Environment and Health, Ministry of Education and State Key Laboratory of Environmental Health (Incubating), School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China, 430030; ^fDepartment of Epidemiology and Biostatistics, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030 Hubei, China, 430030; ^gDepartment of Biostatistics, T. H. Chan School of Public Health, Harvard University, Boston, MA 02115; ^hSingapore Institute for Clinical Sciences, Singapore 117609; ⁱDepartment of Developmental Neurobiology, National Institute of Perinatology, Mexico City 11000, Mexico; ^jDepartment of Environmental Health, T. H. Chan School of Public Health, Harvard University, Boston, MA 02115; ^kDivision of Occupational & Environmental Health, Dalla Lana School of Public Health, University of Toronto, Toronto, ON M5T 3M7, Canada; ^lDepartment of Medicine, University of Toronto, Toronto, ON M5S 1A8, Canada; ^mLi Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, ON M5B 1W8, Canada; ⁿSouthern Ontario Centre for Atmospheric Aerosol Research, Toronto, ON M5S 3E5, Canada; and ^oChanning Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

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.

¹To whom correspondence should be addressed. Email: jiazhong@mail.harvard.edu.

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 B_6 , and B_{12}), 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 $PM_{2.5}$ ($100.6\text{--}287.5 \mu\text{g}/\text{m}^3$; 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 B_6 and B_{12} were $35 \text{ nmol}/\text{L}$ (IQR: $14 \text{ nmol}/\text{L}$), $41 \text{ nmol}/\text{L}$ (IQR: $16 \text{ nmol}/\text{L}$), and $292 \text{ pmol}/\text{L}$ (IQR: $72 \text{ pmol}/\text{L}$) before sham experiment, respectively. After volunteers took placebos for 4 wk, their median plasma concentrations were similar: $39 \text{ nmol}/\text{L}$ (IQR: $24 \text{ nmol}/\text{L}$) for folic acid ($P = 0.82$), $37 \text{ nmol}/\text{L}$ (IQR: $18 \text{ nmol}/\text{L}$) for vitamin B_6 ($P = 0.75$), and $262 \text{ pmol}/\text{L}$ (IQR: $214 \text{ pmol}/\text{L}$) for vitamin B_{12} ($P = 0.42$). B-vitamin supplementation significantly increased the median plasma concentrations of folic acid ($56 \text{ nmol}/\text{L}$; IQR: 13 ; $P = 0.02$), vitamin B_6 ($428 \text{ nmol}/\text{L}$; IQR: 321 ; $P = 0.004$), and vitamin B_{12} ($511 \text{ pmol}/\text{L}$; 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%,

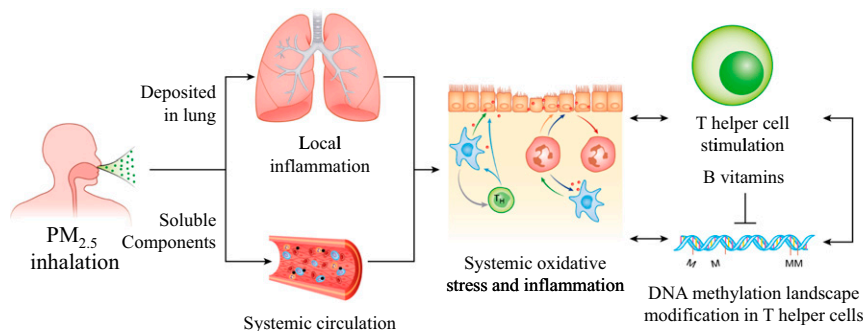


Fig. 1. Proposed conceptual model linking fine particulate matter ($PM_{2.5}$), systemic oxidative stress and inflammation, and altered DNA methylation landscape in Th cells. We hypothesized that $PM_{2.5}$ inhalation triggers local and systemic inflammation and oxidative stress, which alters the DNA 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 $PM_{2.5}$.

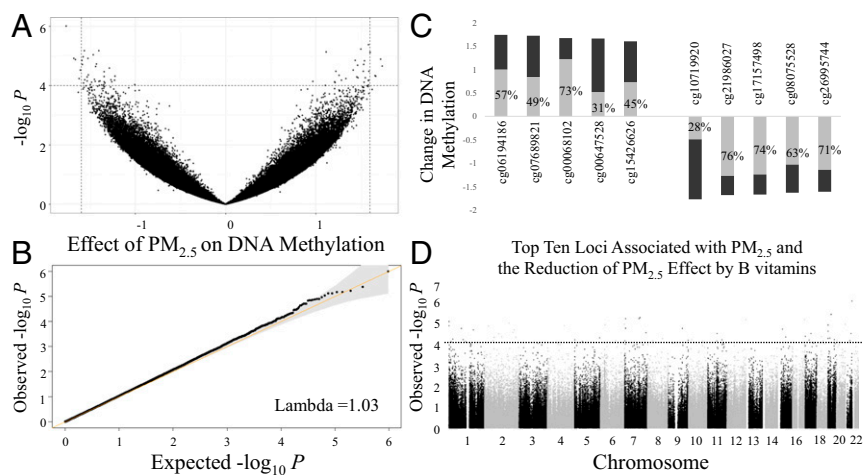


Fig. 2. The effect of 2-h exposure to PM_{2.5} on DNA methylation landscape and the reduction of PM_{2.5} effect by B-vitamin supplementation. **A** is the Volcano plot depicting the distribution of estimated effect of PM_{2.5} exposure on the epigenome. Each dot represents the estimated PM_{2.5} effect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

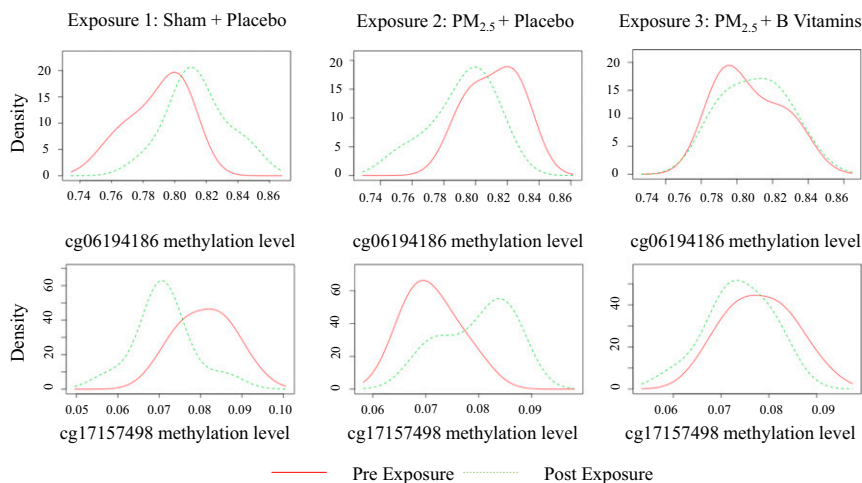


Fig. 3. Distribution of DNA methylation levels of cg06194186 and cg17157498 before and after each exposure experiment. Red solid lines and green dashed lines indicate the distribution of methylation levels measured before and after exposure, respectively.

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 immunoenzymatic assay (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.

- Bell ML, Davis DL (2001) Reassessment of the lethal London fog of 1952: Novel indicators of acute and chronic consequences of acute exposure to air pollution. *Environ Health Perspect* 109(Suppl 3):389–394.
- World Health Organization (September 27, 2016) WHO releases country estimates on air pollution exposure and health impact. Press release. Available at www.who.int/mediacentre/news/releases/2016/air-pollution-estimates/en/. Accessed February 24, 2017.
- Lielveld J, Evans JS, Fnaiss M, Giannadaki D, Pozzer A (2015) The contribution of outdoor air pollution sources to premature mortality on a global scale. *Nature* 525(7569):367–371.
- Brook RD, et al.; American Heart Association Council on Epidemiology and Prevention, Council on the Kidney in Cardiovascular Disease, and Council on Nutrition, Physical Activity and Metabolism (2010) Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. *Circulation* 121(21):2331–2378.
- World Health Organization (2006) *Air Quality Guidelines: Global Update 2005: Particulate Matter, Ozone, Nitrogen Dioxide, and Sulfur Dioxide* (World Health Organization, Copenhagen).
- Richmond-Bryant J, Saganich C, Bukiewicz L, Kalin R (2009) Associations of PM_{2.5} and black carbon concentrations with traffic, idling, background pollution, and meteorology during school dismissals. *Sci Total Environ* 407(10):3357–3364.
- Bender A, et al. (2009) *Understanding Episodes of High Airborne Particulate Matter in Iowa* (Bistate Regional Commission in Eastern Iowa, Iowa City, IA).
- Ramchandani S, Bhattacharya SK, Cervoni N, Szyf M (1999) DNA methylation is a reversible biological signal. *Proc Natl Acad Sci USA* 96(11):6107–6112.
- Panni T, et al. (2016) A genome-wide analysis of DNA methylation and fine particulate matter air pollution in three study populations: KORA F3, KORA F4, and the Normative Aging Study. *Environ Health Perspect* 124(7):983–990.
- Baccarelli A, et al. (2009) Rapid DNA methylation changes after exposure to traffic particles. *Am J Respir Crit Care Med* 179(7):572–578.
- Houseman EA, et al. (2012) DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics* 13:86.
- Rakyan VK, Down TA, Balding DJ, Beck S (2011) Epigenome-wide association studies for common human diseases. *Nat Rev Genet* 12(8):529–541.
- Liu J, et al. (2008) Combined inhaled diesel exhaust particles and allergen exposure alter methylation of T helper genes and IgE production in vivo. *Toxicol Sci* 102(1):76–81.
- Bruniquel D, Schwartz RH (2003) Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nat Immunol* 4(3):235–240.
- Cropley JE, Suter CM, Martin DI (2007) Methyl donors change the germline epigenetic state of the A(vy) allele. *FASEB J* 21(12):3021, author reply 3021–3022.
- Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* 114(4):567–572.
- Sinclair KD, et al. (2007) DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proc Natl Acad Sci USA* 104(49):19351–19356.
- Waterland RA, et al. (2006) Maternal methyl supplements increase offspring DNA methylation at Axin Fused. *Genesis* 44(9):401–406.
- Weaver IC, et al. (2005) Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: Altering epigenetic marking later in life. *J Neurosci* 25(47):11045–11054.
- Kotsopoulos J, Sohn KJ, Kim YI (2008) Postweaning dietary folate deficiency provided through childhood to puberty permanently increases genomic DNA methylation in adult rat liver. *J Nutr* 138(4):703–709.
- Jacob RA, et al. (1998) Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. *J Nutr* 128(7):1204–1212.
- Dolinoy DC, Huang D, Jirtle RL (2007) Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *Proc Natl Acad Sci USA* 104(32):13056–13061.
- Maccani JZ, et al. (2015) Placental DNA methylation related to both infant toenail mercury and adverse neurobehavioral outcomes. *Environ Health Perspect* 123(7):723–729.
- Hyslop SJ, Duncan AM, Pitkanen S, Robinson BH (1996) Assignment of the PSST subunit gene of human mitochondrial complex I to chromosome 19p13. *Genomics* 37(3):375–380.
- Wei S, et al. (2002) Identification and characterization of three members of the human metallocoxy-peptidase gene family. *J Biol Chem* 277(17):14954–14964.
- Butler CC, et al. (2006) Oral vitamin B₁₂ versus intramuscular vitamin B₁₂ for vitamin B₁₂ deficiency: A systematic review of randomized controlled trials. *Fam Pract* 23(3):279–285.
- Ghio AJ, Carraway MS, Madden MC (2012) Composition of air pollution particles and oxidative stress in cells, tissues, and living systems. *J Toxicol Environ Health B Crit Rev* 15(1):1–21.
- Bellavia A, et al. (2013) DNA hypomethylation, ambient particulate matter, and increased blood pressure: Findings from controlled human exposure experiments. *J Am Heart Assoc* 2(3):e000212.
- Turrens JF (2003) Mitochondrial formation of reactive oxygen species. *J Physiol* 552(Pt 2):335–344.
- Smeitink J, van den Heuvel L (1999) Human mitochondrial complex I in health and disease. *Am J Hum Genet* 64(6):1505–1510.
- Cooper G (2000) *The Cell: A Molecular Approach* (Sinauer Associates, Sunderland, MA), 2nd Ed.
- Lee HC, Wei YH (2005) Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *Int J Biochem Cell Biol* 37(4):822–834.
- Attardi G, Schatz G (1988) Biogenesis of mitochondria. *Annu Rev Cell Biol* 4:289–333.
- Zhong J, et al. (2015) Traffic-related air pollution, blood pressure, and adaptive response of mitochondrial abundance. *Circulation* 133(4):378–87.
- Kim I, Rodriguez-Enriquez S, Lemasters JJ (2007) Selective degradation of mitochondria by mitophagy. *Arch Biochem Biophys* 462(2):245–253.
- Janssen BG, et al. (2012) Placental mitochondrial DNA content and particulate air pollution during in utero life. *Environ Health Perspect* 120(9):1346–1352.
- Pufulete M, et al. (2005) Effect of folic acid supplementation on genomic DNA methylation in patients with colorectal adenoma. *Gut* 54(5):648–653.
- Behbod B, et al. (2013) Endotoxin in concentrated coarse and fine ambient particles induces acute systemic inflammation in controlled human exposures. *Occup Environ Med* 70(11):761–767.
- Brook RD, et al. (2002) Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. *Circulation* 105(13):1534–1536.
- Urch B, et al. (2004) Relative contributions of PM_{2.5} chemical constituents to acute arterial vasoconstriction in humans. *Inhal Toxicol* 16(6–7):345–352.
- Demokritou P, Gupta T, Ferguson S, Koutrakis P (2003) Development of a high-volume concentrated ambient particles system (CAPS) for human and animal inhalation toxicological studies. *Inhal Toxicol* 15(2):111–129.
- Lonn E, et al.; Heart Outcomes Prevention Evaluation (HOPE) 2 Investigators (2006) Homocysteine lowering with folic acid and B vitamins in vascular disease. *N Engl J Med* 354(15):1567–1577.
- Ullegaddi R, Powers HJ, Gariballa SE (2004) B-group vitamin supplementation mitigates oxidative damage after acute ischaemic stroke. *Clin Sci (Lond)* 107(5):477–484.
- Willett WC, et al. (1985) Reproducibility and validity of a semiquantitative food frequency questionnaire. *Am J Epidemiol* 122(1):51–65.
- Lowe R, Morris T (2013) Report on the 2nd annual Infinium HumanMethylation450 array workshop: 15 April 2013 QMUL, London, UK. *Epigenetics* 8(10):1123–1124.
- Sandoval J, et al. (2011) Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics* 6(6):692–702.
- Aryee MJ, et al. (2014) Minfi: A flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics* 30(10):1363–1369.
- Pidsley R, et al. (2013) A data-driven approach to preprocessing Illumina 450K methylation array data. *BMC Genomics* 14(1):293.
- Bates D, Maechler M, Bolker B (2012) lme4: Linear mixed-effects models using Eigen and syntax. *J Stat Software* 67(1).
- Kuznetsova A, Brockhoff PB, Christensen RHB (2013) lmerTest, R Package Version 2(6). Available at <https://cran.r-project.org/web/packages/lmerTest/lmerTest.pdf>. Accessed February 24, 2017.
- Peng C, et al. (2016) Particulate air pollution and fasting blood glucose in nondiabetic individuals: Associations and epigenetic mediation in the Normative Aging Study, 2000–2011. *Environ Health Perspect* 124(11):1715–1721.
- Bind M-AC, Vanderweele TJ, Coull BA, Schwartz JD (2016) Causal mediation analysis for longitudinal data with exogenous exposure. *Biostatistics* 17(1):122–134.