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World Trade Center (WTC) dust exposure in mice is associated with inflammation, oxidative stress and epigenetic changes in the lung

Vasanthi R. Sunil^{a,*}, Kinal N. Vayas^a, Mingzhu Fang^b, Helmut Zarbl^b, Christopher Massa^a, Andrew J. Gow^a, Jessica A. Cervelli^a, Howard Kipen^b, Robert J. Laumbach^b, Paul J. Liroy^b, Jeffrey D. Laskin^b, and Debra L. Laskin^a

^aDepartment of Pharmacology and Toxicology, Rutgers University, Ernest Mario School of Pharmacy, Piscataway, NJ, United States

^bDepartment of Environmental and Occupational Health, Rutgers School of Public Health, Piscataway, NJ, United States

Abstract

Exposure to World Trade Center (WTC) dust has been linked to respiratory disease in humans. In the present studies we developed a rodent model of WTC dust exposure to analyze lung oxidative stress and inflammation, with the goal of elucidating potential epigenetic mechanisms underlying these responses. Exposure of mice to WTC dust (20 µg, i.t.) was associated with upregulation of heme oxygenase-1 and cyclooxygenase-2 within 3 days, a response which persisted for at least 21 days. Whereas matrix metalloproteinase was upregulated 7 days post-WTC dust exposure, IL-6RA1 was increased at 21 days; conversely, expression of mannose receptor, a scavenger receptor important in particle clearance, decreased. After WTC dust exposure, increases in methylation of histone H3 lysine K4 at 3 days, lysine K27 at 7 days and lysine K36, were observed in the lung, along with hypermethylation of Line-1 element at 21 days. Alterations in pulmonary mechanics were also observed following WTC dust exposure. Thus, 3 days post-exposure, lung resistance and tissue damping were decreased. In contrast at 21 days, lung resistance, central airway resistance, tissue damping and tissue elastance were increased. These data demonstrate that WTC dust-induced inflammation and oxidative stress are associated with epigenetic modifications in the lung and altered pulmonary mechanics. These changes may contribute to the development of WTC dust pathologies.

Keywords

WTC dust; Lung; Oxidative stress; Inflammation; Epigenetics; Lung function

*Corresponding author at: Rutgers University, Ernest Mario School of Pharmacy, 160 Frelinghuysen Road, Piscataway, NJ 08854, United States. sunilva@pharmacy.rutgers.edu (V.R. Sunil).

Conflict of interest

The authors declare that there are no conflicts of interest.

1. Introduction

The collapse of the World Trade Center (WTC) in New York City on September 11, 2001 resulted in high concentrations of a complex mixture of airborne pollutants, consisting of coarse and fine particles from building materials and combustion products in the ensuing plume. This included, but was not limited to cement, glass fibers, asbestos, metals, organic and inorganic particles and gases. Analysis of WTC dust revealed the presence of particles small enough to penetrate deep into the lungs of first responders, recovery workers and residents, reaching the distal airways and alveoli (Lioy et al., 2002; Gavett, 2003; McGee et al., 2003; Lioy and Georgopoulos, 2006). Evidence suggests a link between exposure to WTC dust-derived airborne materials and respiratory disease (Guidotti et al., 2011; Wisnivesky et al., 2011; Berger et al., 2013). Upper and lower respiratory tract pathologies including chronic rhinosinusitis, bronchitis, asthma and bronchiolitis have been reported in individuals exposed to WTC dust (Guidotti et al., 2011). Sarcoid-like granulomatous inflammation has also been noted in rescue and recovery workers (Izbicki et al., 2007; Crowley et al., 2011). To characterize these responses, we developed an animal model of WTC dust exposure, with the goal of elucidating potential epigenetic mechanisms underlying disease pathogenesis.

Epigenetic changes encompass an array of molecular modifications to both DNA and chromatin. The most extensively investigated modifications are DNA methylation at the carbon-5 position of cytosine in CpG dinucleotides, and alterations in chromatin packaging of DNA by posttranslational histone modification, such as methylation and acetylation (Jirtle and Skinner, 2007). These are known to be involved in regulating macrophage activation and inflammatory gene expression (Jirtle and Skinner, 2007; Kapellos and Iqbal, 2016). Exposure to diesel exhaust particles, cigarette smoke and ozone has been reported to cause epigenetic changes in the respiratory tract, which have been implicated in the development of inflammatory lung diseases (Rajendrasozhan et al., 2008; Baccarelli et al., 2009; Ho et al., 2012). DNA methylation and histone modifications are thought to be the major epigenetic mechanisms leading to transcriptional silencing of genes relevant to the development of airway inflammatory and allergic diseases following environmental exposures (Adcock et al., 2007; Kuriakose and Miller, 2010; Ho et al., 2012; Lepeule et al., 2012; Tzouveleakis and Kaminski, 2015).

In the present studies, we analyzed the effects of WTC dust exposure on pulmonary inflammation and oxidative stress, and on lung function in an experimental rodent model. We also determined if WTC dust-induced lung changes were associated with DNA methylation and histone modifications. Elucidating mechanisms underlying WTC dust disease may be useful in understanding inflammatory disease pathogenesis in humans and developing efficacious therapeutic approaches.

2. Materials and methods

2.1. Materials

A bulk sample of settled WTC dust was collected from a single location on Market Street in New York City in a protected area near ground zero on day 5 after the attack. The physical

characteristics and chemical composition of the sample have been described previously (Lioy et al., 2002). The sample represented total settled dust and was used in these experiments without pre-sizing or other modifications.

2.1.1. Animals, treatments and sample collection—Female specific pathogen-free C57BL/6J mice (8–10 weeks, 17–20 g) were obtained from The Jackson Laboratories (Bar Harbor, ME). Animals were housed in filter-top microisolation cages and maintained on food and water *ad libitum*. All animals received humane care in compliance with the institution's guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health. Animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) and then placed on a tilting rodent work stand (Hallowell EMC, Pittsfield, MA) in a supine position and restrained using an incisor loop. The tongue was extruded using a cotton tip applicator and the larynx visualized by a hemi-sectioned 3 mm diameter speculum attached to an operating head of an otoscope (Welch Allyn, Skaneateles Falls, NY). WTC dust (1 mg) was resuspended in sterile PBS (2.5 ml) and vortexed for 1 min immediately before use. PBS or PBS containing WTC dust was administered intratracheally (i.t.) via Clay Adams Intramedic PE-10 (I.D. 0.76 mm, O.D. 1.22 mm) polyethylene tubing (Becton, Dickinson and Company, Franklin Lakes, NJ), attached to a 27^{1/2} gauge hypodermic needle (0.4 × 13 mm). The tubing was advanced approximately 10 mm past the epiglottis and 50 µl of PBS control or WTC dust suspension (20 µg/animal) was instilled into the trachea. The tubing and speculum were withdrawn immediately after instillation. Animals were then removed from the work stand and maintained in a vertical position until normal respiration was observed (b1 min). Animals were euthanized 3 d, 7 d or 21 d after treatment by intraperitoneal (i.p.) injection of pentobarbital (200 mg/kg). Bronchoalveolar lavage (BAL) fluid and lung tissue were collected as previously described (Sunil et al., 2015). The largest lobe of the lung was fixed with 3% paraformaldehyde and used for histological analysis.

2.1.2. Immunohistochemistry—Tissue sections (4 µm) were deparaffinized with xylene (4 min, × 2) followed by decreasing concentrations of ethanol (100%–50%) and then water. After antigen retrieval using citrate buffer (10.2 mM sodium citrate, 0.05% Tween 20, pH 6.0) and quenching of endogenous peroxidase with 3% H₂O₂ for 10–30 min, sections were incubated for 1–4 h at room temperature with 5–100% goat serum to block nonspecific binding. This was followed by overnight incubation at 4°C with rabbit IgG or rabbit polyclonal anti-heme oxygenase (HO)-1 (1:100, Enzo Life Sciences, Farmingdale, NY), anti-mannose receptor-1 (1:400, Abcam, Cambridge, MA), or anti-cyclooxygenase (COX)-2 (1:400, Abcam) antibodies. Sections were then incubated with biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 30 min at room temperature. Binding was visualized using a Peroxidase Substrate Kit DAB (Vector Labs). Positively stained macrophages (HO-1 and mannose receptor) and Type II cells (COX-2) were enumerated in 450 fields/lung section (4–6 mice/treatment group), and assigned an intensity score of 0 to 3, with 0 = no staining, 0.5 = minor staining, 1 = light staining, 2 = medium staining and 3 = dark staining.

2.1.3. Western blotting—Lung lysates (25 µg) or nuclear extracts (5–10 µg) were fractionated on 7.5%–15% SDS-PAGE gels (Bio Rad, Hercules, CA), and transferred to polyvinylidene fluoride membranes (PVDF, 0.45 µm pore size; Millipore, Billerica, MA). Nonspecific binding was blocked by incubation of the blots for 1 h at room temperature with 5% non-fat dry milk in TBS-T (0.02 M Tris base, 0.137 M sodium chloride, 0.1% Tween-20) buffer. Blots were incubated overnight at 4 °C with anti-matrix metalloproteinase (MMP)-12 antibody (1:500, Santa Cruz Biotechnology, CA), Histone H3 (1:4000, Cell Signaling, Danvers, MA), mono-methyl histone H3meK4 (1:1000, Cell Signaling), pan-methyl histone H3meK9 (1:1000, Cell Signaling), tri-methyl histone H3meK27 (1:1000, Cell Signaling), or dimethyl histone H3meK36 (1:1000, Cell Signaling). After 5 washes in 0.1% TBS-T buffer, blots were incubated with anti-rabbit HRP-conjugated secondary antibody (1:20,000, Cell Signaling) for 1 h at room temperature. Bands were visualized using an ECL+ detection system (GE, Piscataway, NJ). Band densities were analyzed using a Bio-Rad Imager and quantified using Quantity One software (Hercules, CA). Band densities were normalized to histone H3 and data presented as the ratio of methylated histone H3 (H3me) to unmethylated histone H3.

2.1.4. Reverse transcriptase qPCR—Total RNA was extracted from mouse lung using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA), and then digested with Qiagen RNase-free DNase. Relative mRNA expression was determined by RT-PCR as previously described (Sunil et al., 2012). GAPDH was used as an endogenous control. The comparative Ct method was used to analyze relative mRNA expression. Forward and reverse primer sequences were IL-6 (5'-CTA CCC CAA TTT CCA ATG CT-3'; 5'-ACC ACA GTG AGG AAT GTC CA-3'), IFN γ (5'-CAA AAG GAT GGT GAC ATG AA-3'; 5'-TTG GCA ATA CTC ATG AAT GC-3'), TNF α (5'-CCC ACT CTG ACC CCT TTA CT-3'; 5'-TTT GAG TCC TTG ATG GTG GT-3'), IL-6RA1 (5'-CAG AAA AGC CTT TGG TTT CA-3'; 5'-CCT CGG TGA CCA CTA ACA TC-3'), IL-6RA2 (5'-CAA GAA TCC TCG TCC ATG TC-3'; 5'-ACG GTA TTG TCA GAC CCA GA-3'), IL-1 α (5'-CGG GTG ACA GTA TCA GCA AC-3'; 5'-GAC AAA CTT CTG CCT GAC GA-3').

2.1.5. DNA methylation analysis—Global DNA methylation of B1 and Line-1 elements and gene-specific DNA methylation in the promoter regions of IL-4, IL-6, IFN γ , PTGS2, TNF α , and IL-6RA1 genes were assessed by pyrosequencing. Briefly, genomic DNA samples were extracted from the lung and subjected to bisulfite modification. Samples were then amplified by PCR and analyzed using a PSQHS96 Pyrosequencing System (EpigenDx, Hopkinton, MA). The degree of methylation is expressed as the percentage of methylated cytosines (%5mC) for mouse B1 and Line-1, and in CpG sites within the promoter regions selected in each gene, respectively. For all assays, positive methylation and negative unmethylated DNA standards were used to generate a standard curve verifying bisulfite conversion and PCR amplification. For each CpG site tested, %5mC was calculated based on the standard curve.

2.1.6. Measurement of pulmonary mechanics—Animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg; i.p.). After 5 min, a tracheotomy was performed using an 18 gauge cannula and the animals connected to a SciReq flexiVent small

animal ventilator (Montreal, Canada) to measure respiratory mechanics at positive end-expiratory pressures (PEEPs) ranging from 0–9 cm H₂O (Groves et al., 2012). Data from the impedance spectra were fit to a constant phase model, allowing for calculation of frequency-independent central airway resistance (R_{aw}), and the coefficients of tissue damping (G) and tissue elastance (H) in the tissue compartment (Suki et al., 1991). Data were analyzed using flexiVent software version 5.2.

2.2. Statistics

All experiments were repeated at least 3 times. Data were analyzed by 2-way or 3-way ANOVA followed by post hoc analysis using the Holm-Sidak test. A *p* value of 0.05 was considered statistically significant.

3. Results

3.1. Effects of WTC dust on markers of oxidative stress, inflammation and injury

Treatment of mice with WTC dust resulted in oxidative stress, as indicated by increases in HO-1 expression in alveolar macrophages and Type II cells (Fig. 1 and Table 1); this was observed at all post-exposure times examined. WTC dust exposure was also associated with a significant increase in the percentage of BAL neutrophils at 7 d and 21 d (Table 2). In contrast, total BAL cell and protein content were unaffected by WTC dust exposure (data not shown).

Mannose receptor is a scavenger receptor important in macrophage uptake of particles (Geiser, 2010). Alveolar macrophages from control animals constitutively expressed mannose receptor (Fig. 2 and Table 1). Treatment of mice with WTC dust resulted in down regulation of mannose receptor expression at 3 d and 7 d post-exposure; by 21 d, mannose receptor expression was at control levels. COX-2 is an enzyme mediating production of pro-inflammatory eicosanoids (Park and Christman, 2006; Lee and Yang, 2013). In control mice, low level constitutive expression of COX-2 was noted, predominantly in Type II epithelial cells (Fig. 3 and Table 1). Following WTC dust exposure, expression of COX-2 increased in Type II cells, beginning at 3 d and persisting for at least 21 d; a subset of macrophages expressing COX-2 was also identified 3 d post-exposure (Fig. 3). MMP-12 is a protease involved in inflammation and wound healing; it has also been implicated in the development of fibrosis (Matute-Bello et al., 2007; Crouser et al., 2009; Stawski et al., 2014). MMP-12 was upregulated in lungs of animals treated with WTC dust, but only at 7 d post-exposure (Fig. 4). Expression of inflammatory genes including IL-6, IFN γ , TNF α , IL-6RA1, IL-6RA2 and IL-1 α was also analyzed after WTC dust exposure. At 21 d post-exposure, a significant increase in IL-6RA1 mRNA expression was observed in the lung (Fig. 5). No significant effects were noted on any other inflammatory genes.

3.2. Effects of WTC dust on epigenetic markers

In further studies we determined if WTC dust exposure resulted in alterations in global and inflammatory gene-specific DNA methylation. Significant increases in global methylation of Line 1 at each of the 5 CpG sites tested were noted at 21 d following WTC dust exposure (Table 3). Conversely, B1 element and inflammatory gene-specific methylation were not

significantly altered by WTC dust. Methylation of histone H3 proteins was also analyzed. Low level histone H3 methylation on lysine 4, 9, 27 and 36 was observed in lungs of control mice (Fig. 6). WTC dust exposure significantly increased histone H3 methylation on lysine 4 at 3 d, lysine 27 at 7 d and lysine 36 at 21 d, with no major effects on lysine 9. At 21 d post-WTC dust exposure, histone H3 lysine 4 methylation was reduced relative to PBS control (Fig. 6).

3.3. Effects of WTC dust on pulmonary mechanics

We next analyzed the effects of WTC dust exposure on airway and parenchymal resistive and elastic properties by measuring responses to increasing PEEP (Groves et al., 2012). In mice exposed to WTC dust, significant decreases in overall total lung resistance and overall tissue damping were noted at 3 d relative to PBS control mice when analyzed across all levels of PEEP (Fig. 7). Conversely, at 21 d post-exposure, significant increases in overall total lung resistance, central airway resistance, tissue damping and elastance were observed across all levels of PEEP (Fig. 7).

4. Discussion

WTC dust is comprised of three major components: gypsum, concrete and man-made fibers (Lowers et al., 2009). The high pH (9–11) and the small size of many of the particles makes WTC dust a potent respiratory tract irritant (Landrigan et al., 2004; Lioy and Georgopoulos, 2006; Guidotti et al., 2011). Thus, it is not surprising that more than a decade after the collapse of the WTC towers, there remain respiratory disorders in rescue and recovery workers who responded during the first 72 h. One common pathology observed in the lungs of these individuals is granulomatous inflammation, characterized by the appearance of clusters of enlarged, vacuolated macrophages that are highly positive for markers of oxidative stress and activation (Izbicki et al., 2007; Crowley et al., 2011). In the present studies, we developed a mouse model of WTC dust exposure to characterize inflammatory and functional responses, and potential epigenetic regulatory pathways. Our findings that WTC dust-induced oxidative stress and inflammation are associated with specific epigenetic changes in the lung suggest a potential mechanistic pathway underlying pulmonary toxicity and disease pathogenesis.

A characteristic response to oxidative stress is upregulation of HO-1, a phase II stress response enzyme with anti-oxidant and anti-inflammatory activity (Otterbein et al., 1999). Following exposure to WTC dust, expression of HO-1 increased in alveolar macrophages and Type II cells. These findings suggest that both cell types are targets for oxidative damage, and may be a significant source of cytotoxic oxidants following exposure to WTC dust. This is supported by findings that alveolar macrophages and Type II cells generate reactive oxidants following exposure to WTC dust (Payne et al., 2004). Evidence suggests that HO-1 also plays a role in macrophage activation towards an alternatively activated anti-inflammatory/wound repair phenotype (Lee and Chau, 2002; Alcaraz et al., 2003). This may be important in limiting inflammatory responses to WTC dust. Our findings that WTC dust induces oxidative stress are in accord with a recent report in a rat model of inhalation exposure (Cohen et al., 2015). We also found that numbers of neutrophils in BAL were

increased at 7 d and 21 d after exposure of mice to WTC dust. These cells are known to contribute to oxidative stress induced by organic dust and other particulate matter, and they may play a similar role in the pulmonary response to WTC dust (Mantecca et al., 2010; McGovern et al., 2015; McGovern et al., 2016).

Mannose receptor is a C-type lectin important in macrophage uptake and clearance of bacteria, glycoproteins, and particulates (Gazi and Martinez-Pomares, 2009; Geiser, 2010). The present studies show that mannose receptor is constitutively expressed on resident alveolar macrophages, which is consistent with previous reports (Laskin et al., 2015). Following WTC dust exposure, macrophage expression of mannose receptor was down regulated at 3 d and 7 d, suggesting an early impairment of phagocytosis. This may play a role in prolonging exposure of the lung to WTC dust. Mannose receptor expression has been shown to be reduced in allergic asthmatics and in a murine model of COPD (Ganesan et al., 2012; Geiser et al., 2013). Impaired mannose receptor mediated endocytosis by macrophages may contribute to the development of these pathologies following WTC dust exposure in humans.

We also found that COX-2 expression was upregulated in the lung within 3 d of exposure to WTC dust, consistent with an early pro-inflammatory response. Findings that it remained elevated for at least 21 d are in accord with the persistent inflammatory response observed in exposed humans (Yoon et al., 2013; Liu et al., 2015). The fact that COX-2 was mainly expressed by Type II cells provides support for the notion that these cells participate in inflammatory responses to tissue injury (Cheng et al., 2016). IL-6RA1 mRNA was also upregulated in the lung 21 d post-WTC dust exposure. IL-6RA1 is the major receptor for IL-6, an inflammatory cytokine involved in chronic inflammation and inflammation-driven carcinogenesis (Waldner and Neurath, 2014; Rath et al., 2015). Increases in IL-6RA1 following exposure to WTC dust may be important in the development of granulomatous pulmonary inflammation, as well as increased cancer risk in exposed individuals (Boffetta et al., 2016). After WTC dust exposure, we also found that MMP-12 increased in the lung 7 d post-exposure. However, as MMP-12 promotes both inflammation and wound repair, its role in WTC dust-induced lung injury and disease pathogenesis is unclear (Nenan et al., 2005; Iwanami et al., 2009; Kim et al., 2014). Findings that the risk of developing WTC dust induced lung disease is reduced in exposed individuals with elevated serum levels of MMP-12 suggest that it may be protective (Kwon et al., 2013); however, this remains to be investigated.

Evidence suggests that inflammatory gene expression is regulated, in part, by global and gene-specific DNA methylation and histone modifications (Adcock and Lee, 2006; Tommasi et al., 2012). Alu repetitive elements (B1 element in mouse) and Line-1 element have been used as surrogate markers for estimating global DNA methylation levels (Yang et al., 2004; Weisenberger et al., 2005). Following WTC dust exposure, we observed significant increases in global methylation of Line-1 element at 21 d. Increases in global methylation of Line-1 element have been reported in rodents after exposure to carbon black, nitrogen dioxide, particulate matter and ozone, pulmonary irritants also known to induce oxidative stress (De Prins et al., 2013; Carmona et al., 2014). These findings suggest that global methylation of Line-1 element may be a general response to cytotoxic pulmonary irritants. This is

supported by early reports that in human blood cells, changes in Line-1 methylation correlate with susceptibility to the adverse effects of air pollution (Baccarelli et al., 2009; Bind et al., 2012). Line-1 methylation is thought to be a pre-diagnostic marker for renal cell carcinoma and prostate cancer (Andreotti et al., 2014; Barry et al., 2015; Karami et al., 2015). It remains to be determined if it is also an early marker of lung cancer after exposure to WTC dust.

Histones are proteins that facilitate the assembly of DNA into nucleosomes. Specific amino acids at the N-terminal ends of histones undergo posttranslational modifications including methylation, which leads to changes in gene transcription (Ho et al., 2012). Many environmental pollutants have been shown to induce histone modifications, a response thought to be mediated by oxidative stress (Baccarelli and Bollati, 2009; Baccarelli et al., 2009). Histone methylation at lysine (K) residues by specific histone methyl transferases results in either gene activation (H3K4, H3K36 and H3K79), or gene repression (H3K9, H3K27 and H3K20) (Sundar et al., 2013). Exposure of mice to WTC dust resulted in increased methylation of H3K4 at 3 d, H3K27 at 7 d, and H3K36 at 21 d, but decreased H3K4 methylation at 21 d. Similar methylation patterns have been observed in lungs of mice exposed to cigarette smoke (Sundar et al., 2014). Increases in H3K4 have been reported in humans after prolonged exposure to metal components of particulate matter air pollution (Cantone et al., 2011). While early increases in H3K4 methylation 3 d after exposure to WTC dust are likely linked to acute inflammation, decreases at 21 d are consistent with chronic inflammation (Bam et al., 2016). COX-2 gene expression has been reported to be regulated, in part, by chromatin modifications (Cao et al., 2007). Our findings of persistent COX-2 expression in the lung following WTC dust exposure may be due to increased methylation of histone H3 at lysine K4, K27 and K36; however, further studies are needed to directly evaluate this possibility.

To assess the effects of exposure to WTC dust on lung function, we employed a forced oscillation technique at varying levels of PEEP. Both PBS and WTC dust exposed mice responded relatively normally to increasing PEEP. However, small but significant differences in lung mechanics were noted between the groups. Thus, at 3 d post-exposure, overall total lung resistance across all PEEPs was reduced in WTC dust exposed mice, when compared to control mice, a response coordinate with overall decreases in both tissue damping and tissue elastance. The fact that these changes were not normalized by high PEEP indicates that changes in pulmonary mechanics after WTC dust exposure are not the result of derecruitment of respiratory units. These findings are consistent with the mild inflammation observed in WTC dust-exposed mice. Alterations in tissue damping have been attributed to heterogeneity in the lung (Bates and Allen, 2006), suggesting either tissue destruction or altered surfactant function (Wright et al., 2001). At 21 d post-WTC dust exposure, overall increases in total lung resistance, central airway resistance, tissue damping and tissue elastance relative to control mice, across all PEEPs were observed. Moreover, there was an overall increase in central airway resistance, tissue damping and tissue elastance relative to 3 d post-WTC dust exposure. Changes in pulmonary mechanics at 21 d were also consistent across all levels of PEEP, indicative of a “stiffer” lung. Based on these findings, we speculate that the small changes in lung function between 3 d and 21 d reflect normal tissue repair.

In summary, the present studies show that pulmonary exposure of mice to WTC dust causes oxidative stress in alveolar macrophages and Type II epithelial cells, a response associated with a persistent neutrophilic inflammatory response and upregulation of inflammatory markers. WTC dust exposure also causes changes in DNA methylation and histone modifications, as well as alterations in lung function. These findings may have clinical relevance, as they identify potential targets for the development of therapeutics aimed at treating WTC dust disease in humans.

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Abbreviations

WTC	World Trade Center
i.t.	intratracheal
i.p.	intraperitoneal
HO-1	heme oxygenase-1
COX-2	cyclooxygenase-2
MMP-12	matrix metalloproteinase-12
BAL	bronchoalveolar lavage
PEEP	positive end expiratory pressure
COPD	chronic obstructive pulmonary disease
K	lysine

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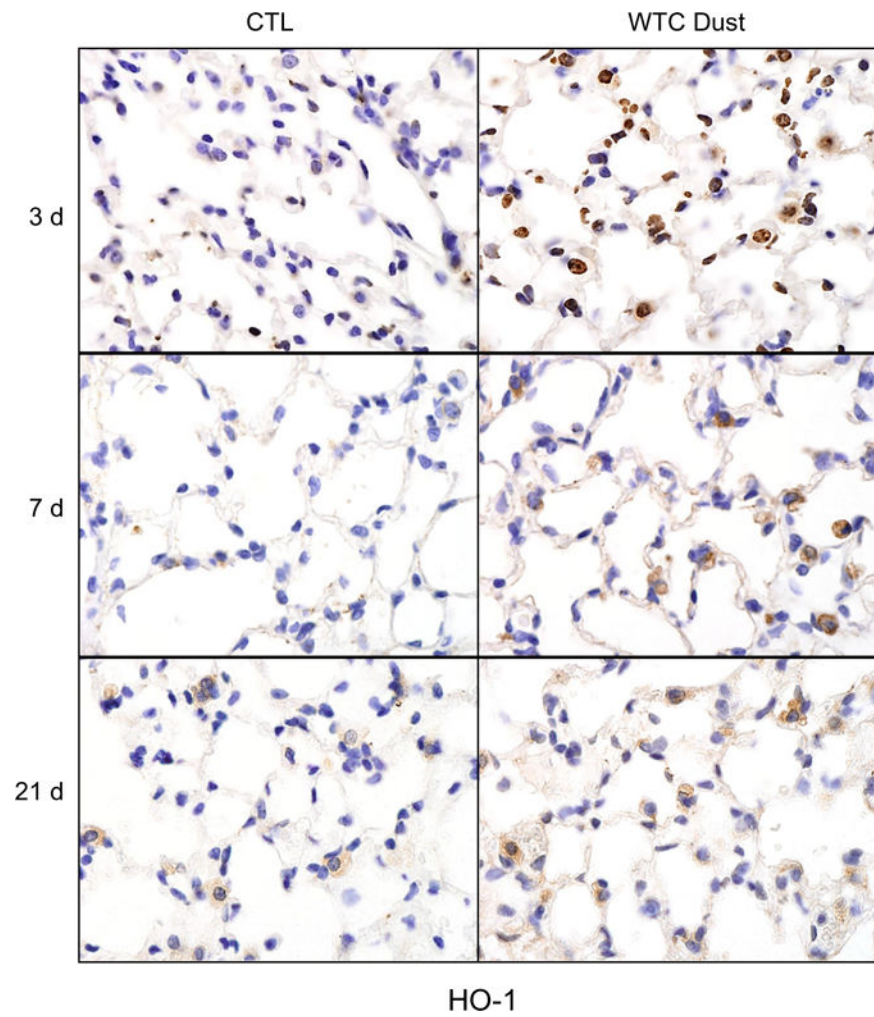


Fig. 1. Effects of WTC dust on HO-1 expression. Tissue sections, prepared 3 d, 7 d and 21 d after exposure of mice to PBS (CTL) or WTC dust, were stained with antibody to HO-1. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 4–6 mice per treatment group is shown (Original magnification, $\times 600$).

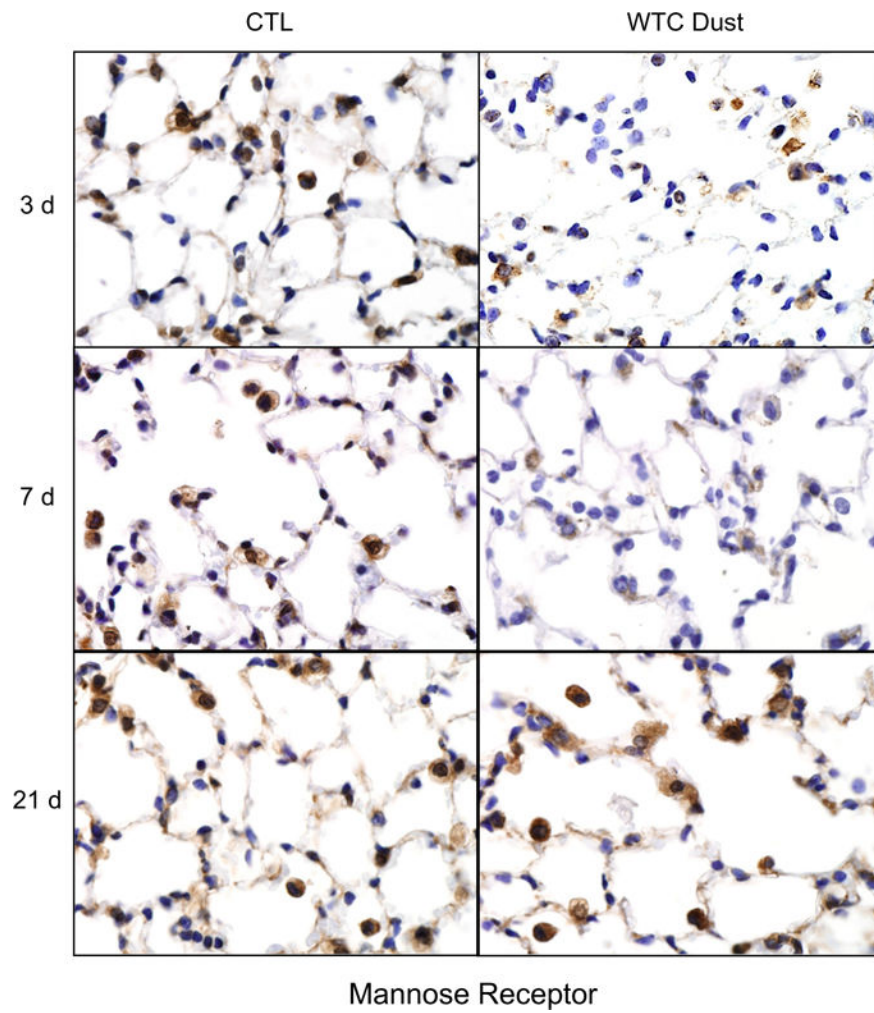


Fig. 2. Effects of WTC dust on mannose receptor expression. Tissue sections, prepared 3 d, 7 d and 21 d after exposure of mice to PBS (CTL) or WTC dust, were stained with antibody to mannose receptor. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 4–6 mice per treatment group is shown (Original magnification, $\times 600$).

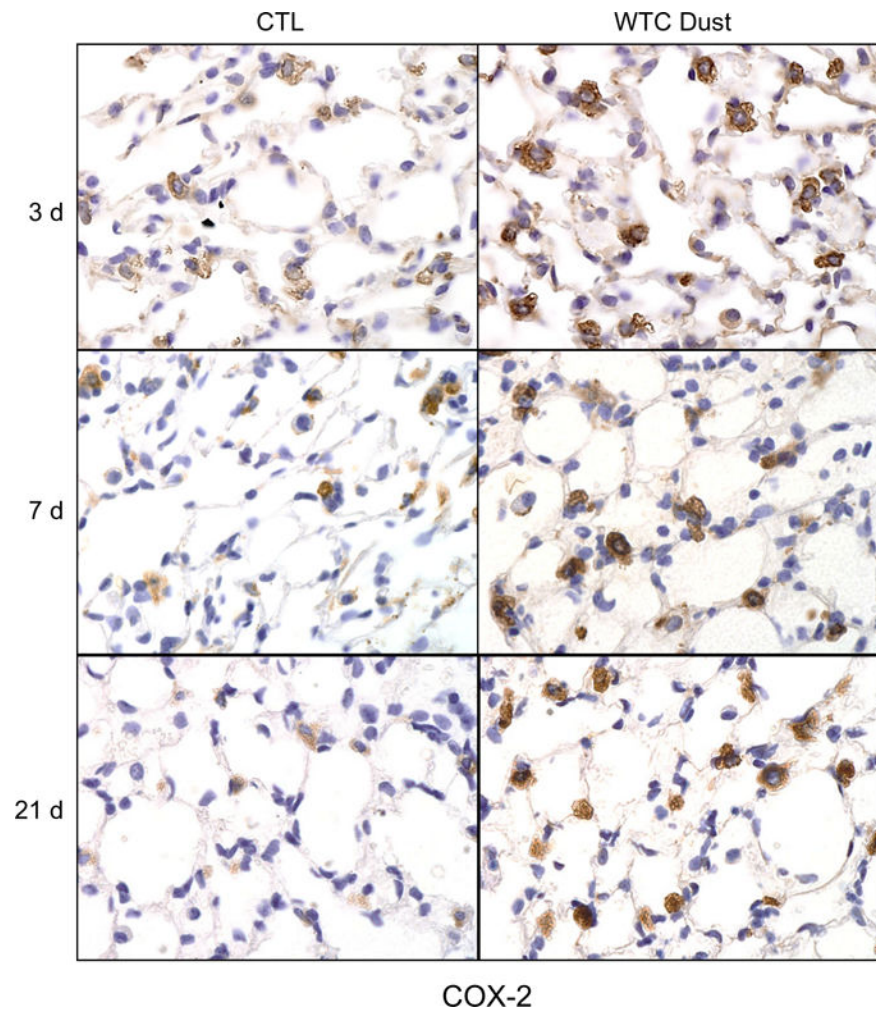


Fig. 3. Effects of WTC dust on COX-2 expression. Tissue sections, prepared 3 d, 7 d and 21 d after exposure of mice to PBS (CTL) or WTC dust, were stained with antibody to COX-2. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 4–6 mice per treatment group is shown (Original magnification, $\times 600$).

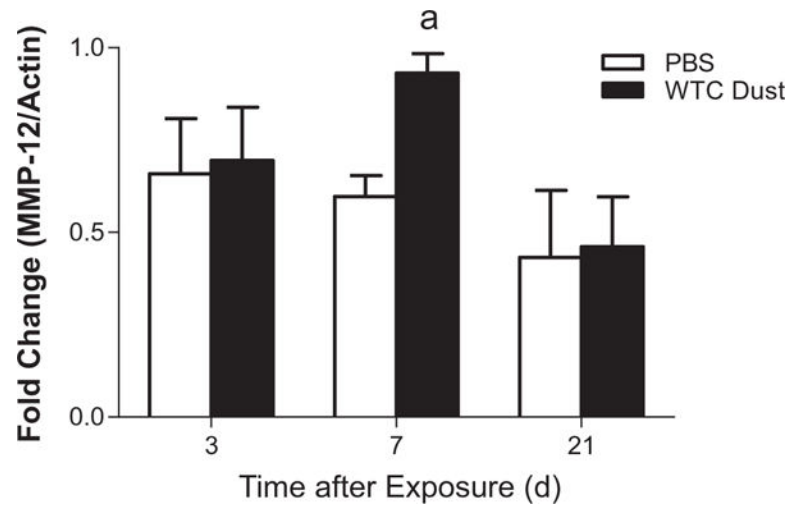


Fig. 4. Effects of WTC dust on lung MMP-12 expression. Lung lysates, prepared 3 d, 7 d and 21 d after exposure of mice to PBS or WTC dust, were analyzed for MMP-12 by Western blotting. Band densities were normalized to actin. Data are presented as the ratio of MMP-12 to actin. Bars, mean \pm SE ($n = 4$ mice/group). ^aSignificantly different ($p < 0.05$) from PBS control.

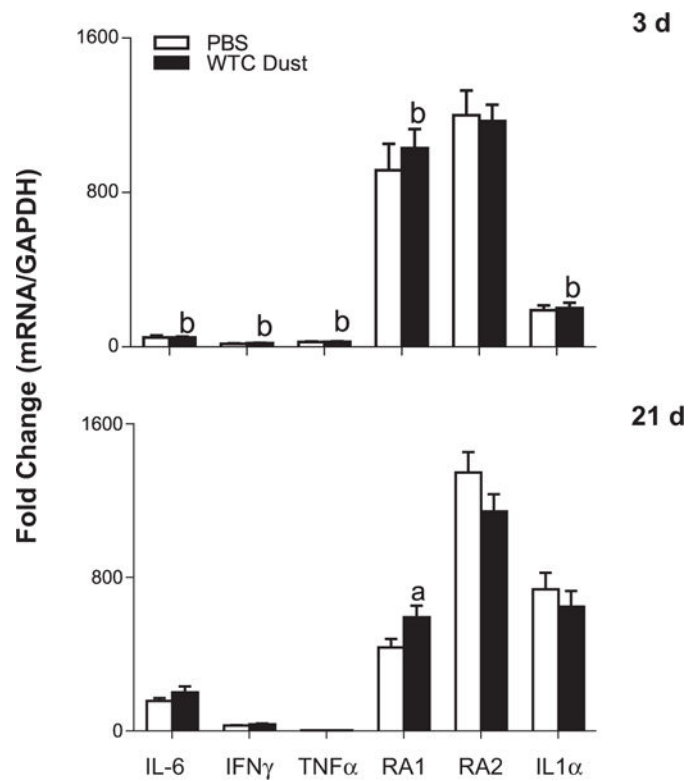


Fig. 5. Effects of WTC dust on inflammatory gene expression. Total RNA, extracted from the lung 3 d or 21 d after exposure of mice to PBS or WTC dust, were analyzed in triplicate for IL-6, IFN γ , TNF α , IL-6RA1 (RA1), IL-6RA2 (RA2) and IL-1 α mRNA expression by real time RT-PCR. Data were normalized to GAPDH and presented as the ratio of gene specific mRNA to GAPDH. Data were analyzed using 2-way ANOVA followed by post hoc analysis using Holm-Sidak test. Bars, mean \pm SE ($n = 5$ mice/group). ^aSignificantly different ($p < 0.05$) from PBS control; ^bSignificantly different ($p < 0.05$) from 21 d.

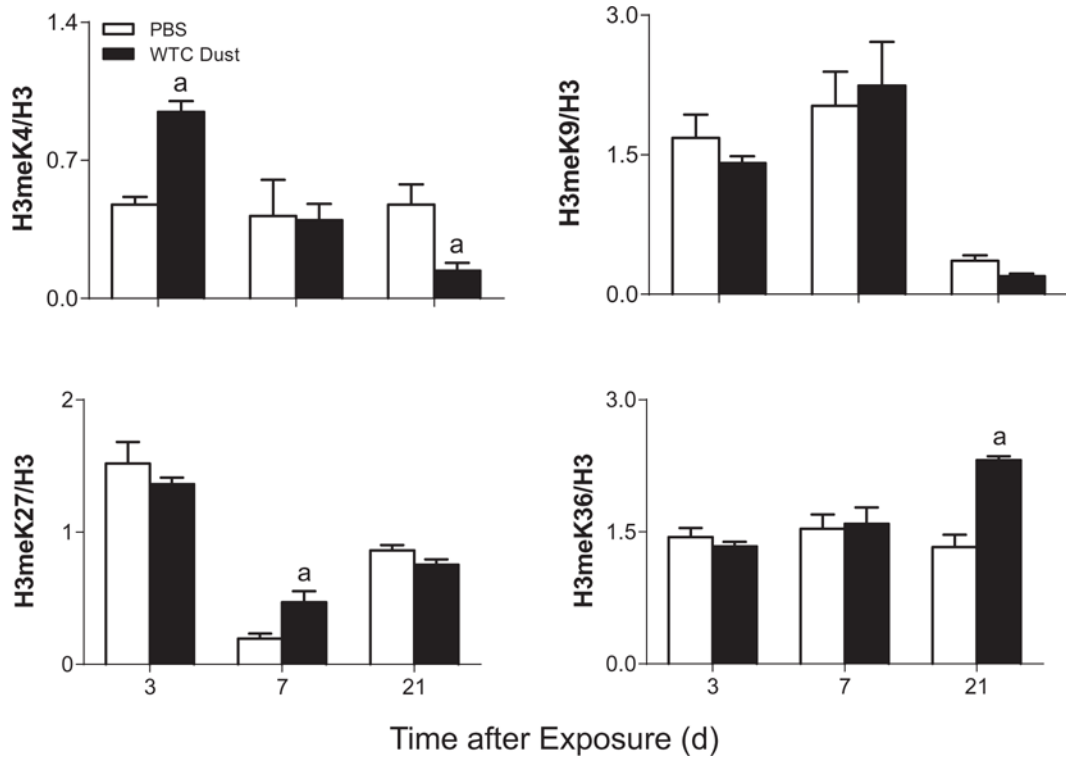


Fig. 6. Effects of WTC dust on histone H3 lysine methylation. Nuclear extracts, prepared 3 d, 7 d and 21 d after exposure of mice to PBS or WTC dust, were analyzed for histone H3 lysine methylation by western blotting. Band densities were normalized to histone H3. Data are presented as the ratio of methylated histone H3 (H3me) to unmethylated histone H3. Bars, mean \pm SE ($n = 4$ mice/group). ^aSignificantly different ($p < 0.05$) from PBS control.

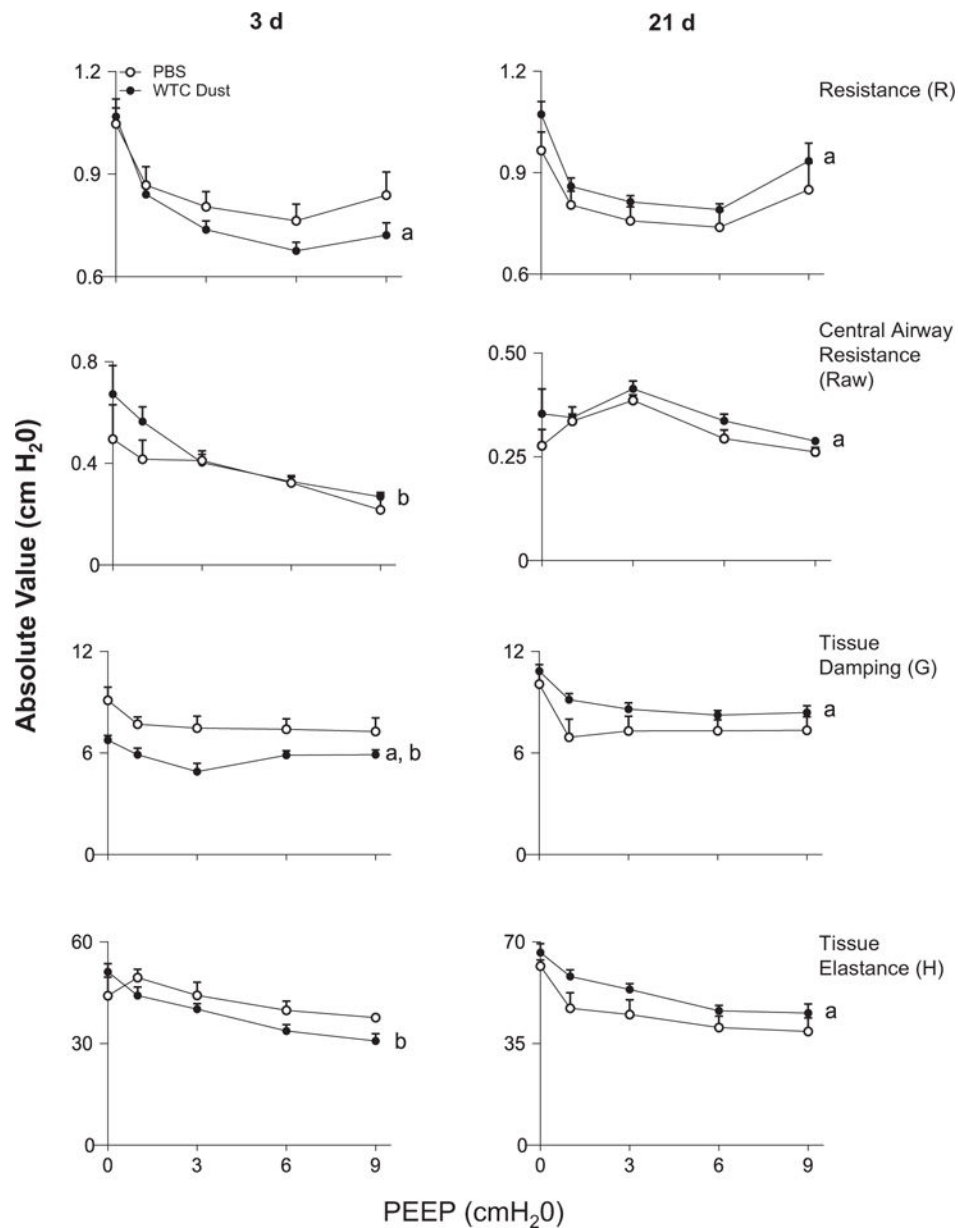


Fig. 7. Effects of WTC dust on pulmonary mechanics. Tissue resistance (R), central airway resistance (Raw), tissue damping (G) and tissue elastance (H) were assessed in triplicate at PEEPs ranging from 0 cm to 9 cm H₂O, 3 d and 21 d following exposure of mice to PBS or WTC dust. Data, mean \pm SE ($n = 5$ mice/group). For each lung parameter, significant differences between the lines were determined by a 3-way ANOVA comparison of treatment, post-exposure time and PEEP. ^aOverall line is significantly different ($p < 0.05$) from PBS control; ^bOverall line is significantly different ($p < 0.05$) from 21 d.

Table 1

Quantification of immunohistochemistry.

	Time after treatment	CTL	WTC dust
HO-1	3 d	0.38 ± 0.13	1.00 ± 0.16 ^a
	7 d	0.25 ± 0.14	0.42 ± 0.08 ^b
	21 d	0.25 ± 0.14	0.10 ± 0.10 ^b
MR-1	3 d	3.13 ± 0.13	1.50 ± 0.42 ^a
	7 d	1.88 ± 0.66	0.75 ± 0.17
	21 d	1.30 ± 0.44	2.20 ± 0.52 ^c
COX2	3 d	2.25 ± 0.48	2.40 ± 0.25
	7 d	1.63 ± 0.32	2.08 ± 0.44
	21 d	1.75 ± 0.25	2.60 ± 0.60

Alveolar macrophages (AM) staining positive for heme oxygenase (HO)-1 and mannose receptor (MR)-1, and Type II cells staining for cyclooxygenase (COX)-2, were enumerated in 450 fields (40)/lung section and assigned a staining intensity score of 0 to 3 (0, no staining; 0.5, minor staining; 1, light staining; 2, medium staining; 3, dark staining). Values are the mean intensity score ± SE of 4–6 mice/treatment group. Data were analyzed by 2 way ANOVA.

^aSignificantly different ($p < 0.05$) from CTL.

^bSignificantly different ($p < 0.05$) from 3 d.

^cSignificantly different ($p < 0.05$) from 21 d.

Table 2

Effects of WTC dust on the percentage of BAL neutrophils.

Time after treatment	CTL	WTC dust
3 d	0.38 ± 0.20	0.60 ± 0.20
7 d	0.40 ± 0.10	1.00 ± 0.10 ^a
21 d	0.47 ± 0.10	0.92 ± 0.10 ^a

BAL cells were analyzed 3 d, 7 d and 21 d after treatment of mice with PBS (CTL) or WTC dust. A total of 300 cells was counted by light microscopy. Data represent the percentage of neutrophils in BAL.

^aSignificantly different ($p < 0.05$) from CTL.

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Table 3

Effects of WTC dust on DNA methylation.

Marker	Global methylation				Gene specific methylation				
	BI Element	Line-1	IL-4	IL-6	IFN γ	PTGS2	TNF α	IL-6RA1	
# of CpG sites	4	5	6	3	6	14	4	9	
Change of average (%)									
3 d	NA	-(0.5)	NA	+(1.5)	+(1.8)	NA	-(1.9)	ND	
21 d	+(0.2)	+(0.8) ^a	+(0.5)	-(2.7)	-(4.0)	ND	-(0.3)	ND	

For each gene, the average of the percentage of methylated CpG sites was compared between control and treated groups using 2-way ANOVA. +, increase; -, decrease. NA, not analyzed; ND, not detected.

^aSignificantly ($p < 0.01$) from control (PBS).