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RESEARCH ARTICLE

Impact on rats from acute intratracheal inhalation exposures to WTC dusts

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ABSTRACT

Background: Studies have revealed the increased incidence of health disorders in First Responders (FR) who were at Ground Zero over the initial 72 hr after the World Trade Center (WTC) collapses. Previous studies in rats exposed to WTC dusts using exposure scenarios that mimicked FR mouth-breathing showed exposure led to altered expression of genes whose products could be involved in lung ailments. Nevertheless, it was uncertain if repeated exposures (as occurred in earliest days post-disaster) might have given rise to long-term changes in the lungs/other organs, in white blood cell (WBC) profiles, and/or systemic expression of select (mostly immune-related) proteins.
Methods: To examine this, rats were exposed on 2 consecutive days (2 hr/d, intratracheal inhalation) to WTC dusts and then examined over a 1-yr period thereafter. At select times post-exposure, organ (lung, heart, liver, kidney, spleen) weights, WBC profiles, and blood levels of a variety of proteins were evaluated.
Results: The study showed that over the 1-yr period, there were nominal effects on organ weights (absolute, index) as a result of the dust exposures. There were significant changes (relative to in naïve rats) in WBC profiles, with exposed rats having increased monocyte-macrophage and decreased lymphocyte percentages. The study also found that dust exposure led to significant systemic increases in many proteins, including MCP-1, RANTES, MMP-9, RAGE, and Galectin-3.
Conclusions: These results provide further support for our longstanding hypothesis that the WTC dusts could potentially have acted as direct inducers of many of the health effects that have been seen in the exposed FR.

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WTC; World Trade Center; dust; heart; lung; spleen; liver; kidney; serum

Introduction

In the years since the 9/11 disaster, chronic health problems continue to become evident among firefighters and rescue personnel (First Responders; FR) who were at Ground Zero for repeated or prolonged periods in the initial 72 h (Webber et al. 2009; Gibbs et al. 2010, 2011; Weakley et al. 2011). Evidence from clinical studies and data accumulated from the World Trade Center (WTC) Health Registry have noted increases in the incidence of chronic lung disorders (certain forms of granulomatous diseases, persistent airway hyperreactivity, and asthma) and a greater risk of development of atherosclerosis and heart diseases (Caplan-Shaw et al. 2011; Crowley et al. 2011; Guidotti et al. 2011; Jordan et al. 2011a, 2011b, 2011c; Nolan et al. 2012; Weiden et al. 2013; Schenck et al. 2014). Readers are directed to (apart from these citations) several other important papers (e.g. Gibbs et al. 2010, 2011; Lippmann et al. 2015; Moline et al. 2016; Yip et al. 2016; Cleven et al. 2017; Landgren et al. 2018; Gargano et al. 2018; Li et al. 2019) for details on specific disorders found in these subjects. Attempts to understand etiologies of these health problems initially focused on immediate effects from dusts derived from the disaster. However, underlying reasons for development of the chronic disorders in FR remain poorly defined.

Ground Zero air in the initial 72 h following the collapse was comprised of a mixture of fine, coarse, and (predominantly) alkaline supercoarse particles (>95% of mass was 10–53 μm) bearing a wide variety of toxic metals and organic agents (Gavett et al. 2003; McGee et al. 2003; Maciejczyk et al. 2005; reviewed in Lippmann et al. 2015;, Cohen et al. 2019). Apart from building-derived materials (collectively termed WTC dusts), other pollutants were also present in the air on those earliest days, like metal cutting fume particles (CFP, by-products of steel cutters) and diesel exhaust particles (DEP) from trucks and cranes). In general, particle densities on-site during those first 72 h were initially quite high. Estimates for 11–13 September 2001 were hundreds of mg/m^3; levels 'dropped' to mg – hundreds of μg/m^3 after ≈3 weeks (CDC (Centers for Disease Control) 2002; Geyh et al. 2005; Maciejczyk et al. 2005). As a result, it was deemed arrival time and respirator use vs. nonuse at Ground Zero in the initial 72 h were key factors for

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develop-ment of health problems in the FR (Feldman et al. 2004; Gibbs et al. 2010, 2011; Antao et al. 2011).

Under normal circumstances, inhaled particles have a relatively limited period to induce toxicity. However, until cleared, constituents of the entrained particles are capable of inducing local and systemic toxicities. Because inhaled alkaline agents can damage respiratory epithelium and give rise to increased epithelial cell death, altered cilia beat frequency, and ciliostasis (Holma et al. 1977; Bui et al. 1998; Clary-Meinesz et al. 1998), it is possible entrained super-coarse WTC particles could have caused altered clearance activities in lungs of the exposed FR. Findings from recent studies showed that acute exposures to the WTC dusts led to a significant decrease in ciliated cell levels and increases in the presence of hyperplastic goblet cells (Cohen et al. 2015). That study also showed there was only a nominal insignificant decrease (6–11%) in WTC dust burden over a 1-year period post-exposure.

If entrained WTC dusts facilitated a retention of CFP/DEP co-pollutants from Ground Zero air, the respective constituents in the latter would have more time to exert toxicities that ultimately give rise to chronic anomalies, including (cardio)pulmonary, hepatic, renal, and blood diseases, etc. In fact, these particular co-pollutants are known to induce by themselves many of the health problems increasingly being documented among exposed FR (Antonini et al. 2004; Hansen et al. 2007; Toren et al. 2007; Lillienberg et al. 2008; Huang et al. 2010; Ibfelt et al. 2010; Maes et al. 2010; Quan et al. 2010; Erdely et al. 2011; Kim et al. 2011; Kim et al. 2012; Riedl et al. 2012). While toxicities from the DEP and/or CFP could be at the root of the diseases in affected FR, to date, it is still not clear if the dusts might *themselves* have also imparted toxicities.

To begin to address this, studies were undertaken to establish if exposures to the WTC dusts alone (under conditions designed to specifically mimic exposures faced by mouthbreathing FR during initial 72 h period at Ground Zero) could cause time-related toxicities (reflected as changes in absolute weights) in key organs such as the lungs, heart, liver, kidney, and spleen. Blood cell (WBC) populations were also examined to see if exposures led to any shifts in cell profiles over time – as occurrence of leukemias has also become increasingly evident among FR who were exposed to the dusts in that critical first 72 h period. Changes in an array of proteins (mostly immune system-related) were also evaluated as altered expression of many of these agents can contribute to the ultimate onset of the array of pathologies increasingly being noted in the FR repeatedly exposed to the dusts (and other airborne agents) in the first week post-disaster.

Materials and methods

WTC dusts

Dusts were collected at representative sites on/around Ground Zero on 12–13 September 2001. All samples were stored in airtight containers in the dark to minimize potential light, heat, or ambient gas-induced changes in physicochemical properties. Parent WTC dusts were sieved to yield all particles $\leq$53 μm (i.e. WTC_{53-}) for use in exposures (WTC_{53-} referred to hereafter as 'WTC dust'). Details about preparation of WTC_{53-} materials, as well as their physicochemical properties, were previously reported (Gavett et al. 2003; McGee et al. 2003; Maciejczyk et al. 2005; Vaughan et al. 2014; Cohen et al. 2015, 2015).

Experimental design

Male SHR rats (10-weeks-old); purchased from Harlan Labs (Frederick, MD) were placed in polycarbonate cages with corncob bedding in a facility maintained at 23 °C with a 30–50% relative humidity and 12-h interval light/dark cycle. Food (Purina lab chow) and tap water were provided *ad libitum*. All rats were acclimated 2 weeks prior to use. All procedures were conducted under a protocol approved by the NYU Institutional Animal Care and Use Committee (IACUC).

In the study, rats were exposed for 2 h periods on two consecutive days to WTC_{53-} dusts while under isoflurane anesthesia (ISO; IsoFlo, Abbott Laboratories, North Chicago, IL) in O_2 carrier gas (2.5% final concentration after mixing with house air); two controls were also used, i.e. rats exposed to ISO only (2.5% in O_2) or naïve hosts. An atmosphere of ≈33–36 mg WTC dust/m^3 (aerodynamic diameter $\leq$53 μm) was used in each exposure; this level was calculated (using Multiple Path Particle Deposition Model program [MPPDep v.1.11, CIIT, Research Triangle Park, NC and RIVM, Bilthoven, the Netherlands]) to conservatively model a 2 h rat exposure that would correspond to one likely to have occurred in mouthbreathing FR exposed to Ground Zero levels of ≈250 mg WTC dust/m^3 during 11–13 September 2001 (Lorber et al. 2007). This value was also based on a conversion to "rat equivalents" (accounting for differences in dose/surface area of trachea, bronchial, and alveolar regions of human and rat respiratory tracts) and on equations reported in Jarabek et al. (2005) that deal with polydisperse atmospheres. Ultimately, each 2 h exposure would correspond to exposures FR encountering 250 mg WTC dust/m^3 atmospheres underwent in a reference 4 h period at/on/around Ground Zero during the period from 12–17 September 2001. This 4 h value has been deemed a "reference person exposure" (Mayor's WTC Medical Working Group; Personal Communication).

All rats were exposed to WTC_{53-} dusts or ISO alone in an intratracheal inhalation (ITIH) integrated system (Vaughan et al. 2014) that allowed the WTC particles (including those of >2.5 μm diameters) to circumvent the rat nasal region so they were introduced/deposited in the rats' lungs in a manner that mimicked the mouthbreathing FR. Protocols for all steps of the exposures (i.e. anesthesia, intubation, exposure) were described in detail in both Vaughan et al. (2014) and Cohen et al. (2015, 2015). At various timepoints post-final exposure, rats from each treatment group (dust, ISO, or naïve) were removed, weighed, and euthanized by injection with Sleepaway (500 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). Blood was

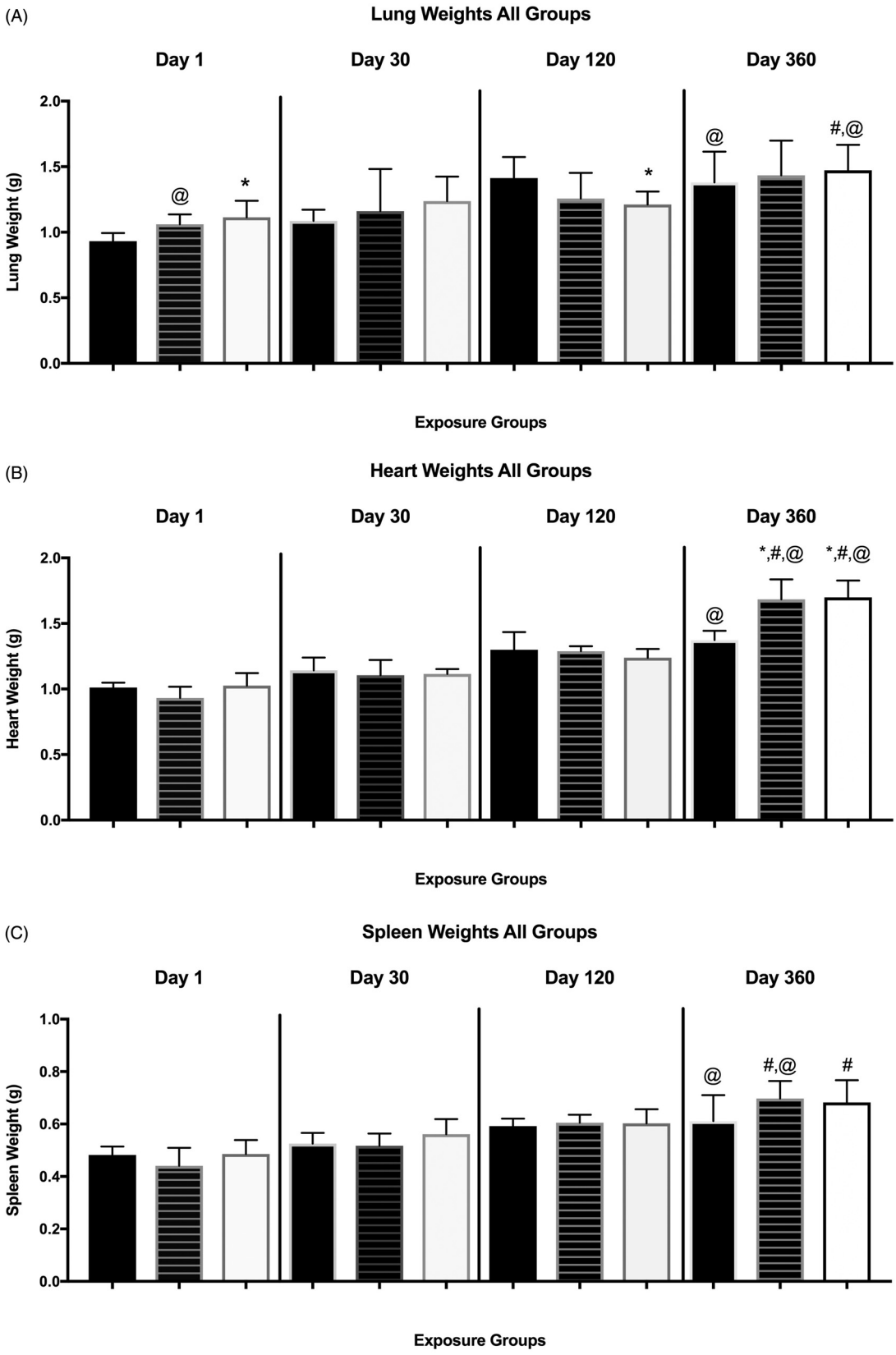

Figure 1. Absolute weights of select organs at indicated post-exposure timepoints. (A) Lungs. (B) Heart. (C) Spleen. (D) Liver. (E) Kidneys. Left to right within each time subset: Naïve (black bar), ISO only (hatched bar), WTC dust only (white bar). All values are reported as gram organ weight. Values shown are mean (±SD) $n = 4$–6/set. At given timepoint, significantly different from naïve control at $^{*}p < 0.05$. Within treatment, significantly different from Day 1 value at $^{@}p < 0.05$. Within treatment, significantly different from Day 120 value at $^{\#}p < 0.05$.

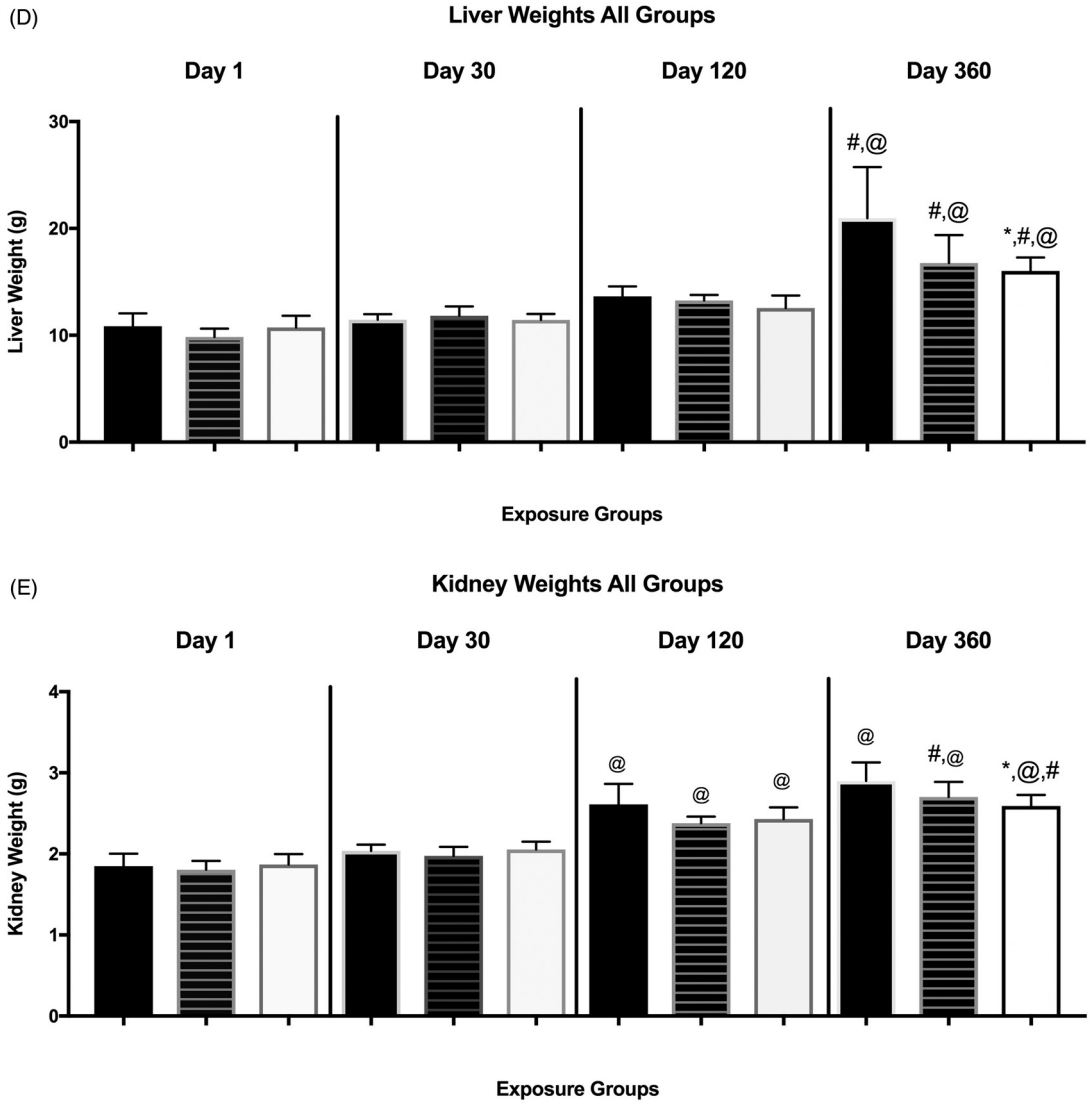

Figure 1. (Continued).

collected from the abdominal aorta to perform immune cell differentials (using standard smear preparation, staining, and evaluation via light microscopy); serum/plasma was isolated for assessments of the expression of a variety of proteins ($n = 79$) in a Proteome Profiler™ Array (Rat XL Cytokine Array kit; R&D Systems, Minneapolis, MN) according to manufacturer protocols. The lungs, heart, kidneys, spleen, and liver were then removed, trimmed, and weighed; organ weight/body weight was used to calculate organ indices. Each sample was then further processed for use in biological assays (described in other papers) or frozen/fixed and then stored (i.e. archived) in the CDC/NIOSH BioBank maintained by Mt. Sinai and NYU (Lieberman-Cribbin et al. 2019).

Statistics

All data were analyzed using unpaired Student's *t*-tests with exposure group (naïve, ISO only, or WTC dust/ISO) as the main factor. Prior to performing ANOVA, all data were tested to assure assumptions of normality and homogeneity of variance were met. Data were also screened for outliers using both Dixon and Grubbs analyses (Taylor 1990). Statistical significance in all cases was considered at $p < 0.05$. Statistical analyses were performed using Prism software (v5.0, Graphpad Inc., San Diego, CA).

Results

WTC dust generation

Based on filter measures taken before and immediately after each 2 h exposure (i.e. no concurrent measures due to impact on flow delivery to rats), the rats in the various post-exposure groups were exposed to at an average of 35.45 (±4.21; SE) mg WTC dust/m^3. The mass median aerodynamic diameter (MMAD) of the dust was confirmed (via horizontal elutriation) to be 23 µm ($\sigma_g = 1.45$). As the dusts used for each exposure were comprised of all particles of diameters ≤53 µm, this meant the materials introduced into the rats' lungs would nominally have been comprised of >95% particles of 10–53 µm (of remainder, 1.1% of all

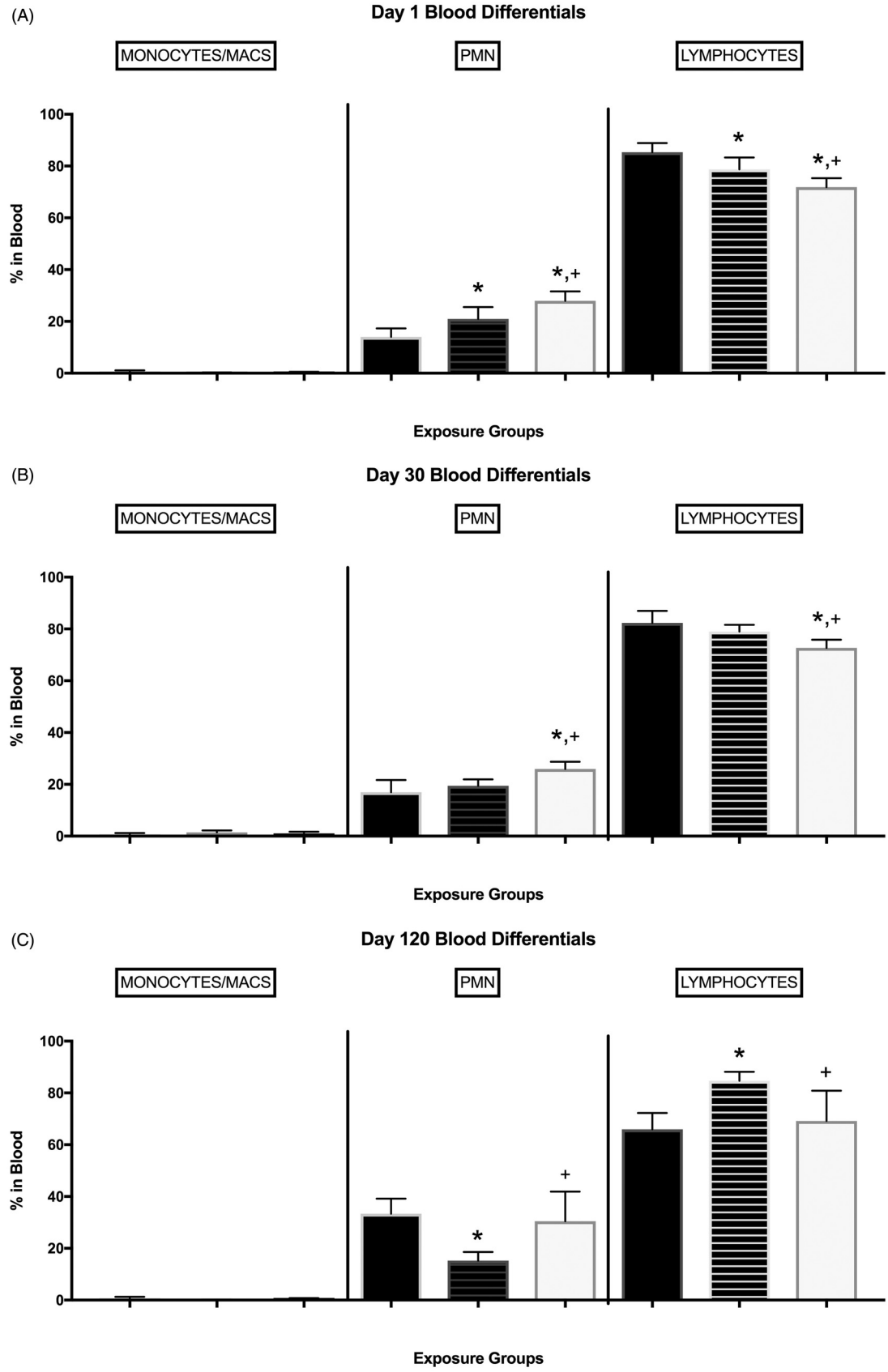

Figure 2. Blood differentials at various timepoints post-exposure. (A–D) Timepoints indicated. Left to right within each time subset: Naïve (black bar), ISO only (hatched bar), WTC dust only (white bar). Values are mean (±SD) $n = 4$–5/set. At given timepoint, significantly different from naïve control at $^{*}p < 0.05$. At given timepoint, significantly different from ISO only at $^{+}p < 0.05$. (E) Comparison of Day 120 vs. Day 360 values. For given exposure, significantly different from corresponding cell type Day 120 value at $^{*}p < 0.05$.

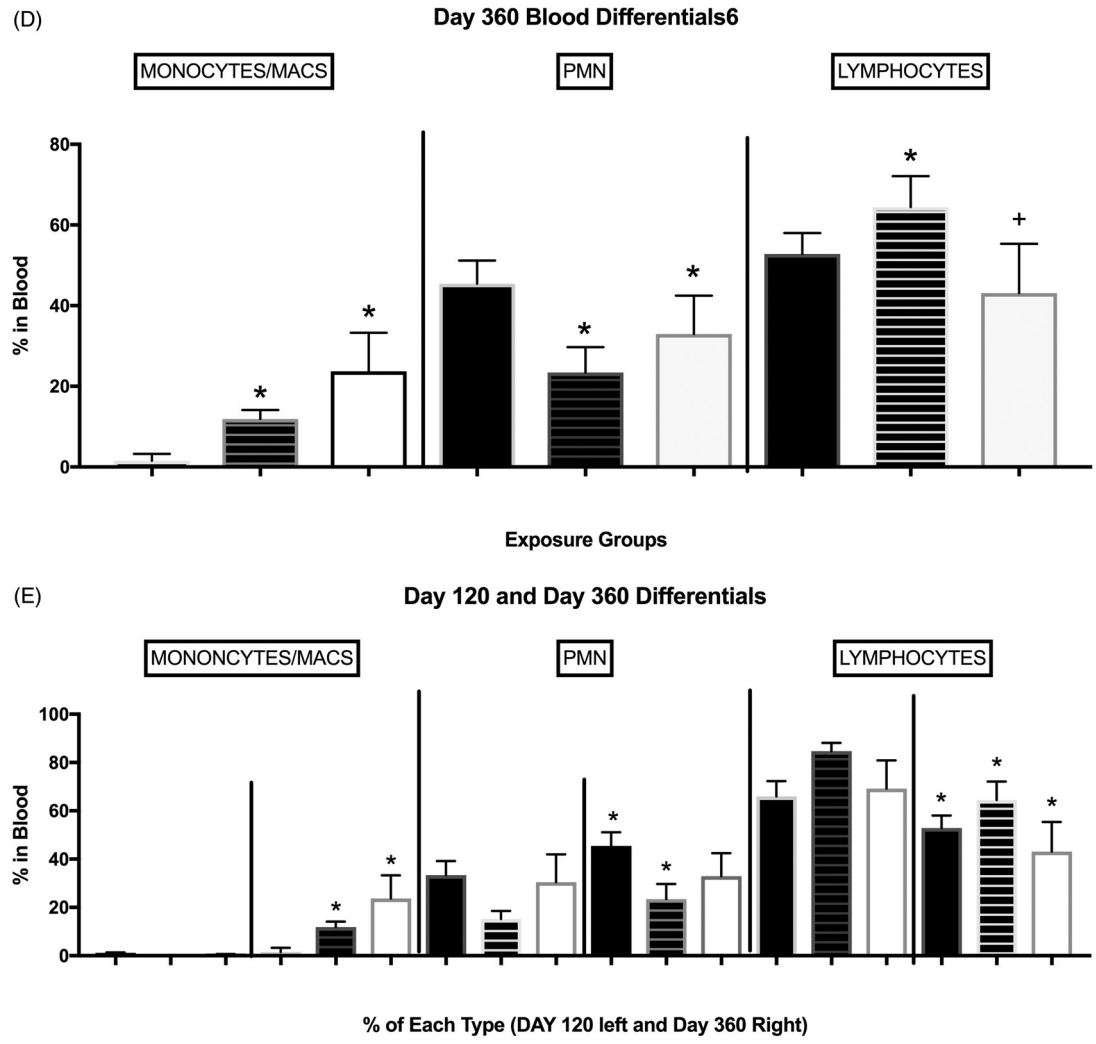

Figure 2. (Continued).

particles were in 2.5–10 µm range and ≈3.5% were <2.5 µm). The average dust level provided the rats in *each* 2 h exposure here (i.e. 32–37 mg/m^3) was expected to and did approximate an ≈250 mg dust/m^3 atmosphere that was likely experienced by FR and others at Ground Zero over a 4 h period in the initial 72 h period (reference exposure: Mayor's WTC Medical Working Group (Gibbs et al. 2011)).

Effects of exposures on organ weights/indices

The impact of the two 2-hr WTC dust exposures on absolute weights of the liver, spleen, lungs, heart, and kidneys of the rats over a 1-year post-exposures period was evaluated. With the lungs, although initially the weights of the lungs of the dust-exposed rats were higher than the naïve controls from Day 1 until Day 30, any differences subsided over time and there were no significant differences by the end of 1 year (Day 360; Figure 1(A)). With the heart, in contrast, there were no significant differences among the groups up until Day 360 (Figure 1(B)). In this case, the weights of hearts from dust- and ISO-only-exposed rats were each significantly greater than those of the naïve control rats (each by 22–23%). Spleen weights were not significantly different among all three groups over the entire course of the 1-year post-exposure period (Figure 1(C)). Interestingly, with both liver and kidneys, it was seen that while absolute weights of each did not significantly differ among the groups over the first 240 days post-exposure, by Day 360 liver weights of dust-exposed rats were now 23.5% lower than naïve controls (Figure 1(D)). Similarly, kidney weights were now lower by 10.8% (Figure 1(E)). Oddly, while exposure to ISO alone again gave rise to a similar effect-trend as WTC dust, levels of change at these two sites as compared against naïve ras were less (i.e. 6.8% and 19.0% for kidney and liver, respectively).

Body weights (and net gains) over the 1-year post-exposure period did not differ between dust-exposed and naïve rats. Weights of naïve rats increased by 51.2 [±4.3]% from Day 1, while those of the WTC dust rats increased by 48.1 [± 2.3]%. In comparison, weights of ISO-only rats increased by 65.3 [±4.8]%; while this was significantly more than that of the dust rats ($p < 0.002$), it was nominally and insignificantly more than that by naïve rats ($p \approx 0.06$). In light of this, all organ weights were reevaluated in terms of corresponding indices. In general, the same inter-group differences noted above still existed though some significant differences appeared that were not detected in the absolute weight data. For example, naïve rats showed significant reduction over

time in splenic indices, but there was no similar effect in ISO and WTC dust rats.

Effects of exposures on blood cell differentials

The impact of the two 2-h WTC dust exposures on white blood cell (WBC) differentials was also examined. No evaluations of red blood cells, platelets, etc. were performed. The results indicate that within 24 h of completion of the second exposure, the relative percentages of neutrophils (PMN) in the blood of both the WTC dust (27.9 [±1.8]%) and ISO (20.9 [±2.0]%) rats were significantly increased by 99.4 and 49.5%, accordingly, compared to naïve control levels (14.0 [±1.5]%) (Figure 2(A)). In the absence of any significant changes in levels of monocytes/macrophages, percentages of lymphocytes correspondingly significantly decreased in both of these exposed groups by 15.8 and 7.8%, accordingly (WTC dust = 71.8 [±1.7], ISO = 78.7 [±2.1], naïve = 85.3 [±1.6]%). Analyses indicated that these Day 1 PMN and lymphocyte percentages differed significantly between the ISO and dust rats.

By Day 30 (Figure 2(B)), the impact of the ISO exposure seemed to have mitigated and compared to the naive controls, the percentages of blood PMN (ISO = 19.5 [±1.1]%, naïve = 17.0 [±1.9]%) and lymphocytes (ISO = 79.0 [±1.2]%, naïve = 82.3 [±1.9]%) no longer differed significantly. However, values for both cell types in the WTC dust-exposed rats were still significantly different from controls (and now from ISO rats as well; PMN = 25.9 [±1.1]%, lymphocytes = 72.7 [±1.3]%). While these represented, accordingly, significant increases of 52.4% and decreases of 11.7% vs. naive control levels, it showed a lessening of the initial impact of the dust exposure on the blood cell differentials with time.

By Day 120, there now seemed to be a shift in WBC subtype distributions (Figure 2(C)). Specifically, the percentages of PMN and lymphocytes in the blood of the WTC dust rats no longer significantly differed from naïve control values (across both groups, PMN = 30–33% and lymphocytes = 66–69%). Oddly, values for the ISO-only rats became significantly different from both other groups (i.e. PMN = 15.2 [±1.4]%, lymphocytes = 84.7 [±1.4]%). This shift appeared to presage an overall change in subtypes by Day 360 (Figure 2(D)), with monocytes-macrophages increasingly evident in the blood of both exposed groups. By Day 360, the percentages of this cell type reached 23.7 [±2.3]% in the dust rats and 11.9 [±1.3]% in the ISO rats (vs. PMN = 1.6 [±0.7]% for naïve controls; these equated to 14.8- and 7.4-fold increases, respectively.

To discern if increases in monocytes-macrophages were due to relative changes in levels of other particular WBC subtypes, the data was re-analyzed to assess shifts from Day 120 to Day 360 (Figure 2(E)). Both naïve and ISO rats had substantive (significant) increases in PMN percent-ages in this period (36.3 and 54.1%, respectively); WTC dust-exposed rats only had an increase of 8.3% (not significant). Though this general trend in increases in percentages of PMN led to concern about if underlying infections had developed in the rats over the course of the 1-year post-exposure period, no increases in overt illness nor in morbidities were reported by the veterinarians who examined the rats daily. As we now know, substantive age-related increases in levels of circulating PMN are common in this breed of rats (Schmid-Schonbein et al. 1991).

In comparison to the PMN observations, all groups had significantly reduced percentages of lymphocytes in their blood. However, the largest change occurred in WTC dust rats (37.8%); changes over this period were 19.7 and 23.6% for the naïve and ISO rats, respectively. Thus, it would seem that with increasing time post-exposure, there was a clear increase in monocyte-macrophage 'relative percentages' with corresponding changes in those of lymphocytes in the blood of all rats. However, this seems most amplified as a result of exposure to the WTC dusts.

The Authors are cognizant that evaluations like above that indicate changes in percent-ages of lymphocytes could reflect a potential 'crowding out' of this cell type within a given number of cells on a slide without there actually being an absolute reduction in these cells in a host. When all data was re-examined in terms of each cell type/ml blood, the same trend patterns at each timepoint for monocyte-macrophages, PMN, and lymphocytes was apparent (see Supplemental Figures). The only notable differences from above were in relative magnitudes of change among the different exposure groups (and in some cases, level of statistical significance). Clearly, future evaluations of blood cell types after exposure to WTC dust (and ISO) will require use of more quantitative approaches than blood smear analyses (i.e. flow cytometry/sorting, etc).

Effects of exposures on blood levels of select proteins

The impact of the dust exposures on systemic levels of a variety of proteins, including several related to immune system function, was also examined. The results indicate that by the end of the post-exposure period, levels of several proteins related to potentially dysregulated immune systems were significantly elevated in the dust rats compared to in naïve controls. Plasma MCP-1, MMP-9, RAGE, RANTES, and Galectin-3 levels were all significantly elevated at Day 360 due to dust exposures (Figures 3(A–E)). MCP-1, MMP-9, RAGE, RANTES, and Galectin-3 levels were increased by, respectively, 37.9, 96.2, 114.1, 101.1, and 16.9% vs. the naïve controls. Other proteins were also up-regulated at Day 360 (Table 1). For monocyte-derived chemokine (MDC/CCL22)

Table 1. Plasma proteins elevated from naïve control levels by Day 360 due to dust exposures.

CCL2/JE/MCP-1
CCL5/RANTES
CCL22/MDC
Galectin-1
Galectin-3
LIX
MMP-9
NOV/CCN3
PDGF-BB
RAGE
TWEAK/TNFSF12

The *p*-values for MDC and Galectin-1 (pink shading) were both ≈ 0.06 vs. naïve control levels. All other protein values shown (red shading) were at $p < 0.05$.

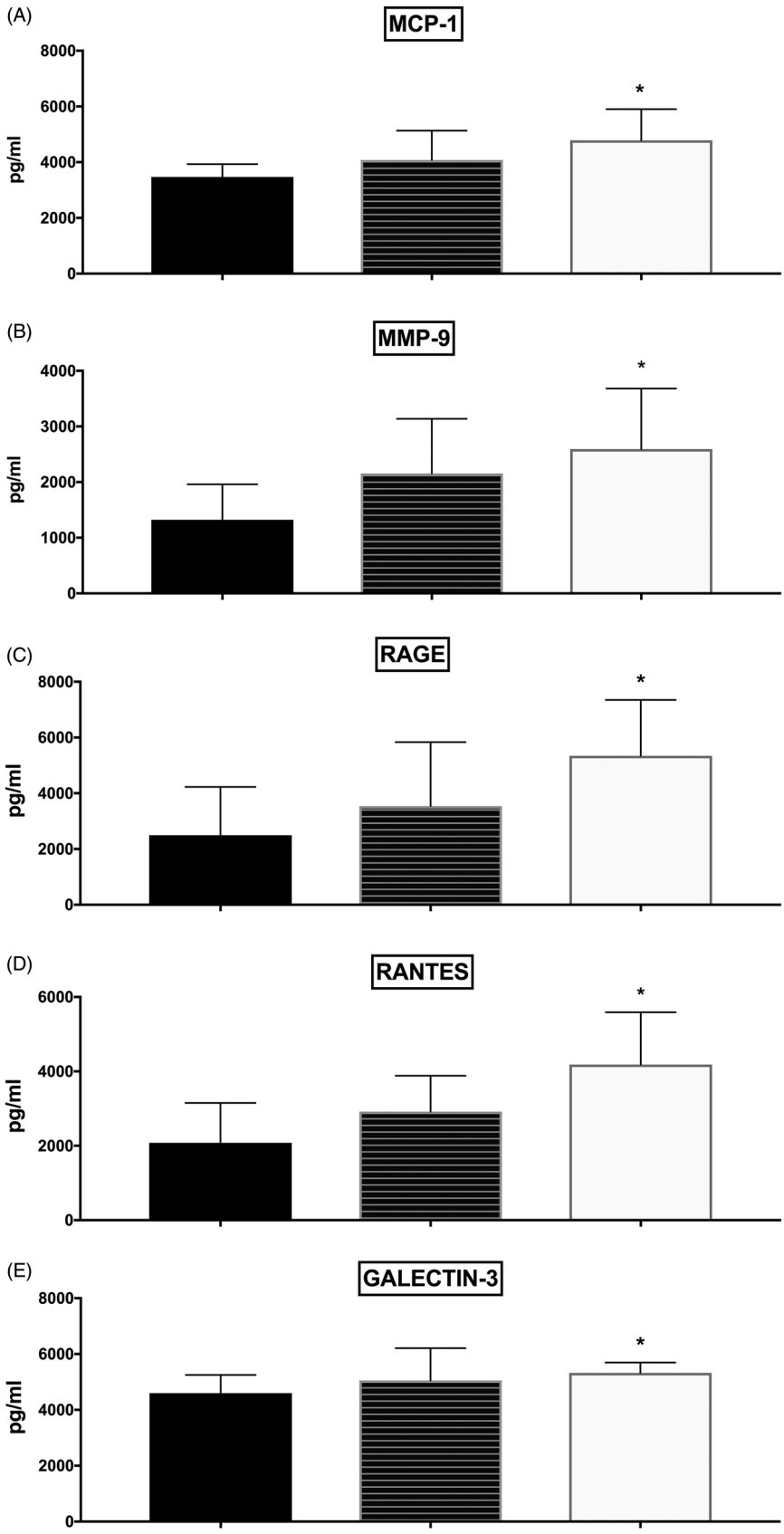

Figure 3. Day 360 post-exposure plasma levels of select proteins. (A) MCP-1. (B) MMP-9. (C) RAGE. (D) RANTES. (E) Galectin-3. Left to right within each protein subset: Naïve (black bar), ISO only (hatched bar), WTC dust only (white bar). All values are reported as pg/ml. Values shown are mean (±SD) $n = 4$–6/set. *Significantly different from naïve control at $p < 0.05$.

and Galectin-1, levels were increased vs. naïve rat values by 48.9 and 37.3%; however, due to large inter-animal variations, these levels ultimately just missed the cutoff to accept significance (i.e. $p \approx 0.06$). It is again of note that ISO alone also seemed to impart long-term effects on the SHR rats. However, unlike with organ weights, in this case while there was the appearance of a trend toward increased expressions of the proteins shown in Figure 3, the ISO-only rat levels never *significantly* differed from those of the naïve controls.

Discussion

This study was done to see if initial deposits of WTC dusts in lungs of rats exposed in manner that mimicked mouth-breathing FR paradigms could impart long-term effects in the lungs and other organs/cell systems. Findings here could help explain why there was (and continues to be) an increased incidence of pathologies among FR who were at Ground Zero over the first 72 h. Over the years, there have only been a handful of animal model studies to assess toxicities (pulmonary and extra-pulmonary) from WTC dusts. In many cases, studies used mice exposed to just the fine fraction (only represented <5% of all particles in Ground Zero air at time) (Gavett et al. 2003) or dusts collected at later timepoints (after rainstorms and truck traffic affected dust composition) (Sunil et al. 2017). Further, in the mouse and some rat studies, hosts received dust by instillation (Soffritti et al. 2013; Caraher et al. 2017), a manner not reflective of entrainment by the FR.

A lack of consistent changes in lung weights here was not in keeping with changes expected to occur with the lung diseases reported in FR, i.e. sarcoid-like granulomatous disease and pulmonary fibrosis. In these pathologies there is increased formation of cell-filled nodules or collagen deposition; these are accompanied by inflammatory cell recruitment to the lungs/sites of damage in the airways, all of which can contribute to increases in organ mass (Gharaee-Kermani et al. 2005; Aiso et al. 2010; Nagano et al. 2011; Jiang et al. 2016; Hu et al. 2017). The lack of increase in lung weight due to WTC dusts is perplexing in that it is already known that >90% of the entrained dust mass is retained for this 1-year period (Cohen et al. 2015).

In contrast, there were long-term increases in heart weights in these rats. It is known that exposures that occurred at or near Ground Zero by FR, community members, and cleanup workers are associated with increases in "heart disease" or "cardiovascular disease" (Jordan et al. 2013; Remch et al. 2018). Nevertheless, a conclusive linking of these to exposure to/burdens of WTC dusts remains in question; data pertaining to cardiomegaly in these populations is lacking. Still, the increased heart mass over time seen here would correspond to other findings wherein changes (increases) were seen in expression of several heart-related proteins, including cardiac gap junction proteins (Tanwar et al. 2019) and SERCA-2/calcium-handling proteins (Wold, in press), and for genes for a variety of proteins (transcriptome analyses; Park, unpublished data).

The findings of significant long-term reductions in liver and kidney weights in the dust-exposed rats (vs. naïves) is interesting. There is no published information on hepatic/renal pathologies (apart from significant increases in renal cancer incidence rate (CIR] among NYC police department members [Kleinman et al. 2015]). Thus, for now, it is not clear what might be the underlying reason(s) for these changes induced by the two acute dust exposures. This issue will be re-visited as data from ongoing epidemiologic studies becomes available.

Data here also revealed significant increases in splenic indices in the dust-exposed rats. There were also significant increases in monocyte/macrophage percentages (and levels) in these rats by the end of the 1-year period. Such findings ultimately could reflect long-term dust effects on the heart. The spleen can generate reserves of these types of cells (stored in red pulp) for release during host infection or inflammation. During cardiac events, monocytes are released; in the heart, they help manage inflammation and promote tissue healing (Sager et al. 2017; Honold and Nahrendorf 2018). Swirski et al. (2009) showed spleen have two subclasses of *bona fide monocytes* (that co-exist with, but differ from, macrophages and dendritic cells) that can be present at levels that can outnumber those of circulating monocytes. Ly-6C^{high} (Gr-1^{+}) types are phagocytic, proteolytic, and inflammatory, and migrate to injured/infected sites, while Ly-6C^{low} (Gr-1^{-}) types patrol the vasculature, populate inflammatory sites, and aide in resolving inflamma-tion and wound healing (Swirski et al. 2007; Tacke et al. 2007). During infarct, there is an early release of Ly-6C^{high} cells to help in digestion of damaged tissues; this is later followed by increased release in Ly-6C^{low} monocytes to promote tissue healing (Nahrendorf et al. 2007). While an infarct would deplete these monocyte stores (Leuschner et al. 2012) – and one would expect concurrent decreases in spleen weight, ongoing states that lead to heart pathologies allow for increases in these stores (via extramedullary monopoiesis) (Robbins et al. 2012). Thus, it could be that the dust-exposed rats here are undergoing *development* of cardiac/cardiovascular pathologies that would cause their spleens to increase in size. Re-analysis of archived spleens (red pulp) and blood smears for Ly-6C^{high} and Ly-6C^{low} monocytes will let us verify if increases in splenic indices at 1 year were due to potential cardiac pathology-driven effects.

That dust-exposed rats also had significant increases in circulating RANTES and MCP-1 was not unexpected. Studies in heart failure/coronary artery disease (CAD) patients and angina pectoris (AP) patients noted elevated blood MCP-1, RANTES, and macrophage inflammatory protein (MIP)-1α levels (Aukrust et al. 1998; Zhong et al. 2015). Because MCP-1, RANTES, and fractalkine can promote atherosclerotic plaque instability, based on the above (and studies of heart disease/AP patients [Li et al. 2012]), it has been concluded that circulating these proteins help modulate plaque vulnerability and plaque rupture. Increased expression of these three also occurs in rodents with developing/present heart pathologies (Fuse et al. 2001; Yun et al.

2001; Hayasaki et al. 2006; Montecucco et al. 2012). The Montecucco group also showed that elevated matrix metalloproteinase (MMP)-9 levels (in heart itself) were associated with these pathologies. MMP-9 contributes to collagen breakdown in atherosclerotic plaques to promote adverse cardiac episodes (Busti et al. 2010; Mittal et al. 2014; Newby 2016). Brunner et al. (2010) showed in CAD patients that disease progression was mirrored by increased ratios of MMP-9/TIMP (tissue inhibitor of metalloproteinase)-1 in circulating monocytes. In patients with advancing CAD, elevated serum MMP levels and/or MMP/TIMP imbalances routinely occurred (Blankenberg et al. 2003; Oliveira et al. 2009). Thus, in the WTC dust-exposed rats here, it is likely the increases in circulating MMP-9 noted at Day 360 might be associated with the observed increases in circulating monocytes and that each, in turn, may impact the cardiac/cardiovascular system. Further analyses of the heart and vasculature from these rats will substantiate or refute this.

Among the other proteins found elevated in the blood of WTC dust-exposed rats at Day 360, Galectin (Gal)-3 has a role in cell-cell adhesion, macrophage activation, angiogenesis, and apoptosis (de Oliveira et al. 2015; Sciacchitano et al. 2018). It is also involved in inflammation and heart disease (Dumic et al. 2006; Henderson and Sethi 2009; Suthahar et al. 2018). Elevated Gal-3 levels are significantly associated with higher risk of death in both acute and chronic heart failure populations. Gal-3 levels are low in normal human, mouse, and rat cells; as heart disease progresses, significant up-regulation of Gal-3 occurs in the myocardium (Sharma et al. 2004; de Boer et al. 2009; Lok et al. 2010). As a result, there is interest in circulating Gal-3 levels as a next-generation biomarker for heart failure in at-risk populations (Magnussen and Blankenberg 2018; Lyngbakken et al. 2019). As with MMP-9, further analyses of heart and vasculature collected here will let us substantiate if elevations in Gal-3 were associated with present/ongoing pathologic changes in the dust-exposed rats. Several other proteins were significantly elevated in the blood of the WTC dust-exposed rats at Day 360 (see Table 1). Readers seeking a detailed discussion on some of these particular findings (e.g. for MDC/CCL22, NOV/CCN3) in the context of heart diseases, cardiac pathologies, etc., are directed to the Supplemental Discussion.

When all the data here about time-post-exposure changes in organ weights, WBC differentials , and circulating cytokines/chemokines are taken together, a picture emerges of a long-term gradual development of changes in rats exposed to WTC dusts in a repeated acute manner. Such changes seem to mimic what has been seen in FR being monitored as part of ongoing surveillance-health monitoring programs. Of particular note are the apparent changes evolving in the hearts of the rats. These would be in line with the increased reporting of heart/cardiovascular diseases in FR – primarily among those who had been repeatedly exposed at Ground Zero over the course of the first week post-disaster. As we have noted in our other papers, we do not yet have the ability to state with certainty the WTC dusts *themselves* were/are causative of many of the documented pathologies in FR. Indeed, co-toxicities from inhaled/retained metal cutting fume particles (CFP) and diesel exhaust particles (DEP) that were also present at high levels in Ground Zero air are currently being investigated. Nevertheless, the present studies do show that even repeated acute (2 h) exposures to the WTC dusts that had been in the air from 11 September to 13 September 2001 were able to impart long-term toxicities – both in the lungs and in secondary sites.

We are cognizant the data here indicated many changes in measured parameters due to the ISO required for the WTC dust exposures. It is tempting to state effects here were ISO-driven and only nominally impacted further by the dusts. However, there are many cases where changes due to dust were significantly different than in ISO only-exposed rats. Transcriptomic analyses (Park, unpublished data) have indicated in many cases, patterns of change in RNA expression in heart tissues were completely opposite in ISO and WTC dust rats. For now, we cannot conclude if (A) ISO and WTC dusts act in some synergistic or additive manner, (B) ISO itself is impeding 'actual' effects of the dusts, or (C) WTC dusts impart effects that overwhelm any contribution to the given endpoint by the ISO alone. Further analyses are attempting to resolve this conundrum.

Disclosure statement

The Authors report no conflicts of interest. The Authors are alone responsible for the content and writing of the paper.

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