Chronic Fine Particulate Matter Exposure Induces Systemic Vascular Dysfunction via NADPH Oxidase and TLR4 Pathways

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Rationale: Chronic exposure to ambient air-borne particulate matter of <2.5 μm (PM$_{2.5}$) increases cardiovascular risk. The mechanisms by which inhaled ambient particles are sensed and how these effects are systemically transduced remain elusive.

Objective: To investigate the molecular mechanisms by which PM$_{2.5}$ mediates inflammatory responses in a mouse model of chronic exposure.

Methods and Results: Here, we show that chronic exposure to ambient PM$_{2.5}$ promotes Ly6C$^{\text{high}}$ inflammatory monocyte egress from bone-marrow and mediates their entry into tissue niches where they generate reactive oxygen species via NADPH oxidase. Toll-like receptor (TLR)4 and Nox2 (gp91$^{\text{phox}}$) deficiency prevented monocyte NADPH oxidase activation in response to PM$_{2.5}$ and was associated with restoration of systemic vascular dysfunction. TLR4 activation appeared to be a prerequisite for NADPH oxidase activation as evidenced by reduced p47$^{\text{phox}}$ phosphorylation in TLR4 deficient animals. PM$_{2.5}$ exposure markedly increased oxidized phospholipid derivatives of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (oxPAPC) in bronchoalveolar lavage fluid. Correspondingly, exposure of bone marrow–derived macrophages to oxPAPC but not PAPC recapitulated effects of chronic PM$_{2.5}$ exposure, whereas TLR4 deficiency attenuated this response.

Conclusions: Taken together, our findings suggest that PM$_{2.5}$ triggers an increase in oxidized phospholipids in lungs that then mediates a systemic cellular inflammatory response through TLR4/NADPH oxidase–dependent mechanisms. (Circ Res. 2011;108:716-726.)

Key Words: particulate matter ■ monocyte ■ Toll-like receptor 4 ■ superoxide ■ oxidized phospholipids

Particulate matter (PM) has been consistently linked to morbidity and mortality from ischemic cardiovascular events and reduced life expectancy in epidemiological studies.\textsuperscript{1-4} Particles less than 2.5 μm (PM$_{2.5}$) in diameter represent the size fraction that has been most consistently implicated in the pathogenesis of cardiovascular disease.\textsuperscript{5} Several new studies have also demonstrated that residing in locations with higher long-term average PM levels elevates the risk for cardiovascular morbidity and mortality with the risks associated with chronic exposure to PM$_{2.5}$ (years) vastly exceeding the risks noted with short term exposure (days).\textsuperscript{6,7} PM$_{2.5}$ air pollution has been linked with endothelial dysfunction, systemic inflammatory and oxidative stress responses and the progression of atherosclerosis.\textsuperscript{8,9} Seaton et al first proposed that deposition of particles in the lung provoked a low-grade alveolar inflammation with a secondary systemic inflammatory response.\textsuperscript{10} Since then, numerous studies have supported this inflammatory hypothesis and further expanded on it.\textsuperscript{11,12} However, the precise mechanisms by which particulates are sensed and the responses transduced remain elusive. It is increasingly apparent that biological systems commonly use evolutionarily conserved mechanisms to sense a variety of environmental signals.\textsuperscript{13,14} Toll-like receptors (TLRs) play a central role in the recognition of a broad diversity of environmental and pathogen associated molecular patterns.\textsuperscript{15,16} We therefore

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hypothesized that PM$_{2.5}$ either directly or via biological intermediates modulates systemic effects via TLR4 pathways. Accordingly, we performed exposures to PM$_{2.5}$ using a system that exposes animals chronically to 8 to 10 fold higher levels of ambient particulates.$^{17,18}$ Our findings suggest that TLR4 and Nox2 may be involved in PM$_{2.5}$ induced systemic inflammation.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org. Briefly, this study used male mice at the age of six weeks of following different strains: C57BL/6, Nox2$^{-/-}$ (C57BL/6 background), Balb/c (TLR4 wt), Tlr4$^{-/-}$ (background strain BALB/cAnPt), and c-fms$^{YFP}$ transgenic mice (FVB/N background).

Animal exposure and the monitoring of exposure atmosphere and ambient aerosol were performed as previously described using a versatile aerosol concentration enrichment system that was modified for long-term exposures.$^{19}$ The Ohio State University Committee on Use and Care of Animals approved all experimental procedures.

**Results**

**PM$_{2.5}$ Concentration and Exposure Protocol**

The exposure metrics are described in Online Table I. Ambient mean daily PM$_{2.5}$ concentration at the exposure facility was 10.7±2.1 µg/m$^3$. Mean concentration of PM$_{2.5}$ in the chamber was 92.4±2.1 µg/m$^3$ (Online Figure I). Because the mice were exposed for 6 hours a day, 5 days a week, the normalized PM$_{2.5}$ concentration was 24.7 µg/m$^3$ which is close to the annual average PM$_{2.5}$ National Ambient Air Quality Standard (NAAQS) of 15 µg/m$^3$. The total PM$_{2.5}$ dose inhaled during the exposure corresponded to 104±20 µg assuming a ventilation rate of 105 breaths/min and 0.2 cc/breath in mice.$^{21}$ LPS levels in the serum and BAL did not differ between the FA and PM$_{2.5}$ group (55.2±19.4 pg/mL versus 49.8±13.6 pg/mL in the serum and 423±233 pg/mL versus 654±262 pg/mL in the BAL, respectively).

**PM$_{2.5}$ Exposure Promotes Inflammatory Monocyte Egress From Bone Marrow to Blood via TLR4 Pathways**

To determine whether chronic PM$_{2.5}$ exposure had effects on monocyte subpopulations, we analyzed peripheral blood, bone marrow and splenic cell populations. PM$_{2.5}$ increased inflammatory monocytes in the periphery in TLR4 wt mice, whereas deficiency of TLR4 diminished the effect (Figure 1B). The spleen and bone marrow played a differential role in

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
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<tr>
<td>d</td>
<td>deficient</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>PAPC</td>
<td>1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine</td>
</tr>
<tr>
<td>PGPC</td>
<td>1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PM$_{2.5}$</td>
<td>particulate matter of &lt;2.5 µm</td>
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<tr>
<td>P0VPC</td>
<td>1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
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<td>YFP</td>
<td>yellow fluorescent protein</td>
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**Figure 1.** PM$_{2.5}$ exposure promotes inflammatory monocyte egress from bone marrow to blood via TLR4 pathways. Inflammatory monocyte population of TLR4$^{wt}$ and TLR4$^{d}$ mice in spleen (A), peripheral blood (B), and bone marrow (C). Data are means±SD (n=4 to 8/group; *P<0.05). Exposure duration of 20 weeks.
contribution of inflammatory monocytes to the systemic circulation (Figure 1A and 1C).

Chronic PM2.5 Exposure Increases NADPH Oxidase–Derived Superoxide in Monocytes, Aortic Tissue, and Perivascular Fat Through TLR4

We investigated the systemic effects of PM2.5 exposure. Figure 2 depicts NADPH oxidase–derived superoxide ($O_2^-$) generation in F4/80$^+$ monocytes and systemic vasculature. NADPH oxidase–derived $O_2^-$ production increased in aorta and perivascular fat in response to PM$_{2.5}$. TLR4 deficiency attenuated but did not abolish the effects of PM$_{2.5}$ (Figure 2A). We then used mice deficient in Nox2 (gp91$^{phox}$/−/−), to address the relative role of this sub-unit in upregulated NADPH oxidase activity in response to PM$_{2.5}$. Nox2$^{−/−}$ mice were exposed for 20 weeks to either FA or PM$_{2.5}$. NADPH oxidase–derived $O_2^-$ production was decreased in Nox2$^{−/−}$ mice in F4/80 cells but not in aorta or perivascular fat of FA exposed animals. Nox2 deficiency abrogated the effects of PM$_{2.5}$ on $O_2^-$ production in F4/80$^+$ cells, aorta and perivascular fat (Figure 2B). In contrast TLR4$^d$ abolished the increase in F4/80 cells in response to PM$_{2.5}$ in the peri-vascular fat in the aorta. These findings suggest that incursion into the perivascular space by monocytes may contribute to increased $O_2^-$ in response to PM$_{2.5}$ and support a functional role for Nox2 in PM$_{2.5}$ mediated effects.

PM$_{2.5}$ Impairs Macrovascular Tonal Responses and Influences Leukocyte Trafficking in the Microvasculature

As perivascular fat is an important portal of entry for inflammatory cells and has been shown to influence vascular tone, we investigated vascular responses in the presence and absence of perivascular fat. Segments with and without fat had different levels of tension following application of a preconstrictor dose of phenylephrine (before acetylcholine) and are depicted separately in Figure 3. Chronically PM$_{2.5}$ exposed TLR4$^w_t$ mice demonstrated increased constriction to phenylephrine in the presence of perivascular fat (Figure 3A), an effect that was noted in the absence of fat as well. (Figure 3B). TLR4 deficiency normalized the heightened constriction in response to PM$_{2.5}$. PM$_{2.5}$ exposed TLR4$^w_t$ mice demon-
Tissue in TLR4wt Mice
Receptor–Dependent Gene Expression in Aortic
no change versus the TLR4wt FA control group are

deficiency attenuated the effects of PM2.5 exposure (Figure 3C and 3D). Online Figure II allows the interpretation of the Δ change in the relaxation and constriction maxima in segments with and without fat. We studied the vasomotor response in mice with and without intact Nox2. Online Table III depicts the responses in vascular segments from these mice. These results show a significantly higher constriction in Nox2wt mice in response to PM2.5, whereas deficiency of Nox2 abrogated this effect. These results suggest that both TLR4 and NADPH oxidase mediate the systemic vascular effects of PM2.5 exposure.

To test the effect of PM2.5 on leukocyte trafficking in the microvasculature, we performed in vivo intravital microscopic experiments on the cremasteric muscle as a surrogate for the perivascular microcirculatory environment. Here, we found a significant increase of leukocyte adherence (Online Figure III) in response to chronic exposure to PM2.5.

PM2.5 Exposure Increases Toll-Like Receptor–Dependent Gene Expression in Aortic Tissue in TLR4wt Mice
To determine the molecular basis of PM2.5 exposure effects in the vasculature, we examined a panel of genes involved in TLR signaling in aortic tissue. We used a PCR array, profiling the expression of genes related to TLR-mediated signal transduction. Although in TLR4wt mice, 8 genes changed significantly, this induction was abolished in TLR4Δ mice (Figure 4).

PM2.5 Exposure Increases Monocyte Homing and Adherence in Tissue Niches
To provide additional evidence of recruitment of monocytes with chronic PM2.5 exposure in tissue niches, we used a transgenic reporter mouse model expressing YFP under the control of a c-fms promoter (c-fmsYFP) that were exposed to FA or PM2.5 initially over a duration of 20-weeks. We first examined the number of adherent YFP+ cells in the cremasteric and mesenteric adipose tissue. Significantly more adherent YFP+ cells were found in the cremasteric endothelial wall in response to chronic PM2.5 (Figure 5A). Figure 5B indicates similar findings in the mesenteric adipose tissue; representative images of mesenteric tissue from c-fmsYFP mice exposed to either FA or PM2.5, respectively are depicted in Online Videos I through IV. Impairment in contractile properties of isolated thoracic aortic rings with and without perivascular fat in c-fmsYFP mice are shown in Online Figure IV in response to PM2.5 exposure. An increase of YFP+ cell-infiltration into the perivascular adipose tissue was observed in response to PM2.5 (Figure 5C). YFP+ cells in the BAL, lung and epididymal fat were noted in response to PM2.5 (Figure 5D through 5F). In additional experiments we exposed c-fmsYFP mice over a shorter 12 week duration to PM2.5 or FA. Flow-cytometric analysis of YFP+ cells coexpressing CCR2, CCR3 and CXCR3 in the bone marrow and spleen revealed a significant increase in YFP+CCR2+ cells in response to PM2.5 exposure in bone marrow (Online Table IV). In light of increased YFP+ monocytes and F4/80 macrophages in the lung we hypothesized that the inflammatory milieu in the lung may provide a substrate for the generation of mediators that in turn could participate in TLR signaling and NADPH oxidase activation.

TLR4 Deficiency Normalizes Inflammatory Cytokine and MCP-1 Release in Response to PM2.5
TLR4wt mice demonstrated an increase in TNFα, MCP-1 and IL-12p70 and a decrease of IL-10 levels in the lung (Figure 6A). TLR4 deficiency attenuated TNFα, MCP-1 and IL-12p70 levels in response to PM2.5. Corresponding plasma measurements demonstrated an increase in TNFα and MCP-1 with PM2.5 exposure with normalization in TLR4Δ (Figure 6B). We then investigated if TLR4 activation by PM2.5 leads to downstream NADPH oxidase activation.23–25 We chose phosphorylation of the p47phox subunit in the lung as an index of NADPH oxidase activation in response to PM2.5.12,26 TLR4wt showed a significant increase in p47phox phosphorylation in lung following PM2.5 exposure, compared to FA mice, whereas TLR4 deficiency prevented this effect (Figure 6C).

Airborne Particulate Matter Causes Increased Levels of Two Oxidized PACP Derivatives in BAL Fluid of PM2.5-Exposed Mice
Prior studies have indicated a role for surfactant phospholipids in mitigating inflammatory responses, whereas generation of oxidized derivatives of surfactant phospholipids has been shown to potentiate inflammatory signaling.14,27,28 At first, we analyzed 1-palmitoyl-2-arachidonoyl-sn-glycero-3-
phosphocholine (PAPC), the dominant phospholipid in BAL and its oxidized derivatives by means of liquid chromatography-electrospray mass spectrometry. Representative LC-MS images are shown in Figure 7A (TLR4\textsuperscript{wt} FA), Figure 7B (TLR4\textsuperscript{wt} PM\textsubscript{2.5}), Figure 7C (TLR4\textsuperscript{4} FA), and Figure 7D (TLR4\textsuperscript{4} PM\textsubscript{2.5}) and depict a peak shift from PAPC to the oxidized derivatives in response to PM\textsubscript{2.5} exposure. Figure 7E illustrates the oxidation steps from PAPC through POVPC (1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine) to PGPC (1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine). Mice deficient of TLR4 had lower amounts of oxidized phospholipids in response to PM\textsubscript{2.5} (Figure 7F and 7G). To address if the oxidation products were a consequence of oxidative chemistry relating to PM\textsubscript{2.5} constituents or related to cellular oxidative end-products, we incubated PAPC with or without added PM\textsubscript{2.5} at 37°C for duration of 24 to 120 hours. Our data demonstrates that PM\textsubscript{2.5} alone is not the primary cause of oxidation. Thus, we have found no difference in the degree of oxidation products of PAPC between PM\textsubscript{2.5} group and control group. The increasing levels of these products over time in both groups suggest PAPC auto-oxidation (Figure 7H and 7I).

**TLR4 Triggers Inflammatory Gene Expression and Cytokine Release and Promotes IRAK-Modulated p47\textsuperscript{phox} Phosphorylation in Response to Oxidized Phospholipid Treatment in BMDM**

To test the hypothesis that oxidized phospholipids may activate inflammatory pathways via TLR4, we treated...
BMDM with and without intact TLR4 to oxidized PAPC derivatives. Online Figure V shows the gene expression profile of cells treated with oxidized PAPC derivatives. oxPAPC strongly induced the expression of TNFα, various components of the NADPH oxidase including Nox2 and p67phox and the homing receptors CCR1, CCR2 and CCR5. The cytokine profile of BMDM treated with oxidized and nonoxidized PAPC is outlined in Figure 8A. TLR4wt BMDM exhibited a significant increase of TNFα and MCP-1 but a marginal increase of IL-6 in response to oxPAPC treatment. Figure 8B shows that oxidized PAPC phosphorylates the p47phox subunit of NADPH oxidase. This response was...
abrogated by an Interleukin-1 Receptor-associated kinase (IRAK) inhibitor. TLR4 deficiency in BMDM abolished the effects of oxPAPC.

**Discussion**

The inhalation of airborne pollutants is linked to lung and systemic inflammation. The mechanisms by which dust is recognized and how chronic inflammatory diseases are triggered are poorly understood. Here we show that: (1) chronic PM$_{2.5}$ exposure promotes monocyte egress from bone marrow into the systemic circulation; (2) increased presence of inflammatory monocytes in the circulation corresponds to ingress of these cells into vascularized tissue niches such as perivascular fat and visceral adipose; (3) alteration in macrovascular and microvascular function with PM$_{2.5}$ is related to vascular infiltration in the perivascular fat and superoxide generation by monocytes. 4. Activation of TLR4 and NADPH oxidase in monocyte/macrophages by oxidized phospholipids may represent one potential mechanism by which PM$_{2.5}$ mediates systemic inflammation.

**Chronic PM$_{2.5}$ Promotes Monocyte Egress From Systemic Reservoirs**

Our results add to the growing body of evidence that chronic PM$_{2.5}$ exposure modulates systemic inflammatory effects and provides important insights into the mechanisms and mediators of these responses. Prior cohort and panel studies have postulated an effect of PM on the bone marrow to enhance the systemic release of inflammatory cells. A few controlled exposure studies have corroborated a cellular proinflammatory response, manifested as increase in circulating white blood cell counts or immune cell infiltration. Our results provide mechanistic proof of the existence of a significant contribution of the bone marrow and potentially spleen in response to chronic PM$_{2.5}$ exposure. Blood monocytes are heterogenous and comprise of distinct subsets...
with specific migratory properties. In the mouse, monocyte subsets can be distinguished on the basis the Ly6C antigen expression. Ly6C high cells (F4/80, CD11b, CD115) cells, originally called “inflammatory” are recruited to tissue niches in response to chemokine signals. In contrast, Ly6C low monocytes represent resident cells with lower recruitment to sites of inflammation. Prior studies have suggested an important role for the corresponding ligands in promoting the egress of monocytes into the peripheral circulation. In keeping with these results there was increase in lung and circulating levels of MCP-1 (CCL2). Increase in MCP-1 could represent one mechanism for the enhanced flux of monocytes from PM2.5. However it is possible that other chemokines or mechanisms may be responsible, as other studies have reported that MCP-1 does not contribute to macrophage infiltration into adipose tissue. We hypothesize that other mechanism might drive those inflammatory monocytes to adipose tissue niches. The results with the c-fms YFP mouse model provide additional evidence that monocytes mediate tissue infiltration in response to chronic PM2.5 exposure and suggest that MCP-1 release may indeed result in homing of CCR2 cells from the bone-marrow. Ly6C high cells almost always expressed YFP in this study. The results in the YFP model are partially supported by additional exposure studies where there was an increase in CCR2+ monocytes with exposure durations as brief as 10-weeks.

**TLR4 Activation Is Critical to the Transduction of PM2.5 Systemic Response**

The upregulation of multiple genes involved in TLR4 signaling in the vasculature in response to PM2.5 and abrogation in TLR4 mice is suggestive of a specific interaction of PM2.5 with this pattern recognition receptor. Our data also seem to suggest an important contribution of the lung as MCP-1 and TNFα levels are elevated. These changes in lung cytokine content were accompanied by increased phosphorylation of p47phox subunit of NADPH oxidase in the lung and heightened circulating levels of MCP-1 and TNFα. MCP-1 may then potentially lead to the egress of subsets of monocytes from the bone marrow through CCR2 dependent mechanisms.

**Role of the NADPH Oxidase in PM2.5 Effects**

Our findings may have important consequences for regulation of vascular tone. Although many studies have demonstrated an important role for vascular NADPH oxidase in maladaptive responses in the vessel wall; the contribution of NADPH oxidase in inflammatory cells, versus those of resident vascular cells has only been recently appreciated. Importantly, it has been shown that infiltrating macrophages in the peri-adventitial fat may release cytokines and O2- in a Nox2 dependent manner, which by itself, or via activation of Nox1/Nox2 in vascular cells may then contribute to adverse tonic responses. We believe that our studies provide evidence that monocyte NADPH oxidase-derived O2- in response to PM2.5 may alter redox balance and promote vasoconstrictive responses. Although p47phox phosphorylation is required for the coordinate generation of superoxide by both Nox1 (present only in vascular cells) and Nox2 (present in myeloid cells and vascular cells), it is likely that upstream TLR4 activation in monocytes/macrophages was contributing to Nox2 activation in these cells, rather than Nox1. We base this conclusion on the fact that ablation of Nox2 virtually abolished the O2- in monocytes both in response to PM2.5 as...
well as in the FA group. These results in conjunction with monocyte infiltration in perivascular fat are highly suggestive of a monocyte source for ROS generation in response to PM$_{2.5}$. The magnitude of O$_2^-$ production was equivalent in the perivascular fat compared to the actual vessel wall (data not shown) and is further supportive of the concept that the vasa vasorum coursing through the perivascular fat may represent important entry points for inflammatory cells.$^{45,47}$

Role of NADPH Oxidase in TLR4-Mediated Responses

Our results suggest that NADPH oxidase activation occurs in the lung as well in the systemic circulation. Based on in vitro studies in cultured macrophages, inhibition of IRAK, prevented phosphorylation of the $p47^{phox}$ subunit of NADPH oxidase, suggesting that TLR4 activation occurs upstream of NADPH oxidase. Pacquelet et al demonstrated that NADPH oxidase is activated as a result of the phosphorylation of $p47^{phox}$ by IRAK4 and identified the residues of $p47^{phox}$ as targets of IRAK4 phosphorylation.$^{23}$ In our in vivo experiments IRAK2 (but not IRAK4) was up regulated transcriptionally in the aorta. Both IRAK1 and IRAK4 are active serine/threonine kinases, and phosphorylation of IRAK1 or IRAK2 is crucial for their activation during TLR/MyD88 signaling. Although IRAK1 is the dominant kinase, shown by many studies to be involved in TLR4 signaling, our results suggest that IRAK2 may be important in PM$_{2.5}$ exposure. The upregulation of caspase-8 may represent a nonapoptotic role of this protein as it has been shown that TLR4 activation results in the recruitment of caspase-8 to a complex containing IKK.$^{48}$ The ubiquitin-conjugating enzyme E2N (Ube2N) may represent a general homeostatic response to inflammatory injury as has been previously demonstrated with other toxins.$^{49}$

Exposure Considerations and Limitations

The exposures used in this protocol are broadly relevant to human health as they mimic “real-world” ambient air at doses that are commonly encountered in many parts of the world without requiring invasive methods or the generation of artificial particles.$^{50}$ Our results provide mechanistic rationale for inflammatory effects of PM$_{2.5}$ exposure and why even relatively low levels of particle exposure (when compared to cigarette smoking for instance) results in relatively robust systemic inflammatory effects.$^3$ Several important limitations must be acknowledged. Although we did not test for the involvement of other pattern recognition receptors, it is conceivable that the Nalp3 inflammasome and other TLR receptors such as TLR3 pathways may also be involved.$^{13}$ Although we have shown an involvement of oxidized PAPC, it is conceivable that alternate/additional TLR4 ligands may participate such as modified matrix components, hyaluronan, or even HMGB1. We have not provided definitive evidence that non-MyD88 pathways are not involved (TRAF6/TRIF). Another limitation stems from the fact that we performed exposures in different strains. It is well known that different strains may have widely different responses to inflammatory stimuli. Thus, we cannot conclude that these pathways are conserved and identical across strains. A final limitation is that although we have tried to characterize the precise locus of activation of inflammatory cells in response to PM$_{2.5}$, we have not been able to definitively conclude that the lung is integral to this response. Our findings reveal that PM$_{2.5}$ may represent a chronic inflammatory stimulus and may contribute to the pathogenesis of cardiometabolic disease.

Hypothetical Model

This work emphasizes on the role of TLR4 in mediating systemic inflammation in response to PM$_{2.5}$ exposure. Online Figure VI shows the hypothetical model of TLR4/NADPH oxidase interaction.

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Disclosures

None.

References


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Novelty and Significance

What Is Known?

- Chronic exposure to PM$_{2.5}$ (particulate matter of $\leq 2.5\ \mu m$) is associated with heightened risk for cardiovascular events.
- The mechanisms are believed to involve inflammatory responses triggered by PM$_{2.5}$. However, the locus, differential contribution of the lung, and the pathways remain poorly defined.

What New Information Does This Article Contribute?

- Identification of monocyte subsets (Ly6C$^{high}$) that increase in response to chronic inhalational PM$_{2.5}$ exposure.
- TLR4 and Nox2 mitigates the systemic effects of chronic PM$_{2.5}$ exposure.
- Chronic PM$_{2.5}$ exposure increases oxidized phospholipid derivatives of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine in bronchioalveolar lavage fluid.
- Exposure of bone marrow–derived macrophages to oxPAPC but not PAPC recapitulated effects of chronic PM$_{2.5}$ exposure, whereas TLR4 deficiency attenuated this response.

Epidemiological investigations have provided a compelling association between exposure to inhaled particulate matter and cardiovascular disease. The biological mechanisms that underpin this association and provide insights into how an inhalational stimulus can trigger systemic effects remain to be fully characterized. Prior studies have suggested an inflammatory component in the lung in acute studies using intratracheal installation with high doses of particles. However, the inflammatory pathways and the role of the lung in chronic inhalational delivery poorly defined. In this study, we demonstrate that chronic inhalational exposure to PM$_{2.5}$ results in generation of oxidized phospholipid byproducts in the lung that may then trigger a systemic inflammatory response via TLR4/NADPH oxidase pathways. This work highlights the role of modified phospholipid derivatives in lung surfactant as drivers of inflammatory effects in response to inhaled particles.
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Material and Methods

Animal Models
Six-week-old male C57BL/6, Nox2<sup>−/−</sup> (C57BL/6 background), Balb/c (TLR4<sup>wt</sup>), Tlr4<sup>Lps-d</sup> (TLR4<sup>d</sup>, background strain BALB/cAnPt) were obtained from Jackson Laboratories (Bar Harbor, ME). Tlr4<sup>Lps-d</sup> mice harbor a spontaneous mutation in the toll-like receptor gene. c-fms<sup>YFP</sup> transgenic mice (FVB/N background) were obtained as previously described (1), All mice were randomized to exposure (see below). The Committee on Use and Care of Animals from the Ohio State University (OSU) approved all experimental procedures.

Exposures to PM<sub>2.5</sub> Ambient Whole-Body Inhalational Protocol
Animal exposure and the monitoring of exposure atmosphere and ambient aerosol were performed as previously described using a versatile aerosol concentration enrichment system that was modified for long-term exposures (2). Briefly, mice were exposed to concentrated PM<sub>2.5</sub> or filtered air (FA) in a mobile trailer at The Ohio State University in Columbus, OH (OASIS-1 chamber, “Ohio air pollution exposure system for the interrogation of systemic effects”). FA-exposed mice received an identical protocol with the exception of a high-efficiency particulate air filter (Pall Life Sciences, East Hills, NY) positioned in the inlet valve to remove PM<sub>2.5</sub> in the filtered air stream, as detailed described previously (3). The exposure protocol comprised of exposures for 6 h/day, 5 days/wk. The TLR4<sup>wt</sup> and TLR4<sup>d</sup> mice were exposed for a total duration of 20 weeks from March to July 2009. The Nox2<sup>wt</sup> and Nox2<sup>−/−</sup> mice were exposed from November 2008 – March 2009 (20 weeks) and the c-fms<sup>YFP</sup> mice from October 2008 – March 2009 (23 weeks). Supplemental Table 4 shows the plasma lipid profile among the different groups after exposure.

Analysis of PM<sub>2.5</sub> Concentration in the Exposure Chamber
To calculate exposure mass concentrations of PM<sub>2.5</sub> in the exposure chambers, samples were collected on Teflon filters (PALL Life Sciences Teflo, 37 mm, 2 μm pore, Ann Arbor, MI) and weighed before and after sampling in a temperature- and humidity-controlled weighing room using an oscillating microbalance (Tapered-Element Oscillating Microbalance, model 1400, Rupprecht and Patashnick). Weight gains were used to calculate exposure concentrations.

Flow Cytometry.
Whole blood was collected to assess the expression of inflammatory monocytes and YFP positive cells in peripheral leukocytes in TLR4<sup>wt</sup>, TLR4<sup>d</sup> and c-fms<sup>YFP</sup> mice. About 1 mL of blood was treated with red blood cell lysis buffer (Biolegend, San Diego, CA) for 5 minutes (2 times). Bone marrow cells were collected by flushing femur and tibia with 10 mL PBS. Monocytes were isolated from bone marrow with Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). Isolated leukocytes from both blood and bone marrow were resuspended in flow buffer (PBS containing 5% bovine serum albumin (BSA) and 0.02% NaN<sub>3</sub>) and washed twice. This was followed by incubation with PE-Cy7 anti-Ly6G (eBioscience, San Diego, CA), FITC anti-CD3 (Pharmingen, San Jose, CA), APC-Cy7 anti-CD45.BR (Pharmingen, San Jose, CA), PE-Cy5 anti-F4/80 (Biolegend, San Diego, CA), PE-Cy5 anti-Ly6C (Biolegend, San Diego, CA), PE anti-CD11b (Biolegend, San Diego, CA); CCR2 ((primary antibody (rabbit monoclonal) and secondary antibody, donkey polyclonal to rabbit (PE) both Abcam)), PE-Cy5 anti-CCR5 (Biolegend, San Diego, CA) and PE anti-CxCr3 (Biolegend, San Diego, CA) with 1 μg per million cells for 30 minutes. Cells were subsequently washed with flow buffer, resuspended in 1% natural buffered formaline and analyzed by flow cytometry (BD FACS LSR II™ flow cytometer, Becton Dickinson, San Jose, CA) and the data were analyzed on BD FACS Diva software (Becton
Dickinson, San Jose, CA). To detect YFP⁺ monocyte infiltration in lung and epidydimal fat cells were isolated as mentioned and analyzed by flow cytometry. Broncheolar alveolar lavage (BAL) cells were isolated by flushing the lung with 1 ml PBS for three times. Epidydimal fat and lung tissue were digested with Collagenase II (Sigma-Aldrich, St. Louis, MO) for one hour and samples were stained for flow cytometry afterwards.

Cytokine Measurements in Tissues and Bone Marrow-Derived Monocyte Conditioned Media

Lung tissue homogenates and BMDM supernatants were analyzed for 6 different cytokines by Mouse Inflammation 6-Plex Kit from BD Bioscience (San Diego, CA). The lung lobes were snap-frozen (in liquid nitrogen) and stored at -80°C until further analysis. The snap-frozen lungs were thawed, weighed, transferred to different tubes on ice containing 1 ml of T-PER containing Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany) tablets at a proportion of 1 tablet/10 ml of T-PER stock reagent. The lung tissues were homogenized at 4°C. Lung homogenates were centrifuged at 9,000 × g for 10 minutes at 4°C. Supernatants were transferred to clean microcentrifuge tubes, frozen on dry ice and thawed on ice. Total protein concentrations in the lung tissue homogenates were determined using a BCA kit. Lung tissue homogenates were diluted with 50% assay diluents (provided in the BD™ Cytometric Bead Array (CBA), Mouse Inflammation 6-Plex Kit, BD Bioscience, San Jose, CA) and 50% TPER reagent to a final protein concentration of 500 μg/ml.

Superoxide Measurements

NADPH oxidase derived superoxide production was measured by lucigenin chemiluminescence. Bone marrow derived F4/80⁺ (Miltenyi Biotec, Bergisch Gladbach, Germany) monocytes were sorted by immuno magnetic bead sorting MiniMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). The cell number was adjusted at 1x10⁶ cells per well. Additionally, mouse aorta without perivascular fat and perivascular fat alone were placed in chilled, modified Krebs-Hepes Buffer (pH = 7.4; initially gassed with 95% O₂, and 5% CO₂), cleaned of excessive adventitial tissue and cut into 2 to 3 mm segments. The vessel segments were placed in a microtiter plate (Berthold Technologies, Bad Wildbad, Germany) containing 300 μl of Krebs-Hepes buffer with 5 μM Lucigenin in each well were placed into an Berthold CENTRO LB 960 Luminometer (Berthold Technologies, Bad Wildbad, Germany). Scintillation counts were recorded every minute for a duration of one second. After baseline measurements for 10 min, 100 μM NADPH (Sigma-Aldrich, St. Louis, MO) was added to each well and measurements made over additional 10 minutes. Values were reported after subtracting respective background counts. Aortic tissue and perivascular fat tissue were quantified per/mg wet weight.

Myography and Intravital Microscopy

Thoracic aortas were dissected from animals and immediately immersed in physiological salt solution buffer at room temperature. In some experiments aortas were then cleaned of adhering fat and connective tissue under a microscope while as in other experiments aortas were left with adipose tissue around them. The blood vessels were cut into rings of 2 mm to 3 mm length and were mounted in a standard 5 ml organ bath (DMT 700 MO, Atlanta, GA) filled with physiological saline solution (PSS) buffer. The bath medium was maintained at 37°C with a pH of 7.4 and aerated continuously with 95% oxygen and 5% carbon dioxide. Extra care was taken to ensure that the endothelium was not damaged during the whole process of tissue preparation and mounting. Aortic rings were subject to graded doses of vasoconstrictor agonists and the endothelium dependent dilator acetylcholine as described previously (4). For intravital microscopy mice were anesthetized by i.p. injection of a mixture of 20 mg/kg xylazine (VET TEK, Blue Springs, MO) and 100 mg/kg ketamine hydrochloride (Bioniche Pharma USA LLC,
Lake Forest, IL). Cremaster muscle or mesenteric adipose tissue were stretched on an optically coherent mount. The muscle or the mesenteric adipose tissue were superfused with Ringers Lactate at 37°C and leukocyte endothelial interactions in 10-15 venules obtained using a Nikon Eclipse FN1 microscope (Nikon, Japan) with a 40x/0.80 W (muscle) and 20x/0.50 W (mesenteric tissue) water immersed objective with 2.0 mm working distance. Video images were captured by a monochrome QImaging Rolera-XR camera (Surrey, BC, Canada) at a speed of 20 FPS and digitalized to 12-bit TIF images using Metamorph software (version 7.1.2.0, Metamorph, Downingtown, PA). c-fms<sup>YFP</sup> mice were imaged using a fluorescent filter (YFP emission 527 nm). Rolling leukocytes or YFP positive cells were counted per minute for different vessel diameters and vessel segments. All leukocytes or YFP<sup>+</sup> cells per 100 μm of vessel length that were immobile for at least 30 s were interpreted as adherent cells (5). The number of rolling and adherent cells was then imputed for a theoretical 30 micron vessel, assuming a linear dependency between the vessel diameter and leukocyte adherence/rolling. Calculations were performed using OptiTest (Version 1.4.1.0).

Immunohistochemistry
Segments of thoracic aorta with perivascular fat were frozen in liquid nitrogen and embedded in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA). Rat anti-mouse F4/80 antibody (AbD Serotec, Raleigh, NC) was used to determine macrophage infiltration. Immunohistochemical 3,3′-Diaminobenzidine (DAB) staining was performed by using the primary antibodies (1:200 concentration) and a detection system using Peroxidase-conjugated AffiniPure goat anti-rat antibodies (Immunoperoxidase Secondary Detection System; Jackson ImmunoResearch Laboratories, West Grove, PA). Images were analyzed and quantified with Metamorph software (Metamorph) after digitization of the images with a color QImaging Micro Publisher 5.0 RTV camera (Surrey, BC, Canada) at 200x. Data are expressed as the percentage of the total analyzed area. For estimation of infiltrated F4/80 positive cells per area, four successive sections were collected on the same slide, and at least 10 sections from three consecutive slides per area per mouse were examined.

Total RNA extraction and quantitative RT-PCR
Pathway-focused gene expression profiling was performed using the RT<sup>2</sup> Profiler™ PCR Array System - Mouse Toll-Like Receptor Signaling Pathway according to manufacturer’s instructions (SuperArray Bioscience Corporation, Frederick, MD). Total RNA was isolated with Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA) according to the manufacture’s instructions. cDNA was synthesized using 500 nanogram of total RNA and Transcriptor™ reverse transcriptase (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer’s instructions. Real time reactions (minus primers) were prepared with a SYBR green master mix, loaded onto the array plate containing pre-loaded primer sets for 384 genes, and run on a Lightcycler 480. Expression was determined relative to 7 housekeeping genes using proprietary Superarray software. For additional confirmatory PCRs we used the following primers: CCR2 5′-GCACCTTAGACCAGGCCATGC-3′ (forward) and 5′-GCTCCCTCCTCCTGCTTTA-3′ (reverse); CCR1 5′-CATGCAGTGCTGACTGATTG-3′ (forward) and 5′-TTGAAAAGCTGCAAGGTAC-3′ (reverse); CCR5 5′-CTGGCTCTTGAGGATGTATT-3′ (forward) and 5′-ATGGCCAGGTACGAGGTAG-3′ (reverse); Cx3Cr1 5′-TCTCTCCCAGCTGCTTTG-3′ (forward) and Cx3Cr1 5′-AAGGAGGCCAGACCCTG-3′ (reverse); Hmg14 5′-GCAGAAAATGGAGAGACGGAAAACC-3′ (forward) and 5′-AAGGAGGCCAGACCCTG-3′ (reverse); Nox2 5′-ACTCCTTGGTCCAGACTGTTG-3′ (forward) and 5′-GCCGCTTGACGGTCCAG-3′ (reverse); p67<sub>phox</sub> 5′-CAGTCCTTAGAGGAAATG-3′ (forward) and 5′-TCTGCCATAGCTGGACAGTG-3′ (forward); p47<sub>phox</sub> 5′-ACCTGTCGGAGAAATG-3′ (forward) and 5′-TAGTCTGAAGGATGTGGG-3′ (forward); and 5′-CAGTCCAAAGGAAATGAGG-3′ (reverse); 5′-ACCTGTCGGAGAAATG-3′ (forward) and 5′-TAGTCTGAAGGATGTGGG-3′ (forward).
GGTTGGTAGGTTGGCTGCTTGATGG-3' (reverse); Rac1 5'-TGGGACACAGCTGGACAAGAAGAT-3' (forward) and 5'-TCAGGATACCACCTTGACCCGACA-3' (reverse); Rps3 5'-ATCAGAGAGTTGACCGCAGTTG-3' (forward) and 5'-AATGAACCGAAGCACACCATAGC-3' (reverse). Quantitative real-time PCR was performed with a Lightcyler 480 (Roche Applied Sciences, Penzberg, Germany) using SYBR Green I Master Mix (Roche Diagnostics GmbH, Mannheim, Germany). The relative quantification values for these gene expressions were calculated from the accurate threshold cycle (C_T), which is the PCR cycle at which an increase in reporter fluorescence from SYBR green dye can first be detected above a baseline signal. The C_T values for Hmg14 and Rps3 were averaged and subtracted from the C_T values for the gene of interest in each well to calculate ΔC_T. The duplicate ΔC_T values for each sample were averaged.

In-vitro Experiments in Cultured Bone Marrow Derived Monocytes
Primary bone marrow derived monocytes (BMDM) were isolated by flushing the femur and tibia by a 26-gauge needle and (PBS, 5% FBS). Afterwards, transfer the cell suspension on a 150 mm suspension culture dish (Corning Incorporated, Corning, NY). Cells are cultured in L-cell conditioned media plus D-MEM at 37°C for 5 days and harvested by trypsinization (Sigma-Aldrich, St. Louis, MO). BMDM were isolated from mice whereby TLR4 is intact and deficient as described previously. After harvesting the BMDM by trypsinization, 1x10^5 cells were plated in a 96-well plate (Microtest, Becton Dickinson Labware, NJ) and cultured overnight. Triplet or quadruplet samples per group were either untreated, treated with 25 μM PAPC (Avanti Polar Lipids Inc., Alabaster, AL) or a mixture of 12.5 μM POVPC (Avanti Polar Lipids Inc., Alabaster, AL) and 12.5 μM PGPC (Avanti Polar Lipids Inc., Alabaster, AL) for 0.5 h (western blot) or 4 h (cytokine release or RT-PCR) at 37°C. For western blot and RT-PCR experiments cells were lysed as described previously and stored at -80°C until further procession. In experiments detecting the cytokine release cells were washed 3 times with PBS, new DMEM media with 20% FBS was added and further incubated for 48 h at 37°C. After treatment with phospholipids cells were incubated in L-cell conditioned free media for 48 h. Then supernatants were collected and stored in -80°C until further procession.

Western Blot in Lung Homogenates and Bone Marrow Derived Monocytes
Lung homogenates were received as described previously. Cells were briefly washed with ice-cold PBS. The cells were then scraped in 500 μl extraction lysis buffer containing 20 mM Tris pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, 0.5 mM PMSF and 0.5% protease inhibitor cocktail (Thermo Fisher Scientific Inc., Waltham MA). Cell debris was removed by micro centrifugation. The protein concentration of the cell lysates were determined using Bio-Rad DC (Bio-Rad Laboratories, Hercules, CA) protein assay according to the manufacturer's instructions. The supernatants were then boiled with Laemmli sample buffer for 5 minutes. Samples (25 μg of protein per lane) were separated on 8% sodium dodecyl sulfate-polyacrylamide gels at 30 mA for 3 hours. Separated proteins were transferred electrophoretically into polyvinylidene difluoride membrane (Immuno-Blot; Bio-Rad Laboratories, Hercules, CA) at 160 mA for 90 minutes. Membranes were blocked with blocking buffer phosphate-buffered saline and 0.1% Tween 20 (Acros Organics, NJ) containing 5% BSA at 4°C overnight. For detection of phosphorylated proteins, membranes were incubated with anti-phosphoserine antibody (Abcam, Cambridge, MA) overnight at 4°C. After washing, membranes were incubated with the horseradish-peroxidase conjugated anti-mouse antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, dilution of 1:500) at room temperature for 90 minutes for detection of p47phox membrane was stripped and reincubated with rabbit anti-p47phox monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight. Prestained markers (Precision Plus Protein; Bio-Rad Laboratories, Hercules, CA) were used for molecular mass
determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Super Signal West Pico; Thermo Scientific, Rockford, IL).

**Liquid Chromatography Mass Spectrometry of Oxidized Phospholipids**
Broncholar alveolar lavage fluid (BALF) was isolated by flushing the lung three times with 2 ml PBS. The samples of 5 mice were pooled and further processed. Lipids were extracted three times with chloroform / methanol mixture (1:1) and combined extracts were evaporated to dryness under stream of nitrogen. Samples were stored under nitrogen atmosphere at -80°C until analysis. Mass spectra were acquired in positive ion mode using Applied Biosystems 3200 QTRAP system coupled with electrospray ionization (TurbolonSpray) source. The spectrometer was first optimized by infusion of PAPC (25 nmol/ml), POVPC (5 nmol/ml) and PGPC (2 nmol/ml). All phospholipids were purchased from Avanti Polar Lipids Inc, (Alabaster, AL). The source parameters were set as follows: curtain gas (nitrogen), 10 psi; collision gas (nitrogen), medium; ion spray voltage 5000 V; temperature 550°C, ion spray voltage, 5000 V; ion source gas 1 and 2, 30 and 50 psi, respectively. Optimized parameters for all phospholipids were: declustering potential, 50 V; entrance potential, 10 V and collision energy, 50 eV. For analysis of BAL extracts, samples were dissolved in mobile phase consisting of chloroform, methanol, water and trifluoroacetic acid (65:25:4:0.1, by vol). Lipids were characterized after isocratic separation on 5 μm Zorbax RX-SIL 4.6 mm x 250 mm HPLC column (Agilent Technologies, Santa Clara, CA) at 0.4 ml/min flow rate using Shimadzu LC-20AD pump interfaced to a Shimadzu CBM-20A system controller. Mass spectrometer was operated in multiple reactions monitoring (MRM) positive ionization mode. Specific monitor Q1/Q3 ion pairs were m/z 782→184 for PAPC, m/z 594→184 for POVPC and m/z 610→184 for PGPC. Standard curves for all phospholipids were obtained in the same set of experiments by infusion of serially diluted PAPC (from 100 to 1000 ng/ml), POVPC (1 to 100 ng/ml) and PGPC (1 to 100 ng/ml). All data were acquired and processed by Analyst software (version 1.4.2, Applied Biosystems, Foster City, CA).

**Endotoxin-Detection**
Lipopolysaccharide (LPS) levels were analyzed in BALF and Serum by using ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript, Piscataway, NJ, USA). From each sample 4 μl were used as substrate and further processed the according manufactur’s instructions.

**Statistical Analysis**
All data are expressed as means ± standard deviation (SD) unless otherwise mentioned. Statistical tests were performed using one-way ANOVA followed by Mann Whitney test or unpaired t test using GraphPad Prism (version 4.1.2). The α-level was set at 0.05.
References


Supplementary Figure I

PM$_{2.5}$ concentration in the exposure chamber and particulate composition. Ambient and exposure of particulate matter (PM$_{2.5}$) concentrations during the exposure period at The Ohio State University Airport (April – July, 2009).
Supplementary Figure II

Delta change in the relaxation and constriction maxima of isolated aortic rings with and without perivascular fat (PVAT). (n=8-10/group).
Supplementary Figure III

Intravital microscopic leukocyte adherence in the cremaster venular endothelium. Data are mean ± SD. (Original magnification 400x, n=5, *p<0.05).
PM$_{2.5}$ impairs contractile properties of isolated thoracic aortic rings in c-$fms^{YFP}$ mice. Constriction of aortic rings with and without perivascular fat in response to increasing dosages of phenylephrene. (n=4/group; *p<0.01 vs. same group FA; †logEC50 vs. same group FA).
TLR4 triggers inflammatory gene expression in response to oxidized phospholipid treatment in BMDM derived from TLR4<sup>wt</sup> and TLR4<sup>d</sup> mice. BMDM were treated with PAPC and oxPAPC and mRNA levels were quantified by RT-PCR to confirm the specificity of TLR4 deletion. (n=4/group; *p<0.05; **p<0.01).
Supplementary Figure VI

Hypothetical model of TLR4/NADPH oxidase interaction in response to oxidized phospholipids and PM$_{2.5}$ upon stimulation. Preliminary data described a marked increase of oxidized phospholipids in the BAL fluid caused by PM$_{2.5}$ exposure. However, we hypothesize that oxidized phospholipids play a critical role in receptor activation for Toll-Receptor 4. In turn TLR4 signals downstream and promotes NADPH oxidase activation through phosphorylation of the NADPH oxidase subunit p47$^{phox}$ by IRAK. Although, the underlying mechanism has not been understood phosphorylated p47$^{phox}$ and other cytosolic subunits of the NADPH oxidase translocate to the membrane and form with gp91$^{phox}$ and p22$^{phox}$ an active, superoxide producing NADPH oxidase complex. A set of in-vitro experiments on cultured primary macrophages should elucidate the inflammatory in-vivo effect of particulate matter exposure. Furthermore, these experiments should generate a mechanistic link between TLR4 signaling and NADPH oxidase activation.
Supplementary Table I

<p>| | |</p>
<table>
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<tr>
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<tr>
<td>Exposure time (Dates)</td>
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<tr>
<td>Ambient concentration (μg/m³)</td>
<td>10.7 ± 2.1</td>
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<tr>
<td>FA chamber concentration (μg/m³)</td>
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<tr>
<td>PM chamber concentration (μg/m³)</td>
<td>92.4 ± 7.1</td>
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<tr>
<td>PM chamber particle enrichment factor</td>
<td>8.8 ± 1.6</td>
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<tr>
<td>* Estimated total exposure dose in FA group, μg</td>
<td>36.86 ± 5.9</td>
</tr>
<tr>
<td>† Estimated total exposure dose in PM group, μg</td>
<td>103.65 ± 19.48</td>
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</table>

Characteristics of exposure protocol. The exposure protocol describes the time of exposure, ambient concentration and the particulate concentration within the filtered air (FA) and particulate matter (PM_{2.5}) chamber as well as the particle enrichment factor. Assuming a ventilation rate of 105 breaths/min and tidal volume of 0.2 mL for a 25 g mouse (20). *The baseline estimated total dose over the exposure period of 20 weeks. †The estimated total dose of ambient and exposure over the exposure period of 20 weeks. Values shown are mean ± SD.
**Supplementary Table II**

Vasomotor response in TLR4<sup>wt</sup> and TLR4<sup>d</sup> mice in response to PM<sub>2.5</sub> exposure

<table>
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<tr>
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<th>Perivascular Fat</th>
<th>TLR4&lt;sup&gt;wt&lt;/sup&gt; FA</th>
<th>TLR4&lt;sup&gt;wt&lt;/sup&gt; PM&lt;sub&gt;2.5&lt;/sub&gt;</th>
<th>TLR4&lt;sup&gt;d&lt;/sup&gt; FA</th>
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<td>Max. Constrict. To PE</td>
<td>+</td>
<td>74.6 ± 2.5</td>
<td>88.4 ± 3.3 *</td>
<td>70.1 ± 3.6</td>
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<td></td>
<td>-</td>
<td>81.9 ± 3.5</td>
<td>97.9 ± 3.5 *</td>
<td>65.8 ± 2.3</td>
<td>72.4 ± 3.5</td>
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<td>logEC50</td>
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<td>-</td>
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<td>-8.1 ± 0.2</td>
<td>-7.2 ± 0.2</td>
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<tr>
<td>Max. Relax. To Ach</td>
<td>+</td>
<td>-30.6 ± 1.7</td>
<td>-23.3 ± 0.7 *</td>
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<td>-</td>
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<td>-37.9 ± 0.8 #</td>
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(*p<0.01, # p<0.05 vs same group FA)
## Supplementary Table III

Vasomotor response in Nox2\(^{wt}\) and Nox2\(^{-/-}\) mice in response to PM\(_{2.5}\) exposure

<table>
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<tr>
<th></th>
<th>Nox2(^{wt}) FA</th>
<th>Nox2(^{wt}) PM(_{2.5})</th>
<th>Nox2(^{-/-}) FA</th>
<th>Nox2(^{-/-}) PM(_{2.5})</th>
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<tbody>
<tr>
<td><strong>Max. Constrict. To PE</strong></td>
<td>102.4 ± 6.2</td>
<td>163.5 ± 6.4 *</td>
<td>118.3 ± 3.9</td>
<td>121.8 ± 5.4</td>
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<td><strong>logEC50</strong></td>
<td>-6.4 ± 0.1</td>
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<tr>
<td><strong>Max. Relax. To Ach</strong></td>
<td>-30.8 ± 3.1</td>
<td>-23.5 ± 1.8</td>
<td>-33.1 ± 5.7</td>
<td>-24.8 ± 6.7</td>
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<tr>
<td><strong>logEC50</strong></td>
<td>-6.5 ± 0.2</td>
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(*p<0.01 vs same group FA)
**Supplementary Table IV**

Flow cytometry results – homing signals in c-fms$^{YFP}$ mice

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<tr>
<th></th>
<th>YFP*</th>
<th>YFP<em>CCR2</em></th>
<th>YFP<em>CCR5</em></th>
<th>YFP<em>CXCR3</em></th>
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<tr>
<td><strong>BM FA</strong></td>
<td>74 ± 4.5</td>
<td>56.1 ± 3.7</td>
<td>8.5 ± 0.9</td>
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<tr>
<td><strong>BM PM$_{2.5}$</strong></td>
<td>70.5 ± 6.2</td>
<td>63.5 ± 0.2</td>
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<td><strong>Spleen FA</strong></td>
<td>34.8 ± 4.7</td>
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<td><strong>Spleen PM$_{2.5}$</strong></td>
<td>38.6 ± 5.7</td>
<td>26.8 ± 2.9</td>
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(*p<0.05 vs same group FA; n=3-4)
Supplementary Table V

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<th>TLR4&lt;sup&gt;wt&lt;/sup&gt; FA</th>
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<th>TLR4&lt;sup&gt;d&lt;/sup&gt; FA</th>
<th>TLR4&lt;sup&gt;d&lt;/sup&gt; PM&lt;sub&gt;2.5&lt;/sub&gt;</th>
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<tr>
<td><strong>Cholesterol</strong></td>
<td>114.5 ± 9.4</td>
<td>113.3 ± 12.9</td>
<td>118.8 ± 23.4</td>
<td>107.3 ± 9.5</td>
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<tr>
<td>[mg/dl]</td>
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<td><strong>Triglyceride</strong></td>
<td>115.8 ± 60.5</td>
<td>119.8 ± 76.8</td>
<td>101.3 ± 13.8</td>
<td>122.3 ± 53.5</td>
</tr>
<tr>
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</tbody>
</table>

(n=3-4)