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

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Abstract

Rationale—Chronic exposure to ambient air-borne particulate matter <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 $Ly6C^{high}$ 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-4 (TLR4) and Nox2 ($gp91^{phox}$) 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 NAPDH oxidase activation as evidenced by reduced $p47^{phox}$ 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 bronchioalveolar lavage fluid. Correspondingly, exposure of bone-marrow derived

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Disclosure
None.

macrophages to oxPAPC but not PAPC recapitulated effects of chronic $PM_{2.5}$ exposure while 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.

Keywords

particulate matter; monocyte; Toll-like receptor 4; superoxide; oxidized phospholipids

Introduction

Particulate matter (PM) has been consistently linked to morbidity and mortality from ischemic cardiovascular events and reduced life expectancy in epidemiologic studies (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 (5). Several new studies have also demonstrated that residing in locations with higher long-term average PM levels elevates the risk for cardiovascular (CV) 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) (6, 7). $PM_{2.5}$ air pollution has been linked with endothelial dysfunction, systemic inflammatory and oxidative stress responses and the progression of atherosclerosis (8, 9). Seaton and colleagues first proposed that deposition of particles in the lung provoked a low-grade alveolar inflammation with a secondary systemic inflammatory response (10). Since then, numerous studies have supported this inflammatory hypothesis and further expanded on it (11, 12). However, the precise mechanisms by which particulates are sensed and the responses transduced remain elusive. It is increasingly apparent that biologic systems commonly employ evolutionarily conserved mechanisms to sense a variety of environmental signals (13, 14). Toll-like receptors (TLRs) play a central role in the recognition of a broad diversity of environmental and pathogen associated molecular patterns (15, 16). We therefore hypothesized that $PM_{2.5}$ either directly or via biologic intermediates modulates systemic effects via TLR4 pathways. Accordingly, we performed exposures to $PM_{2.5}$ using a system that exposes animals chronically to 8–10 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.

Material and Methods

An expanded methods section is available in the Online Data Supplement.

Briefly, This study utilized male mice at the age of six-weeks of following different strains: C57BL/6, $Nox2^{-/-}$ (C57BL/6 background), Balb/c ($TLR4^{wt}$), $Tlr4^{Lps\text{-}d}$ ($TLR4^{d}$, 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 Committee on Use and Care of Animals from the Ohio State University (OSU) approved all experimental procedures.

Results

$PM_{2.5}$ concentration and exposure protocol

The exposure metrics are described in Supplementary 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 (Supplementary 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 (20). The total $PM_{2.5}$ dose inhaled during the exposure corresponded to 104 ± 20 μg assuming a ventilation rate of 105 breaths/minute 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 vs. 49.8 ± 13.6 pg/ml in the serum and 423 ± 233 pg/ml vs. 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 sub-populations, we analyzed peripheral blood, bone marrow and splenic cell populations. $PM_{2.5}$ increased inflammatory monocytes in the periphery in $TLR4^{wt}$ mice while deficiency of TLR4 diminished the effect (Figure 1B). The spleen and bone marrow played a differential role in contribution of inflammatory monocytes to the systemic circulation (Figure 1A and C).

Chronic $PM_{2.5}$ exposure increases NADPH oxidase derived superoxide ($O_2\cdot^-$) in monocytes, aortic tissue and perivascular fat through TLR4

We investigated the systemic effects of $PM_{2.5}$ exposure. Figure 2 depicts NADPH oxidase derived $O_2\cdot^-$ generation in $F4/80^+$ monocytes and systemic vasculature. NADPH oxidase derived $O_2\cdot^-$ 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 utilized mice deficient in Nox2 ($gp91^{phox-/-}$), to address the relative role of this sub-unit in up-regulated 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 $O_2\cdot^-$ 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 $O_2\cdot^-$ 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 $O_2\cdot^-$ 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 (22), 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 pre-constrictor dose of phenylephrine (prior to acetylcholine) and are depicted separately in Figure 3. Chronically $PM_{2.5}$ exposed $TLR4^{wt}$ 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 $TLR4^{wt}$ mice demonstrated attenuation of peak relaxation and increased EC_{50} dose to acetylcholine compared with FA mice; findings that were also seen in segments without perivascular fat. TLR4 deficiency attenuated the effects of $PM_{2.5}$ exposure (Figure 3C, 3D.

Supplementary Figure II allows the interpretation of the delta 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. Supplementary Table III depicts the responses in vascular segments from these mice. These results show a significantly higher constriction in $Nox2^{wt}$ mice in response to $PM_{2.5}$, while deficiency of Nox2 abrogated this effect. These results suggest that both TLR4 and NADPH oxidase mediate the systemic vascular effects of $PM_{2.5}$ exposure.

To test the effect of $PM_{2.5}$ 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 (Supplementary Figure III) in response to chronic exposure to $PM_{2.5}$.

$PM_{2.5}$ exposure increases Toll-like receptor dependent gene expression in aortic tissue in $TLR4^{wt}$ mice

To determine the molecular basis of $PM_{2.5}$ exposure effects in the vasculature, we examined a panel of genes involved in TLR signaling in aortic tissue. We utilized a PCR array, profiling the expression of genes related to TLR-mediated signal transduction. While in $TLR4^{wt}$ mice 8 genes significantly changed, this induction was abolished in $TLR4^{d}$ mice (Figure 4).

$PM_{2.5}$ exposure increases monocyte homing and adherence in tissue niches

To provide additional evidence of recruitment of monocytes with chronic $PM_{2.5}$ exposure in tissue niches, we used a transgenic reporter mouse model expressing YFP under the control of a c-*fms* promoter (c-fms^{YFP}) that were exposed to FA or $PM_{2.5}$ 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 $PM_{2.5}$ (Figure 5A). Figure 5B indicates similar findings in the mesenteric adipose tissue; representative images of mesenteric tissue from c-fms^{YFP} mice exposed to either FA or $PM_{2.5}$, respectively are depicted in Supplementary Video I–IV. Impairment in contractile properties of isolated thoracic aortic rings with and without perivascular fat in c-fms^{YFP} mice are shown in Supplementary Figure IV in response to $PM_{2.5}$ exposure. An increase of YFP^+ cell-infiltration into the perivascular adipose tissue was observed in response to $PM_{2.5}$ (Figure 5C). YFP^+ cells in the BAL, lung and epididymal fat were noted in response to $PM_{2.5}$ (Figure 5D, E and F). In additional experiments we exposed c-fms^{YFP} mice over a shorter 12 week duration to $PM_{2.5}$ or FA. Flow-cytometric analysis of YFP^+ cells co-expressing CCR2, CCR5 and CXCR3 in the bone marrow and spleen revealed a significant increase in YFP^+CCR2^+ cells in response to $PM_{2.5}$ exposure in bone marrow (Supplementary 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 $PM_{2.5}$

$TLR4^{wt}$ mice demonstrated an increase in TNFα, MCP-1 and IL12p70 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 $PM_{2.5}$. Corresponding plasma measurements demonstrated an increase in TNFα and MCP-1 with $PM_{2.5}$ exposure with normalization in $TLR4^{d}$ (Figure 6B). We then investigated if TLR4 activation by $PM_{2.5}$ leads to downstream NADPH oxidase activation (23–25). We chose phosphorylation of the $p47^{phox}$ subunit in the lung as an index of NADPH oxidase activation in response to $PM_{2.5}$ (12, 26). $TLR4^{wt}$ showed a

significant increase in $p47^{phox}$ phosphorylation in lung following $PM_{2.5}$ exposure, compared to FA mice, while TLR4 deficiency prevented this effect (Figure 6C).

Airborne particulate matter causes increased levels of two oxidized PAPC derivatives in BAL fluid of $PM_{2.5}$ exposed mice

Prior studies have indicated a role for surfactant phospholipids in mitigating inflammatory responses while 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 ($TLR4^{wt}$ FA), 7B ($TLR4^{wt}$ $PM_{2.5}$), 7C ($TLR4^{d}$ FA) and 7D ($TLR4^{d}$ $PM_{2.5}$) and depict a peak shift from PAPC to the oxidized derivatives in response to $PM_{2.5}$ 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 $PM_{2.5}$ (Figure 7F and G). To address if the oxidation products were a consequence of oxidative chemistry relating to $PM_{2.5}$ constituents or related to cellular oxidative end-products, we incubated PAPC with or without added $PM_{2.5}$ at 37°C for duration of 24–120 h. Our data demonstrates that $PM_{2.5}$ alone is not the primary cause of oxidation. Thus, we have found no difference in the degree of oxidation products of PAPC between $PM_{2.5}$ group and control group. The increasing levels of these products over time in both groups suggest PAPC auto-oxidation (Figure 7H and I).

TLR4 triggers inflammatory gene expression, cytokine release and promotes IRAK modulated $p47^{phox}$ phosphorylation in response to oxidized phospholipids 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. Supplemental 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 $p67^{phox}$ and the homing receptors CCR1, CCR2 and CCR5. The cytokine profile of BMDM treated with oxidized and non-oxidized PAPC is outlined in Figure 8A. $TLR4^{wt}$ 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 $p47^{phox}$ 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 (9, 11, 29, 30). Prior cohort and panel studies have postulated an effect of PM on the bone marrow to enhance the systemic release of inflammatory cells (31, 32). A few controlled exposure studies have corroborated a cellular pro-inflammatory response, manifested as increase in circulating white blood cell counts or immune cell infiltration (33, 34). 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 (35, 36). Blood monocytes are heterogenous and comprise of distinct subsets with specific migratory properties (35, 37). 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 (36–38). Prior studies have suggested an important role for the corresponding ligands in promoting the egress of monocytes into the peripheral circulation (39, 40). 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 with $PM_{2.5}$. 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 (50). 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 $PM_{2.5}$ 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 $PM_{2.5}$ systemic response

The up-regulation of multiple genes involved in TLR4 signaling in the vasculature in response to $PM_{2.5}$ and abrogation in $TLR4^{d}$ mice is suggestive of a specific interaction of $PM_{2.5}$ 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 $p47^{phox}$ subunit of NADPH oxidase in the lung and heighted 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 $PM_{2.5}$ 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 (41–44). Importantly, it has been shown that infiltrating macrophages in the peri-adventitial fat may release cytokines and $O_2\cdot^{-}$ in a Nox2 dependent manner, which by itself, or via activation of Nox1/Nox2 in vascular cells may then contribute to adverse tonal responses (44–46). We believe that our studies provide evidence that monocyte NADPH oxidase derived $O_2\cdot^{-}$ in response to $PM_{2.5}$ may alter redox balance and promote vasoconstrictive responses. While $p47^{phox}$ 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 (41). We base this conclusion on the fact that ablation of Nox2 virtually abolished the $O_2\cdot^-$ in monocytes both in response to $PM_{2.5}$ 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 $O_2\cdot^-$ 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 vaso-vasorum coursing through the perivascular fat may represent important entry points for inflammatory cells (44, 46).

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 up-regulation of caspase-8 may represent a non-apoptotic role of this protein as it has been shown that TLR4 activation results in the recruitment of caspase-8 to a complex containing IKK (47). The ubiquitin-conjugating enzyme E2N (Ube2N) may represent a general homeostatic response to inflammatory injury as has been previously demonstrated with other toxins (48).

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 (49).. 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. Supplementary Figure VI shows the hypothetical model of TLR4/NADPH oxidase interaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations and Acronyms

BALF	broncheolar alveolar lavage fluid
d	deficient
PAPC	1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine
PGPC	1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine
$PM_{2.5}$	particulate matter <2.5 μm
POVPC	1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine
TLR4	Toll-like receptor 4
wt	wildtype
YFP	Yellow fluorescent protein

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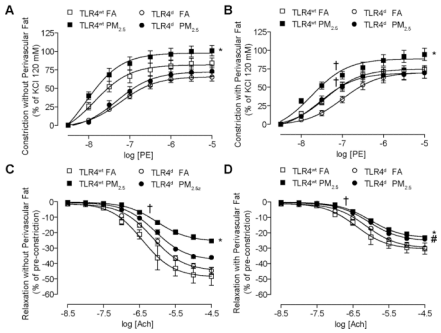

Figure 1.

$PM_{2.5}$ exposure promotes inflammatory monocyte egress from bone marrow to blood via TLR4 pathways. Inflammatory monocyte population of $TLR4^{wt}$ and $TLR4^{d}$ mice (**A**) in spleen **(B)** in peripheral blood, (**C**) in bone marrow. Data are mean ± SD. (n=4–6/group; *p<0.05). Exposure duration of 20 weeks.

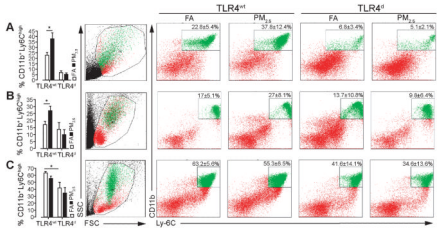

Figure 2.

$PM_{2.5}$ exposure increases NADPH oxidase derived $O_2\cdot^-$ production in monocytes, aortic tissue and perivascular fat in wildtype mice. (**A**) $O_2\cdot^-$ production in response to $PM_{2.5}$ exposure in bone marrow derived F4/80$^+$ cells, aortic and perivascular tissue from TLR^{wt} and TLR4^d mice. **(B)** $O_2\cdot^-$ production in response to $PM_{2.5}$ exposure in F4/80$^+$ cells, aortic and perivascular tissue from Nox2^{wt} and Nox2$^{-/-}$ mice after 20 weeks of exposure. Data are mean ± SD. (n=5, *p<0.05).

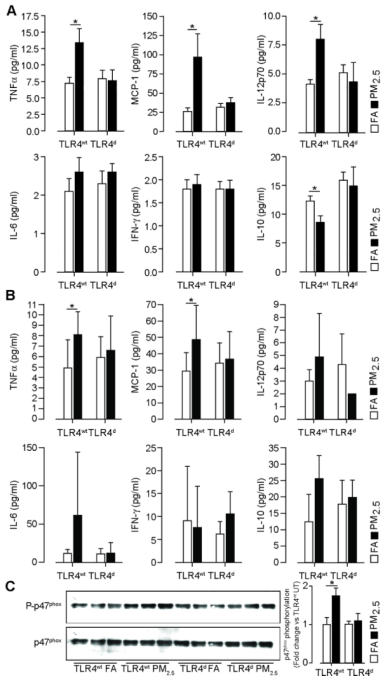

Figure 3.

$PM_{2.5}$ impairs macrovascular tonal responses with chronic exposure to PM2.5 through TLR4 pathways (20 weeks of exposure). (**A**) Constriction of aortic rings without perivascular fat in response to increasing dosages of phenylephrine. **(B)** Constriction of aortic rings with perivascular fat in response to increasing dosages of phenylephrine. **(C)** Relaxation of aortic rings without perivascular fat in response to increasing dosages of acetylcholine. **(D)** Relaxation of aortic rings with perivascular fat in response to increasing dosages of acetylcholine. (n=8–10/group; *$p<0.01$ vs. $TLR4^{wt}$ FA; $^{\#}p<0.05$ vs. $TLR4^{d}$ FA; †logEC50 vs. same group FA).

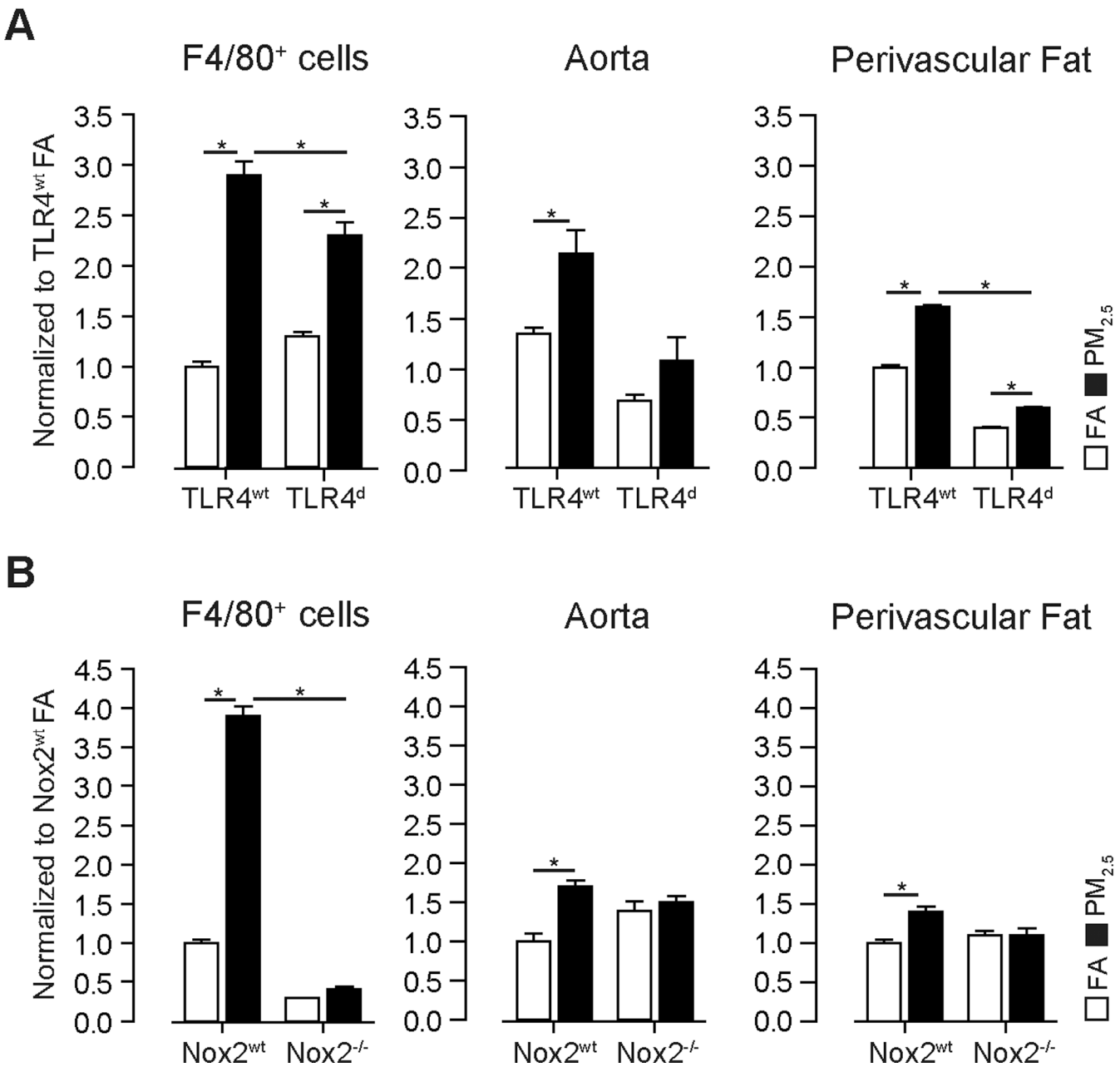

Figure 4.
Chronic $PM_{2.5}$ exposure increases Toll-like receptor dependent gene expression in perivascular tissue in $TLR4^{wt}$ mice. 8 of 82 analyzed genes found to have a significant change vs. the $TLR4^{wt}$ FA control group are depicted. (n=4/group).

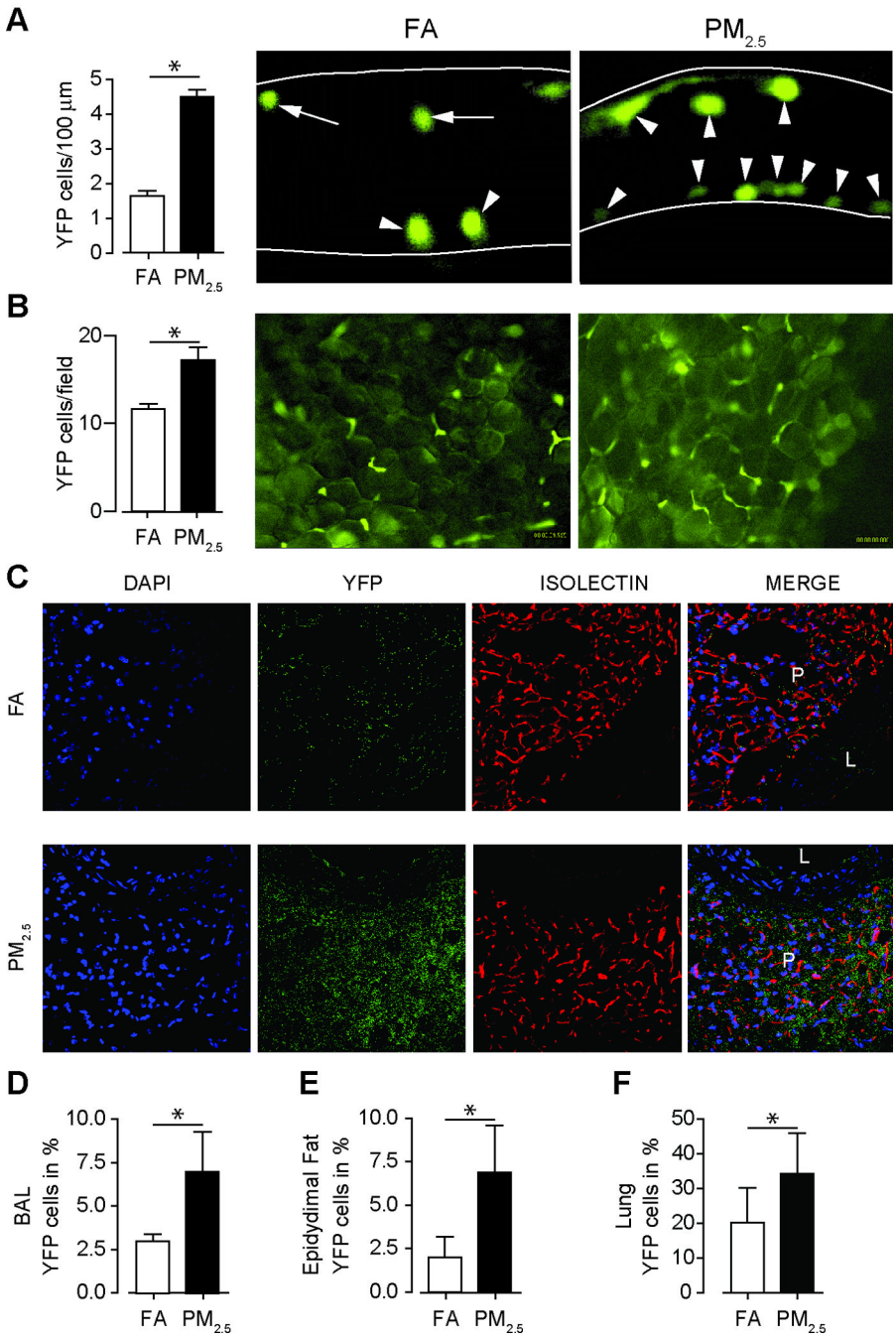

Figure 5.

Chronic $PM_{2.5}$ exposure over 20 weeks increases monocyte adherence within microvasculature and tissue niches in c-fms^{YFP} mice (FVB/N background). (**A**) Representative images and quantification of adherent YFP cells in the cremasteric venular endothelium (open arrow heads adherent monocytes; arrow heads with tail, rolling monocytes). (Original magnification 400×, n=5). **(B)** Representative images and quantification of adherent YFP cells in the mesenteric adipose tissue. (Original magnification 200×, n=5). **(C)** Immunohistochemical staining for YFP positive monocyte infiltration into perivascular fat tissue in c-fms^{YFP} mice exposed to FA or $PM_{2.5}$. Perivascular fat tissue from mice that express a yellow fluorescent protein (c-fms^{YFP},

yellow) was stained with DAPI (blue) and isolectin (red) and visualized by confocal microscopy. (L=lumen; P=perivascular fat). (Original magnification 400×, n=3). Quantification of YFP positive cells in BAL fluid (**D**), in epididymal fat **(E)** and in lung tissue **(F)** (n=4–6). Exposure time was about 20 weeks. Data are mean ± SD. (*$p<0.05$).

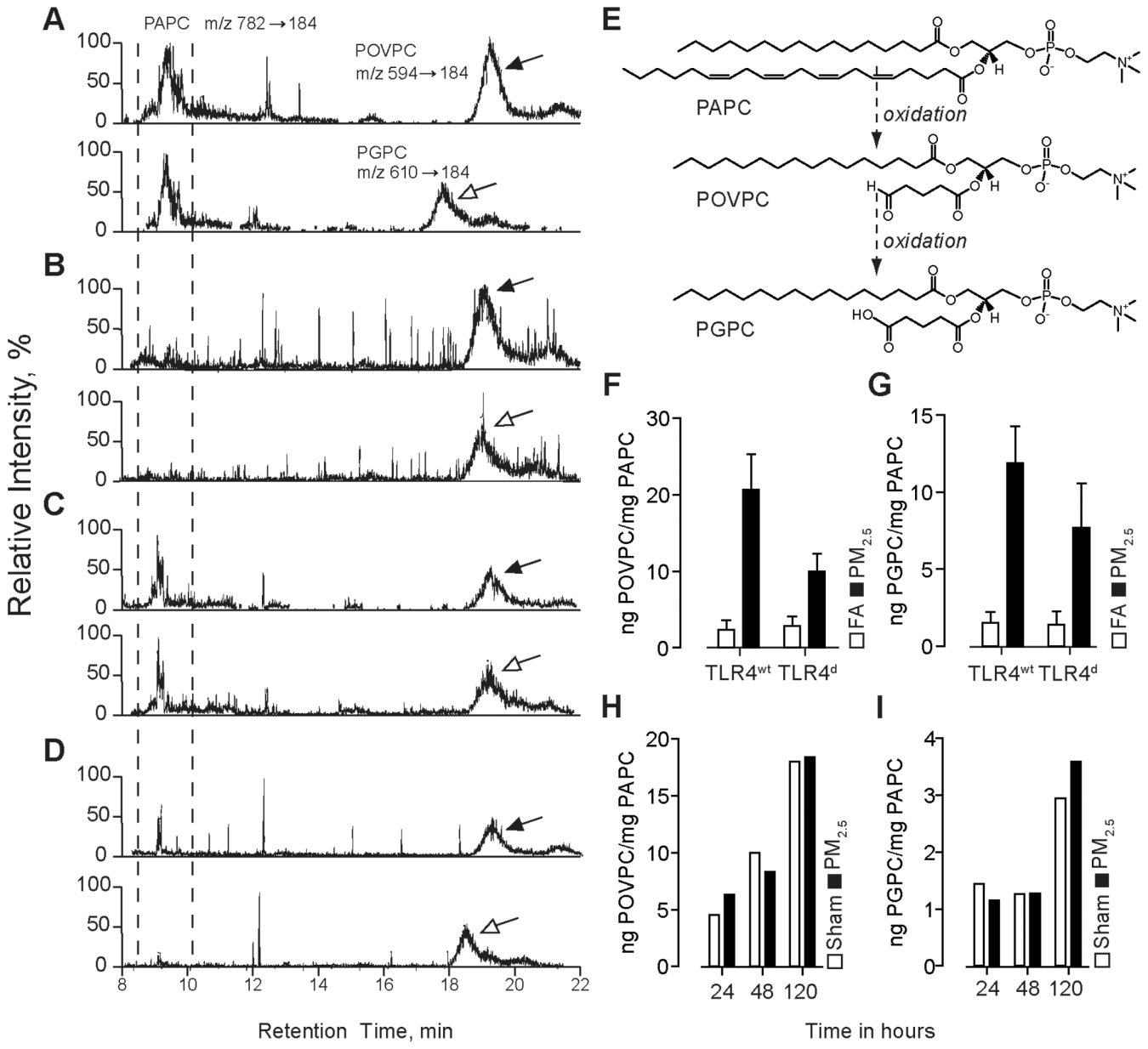

Figure 6.

TLR4 deficiency normalizes inflammatory cytokine release and prevents $p47^{phox}$ phosphorylation in response to $PM_{2.5}$ exposure over 20 weeks. **(A)** Cytokine analysis of lung homogenates (500 μg/ml protein) in $TLR4^{wt}$ and $TLR4^{d}$ mice measured by a cytokine bead array. (n=8–10/group). (**B**) Cytokine analysis of plasma samples in $TLR4^{wt}$ and $TLR4^{d}$ mice measured by a cytokine bead array. (n=8–10/group) **(C)** Immunoblots demonstrating increased $p47^{phox}$ expression in response to $PM_{2.5}$ exposure compared to FA in $TLR4^{wt}$ and normalization of $p47^{phox}$ phosphorylation in $TLR4^{d}$ mice. Lung homogenates from $TLR4^{wt}$ and $TLR4^{d}$ mice were immunoblotted for $p47^{phox}$ and phospho-$p47^{phox}$ (left). The figure on the right represents the photodensitometric quantification of the blots. (n=5/group). Data are mean ± SD. (*$p<0.05$).

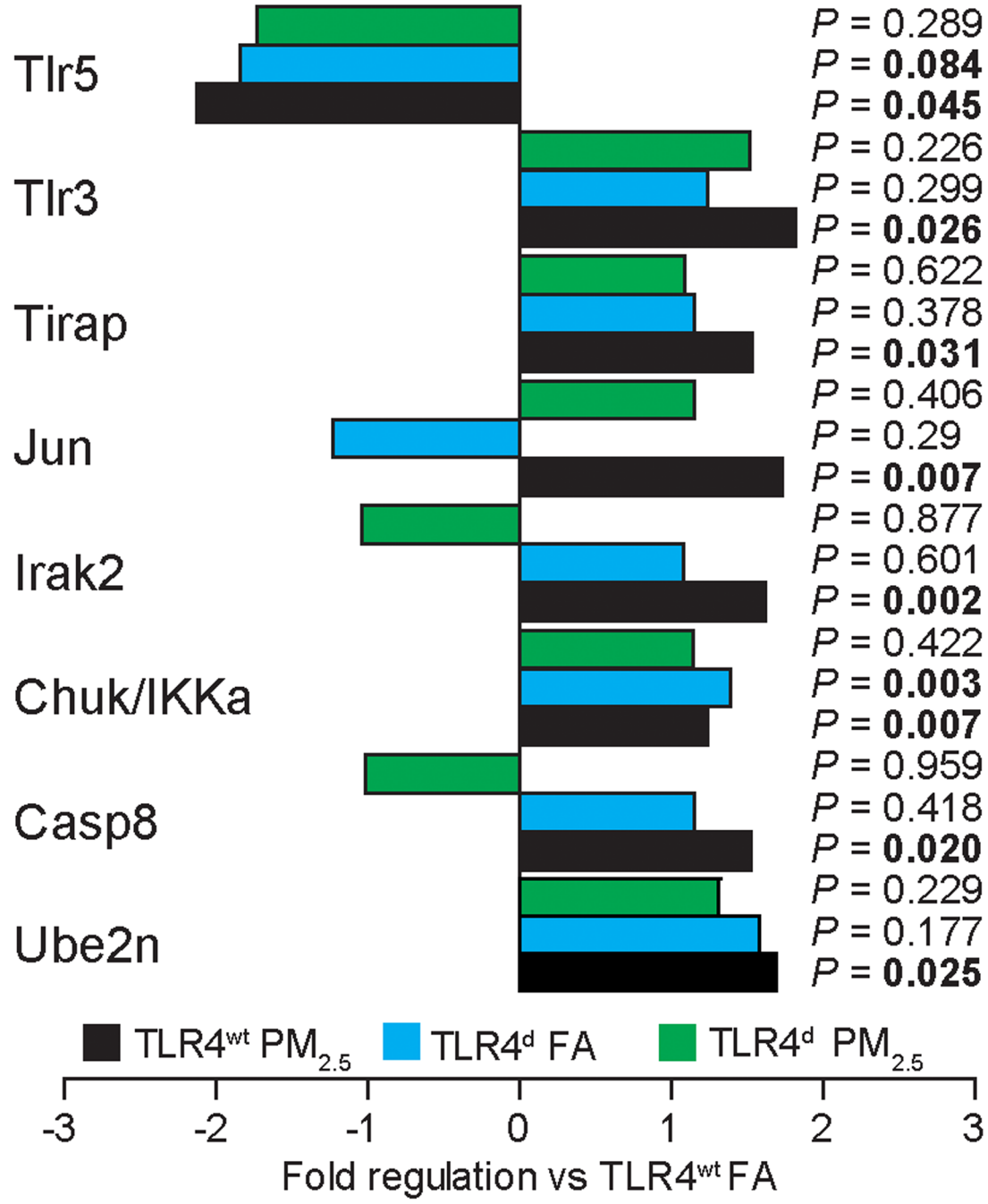

Figure 7.

Airborne particulate matter causes increased levels of two oxidized PAPC derivatives in BAL fluid of $PM_{2.5}$ exposed mice. Lipid extracts from BAL fluid of $TLR4^{wt}$ and $TLR4^{d}$ mice exposed for 20 weeks to FA or $PM_{2.5}$ were analyzed by HPLC with positive electrospray ionization mass-spectrometry operating in multiple reaction monitoring mode. Parent PAPC and oxidized derivatives (POVPC and PGPC) ion pairs were monitored by their characteristic retention time and daughter ions. Corresponding chromatograms were post-processed by extraction of POVPC and PGPC ions for quantitative analysis. Representative LC-MS chromatograms are shown for (**A**) $TLR4^{wt}$ FA, **(B)** $TLR4^{wt}$ $PM_{2.5}$, **(C)** $TLR4^{d}$ FA, **(D)** $TLR4^{d}$ $PM_{2.5}$. **(E)** Chemical structures of monitored phospholipids.

Quantitative analysis of levels of **(F)** POVPC and **(G)** PGPC against PAPC with an exaggerated level of oxidation in the $PM_{2.5}$ exposed mice over 20 weeks. *In-vitro* incubation of PAPC in the presence of $PM_{2.5}$ or with PBS was performed in time-dependent manner followed by quantification of levels of **(H)** POVPC and **(I)** PGPC by LC/MS-MS. BAL fluid of 5 mice per group were pooled for these experiments with extraction of the lipid content. The amount of oxidized phospholipid is set in ratio to non-oxidized phospholipid in order to compare the different groups.

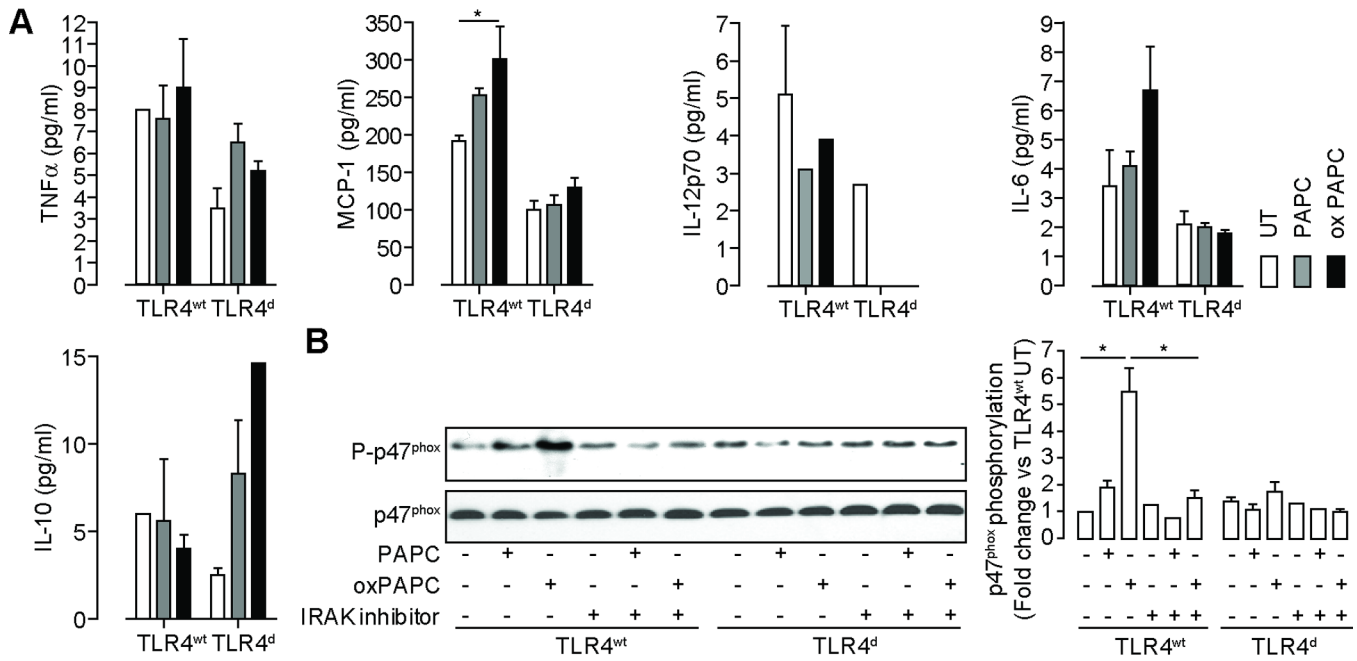

Figure 8.

TLR4 triggers inflammatory cytokine release and promotes IRAK modulated $p47^{phox}$ phosphorylation in response to oxidized phospholipid treatment in BMDM derived from $TLR4^{wt}$ and $TLR4^{d}$ mice. (**A**) BMDM were treated with PAPC and oxPAPC and inflammatory cytokine levels in the supernatant were determined. (n=3/group; *p<0.05) **(B)** These blots show $p47^{phox}$ expression and phosphorylation in response to PAPC and oxidized PAPC treatment. Lysates from bone marrow derived monocytes isolated from $TLR4^{wt}$ and $TLR4^{d}$ mice were immunoblotted for $p47^{phox}$ and phospho-$p47^{phox}$ (left). A subset of experiments was performed in presence of an IRAK inhibitor. The figure on the right represents the photodensitometric quantification of the blots. (n=3) Data are mean ± SD. (*p<0.05).