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# **Air Pollution–Mediated Susceptibility to Inflammation and Insulin Resistance: Influence of CCR2 Pathways in Mice**

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## **Abstract**

**Background:** Epidemiologic and experimental studies support an association between fine ambient particulate matter < 2.5  $\mu\text{m}$  (PM<sub>2.5</sub>) exposure and insulin resistance (IR). A role for innate immune cell activation has been suggested in the pathogenesis of these effects.

**Objectives:** To evaluate the role of CC-chemokine receptor 2 (CCR2) in PM<sub>2.5</sub>-mediated inflammation and IR.

**Methods:** Wild-type C57BL/6 and CCR2<sup>-/-</sup> male mice were fed a high-fat diet and assigned to concentrated ambient PM<sub>2.5</sub> or filtered air for 17 weeks via a whole body exposure system. Glucose tolerance and insulin sensitivity were evaluated. At euthanasia, blood, spleen and visceral adipose tissue (VAT) were collected to measure inflammatory cells using flow cytometry. Standard immunoblots, immunohistochemical methods and quantitative PCR were used to assess pathways of interest involving insulin signaling, inflammation, lipid and glucose metabolism in various organs. Vascular function was assessed with myography.

**Results:** PM<sub>2.5</sub> exposure resulted in whole body IR and increased hepatic lipid accumulation in the liver which was attenuated in CCR2<sup>-/-</sup> mice by inhibiting SREBP1c mediated transcriptional programming, decreasing fatty acid uptake and suppressing p38 MAPK activity. CCR2<sup>-/-</sup> restored abnormal phosphorylation levels of AKT, AMPK in VAT and adipose tissue macrophage content. However, the impaired whole body glucose tolerance and reduced GLUT-4 in skeletal muscle in response to PM<sub>2.5</sub> was not corrected by CCR2 deficiency.

**Conclusions:** PM<sub>2.5</sub> mediates IR by regulating VAT inflammation, hepatic lipid metabolism and glucose utilization in skeletal muscle via both CCR2-dependent and independent pathways. These findings provide new mechanistic links between air pollution and metabolic abnormalities underlying IR.

## INTRODUCTION

Recent epidemiologic studies and experimental evidence supports adverse cardiometabolic consequences of air-pollution exposure by worsening of whole body insulin sensitivity (Rajagopalan and Brook 2012). Studies from our group firstly demonstrated that PM<sub>2.5</sub> (particulate matter < 2.5 μm) exposure exaggerates insulin resistance (IR) and visceral inflammation/adiposity in mice fed on high fat diet (HFD) and normal diet (Sun et al. 2009; Xu et al. 2010). Inflammation in insulin sensitive tissues such as the visceral adipose tissue (VAT) and liver is a central abnormality in obesity/insulin resistance (IR) (Hotamisligil 2006; Ouchi et al. 2011; Shoelson et al. 2006), with recruitment of innate immune cells such as monocytes into adipose tissue and the liver driving the development of glucose and lipoprotein dysregulation (Lumeng et al. 2008; Weisberg et al. 2006; Weisberg et al. 2003; Xu et al. 2003).

CC-chemokine receptor 2 (CCR2) plays a critical role in the entry of innate immune cells into tissue through direct interaction with its ligands, CCL2 (monocyte chemoattractant protein 1, MCP-1), CCL7, CCL8 and CCL12 (Charo and Ransohoff 2006; Proudfoot 2002). Recent studies have shown that the CCR2/MCP-1 system is not only critical to VAT inflammation but in the recruitment of macrophages to the liver in response to an HFD (Oh et al. 2012). Consistent with a central role, CCR2 deficiency ameliorates obesity, VAT inflammation and systemic IR with hematopoietic CCR2 deficiency being essential for improvement (Ito et al. 2008; Weisberg et al. 2006). In light of the obligatory role of the innate immune system in PM<sub>2.5</sub> effects and these previously reported studies, we hypothesized that PM<sub>2.5</sub> exposure mediates its adverse effects in metabolic dysregulation through coordinated effects on the liver and VAT and systematically investigated this question in wild-type and CCR2<sup>-/-</sup> mice subjected to air-pollution exposure.

## **METHODS**

### **Animal Care and Use**

Male C57BL/6 (wild-type, WT) and CCR2<sup>-/-</sup> mice were purchased from Jackson Laboratories (Bar Harbor, Me). All mice at 18-week-old were housed in groups and maintained at 21°C on a 12-h light/12-h dark cycle with free access to water and fed HFD that derived 60% of calories from lipids (Harlan Teklad, Indianapolis, Indiana). The protocols and the use of animals were approved by and in accordance with the Ohio State University Animal Care and Use Committee and the animals were treated humanely and with regard for alleviation of suffering. To avoid sex-dependent differences, only male mice were studied.

### **Ambient Whole-Body Inhalational Protocol**

During HFD feeding, both C57BL/6 and CCR2<sup>-/-</sup> mice were exposed by inhalation to either filtered air (FA) or concentrated PM<sub>2.5</sub> (PM) for 6 h/d, 5 d/wk from November 28, 2011 to March 23, 2012, for a total duration of 117 days (~17 weeks), in a mobile trailer exposure system (“Ohio Air Pollution Exposure System for Interrogation of Systemic Effects,” located at The Ohio State University Animal Facility in Columbus). The animal groups are named WT-FA (*n* = 8), WT-PM (*n* = 9), CCR2-FA (*n* = 9) and CCR2-PM (*n* = 8) for ease of identification throughout the manuscript. Animal exposure and monitoring of the exposure environment and ambient aerosol were performed as previously described (Sun et al. 2009; Xu et al. 2010).

### **PM<sub>2.5</sub> Concentration and Composition 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 (Teflo, 37 mm, 2 μm pore; PALL Life Sciences, Ann Arbor, MI)

every week. Gravimetric determinations were made using a microbalance (MT-5 Mettler Toledo, Columbus OH) in a temperature/humidity-controlled Class 100 clean laboratory. PM samples collected on Teflon filters were wetted with ethanol and extracted in 10% nitric acid solution. Sample extracts were then analyzed for a suite of trace elements using inductively coupled plasma-mass spectrometry (ELEMENT2, Thermo Finnigan, San Jose, CA) as discussed in the previous studies (Morishita et al. 2011).

### **Measurements of Blood Glucose Homeostasis and Insulin Sensitivity**

Four days before exposure and subsequent to the end of exposure to FA or PM<sub>2.5</sub>, mice were fasted overnight and underwent assessment of fasting insulin/glucose levels and intraperitoneal glucose tolerance test (IPGTT). One day before exposure, after 8-week of exposure and one day subsequent to the end of exposure to FA or PM<sub>2.5</sub>, mice were fasted for 4.5 hours and underwent assessment of insulin sensitivity by the insulin tolerance test (ITT) (Hagiwara et al. 2012). The Homeostasis Model Assessment of the IR index (HOMA-IR) was calculated based on 1 mg of insulin as equivalent to 24 IU, using the formula  $HOMA\ IR = \frac{[fasting\ insulin\ concentration\ (ng/ml) \times 24 \times fasting\ glucose\ concentration\ (mg/dl)]}{405}$  (Xu et al. 2010). The Homeostasis Model Assessment of the beta cell function (HOMA-β) was calculated using the formula  $HOMA\ Beta = 360 \times \frac{[fasting\ insulin\ concentration\ (ng/ml)]}{([fasting\ glucose\ concentration\ (mg/dl)] - 63)}\%$ .

### **Measurement of Circulating Inflammatory Biomarkers and Lipid Profile**

Cytokine levels in plasma were determined using a Cytometric Bead Array (BD Biosciences, San Diego, CA). Serum was incubated with beads specific for tumor necrosis factor (TNFα), interferon γ (IFNγ), monocyte chemoattractant protein 1 (MCP-1), interleukin 6 (IL-6), IL-10,

and IL-12p70 according to the manufacturer's instructions. The total amount of cytokines was then determined using a BD LSR II instrument and analyzed by the BD CBA software (Becton Dickinson, San Jose, CA) (Xu et al. 2011). Triglyceride and cholesterol levels in liver and/or blood were measured with triglyceride reagent and cholesterol reagent prepared according to previous studies (Allain et al. 1974; McGowan et al. 1983).

### **Myograph Study**

We sacrificed mice by isoflurane inhalation after IPGTT and ITT measurement subsequent to 17-week of PM<sub>2.5</sub> exposure in conjunction with HFD feeding. Thoracic aorta with adhesive tissue was dissected out and vascular function (acetylcholine and insulin-induced vasorelaxation) was evaluated in a 5-ml chamber on a Myograph (Danish, Myo Technology A/S, Denmark) as previously described (Liu et al. 2009; Sun et al. 2009).

### **Histology and Immunohistochemistry**

Segments of liver were frozen in liquid nitrogen and embedded in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek USA Inc., Torrance, CA) for oil red-O staining. Some deparaffinized sections from liver were stained with hematoxylin eosin (H&E) to observe the tissue morphology; some others from liver and VAT were stained with immunohistochemistry to test F4/80 location (Xu et al. 2011).

### **Immunoblotting**

Adipose tissue and liver were homogenized with M-PER Mammalian protein extraction reagent (Thermo Scientific, Rockford, IL), loaded on 10% SDS-PAGE gel and transferred to immobilon-P polyvinylidene difluoride membranes and incubated with different primary antibodies. Antibodies were procured for P-AKT (phosphorylation at Ser473)/AKT, PI3K, P-AMPK

(phosphorylation at Thr172)/AMPK, P-IRS (phosphorylation at Tyr612)/IRS, P-GSK3 $\beta$  (phosphorylation at Ser9)/GSK3 $\beta$ , MAPK pathway proteins (Cell Signaling Technology, Danvers, MA) and PEPCK (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoblots were then incubated with a secondary antibody conjugated with horseradish peroxidase and visualized with enhanced chemiluminescence, and the autoradiograph was quantitated by densitometric analysis with ImageJ software.  $\beta$ -actin was used as control reference.

### **Quantitative RT-PCR**

RT-PCR was performed using RNA extracted from liver and adipose tissue of the experimental mice as described (Hagiwara et al. 2012; Xu et al. 2011). Gene expression levels were calculated using the  $\Delta$ Ct method relative to  $\beta$ -actin and expressed as relative mRNA levels compared with internal control. The sequences of all primers are available upon request.

### **Flow Cytometric Evaluation of Inflammation in Blood/Tissues**

VAT, spleen and blood from various groups (WT-FA, WT-PM, CCR2-FA and CCR2-PM) were processed as previously described (Kampfrath et al. 2011; Zhong et al. 2013). Then, blood cells and spleen cells were stained with anti-CD11b, anti-7/4 and anti-Ly6G, stromal vascular fraction of VAT was stained with anti-CD11b, anti-CD11c, CD206 and F4/80, and evaluated by flow cytometry (BD FACS LSR II<sup>TM</sup> flow cytometer, Becton Dickinson, San Jose, CA). Data was analyzed using BD FACS Diva software (Becton Dickinson, San Jose, CA). All antibodies were purchased from Biolegend, Miltenyi Biotec, or BD Bioscience.

### **Electrophoretic Mobility Shift Assay**

Nuclear proteins were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) from mouse livers and electrophoretic mobility shift assay (EMSA) were

conducted with LightShift™ kits (Pierce, Rockford, IL) according to manufacturer's instructions. Specificity of the SREBP1c probe (5'-GAT CCT GAT CAC CCC ACT GAG GAG-3') (Seo et al. 2003) was confirmed in assays in which unlabeled SREBP1c probe was added in excess as a competitor and by the supershift of SREBP1c-DNA complexes.

## **Data Analysis**

Data are means  $\pm$  standard error of the mean unless otherwise indicated. Graphpad Prism software (Version 5) was used for one-way ANOVA and Bonferroni's post-hoc test where appropriate. *T* test was only used when there was no significant difference between WT-PM with WT-FA with one-way ANOVA and the exact *P* values were measured. Value of EC<sub>50</sub> stands for the concentration needed to induce 50% of the maximal effect as determined by non-linear regression curve fitting. Concentration-relaxation curves were analyzed by two-way ANOVA followed by Bonferroni's post-tests. *P* value of < 0.05 was considered statistically significant.

## **RESULTS**

### **PM<sub>2.5</sub> Exposure Concentration and Compositional Assessment**

Ambient mean daily PM<sub>2.5</sub> concentration at the study site was 9.56  $\mu\text{g}/\text{m}^3$  (SD, 2.9  $\mu\text{g}/\text{m}^3$ ). Mean concentration of PM<sub>2.5</sub> in the filtered chamber was 2.26  $\mu\text{g}/\text{m}^3$  (SD, 1.9  $\mu\text{g}/\text{m}^3$ ). Mean concentration of PM<sub>2.5</sub> in the exposure chamber was 116.9  $\mu\text{g}/\text{m}^3$  (SD, 34.2  $\mu\text{g}/\text{m}^3$ ) which represents a 12.5-fold concentration of ambient levels (Supplemental Material, Figure S1). Elemental composition can be found in Supplemental Material, Table S1.

## **CCR2 genotype Modulates Metabolic Impairment by PM<sub>2.5</sub>**

There was no significant difference between groups at baseline in body weight, glucose and insulin sensitivity prior to consumption of diet or assignment to exposure protocols (Figure 1A, 1B, 1E, 1G). After 8 weeks of PM<sub>2.5</sub> exposure in conjunction with HFD, there was no significant difference in body weight (Figure 1A), or insulin sensitivity (Figure 1H). Nevertheless, WT-PM at 17 weeks displayed elevated fasting glucose and HOMA-IR index, decreased HOMA-beta function, abnormal glucose tolerance and attenuation of whole-body insulin sensitivity (Figure 1, B-D, F, I). PM<sub>2.5</sub>-induced blood glucose elevation was corrected in CCR2<sup>-/-</sup> mice with lower body weight (Figure 1, A-B). CCR2 deficiency did not alter the IPGTT (Figure 1F) but rectified glucose responses to insulin on ITT (Figure 1I) that was statistically significant at 17 weeks, but not at earlier time points (Figure 1H). Taken together, these results suggest that CCR2<sup>-/-</sup> mice are protected from PM<sub>2.5</sub>-induced abnormalities in whole body insulin sensitivity but CCR2 is not required for regulation of post-prandial glycemic response.

## **CCR2 Deficiency Ameliorates Inflammatory Monocytes in Blood/Spleen**

Since prior studies have demonstrated a central role of CCR2 in egress of monocytes from systemic reservoirs and in depot specific inflammation (Charo and Ransohoff 2006; Tsou et al. 2007), we investigated monocyte response to PM<sub>2.5</sub> exposure. In the present study, we defined monocytes as side scatter-high, forward scatter-low cells expressing the myeloid antigen 7/4 (high populations) and high levels of CD11b but low for the neutrophil marker Gr-1 (Ly6G), which corresponds to Ly6C<sup>hi</sup> monocytes and represents the inflammatory subtype (Combadiere et al. 2008; Henderson et al. 2003; Kampfrath et al. 2011).. We noted an increase in circulating CD11b<sup>+</sup>Gr-1<sup>low</sup>7/4<sup>hi</sup> cells, the inflammatory subtype in response to PM<sub>2.5</sub> exposure (Figure 2A). In contrast, splenic levels of CD11b<sup>+</sup>Gr-1<sup>low</sup>7/4<sup>hi</sup> cells remained unchanged (Figure 2B). The

levels of CD11b<sup>+</sup>Gr-1<sup>low</sup>7/4<sup>hi</sup> in circulation following PM<sub>2.5</sub> inhalation were significantly reduced in CCR2<sup>-/-</sup> mice (Figure 2A) with a corresponding decrease in the spleen (Figure 2B). Supplemental Material, Table S2 depicts circulating cytokines in response to PM<sub>2.5</sub> exposure. The concentration of MCP-1 was significantly higher in CCR2<sup>-/-</sup> mice than WT mice, while there were no differences in other measures.

### **CCR2 Deficiency Does Not Improve PM<sub>2.5</sub>-Impaired Endothelium Function**

PM<sub>2.5</sub>-exposed C57BL/6 mice demonstrated a decrease in relaxation both to acetylcholine and insulin (Supplemental Material, Figure S2, A-B). However, CCR2 deficiency did not correct impaired vascular function by PM<sub>2.5</sub> (Supplemental Material, Figure S2, A-B). These results suggest that abnormalities in endothelium dependent relaxation are not modulated through CCR2 dependent pathways in response to air-pollution exposure.

### **CCR2 Modulates Adipose Inflammation in Response to PM<sub>2.5</sub>**

F4/80 is a well-characterized membrane protein that is the best known marker for mature mouse macrophages. In the present study, PM<sub>2.5</sub> exposure increased F4/80<sup>+</sup> adipose tissue macrophages (ATMs) in VAT, which was normalized by CCR2 deficiency (Figure 3A). This observation was confirmed by mRNA levels of F4/80 and an alternate macrophage marker, CD68 (Figure 3B). PPAR $\gamma$ , a transcription factor required for alternate macrophage differentiation was down-regulated in the VAT of WT-PM mice, which was partially restored in CCR2<sup>-/-</sup> mice (Figure 3B). The expression of adipose-derived mediators was not altered by CCR2 deficiency or in response to PM<sub>2.5</sub> exposure (Supplemental Material, Figure S3A). F4/80<sup>+</sup>/CD11b<sup>+</sup> and F4/80<sup>+</sup>/CD11c<sup>+</sup> are widely used to label ATMs and detected with flow cytometry. Figure 3D-3E depicts an increase

in these two populations in VAT in response to PM<sub>2.5</sub> exposure, which were entirely abolished by CCR2 deficiency.

We found no difference in the VAT weight between groups (data not shown). Consistent with it, there was no change in expression of genes involved in lipolysis and mitochondrial oxidation (Supplemental Material, Figure S3B). Nuclear respiratory factor 1 (NrF1) and mitochondrial transcription factor A (mtTFA) are transcription factors involved in mitochondrial biogenesis. Although neither PM<sub>2.5</sub> exposure nor CCR2 genotype induced a change in mtTFA expression, NrF1 levels were significantly lower in the WT-PM group than that in the WT-FA group, and this was partially restored by CCR2 deficiency (Supplemental Material, Figure S3B).

### **CCR2 Modulates Hepatic Steatosis in Response to PM<sub>2.5</sub>**

CCR2<sup>-/-</sup> mice displayed improvement in lipid deposition (HE staining) and intracytoplasmic lipids (Oil Red O staining) with a trend toward correction of the higher liver weight seen with PM<sub>2.5</sub> exposure (Figure 4, A-B). Hepatic triglyceride levels were elevated and accompanied by an increase in plasma triglycerides with PM<sub>2.5</sub> exposure suggesting increased production of triglyceride containing lipoproteins in the liver (Figure 4B). We next examined genes involved in lipid metabolism in the liver. Expression of key lipid synthesis enzymes, acetyl-CoA carboxylase 2 (ACC2), fatty acid synthase (FAS), diacylglycerol acyl transferase (DGAT2) were all significantly increased in the liver of WT-PM<sub>2.5</sub> mice compared to WT-FA (Figure 4C), while there was no difference in expression of other genes. mRNA level of SREBP1, a key transcription factor involved in activation of lipogenic genes but not SREBP2, was significantly increased in liver of mice from WT-PM group (Figure 4D). EMSA demonstrated a trend towards increase in SREBP1c binding activity in nuclear extracts from the liver in response to PM<sub>2.5</sub>

exposure with restoration in CCR2<sup>-/-</sup> mice (Figure 4E). CCR2 deficiency nearly normalized the increased lipogenic gene expression with PM<sub>2.5</sub> exposure except for DGAT2 (Figure 4C). Next, we examined genes related to fatty acid oxidation and no significant difference was observed (Supplemental Material, Figure S3C). Fatty acid-binding protein 1 (FABP1) but not FABP2, FABP5 or CD36, was significantly decreased in the liver of WT-PM group mice, which was reversed in CCR2<sup>-/-</sup> mice (Supplemental Material, Figure S3C). Expression of genes encoding fatty acid export, including Apob and Microsomal triglyceride transport protein (MTP) were unaffected by exposure to PM<sub>2.5</sub> (Supplemental Material, Figure S3C).

### **Role of CCR2 in PM<sub>2.5</sub>-impaired Hepatic Glucose Metabolism**

To investigate mechanisms of hyperglycemia in response to PM<sub>2.5</sub>, we examined pathways involved in gluconeogenesis and glycolysis. Supplemental Material, Figure S4 (A-B) shows no alteration of a rate limiting enzyme involved in gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK), at both mRNA and protein levels. However, inhibition in expression of G6pase, FBpase and pyruvate carboxylase (PC) in the liver of WT-PM compared to that from WT-FA group was noted (Supplemental Material, Figure S4A). We found no difference in expression of the transcription factor (C/EBP- $\alpha$ ), coactivator (PGC1 $\alpha$ ) or glycogen synthase kinase 3 beta (GSK3 $\beta$ , regulating glycogen synthase) in liver of WT-PM animals (Supplemental Material, Figure S4, A and D). These results suggest that enhanced gluconeogenesis or glycogen synthesis is unlikely to contribute to hyperglycemia in response to PM<sub>2.5</sub> exposure.

There were no differences in glucokinase (GK), a key glycolytic enzyme, in response to PM<sub>2.5</sub>. However GK expression was increased in the liver of CCR2<sup>-/-</sup> mice (both FA and PM group) compared to WT mice (Supplemental Material, Figure S4C). This may partially explain the

reduced glucose levels in  $CCR2^{-/-}$  mice. We found a trend in decrease in expression of another enzyme of glucose metabolism, L-type pyruvate kinase (L-PK). GLUT-2 expression was significantly decreased in the liver of WT-PM mice, which was restored in  $CCR2^{-/-}$  mice. In addition,  $PM_{2.5}$  exposure markedly decreased the transcription factor, carbohydrate response element binding protein (ChREBP), indicative of reduced glucose utilization in the liver. ChREBP level was not modulated by  $CCR2^{-/-}$  (Supplemental Material, Figure S4C). As shown in Supplemental Material, Figure S4E, the GLUT-4 expression in skeletal muscle was decreased by  $PM_{2.5}$  exposure, with no improvement in  $CCR2^{-/-}$  mice exposed to  $PM_{2.5}$ .

### **CCR2 Modulates Hepatic p38 Activation in Response to $PM_{2.5}$**

To further explore mechanisms by which  $PM_{2.5}$  impairs glucose homeostasis and hyperlipidemia, we assessed inflammatory signals implicated in hepatic IR. No differences in F4/80 content in the liver tissue of mice (Figure 5A) in response to  $PM_{2.5}$ , in excess of that induced by HFD was noted, which was confirmed by quantitative real-time PCR analysis (Figure 5B). The alternative (M2) macrophage activation marker galactose-N-acetylgalactosamine-specific lectin (MgI1) was however down-regulated in response to  $PM_{2.5}$  exposure, which was partially alleviated in  $CCR2^{-/-}$  mice (Figure 5B). Immunoblotting analysis demonstrated that activated p38, but not ERK or c-Jun N-terminal kinase (JNK), was increased in the liver of the C57BL/6 mice exposed to  $PM_{2.5}$ , compared to that in the mice exposed to FA (Figure 5D). Levels of phosphorylated p38 appeared to be lower in the liver of  $CCR2^{-/-}$  mice.

### **Defective Insulin Signaling in Liver and VAT**

At 17 weeks, phosphorylated AKT (Ser473) was reduced in VAT with  $PM_{2.5}$  vs. FA exposure, an effect reversed in  $CCR2^{-/-}$  mice (Figure 3C). Phosphorylated AMPK (Thr172) in response to

PM<sub>2.5</sub> exposure was inhibited with PM<sub>2.5</sub> exposure (Figure 3C), which was not significantly altered by CCR2 deficiency. Although no significant difference was shown in the liver, there was a clear trend towards decrease in the phosphorylation levels of AKT at Ser473 site and phosphorylation level of insulin receptor substrate-1 (IRS1) at Tyr612 site in C57BL/6 mice with PM<sub>2.5</sub> exposure, which were reversed in CCR2-PM group (Figure 5C).

## **DISCUSSION**

In the present study, we delineate the effects of PM<sub>2.5</sub> inhalation on multiple aspects of glucose/lipid metabolism and support a contributing role of CCR2 in PM<sub>2.5</sub> mediated effects in conjunction with HFD. Our findings include: (1) Impairment of systemic insulin sensitivity by PM<sub>2.5</sub> and amelioration by CCR2 deficiency; (2) CCR2-dependent potentiation of VAT inflammation and impairment of AMPK, AKT signaling by PM<sub>2.5</sub>; (3) CCR2-dependent enhancement of hepatic lipogenesis/steatosis, activation of p38 MAPK and reduction of insulin signaling by PM<sub>2.5</sub>; (4) Worsening of fasting hyperglycemia via CCR2-independent non-gluconeogenic mechanisms.

We have previously reported an important association between PM<sub>2.5</sub> inhalation with HFD feeding providing evidence for an important interaction between environmental and dietary signals (Sun et al. 2009). An important component of this effect is recruitment and activation of myeloid-cells in tissues such as VAT and liver, where they contribute to adverse metabolic consequences (Oh et al. 2012; Weisberg et al. 2006; Xu et al. 2010; Zheng et al. 2012). Given the importance of the CCR2/MCP-1 system in regulating monocyte/macrophage chemotaxis and inflammation in response to high-fat diet signals, we hypothesized that ablation of CCR2 would mitigate adverse consequences of air-pollution exposure in conjunction with high-fat diet. We

did not study normal diet-conditions in this investigation given that the MCP-1/CCR2 system does not substantially alter inflammation or metabolism in the normal-diet context (Lumeng et al. 2007a; Obstfeld et al. 2010; Weisberg et al. 2006).

PM<sub>2.5</sub> exposure attenuated whole-body insulin sensitivity and glucose homeostasis after a substantial latency period (> 8 weeks). In keeping with our original hypothesis, we noted increased immune cell numbers in the peripheral circulation and VAT in response to PM<sub>2.5</sub> exposure which was ablated by CCR2 deficiency, suggesting a dependence of PM<sub>2.5</sub> on CCR2 in recruitment of innate immune cells (Ito et al. 2008; Tsou et al. 2007; Weisberg et al. 2006). Infiltration of monocytes is enhanced in obesity via local tissue cues with a progressive transformation of these cells to a CD11c<sup>+</sup> status, resulting in a polarization of the local adipose milieu to an M1 state from a predominantly M2 state under conditions of normal diet (Lumeng et al. 2007b; Oh et al. 2012). Given the significantly higher CD11c<sup>+</sup> cells (absolute numbers) with PM<sub>2.5</sub> exposure, our results suggest that these cells in VAT may be a consequence of recruitment rather than polarization of existing cell populations.

A key defect in IR is abnormal insulin signaling through alterations in the IRS1-PI3K-AKT pathway. The reduced phosphorylation of the downstream signaling mediator AKT is well implicated as a key marker of IR and has been strongly linked to inflammatory triggers in the VAT (Lumeng et al. 2007a; Lumeng et al. 2007b; McGillicuddy et al. 2009; Osborn and Olefsky 2012; Sun et al. 2009). Similarly abnormalities in AMP-kinase signaling have been noted as a potential target of inflammation in metabolic diseases (Canto et al. 2009; Salminen et al. 2011; Yu et al. 2010). Reduction in phosphorylated AKT and AMPK in VAT in response to PM<sub>2.5</sub> exposure with restoration in CCR2<sup>-/-</sup> mice suggests a dependence of abnormal signaling in these pathways on inflammation. Similarly, a clear trend towards decrease in levels of liver

phosphorylated AKT and phosphorylated IRS1 at Tyr 612 with PM<sub>2.5</sub> exposure was noted findings which were reversed in CCR2-PM group. These results complement our prior work which clearly demonstrated increased Ser 636 and Ser 1101 phosphorylation in the liver of mice exposed to PM<sub>2.5</sub>, collectively suggesting a PM<sub>2.5</sub>-triggered inhibition of the IRS1 signaling (Zheng et al. 2012).

Obesity is well known to induce hepatic triglyceride accumulation and fatty liver, a process coordinated by broad transcriptional programs governing carbohydrate and lipid metabolism. CCR2/CCL2 has previously been shown to regulate triglyceride accumulation by multiple groups (Baeck et al. 2012; Mandrekar et al. 2011). Triglyceride levels and neutral fat deposition were markedly higher with PM<sub>2.5</sub> compared with that of FA group, which may partly explain the increased liver mass. SREBP1c activation shown in response to PM<sub>2.5</sub> exposure is likely critical to the up-regulation of multiple enzymes involved in triglyceride synthesis. We noticed FABP1, a protein highly expressed in tissues (i.e. liver) active in long-chain fatty acid uptake and metabolism was down-regulated in response to PM<sub>2.5</sub> exposure and was restored by CCR2 deficiency. In line with our study, FABP1 ablated mice were shown to exhibit increased age-dependent obesity (Martin et al. 2008). Taken together, increased lipogenesis and decreased fatty acid uptake, but not fatty acid oxidation or lipid export pathways, account for excess triglyceride accumulation in the liver in response to PM<sub>2.5</sub> exposure.

p38 MAPK belongs to a family of evolutionarily conserved serine-threonine MAPKs that link extracellular signals to intracellular machinery regulating a plethora of cellular processes. Together with JNK, they are activated by environmental or genotoxic stress and described as stress-activated protein kinases (Chang and Karin 2001; Coulthard et al. 2009; Morrison and Davis 2003) Consistent with studies that demonstrated the role of p38 in mediating adverse

consequences (Liu and Cao 2009), p38 was selectively up-regulated in response to PM<sub>2.5</sub>, an effect partially restored by CCR2 deficiency in the present study. However, others have suggested a protective effect where increases in p38 activity may regulate Xbp1 nuclear translocation and activity and thus may represent a compensatory mechanism to maintain homeostatic response (Lee et al. 2011). Therefore, the significance of this finding may need further studies.

Circulating glucose levels reflect a balance between glucose production and utilization. The skeletal muscle accounts for ~80% of insulin-stimulated whole-body glucose disposal, and is by far the most affected organ with respect to impaired insulin-stimulated glucose disposal in states of IR. GLUT-4 expression in skeletal muscle was decreased in response to PM<sub>2.5</sub> exposure in C57BL/6 mice, indicating obstruction in glucose utilization. Interestingly, CCR2<sup>-/-</sup> mice displayed no reverse of the decreased GLUT-4 levels and may represent a potential explanation for lack of improvement in glucose-tolerance on IPGTT. Gluconeogenesis is tightly regulated by insulin signaling (suppressed) with mitigation of this suppression with IR (in the face of continued insulin-mediated lipogenesis). This process requires coordinated activity of four enzymes (PEPCK, G6pase, FBPase and PC) (Jitrapakdee 2012). Surprisingly, we found reduced expression of G6pase, FBPase and PC mRNA levels with no alteration of PEPCK levels in response to PM<sub>2.5</sub> exposure, suggesting an adaptive negative feedback regulation of gluconeogenesis. We found no difference in expression of transcription factors responsible for regulating gluconeogenesis/glycogen synthesis in liver of WT-PM animals suggesting that enhanced gluconeogenesis or glycogen synthesis is unlikely to contribute to hyperglycemia in response to PM<sub>2.5</sub> exposure. Using the DNA motif of the L-PK gene as an affinity tag, the Uyeda group purified a transcription factor from nuclear extracts from liver tissue, which was named as

ChREBP (Uyeda and Repa 2006). Decreased ChREBP in response to PM<sub>2.5</sub> exposure may provide an explanation for a trend of glycolysis inhibition. In contrast, Glut-2, a transporter in liver cells that functions to mediate glucose uptake in the liver for glycolysis, was reduced by PM<sub>2.5</sub> exposure. This may contribute to the attenuated glucose uptake in the liver and PM<sub>2.5</sub>-mediated hyperglycemia in the present study. Although CCR2 deficiency showed no improvement in ChREBP or L-PK, the normalized GLUT-2 expression and GK overexpression in the CCR2 deficiency phenotype may be expected to alleviate glucose dysregulation induced by PM<sub>2.5</sub> exposure. Additional experimentation will be required to clarify the mechanism.

In summary, the present study demonstrates complex effects of PM<sub>2.5</sub> in exaggerating effects of high-fat diet. CCR2 plays important roles in adverse effects of PM<sub>2.5</sub> by modulating VAT inflammation and hepatic steatosis but not glucose utilization in skeletal muscle. These findings provide new mechanistic links between air pollution and metabolic abnormalities.

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## FIGURE LEGENDS

**Figure 1. Effect of PM<sub>2.5</sub> exposure on glucose homeostasis in WT and CCR2<sup>-/-</sup> mice fed an HFD.** **A**, Body weight of mice at baseline, 8-wk, 17-wk exposure to PM<sub>2.5</sub> or FA in conjunction with HFD. **B**, Fasting blood glucose at baseline and at the end of 17-wk PM<sub>2.5</sub> exposure in conjunction with HFD. **C-D**, HOMA-IR (C) and HOMA-β (D) at the end of 17-wk PM<sub>2.5</sub> exposure in conjunction with HFD. **E-F**, GTT in overnight fasted mice before (E) and after (F) 17-wk PM<sub>2.5</sub> exposure in conjunction with HFD. **G-I**, ITT in 4.5 hr fasted mice before (G), after 8-wk (H) and after 17-wk (I) PM<sub>2.5</sub> exposure in conjunction with HFD. GTT and ITT were also analyzed with area under curves. \**p* < 0.05, \*\**p* < 0.01 when WT-PM compared to WT-FA group, #*p* < 0.05, ###*p* < 0.01 when CCR2-PM group compared to WT-PM group. *n* = 7-9 per group.

**Figure 2. Effects of PM<sub>2.5</sub> exposure on inflammatory monocytes in blood and spleen from WT and CCR2<sup>-/-</sup> mice fed an HFD.** **A-B**, Representative flow cytometric dot plots and analysis showing CD11b<sup>+</sup>Gr-1<sup>low</sup>/4<sup>hi</sup> cells from mice blood (A) and mice spleen (B). Data were analyzed by relative percentage or absolute cell counts. \**p* < 0.05 when WT-PM compared to WT-FA group, #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 when CCR2-PM group compared to WT-PM group. *n* = 7-9 per group.

**Figure 3. Effect of PM<sub>2.5</sub> exposure on inflammation and insulin signaling in VAT in WT and CCR2<sup>-/-</sup> mice fed an HFD.** **A**, Representative image and analysis of F4/80<sup>+</sup> staining. The scale bar in the bottom right of the image represents 100 μm. **B**, mRNA levels of genes involved in inflammation including F4/80, CD68, TNFα and PPARγ. **C**, Western blotting for phosphorylated AKT (P-AKT)/total AKT, phosphorylated AMPK (P-AMPK)/total AMPK. **D-E**,

Representative flow-cytometric dot plots showing F4/80<sup>+</sup>/CD11b<sup>+</sup> (D) and F4/80<sup>+</sup>/CD11c<sup>+</sup> (E) with respective analysis by relative percentage or absolute cell counts/g tissue in VAT. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 when WT-PM compared to WT-FA group, #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 compared to WT-PM group. *n* = 7-9 per group.

**Figure 4. Effect of PM<sub>2.5</sub> exposure on lipid homeostasis in WT and CCR2<sup>-/-</sup> mice fed an HFD.** **A**, Representative images of H&E staining of liver sections from mice and the liver weight. The scale bar in the bottom right of the image represents 100 μm. **B**, Representative images of oil red O staining of liver sections from mice and TG levels in liver and plasma. The scale bar in the bottom right of the image represents 25 μm. **C**, mRNA levels of genes involved in de novo lipid synthesis in the liver. **D**, mRNA levels of transcription factors including SREBP1 and SREBP2. **E**, The DNA binding activity of SREBP1c showing SREBP1c activity in the liver. \**p* < 0.05 when WT-PM compared to WT-FA group, #*p* < 0.05 when CCR2-PM compared to WT-PM group. *n* = 7-9 per group.

**Figure 5. Effects of PM<sub>2.5</sub> exposure on inflammation, insulin and MAPK signaling pathways in the liver from WT and CCR2<sup>-/-</sup> mice fed an HFD.** **A**, Representative image and analysis of F4/80<sup>+</sup> staining. The scale bar in the bottom right of the image represents 100 μm. *n* = 7-9 per group. **B**, mRNA levels of genes involved in inflammation including F4/80, TNFα, and MgI1. *n* = 7-9 per group. **C**, Western blotting for phosphorylated AKT (P-AKT)/total AKT, phosphorylated IRS1 (P-IRS1)/total IRS1. *n* = 3-7 per group. **D**, Western blotting of signaling molecules involved in MAPK pathway. *n* = 3-5 per group. \**p* < 0.05 when WT-PM compared to WT-FA group, #*p* < 0.05, ##*p* < 0.01 when CCR2-PM compared to WT-PM group.

□ WT-FA    ■ CCR2-FA  
 ■ WT-PM    ■ CCR2-PM









