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CD36 Mediates Endothelial Dysfunction Downstream of Circulating Factors Induced by O_3 Exposure

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Inhaled pollutants induce the release of vasoactive factors into the systemic circulation, but little information is available regarding the nature of these factors or their receptors. The pattern recognition receptor CD36 interacts with many damage-related circulating molecules, leading to activation of endothelial cells and promoting vascular inflammation; therefore, we hypothesized that CD36 plays a pivotal role in mediating cross talk between inhaled ozone (O_3)-induced circulating factors and systemic vascular dysfunction. O_3 exposure (1 ppm × 4 h) induced lung inflammation in wild-type (WT) mice, which was absent in the CD36 deficient ($CD36^{-/-}$) mice. Acetylcholine (ACh)-evoked vasorelaxation was impaired in isolated aortas from O_3-exposed WT mice but not in vessels from $CD36^{-/-}$ mice. To delineate whether vascular impairments were caused by lung inflammation or CD36-mediated generation of circulating factors, naïve aortas were treated with diluted serum from control or O_3-exposed WT mice, which recapitulated the impairments of vasorelaxation observed after inhalation exposures. Aortas from $CD36^{-/-}$ mice were insensitive to the effects of O_3-induced circulating factors, with robust vasorelaxation responses in the presence of serum from O_3-exposed WT mice. Lung inflammation was not a requirement for production of circulating vasoactive factors, as serum from O_3-exposed $CD36^{-/-}$ mice could inhibit vasorelaxation in naïve WT aortas. These results suggest that O_3 inhalation induces the release of circulating bioactive factors capable of impairing vasorelaxation to ACh via a CD36-dependent signaling mechanism. Although lung inflammatory and systemic vascular effects were both dependent on CD36, the presence of circulating factors appears to be independent of CD36 and inflammatory responses.

Key Words: **ozone; CD36; vascular; inflammation; vasorelaxation; air pollution; endothelial; pattern recognition receptor; scavenger receptor.**

Air pollution remains a serious problem in urban regions throughout the world. Globally, an estimated 800,000 people every year die prematurely due to air pollution (Brook, 2008), mostly from cardiovascular disease (CVD). Ground-level ozone (O_3), considered one of the most important air pollutants, may cause the premature deaths of over 3000 people each year in the United States, alone (WHO, 2002) and costs the economy billions of dollars in medical care and productivity losses (Hubbell *et al.*, 2005). Ground-level O_3 is formed when pollutants emitted by cars, and other sources undergo chemical and photochemical reactions in the atmosphere (Cole and Freeman, 2009). As a result, mortality and morbidity rates associated with O_3 exposure are expected to increase as temperatures continue to rise with global warming, adding urgency to the need for action to limit the damaging effects of air pollution. Uncertainty remains concerning the mechanism(s) underlying systemic responses to inhaled pollutants, negatively impacting our ability to manage vulnerable populations and establish precise, effective regulatory policies.

There is growing evidence suggesting that inhaled pollutants evoke a systemic inflammatory response that ultimately results in endothelial injury and dysfunction—two key features of CVD. Using a variety of air pollutants, including O_3, research in our laboratory and others have documented adverse effects on systemic vascular reactivity both in animals and, to a more limited extent, in humans (Brook *et al.*, 2002; Channell *et al.*, 2012; Cherng *et al.*, 2011; Chuang *et al.*, 2009; Lund *et al.*, 2011). Recent controlled human exposure studies with diesel emissions and nitrogen dioxide (NO_2) note definitive transference of toxicity to the circulation, with the evident induction of as yet unknown factors that have the potential to induce adhesion molecules and cytokine release from primary human coronary artery endothelial cells (Channell *et al.*, 2012).

Given its reactivity, O_3 cannot be directly transferred into the circulation but instead reacts with the surfactant to generate a mix of secondary and tertiary reactants (Frampton *et al.*, 1999; Kafoury *et al.*, 1999; Postlethwait *et al.*, 1998; Pryor *et al.*, 1995). These reactants have the potential to modify lipids or produce

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protein adducts, capable of binding as epitopes to pattern recognition receptors (PRRs), such as lectin-like oxidized low-density lipoprotein receptor-1, Toll-like receptor 4 (TLR4), and cluster of differentiation receptor 36 (CD36) (Garantziotis *et al.*, 2010; Kampfrath *et al.*, 2011; Kumano-Kuramochi *et al.*, 2012; Li *et al.*, 2011). Although lipid peroxidation products present in the lung lining fluid following exposure to particulate matter (PM) have been reported to mediate systemic cellular inflammatory responses through TLR4 (Kampfrath *et al.*, 2011), CD36 has not been well studied with respect to air pollution. CD36, a class B scavenger receptor, recognizes many ligands and is widely expressed on the surface of multiple cell types, including macrophages and endothelial cells (Febbraio *et al.*, 2001; Sawada *et al.*, 2012). CD36 has been strongly implicated in pathological conditions associated with air pollution, including atherosclerosis and inflammation (Febbraio *et al.*, 2001; Kuda *et al.*, 2011; Silverstein and Febbraio, 2009). Increased expression of CD36 in the vasculature was correlated positively with increased entry of monocytes into atherosclerotic plaques following diesel exhaust (DE) exposure (Bai *et al.*, 2011). Currently, this is the only reported role of CD36 in relation to air pollution exposure, and no studies to date have investigated potential links between CD36, vascular dysfunction, and O_3 exposure.

We hypothesized that the CD36 PRR contributes to systemic vascular impairment downstream of O_3 inhalation. To test this hypothesis, we developed an innovative methodology for assessing potential cumulative effects of circulating mediators on vascular function, in both CD36 knockout ($CD36^{-/-}$) and wild-type (WT) mice. In this study, we demonstrate that O_3 inhalation induces the generation of circulating bioactive factors, leading to impaired vascular responses to ACh via a CD36-dependent signaling mechanism.

MATERIALS AND METHODS

Animals. Female C57BL/6 WT mice (Harlan Laboratories) and $CD36^{-/-}$ mice aged 8–10 weeks were used in the study. $CD36^{-/-}$ mice, generated on C57BL/6 background, as previously described (Febbraio *et al.*, 1997), were kindly provided by Dr Maria Febbraio (University of Alberta, Edmonton, Canada). Upon arrival, mice were housed four per cage under controlled environmental conditions (21°C ± 2°C; 12-h light/dark cycle) with access to tap water and standard chow *ad libitum* (Harlan). The mice were allowed to acclimate for at least 1 week prior to the start of experimentation. All procedures were approved by the Institutional Animal Care and Use Committee at the University of New Mexico. Mice were euthanized with an overdose of anesthesia (isoflurane; concentration 5%) or by exsanguination via cardiac puncture while under anesthesia (isoflurane; concentration 1.5–2%).

O_3 exposures. O_3 was generated using an OREC silent arc discharge O_3 generator (Osmonics, Phoenix, AZ). The O_3 concentration was continuously monitored using a photometric O_3 analyzer (TG-501; GrayWolf, Shelton, CT), and temperature was maintained at 21°C ± 2°C. Concentrations of carbon monoxide and nitrogen oxides (NOx) were unaltered. The mice were randomly assigned a group and were exposed to either filtered air (FA) or 1 ppm O_3 for 4 h. During exposures, the mice were singly housed within a sealed chamber (Biospherics) without bedding. Food, but not water, was withheld during the 4-h exposure period to preclude ingestion of ozonation products. Mice were euthanized for tissue collections 24 h after exposure.

Collection and analysis of bronchoalveolar lavage fluid. Bronchoalveolar lavage (BAL) fluid was collected for assessment of lung inflammatory responses of mice following inhalation. Following euthanasia, the lungs were lavaged three times with 0.8 ml of sterile saline. BAL fluid was centrifuged at 1800 × g for 5 min. The supernatant from the first lavage was stored at −80°C until required for biochemical determination. Total protein content was assessed with a bicinchoninic acid assay kit (Pierce, Rockford, IL). The cell pellets from all lavages were resuspended in 0.8 ml of physiological saline and combined. Total cell numbers were determined and 10,000 cells were centrifuged onto cytospin slides for differential staining as described (Robertson *et al.*, 2012).

Ex vivo *vascular function using myography.* Rings from the thoracic and abdominal aorta were isolated from mice 24 h after exposure, and cleaned of connective tissue. Segments of aorta (2–3 mm length) were mounted in a 4-chamber myograph system (610 M; Danish Myo Technology A/S, Aarhus, Denmark). Vessels were submerged in physiological salt solution (composition in millimolar: 119.0 NaCl, 25.0 $NaHCO_3$, 5.5 glucose, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 0.027 EDTA, 2.5 $CaCl_2$) bubbled at 37°C with 21%O_2-5%CO_2-balance N_2 and left to equilibrate at 2 mN of tension for ~30 min. Tension was gradually applied over 10 min to an optimal passive tension of 10 mN. Preliminary experiments showed that this tension produced optimal contraction and relaxation responses. Data from force transducers were processed by a MacLab/4e A-Dl converter displayed through LabChart software (AD Instruments).

Vessel viability was confirmed by a contractile response on addition of high potassium physiological salt solution (KPSS in millimolar: 64.9 NaCl, 25.0 $NaHCO_3$, 5.5 glucose, 58.9 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 0.027 EDTA, 2.5 $CaCl_2$), repeated twice. Cumulative concentration-response curves were constructed for the Thromboxane A2 analogue, U-46619 (Sigma-Aldrich). Following preconstriction with the negative logarithm concentration of U-46619 eliciting 50% of the maximum response to KPSS (58.9nM KCl; EC_{50}), cumulative concentration-response curves were generated with the endothelium-dependent vasorelaxant acetylcholine (ACh; 10^{-9}–10^{-5}M; Sigma-Aldrich). In some experiments, the vessel lumen was rubbed with a strand of moose main to disrupt the endothelium. Successful removal of vascular endothelium was confirmed by the failure of ACh to relax preconstricted aortic rings, before a cumulative concentration-response curve to the NO donor spermine NONOate was generated (10^{-9}–10^{-5}M; Cayman Chemicals).

Effect of serum on the responses of the aorta from naïve WT and $CD36^{-/-}$ mice to ACh. Aortic rings isolated from naïve WT and $CD36^{-/-}$ mice were mounted in a myograph and challenged twice with KPSS as described above. After a 30-min equilibration period, vessels were incubated with 2.5% serum from WT or $CD36^{-/-}$ mice following exposure to FA or O_3. Because addition of serum induced contraction of aortic rings, cumulative concentration-response curves to ACh (10^{-9}–10^{-5}M) were performed after responses to serum had stabilized.

Statistical analysis. Data are expressed as the mean ± SEM. Contractile responses are expressed as a percentage of the maximal contraction induced by KPSS (58.9nM KCl). U-46619 EC_{50} values were calculated with a nonlinear regression analysis. All vasorelaxation responses are expressed as percentage of the precontraction to EC_{50} U-46619 or expressed as a percentage of the response to 2.5% serum, with 100% representing basal tension. Statistical comparisons were performed by two-way ANOVA using the Bonferroni's *post hoc* test, unless otherwise stated. Statistical analyses were performed using GraphPad Prism software (V5.0; GraphPad Software Inc.). $p < 0.05$ was considered to be statistically significant.

RESULTS

Loss of CD36 Blunts Lung Inflammatory Cell Influx Following O_3 Exposure

Consistent with previous findings (Fakhrzadeh *et al.*, 2004; Johnston *et al.*, 2005; Savov *et al.*, 2004), O_3 inhalation

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increased total BAL fluid cell count in WT mice compared with WT FA-exposed mice ($p < 0.05$ compared with WT FA-exposed mice; Fig. 1A). The increase in cell count was mostly macrophages ($39.8 \pm 10.1 \times 10^5$ cells/ml vs. $1.5 \pm 0.3 \times 10^5$ cells/ml in WT FA-exposed mice; Fig. 1B). In contrast, the CD36$^{-/-}$ mice did not display this O_3-induced cell influx in the lungs ($p < 0.05$ compared with O_3-exposed WT mice; Fig. 1A). No significant differences in total and differential cell counts were observed in the CD36$^{-/-}$ mice exposed to FA or O_3 (Figs. 1A and B). As well as cell influx, the total protein concentration in the BAL fluid was measured. The protein concentration of BAL fluid increased significantly in WT mice 24 h after O_3 exposure ($p < 0.05$ compared with WT FA-exposed mice; Fig. 1C). BAL protein levels in the CD36$^{-/-}$ mice did not change significantly between those exposed to FA or O_3 (Fig. 1C); however, baseline protein levels were approximately threefold higher in FA-exposed CD36$^{-/-}$ mice compared with WT (Fig. 1C).

Circulating total white blood cell (WBC) counts and differentials were compared among groups. Total WBC cell counts and the relative proportion of different types of WBCs did not change in WT mice exposed to FA compared with those exposed to O_3 (Supplementary figs. Ia and b). CD36$^{-/-}$ mice had significantly lower levels of total WBCs than WT mice, but there was no change in the proportion of individual types of WBCs (Supplementary fig. Ib). Additionally, there were no statistically significant differences in WBC counts between CD36$^{-/-}$ mice exposed to O_3 compared with those exposed to FA (Supplementary figs. Ia and b). RBC and platelet counts did not differ between strains or across treatment groups (data not shown).

Loss of CD36 Prevents Vascular Endothelial Dysfunction Following Exposure to O_3

There was no change in basal tone of thoracic and abdominal aortic rings among the experimental groups (Supplementary table I). Responses to U-46619 were not influenced by O_3 exposure in both the endothelium-intact thoracic ($p = 0.0543$) and abdominal ($p = 0.1153$) aortas (Supplementary figs. IIa and b; EC_{50} values are presented in Table 1). ACh-induced relaxations were assessed *ex vivo* in U-46619-precontracted thoracic and abdominal aorta rings from mice 24 h after exposure. In the thoracic aorta from FA-exposed WT mice, ACh (10^{-9}–10^{-5}M) relaxed U-46619-preconstricted thoracic aortas, reaching a maximum of $53.2 \pm 5.3\%$ (Fig. 2A). The ACh-induced relaxation was significantly attenuated in WT mice exposed to O_3. The maximum relaxation due to ACh reduced by ~50% in thoracic aortas from WT mice exposed to O_3 compared with their respective FA-exposed control littermates ($p < 0.05$; Fig. 2A). In addition, this impaired relaxing effect to ACh was also observed in abdominal aorta (Fig. 2B). Similar to what has been shown before (Pagano *et al.*, 1999), ACh-induced relaxation was stronger in abdominal aortas from FA-exposed WT mice than in thoracic aorta (Figs. 2A and B). The ability of ACh to cause relaxation of the aorta is mainly attributed to nitric oxide (NO) released from the endothelium, diffusing into the underlying vascular smooth muscle. Relaxation responses to spermine NONOate in endothelium-denuded aortas were generated to investigate whether the impaired ACh-mediated

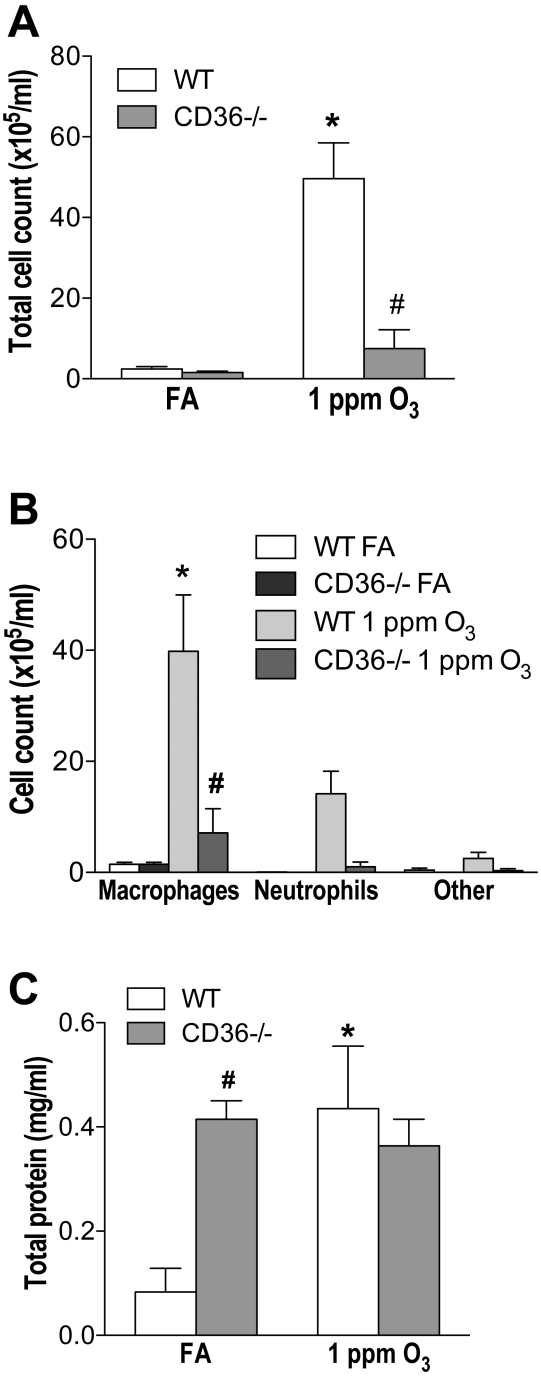

FIG. 1. CD36$^{-/-}$ mice are partially protected from O_3-induced lung inflammation. Female CD36$^{-/-}$ mice or WT animals were exposed to either FA or O_3 (1 ppm) for 4 h. (A) Total cell count, (B) differential cell count, and (C) total protein were analyzed in the BAL fluid 24-h postexposure. Columns represent mean ± SEM ($n = 4$–6). *$p < 0.05$ versus WT FA exposed; #$p < 0.05$ versus WT O_3 exposed; two-way ANOVA followed by Bonferroni's *post hoc* test.

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vascular relaxation following O_3 exposure is due to reduced release of NO from the endothelium or because of a reduced sensitivity of vascular smooth muscle cells in response to NO. There was actually an increased sensitivity of aortas from O_3-exposed mice to spermine NONOate (Supplementary fig. III), thus providing addition support for an effect of O_3 to impair endothelial release of NO.

We next investigated whether O_3 pollution impairs endothelial function through activation of the CD36 receptor. Relaxation in response to ACh in both the thoracic and abdominal aortas did not differ between WT and CD36$^{-/-}$ mice exposed to FA (Figs. 2A and B). However, CD36$^{-/-}$ mice were protected against the O_3-induced impairments of ACh-dependent vasorelaxation in aortic rings (Figs. 2A and B). Whether this protection was due to a role for vascular CD36 or the abrogation of lung inflammation remained unclear from these *in vivo* inhalation studies, necessitating the development of *ex vivo* homologous serum assays.

TABLE 1
–log EC_{50} Values of Concentration-Response Curves Performed for U-46619 in Aortic Rings From WT and CD36$^{-/-}$ Mice

	–log EC_{50} [M]	
	Abdominal aorta	Thoracic aorta
WT		
FA	8.3 ± 0.3	8.2 ± 0.2
1 ppm O_3	8.0 ± 0.2	8.2 ± 0.1
CD36$^{-/-}$		
FA	8.1 ± 0.1	8.1 ± 0.2
1 ppm O_3	7.8 ± 0.2	8.0 ± 0.2

Note. EC_{50}, concentration for half-maximal contraction (obtained using non-linear regression); unit, millimolar (mM). Values are mean ± SEM (n = 4–8).

CD36 Mediates Vascular Endothelium Dysfunction Induced by Circulating Factors Following Exposure to Inhaled O_3

To determine whether changes in the serum after O_3 exposure were a contributor to impaired vascular function, relaxation responses were determined in thoracic aortas from naïve (unexposed) WT mice following incubation with 2.5% serum samples obtained from WT mice 24 h following exposure to FA or O_3. There were no differences in basal tone or constriction to serum between groups (Supplementary table II). Serum samples obtained from WT mice after FA exposure did not modify relaxation to ACh in the aorta (Fig. 3). For serum collected from WT mice previously exposed to O_3, relaxation in response to ACh was impaired in the aorta of WT naïve mice (Fig. 3). Maximum relaxation due to ACh was reduced by ~85% in aortas from WT naïve mice incubated with serum from WT mice previously exposed to O_3 compared with aortas from a FA-exposed WT mouse ($p < 0.05$; Fig. 3).

By contrast, serum obtained from WT mice 24 h after O_3 exposure had no effect on the relaxation response to any concentration of ACh in thoracic aorta of naïve CD36$^{-/-}$ mice (Fig. 4). To investigate whether the O_3-induced circulating factors capable of impairing vascular responses to ACh in WT mice were independent of O_3-induced lung inflammation, relaxation responses were determined in vessels from naïve WT mice following incubation with 2.5% serum samples obtained from CD36$^{-/-}$ mice 24 h following exposure to FA

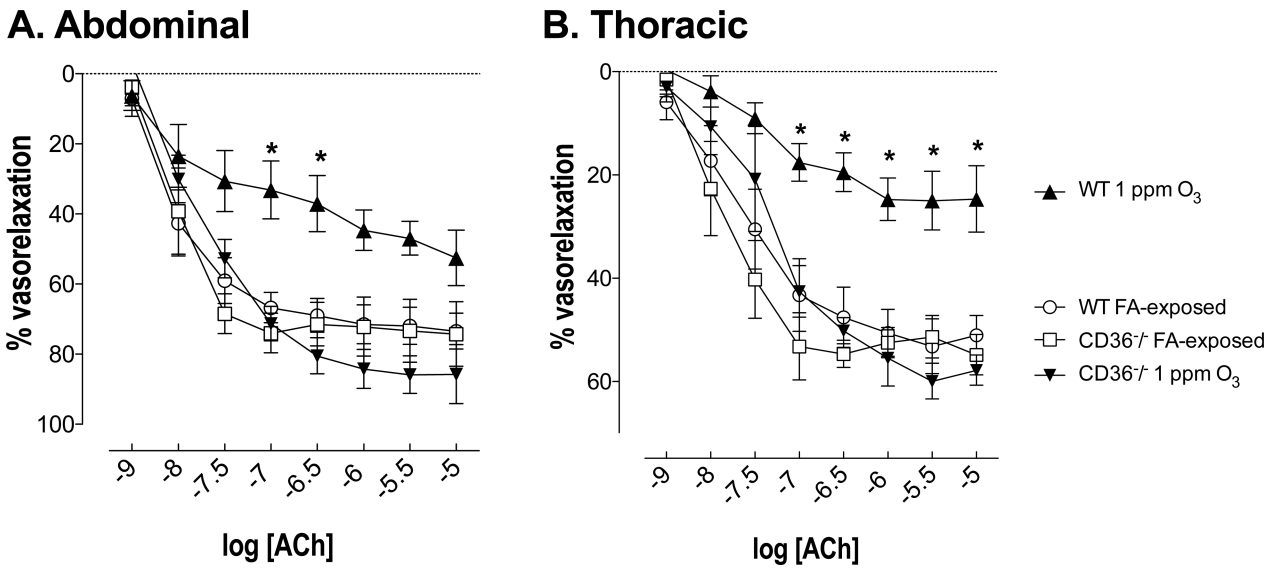

FIG. 2. Loss of the CD36 receptor protects against the impaired ACh-mediated endothelium-dependent relaxation in isolated (A) thoracic aorta and (B) abdominal aorta of O_3-exposed WT mice. Relaxation responses to ACh were reduced in thoracic and abdominal aorta rings from rats exposed to O_3 24 h prior, an effect prevented in the CD36$^{-/-}$ mice. Data are expressed as mean ± SEM (n = 4–5). *$p < 0.05$ versus WT FA exposed; two-way ANOVA followed by Bonferroni's *post hoc* test.

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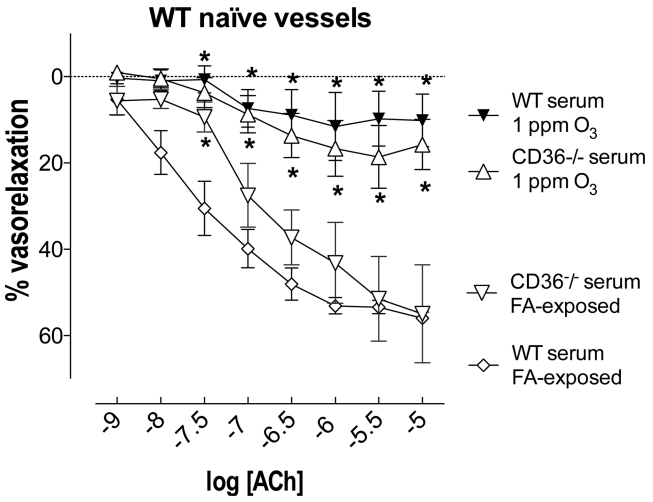

FIG. 3. O_3 exposure induces the secretion of bioactive circulating factors, leading to impaired vascular responses to ACh, independently of CD36 function or pulmonary inflammation. A mixture of 2.5% serum obtained from WT and CD36$^{-/-}$ mice 24 h after 1 ppm O_3 or FA exposure diminished vasorelaxation to ACh in thoracic aortas from naïve (unexposed) WT mice. Data are expressed as mean ± SEM (n = 3–5). *$p < 0.05$ versus WT serum FA exposed; two-way ANOVA followed by Bonferroni's *post hoc* test.

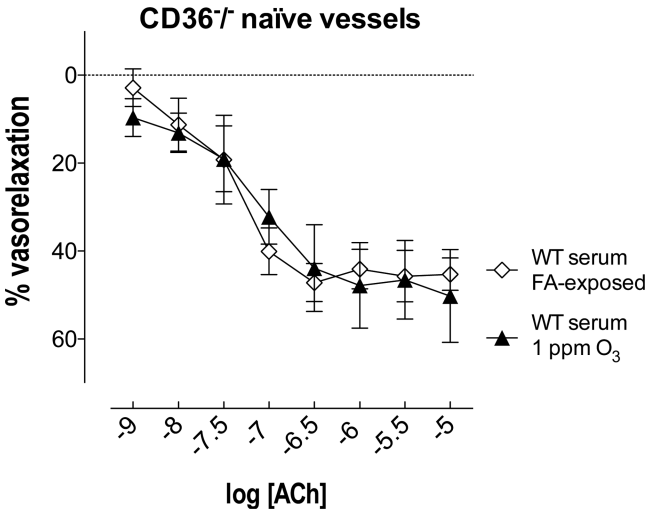

FIG. 4. Vascular CD36 mediates impairments in ACh-mediated vasorelaxation caused by O_3-induced circulating factors. Serum (2.5%) obtained from WT mice 24 h after O_3 exposure had no effect on thoracic aortas of CD36$^{-/-}$ naïve mice. Data are expressed as mean ± SEM (n = 3–5). No significant differences were found between groups (two-way ANOVA).

or O_3. As before, there were no differences in basal tone or constriction to serum in the thoracic aorta of WT naïve mice (Supplementary table II). Serum samples obtained from CD36$^{-/-}$ mice after FA exposure did not modify relaxation to ACh in the thoracic aorta of naïve WT mice (Fig. 3). For serum collected from the O_3-exposed CD36$^{-/-}$, relaxation in response to ACh was impaired in the thoracic aorta of WT naïve mice (Fig. 3). Notably, the constrictor effect of serum collected from CD36$^{-/-}$ mice previously exposed to O_3 was greater on thoracic aortas of naïve WT mice compared with naïve CD36$^{-/-}$ mice (Supplementary table II).

DISCUSSION

In this study, we demonstrate that O_3 inhalation induces circulating vasoactive factors, which can directly impair vasorelaxation to ACh. Moreover, as illustrated in Figure 5, the *ex vivo* homologous serum study design enables us to conclude that the CD36 PRR can influence (1) pulmonary inflammatory response to inhaled O_3 and (2) the vasorelaxation impairments downstream of inhaled O_3, and these responses are independent of one another. Furthermore, the generation of circulating vasoactive factors is independent of CD36, as serum from both the WT and CD36$^{-/-}$ models impaired ACh-mediated relaxation in WT vessels. Our study contributes support to the emerging view that changes in the serum/plasma composition caused by inhaled pollutants facilitate signals in the circulation that can drive vascular inflammatory responses (Channell *et al.*, 2012; Lund *et al.*, 2011).

Consistent with earlier experimental studies (Fakhrzadeh *et al.*, 2004; Johnston *et al.*, 2005; Savov *et al.*, 2004), O_3 exposure induced lung inflammation *in vivo* reflected by inflammatory cell influx and increased total protein in BAL fluid. We further show that this O_3-induced cell influx was absent in CD36$^{-/-}$ mice. These data strongly suggest that the CD36 receptor plays a role in the inflammatory response to O_3 exposure. Although studies have shown that CD36 stimulation mediates key downstream inflammatory signaling pathways (Kuda *et al.*, 2011; Silverstein and Febbraio, 2009), this is the first report on the effects of CD36 on O_3-induced lung inflammation. Unexpectedly, we observed a threefold increase in total protein in the BAL fluid from FA-exposed CD36$^{-/-}$ mice compared with their respective WT control. One may speculate that this could be due to the role of CD36, which is expressed on alveolar macrophages (AMs) (Smith *et al.*, 2007), mediating the phagocytosis of (among other things) anionic phospholipids (Rigotti *et al.*, 1995; Tait and Smith, 1999), fatty acids (Febbraio *et al.*, 2001), and albumin (Baines *et al.*, 2012). AM phagocytosis is essential for the maintenance of lung structure and function. Consequently, it is possible that a reduced capacity of the AMs to clear the lung of inhaled debris is contributing to the increased BAL total protein in mice deficient in CD36. The parallel information obtained between presumed function of CD36 on lung macrophages and on systemic endothelial cells in the present study may provide clues as to the circulating factors produced after O_3 inhalation. Although previously termed "inflammatory spillover," we propose that damage-associated molecular patterns formed by the cleavage of a number of lung phospholipids, proteins, or glycoproteins could, in fact, be a "preinflammatory" message that affects a vascular endothelial response. This concept is analogous to findings of hyaluronan fragment-dependent hyperreactivity

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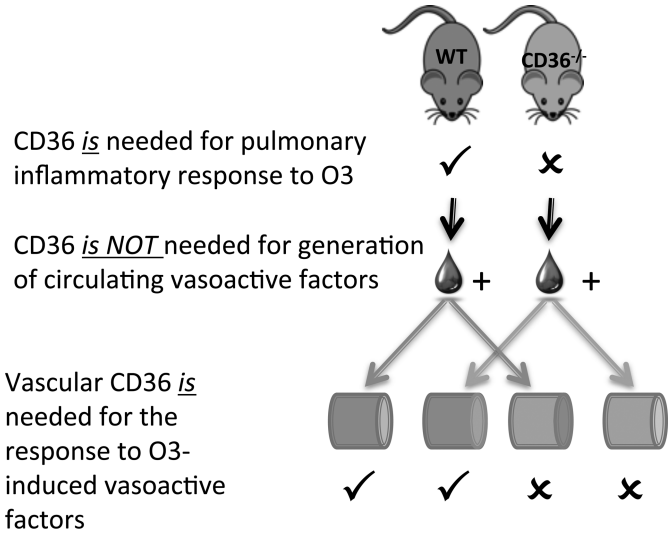

FIG. 5. Summary of homologous serum assay results, highlighting a role for CD36 in mediating (1) the pulmonary inflammatory response to O_3 and (2) the impaired vasorelaxation due to O_3-induced circulating factors. However, the production of such circulating factors was independent of CD36.

that was differentiated from inflammation in a similar model (Li *et al.*, 2010, 2011).

Because the lung inflammatory response was a clear complicating factor in determining the role of vascular CD36, we implemented the homologous serum experiments, treating naïve vessels with serum from control and exposed mice. The results of this experiment revealed two distinct conclusions (1) that circulating components induced by O_3 exposure decreased the vasorelaxation caused by ACh, independent of lung inflammation and (2) that the circulating components act through vascular CD36. Vascular CD36 expression has not been thoroughly studied with respect to air pollution but was found to be closely associated with vascular oxidative stress in response to inhaled DE emissions (Bai *et al.*, 2011). Previously, we reported a possible role of reactive oxygen species (ROS) in mediating the DE-induced vascular toxicity (Cherng *et al.*, 2011). Acute exposure to DE resulted in increased vascular oxidative stress in rats, and pretreatment, *in vitro*, with a free radical scavenger could reverse the diminished NO-dependent vasorelaxation in coronary arteries (Cherng *et al.*, 2011). There is also evidence that DE inhalation may activate a feed-forward mechanism, involving uncoupling of endothelial NO synthase (eNOS), capable of not only impairing NOS-dependent relaxation pathways but those dependent on cyclooxygenase, as well (Cherng *et al.*, 2011). This mechanism may thus contribute to the impaired vasorelaxation following exposure to O_3. However, the involvement of CD36 suggests that somewhat different mechanisms may be involved in the vascular response to O_3. CD36 has been shown to modulate the activity of eNOS via phosphorylation and displacement of the enzyme from the basolateral membrane (Shaul, 2002), and this may be an important avenue for future research. eNOS uncoupling and ROS generation may only partly explain the endothelial dysfunction routinely observed in humans exposed to lower concentrations of pollutants (Barath *et al.*, 2010; Mills *et al.*, 2005). The contribution that pollution-induced circulating factors participate in the relocation and subsequent inhibition of eNOS activity has not yet been explored.

In this study, we have used a novel method to demonstrate a pivotal role of CD36 in mediating the vascular effects induced by the cumulative impact of O_3 exposure in the circulation. The method described in this article is capable of assessing potential cumulative effects of circulating mediators on vascular function and is both anatomically more sophisticated and translationally coherent than comparable methods involving direct application of airborne pollutants to vessels or cultured vascular cells. Although the present application was limited to O_3, this *ex vivo* approach for the evaluation of the vasorelaxant effect of homologous serum samples is clearly applicable to other environmental pollutants. There are, however, some caveats with our overall approach that should be mentioned. The use of a high O_3 concentration was necessary to offset the well-recognized species insensitivity to this reactive air pollutant. Rodents are obligatory nose breathers and exhibit roughly one third of the sensitivity to O_3 compared with primates (Brown *et al.*, 2005; Harkema *et al.*, 2006). It is important to keep in mind, however, that O_3 levels higher than that used in this study have been reported in some metropolitan areas on hot humid days (Alonso *et al.*, 2002; Bytnerowicz *et al.*, 2002a,b). Nevertheless, exposure of humans to O_3 at levels typically encountered on the ground, O_3 does not induce such clear pulmonary inflammation as we have observed in the present study, and it is difficult to entirely rule out this response as a contributor to the systemic vascular effects. However, recent studies utilizing levels below the national ambient air quality standard revealed small but significant lung function decrements without clear evidence of pulmonary inflammation (Kim *et al.*, 2011). The formation of aldehydes in the airways has been demonstrated in humans exposed to only 0.12 ppm (Frampton *et al.*, 1999). Additionally, Brook *et al.* (2002) noted significant narrowing of brachial artery diameter in healthy subjects exposed to 0.12 ppm O_3 and 150 $\mu g/m^3$ concentrated PM. Notably, levels of inhaled NO_2 below what has been previously reported to cause lung inflammation did induce changes in human plasma that led to inflammatory activation of cultured endothelial cells (Channell *et al.*, 2012); thus in human exposures, it may be possible to oxidatively modify serum components at much lower levels of O_3.

To conclude, a role of CD36 as a mediator of vascular impairments following O_3 inhalation has been demonstrated using a straightforward homologous serum technique and global CD36 knockout model. The homologous serum methods further substantiate recent findings in which plasma obtained from human subjects following exposure to NO_2, a pollutant with similar toxic properties as O_3, was able to induce expression of inflammatory adhesion molecules in treated human coronary artery endothelial

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cells (Channell *et al.*, 2012). CD36 additionally has an important role in regulating inflammatory responses in the lung and, quite possibly, clearance of aged proteins from the airways. Far from any consideration of CD36 as a singularly responsible receptor that explains the association between O_3 levels and cardiovascular morbidity, we believe these studies highlight a wide realm of possible interactions between chemically modified blood components and receptors of the systemic endothelium.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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