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Cigarette Smoke Exposure Inhibits Contact Hypersensitivity via the Generation of Platelet-Activating Factor Agonists

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Previous studies have established that pro-oxidative stressors suppress host immunity because of their ability to generate oxidized lipids with platelet-activating factor receptor (PAF-R) agonist activity. Although exposure to the pro-oxidative stressor cigarette smoke (CS) is known to exert immunomodulatory effects, little is known regarding the role of PAF in these events. The current studies sought to determine the role of PAF-R signaling in CS-mediated immunomodulatory effects. We demonstrate that CS exposure induces the generation of a transient PAF-R agonistic activity in the blood of mice. CS exposure inhibits contact hypersensitivity in a PAF-R–dependent manner as PAF-R–deficient mice were resistant to these effects. Blocking PAF-R agonist production either by systemic antioxidants or treatment with serum PAF-acetyl hydrolase enzyme blocked both the CS-mediated generation of PAF-R agonists and PAF-R–dependent inhibition of contact hypersensitivity (CHS) reactions, indicating a role for oxidized glycerophosphocholines with PAF-R agonistic activity in this process. In addition, cyclooxygenase-2 inhibition did not block PAF-R agonist production but prevented CS-induced inhibition of CHS. This suggests that cyclooxygenase-2 acts downstream of the PAF-R in mediating CS-induced systemic immunosuppression. Moreover, CS exposure induced a significant increase in the expression of the regulatory T cell reporter gene in Foxp3EGFP mice but not in Foxp3EGFP mice on a PAF-R–deficient background. Finally, regulatory T cell depletion via anti-CD25 Abs blocked CS-mediated inhibition of CHS, indicating the potential involvement of regulatory T cells in CS-mediated systemic immunosuppression. These studies provide the first evidence, to our knowledge, that the pro-oxidative stressor CS can modulate cutaneous immunity via the generation of PAF-R agonists produced through lipid oxidation. The Journal of Immunology, 2013, 190: 2447–2454.

It has been estimated that half of the world’s population are exposed to environmental or tobacco smoke (1, 2). Among various environmental pollutants, cigarette smoke (CS) exposure (active and passive) has been a leading cause of morbidity and mortality associated with many disorders ranging from chronic lung and vascular diseases to oral and lung cancers (1, 3). In addition to containing various bioactive compounds including nicotine and carcinogens, CS exerts immunomodulatory effects resulting in alterations of both innate and adaptive systemic immunity (4–11). These effects on host immunity are multifaceted involving both proinflammatory and suppressive effects. The absolute effect of CS on the immune system depends on several variables, including the dose and type of tobacco, the route and chronicity of exposure, and the specific inflammatory mediators present at the time of immune cell stimulation. In addition, several studies have demonstrated that CS exposure leads to imbalances in Th1/Th2 responses resulting in T cell anergy (10–13). The mechanism underlying these events has remained largely undetermined. Several studies have claimed nicotine to be a causative factor in CS-mediated suppression of immune cells and increased risk of human cancers (5–7, 13). Other studies have shown contrasting results, indicating that CS-induced effects are independent of nicotine (10, 14). Most importantly, multiple lines of evidence have suggested that the immunomodulatory effects of CS exposure in part are attributed to the ability of CS to induce the production of reactive oxygen species (ROS), which in turn act as an initiating event in modulating host immunity (10, 15–17).

Several groups including ours have characterized the importance of various pro-oxidative stressors including UVB to suppress host immunity through mechanisms involving platelet-activating factor (1-alkyl-2-acyl-glycerophosphocholine; PAF) (reviewed in Refs. 18 and 19). PAF is produced enzymatically in a tightly controlled process (20). In addition, pro-oxidative stressors can act directly on glycerophospholipines (GPCs) to produce oxidized GPC (Ox-GPC) which are potent PAF receptor (PAF-R) agonists (21–23). The ability of various pro-oxidative stressors to suppress host immunity is classically measured by their ability to inhibit contact hypersensitivity (CHS) responses to either chemical Ags such as...
2,4-dinitrofluorobenzene (DNFB) or delayed-type hypersensitivity responses to Ags such as Candida albicans (24–28). Using CHS responses to DNFB in PAF-R expressing C57BL/6 wild-type (WT) and gene-deficient (Ptafr⁻/⁻) mice, it has been previously demonstrated that the ability of Ox-GPCs to mediate systemic immunosuppression is dependent on the presence of the PAF-R and can be blocked by systemic administration of antioxidants, cyclooxygenase-2 (COX-2) inhibitors, PAF-metabolizing enzymes, PAF-acetyl hydrolase (PAF-AH), or neutralizing Abs against the immunosuppressive cytokine IL-10 (24–28). In addition, we have shown that these Ox-GPCs/PAF-R agonists augment experimental murine melanoma tumor growth in a process involving IL-10 and regulatory T cells (Tregs) (29). Because CS exposure acts as a pro-oxidative stressor (15–17) and has been shown to generate PAF-like mediators in hamsters (30), we hypothesized that CS-induced suppression of the host immune system is mediated through PAF/PAF-R.

Delineation of the specific mechanisms by which CS affects host immunity is important to identify potentially novel therapeutic approaches for the management of CS-mediated diseases. Thus, the present studies sought to determine the role of the PAF/PAF-R system in CS-mediated immunomodulatory effects. For these studies, we used several model systems including PAF-R-expressing (KBP) and −deficient (KBM) cells, WT C57BL/6 (WT) and PAF-R−deficient (Ptafr⁻/⁻) mice as well as Foxp3 reporter mice, and a well-characterized DNFB allergen CHS model. These studies provide the first evidence, to our knowledge, that PAF-R signaling is involved in the immunosuppressive effects of CS.

Materials and Methods

Reagents and CS exposure

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise. As previously reported, CS exposure was performed using a total body exposure method (31). Briefly, mice were exposed for the indicated number of days, for 5 h/d, 5 d/wk, in a Teague-10E exposure chamber (Teague Enterprises, Woodland, CA) to a mixture of 90% side-stream and 10% mainstream cigarette smoke. The exposure chamber atmosphere was monitored for total suspended particulates (average, 90 μg/ml) and carbon monoxide (average, 350 ppm). The cigarettes were used were reference-grade cigarettes (1R3F) or low-nicotine cigarettes (1R5F) from the Kentucky Tobacco Research and Development Center (University of Kentucky, Lexington, KY).

Mice

Female C57BL/6-WT mice (PAF-R expressing; age, 6–8 wk) were purchased from the Charles River Laboratories. Age-matched female Ptafr⁻/⁻ mice on a C57BL/6 background, generated as previously described (32), were a gift from Prof. T. Shimizu (Department of Biochemistry, University of Tokyo, Tokyo, Japan). Foxp3EGFP knockin transgenic mice on the C57BL/6 background (age 8–12 wk) were procured from The Jackson Laboratory (33). Foxp3EGFP−Ptafr⁻/⁻ mice were generated to determine the involvement of PAF-R in Treg-mediated inhibition of contact hypersensitivity reactions by CS. In brief, Foxp3EGFP−WT female mice were crossed with Ptafr−/− males, and offspring from this generation was genotyped and crossed to finally obtain Foxp3EGFP−Ptafr⁻/⁻ mice. In some experiments, mice were placed on vitamin C-enriched (10 g/kg; Research Diets, New Brunswick, NJ) and 5 mM N-acetylcysteine (NAC) in water for 10 d prior to CS exposure and for the duration of the study as per our previous studies (28, 29). All mice were housed under specific pathogen-free conditions at the Indiana University School of Medicine. All procedures were approved by the Animal Care and Use Committee of Indiana University School of Medicine.

Measurement of PAF-R agonists by calcium mobilization and IL-8 production

The presence of systemic PAF-R agonists in lipid extracts derived from the blood of treated mice was measured by the ability of the lipid extracts to induce an intracellular Ca²⁺ mobilization response in PAF-R-expressing KBP cells, but not in KBM cells lacking the PAF-R, as described previously (28). In brief, KBP and KBM cells were preloaded with the Ca²⁺-sensitive indicator fura 2-AM (4 μM in HBSS) at 37°C for 90 min, washed, and resuspended in HBSS at room temperature before use. Lipid extracts from whole blood obtained from groups of CS- versus untreated (sham) exposed mice were added to an aliquot of these cells (1.0–1.5 × 10⁶ cells/ml) in a cuvette at 37°C with constant stirring. 1-Hexadecyl-2-N-methylcarbamoyl glycerophosphocholine (CPAF) and endothelin-1 (ET-1) dissolved in ethanol (adjusted to 1 μM) were used as positive controls. Fluorescence and fluorescence changes were measured in a Hitachi F-4010 spectrofluorometer with excitation and emission wavelengths of 331 and 410 nm, respectively. The Ca²⁺ influx in suspensions was calculated as previously described (28) and shown as the percentage of maximal peak calcium flux induced by either CPAF or ET-1. In separate experiments, WT mice were exposed to low-nicotine cigarettes (0.16 mg/cigarette [<10% of standard amounts found in standard reference cigarette]; obtained from University of Kentucky Reference Laboratories), and its effect on PAF agonists production was similarly determined. In some experiments, KBM and KKB cells were exposed to lipid extracts, and supernatants were collected to measure IL-8 protein by ELISA as described previously (34).

Contact hypersensitivity reactions

Contact hypersensitivity (CHS) to DNFB was conducted as described previously (25, 27). In brief, to evaluate the effect of CS on sensitization reactions, WT and Ptafr⁻/⁻ mice were exposed to CS for 5 h/d for 5 d, according to the published protocol (31). Three days following the CS exposure, a 2.5 × 2.5-cm area of distal back skin was shaved under anesthesia and sensitized with the application 25 μL 0.5% DNFB in acetone: olive oil (4:1, v/v). Nine days later, ear thickness was measured, and then, 10 μL 0.5% DNFB was painted on the dorsal sides of one ear, whereas the other ear was painted with vehicle. After 24 h, ear thickness was measured using a digital caliper as a final read out. Intraperitoneal injection of PAF agonist, CPAF (250 ng/mouse), and intradermal injection of histamine (200 ng/mouse) were used as a positive control. In separate experiments, the mice were pretreated with antioxidants, COX-2 inhibitors SC-236 and NS-398, PAF-AH, and neutralizing Abs against CD25 as described in the appropriate figure legend. In CHS experiments, DNFB was added to ears of naive mice for 24 h, which consistently did not elicit an inflammatory response.

Real-time RT-PCR

Total RNA was extracted from lymph nodes of sham versus CS-exposed mice using the RNeasy kit (Qiagen). In brief, tissue was homogenized in RLT buffer containing 2-ME by bullet blender (Next Advance, Averill Park, NY) using carbidie beads following the manufacturer’s protocol (Qiagen). Purified RNA was quantitated with the NanoDrop 2000 (Thermo Fisher Scientific, Lafayette, CO). Reverse transcription of whole RNA was done using SuperScript cDNA synthesis kit (Invitrogen) with random hexamers. Quantitative RT-PCR was performed for EGFP and TGFB expression and normalized against CD3e using the ΔΔCt method on a Step One Real-Time PCR machine (Bio-Rad Laboratories, Hercules, CA). Each assay was performed in triplicate in a 20-μl reaction volume with 2× SYBR green MasterMix (SA Biosciences, Frederick, MD), 2 μl cDNA, and primers at 10 μM. The following PCR conditions were used: 95°C for 15 min, followed by 45 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 30 s, followed by 72°C for a final 10 min. Each PCR was also tested to assure a single product of the predicted size.

Flow cytometry analysis

Tissue samples (draining lymph nodes) were harvested from isotype control Ab or anti-CD25 Ab-treated Foxp3EGFP mice at day 17 (considering 15-d period for CHS experiment including ~2 d of Abs injection) and processed for the flow analysis. In brief, these tissues were dissociated into single-cell suspension in CM-10 media using gentleMACS (Miltenyl Biotec, Auburn, CA). Tissue samples were filtered with cell strainer (40 μm) and centrifuged at 12,000 rpm for 5 min. Cells were washed twice with FACS buffer (PBS containing 2% FBS) and counted. A total of 1 × 10⁶ cells from each organ were incubated with Fc blocking (CD16/32) Ab for 10 min, after which cells were washed twice with FACS buffer. Foxp3-positive cell populations were quantitated based on EGFP as surrogate for FACS. Data files were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

In the current study, at least five mice per group were used in all murine experiments. All statistical calculations were performed using GraphPad Prism 5.0. Nonparametric one-way ANOVA, followed by Bonferroni post
hoc multiple comparison tests, was used to test the statistical significance difference between multiple groups. The data represent mean values with SE. Differences were considered statistically significant when the $p < 0.05$.

**Results**

**CS exposure generates systemic PAF-R agonists**

Previously, Lehr et al. (30) demonstrated that CS exposure generates PAF-like oxidized lipids in hamsters. Our first studies verified that CS induces oxidized lipids with PAF-R activity in mice. Although some of the structures of Ox-GPC PAF-R agonists have been elucidated (19, 22, 23, 35, 36), it appears that numerous as yet undefined bioactive Ox-GPC PAF-R agonistic species exist (36). Hence, our studies measured total PAF-R biochemical activity (28, 35). To test whether CS exposure can stimulate the production of PAF agonists, WT mice were first exposed to CS for 5 h (31). Control mice (SHAM) were not exposed to CS. Groups of mice were then sacrificed at various times post-CS exposure, and blood was drawn by cardiac puncture and underwent lipid extraction (28, 35). These lipids were tested for the presence of total PAF-R agonistic activity by their ability to induce intracellular calcium ion ($Ca^{2+}$) mobilization in PAF-R–expressing KBP cells but not PAF-R–negative KBM cells. We observed that lipid extracts derived from the blood of CS-treated WT mice, but not from sham-treated mice, resulted in an immediate intracellular $Ca^{2+}$ mobilization response in KBP cells (Fig. 1A). However, no $Ca^{2+}$ mobilization response was detected with lipid extracts from sham- or CS-treated mice in KBM cells (Fig. 1B). We then assessed the time course for CS-mediated generation of PAF-R

![FIGURE 1](http://www.jimmunol.org/) Identification of PAF-R agonistic activity in blood from CS-treated mice. Lipid extracts from pooled whole blood from groups of three to four female mice (~2 ml total for each sample) were taken immediately (time 0) or various times following 5 h of cigarette exposure versus sham-treated mice. Twenty-five percent of the sample (0.5 ml blood) from time = 0 was added to KBP cells (A) or KBM cells (B) loaded with Fura 2 dye and fluorescence monitored with spectrophotofluorimeter and converted to intracellular $Ca^{2+}$ levels. Excess (1 μM) CPAF or ET-1 was added at the end of the incubation as positive control. (C) Time course of PAF-R agonist measurement. Lipid extract-induced $Ca^{2+}$ mobilization responses (from 0.5 ml pooled blood) are expressed as a percentage of the maximal response elicited by CPAF (1 μM) in KBP cells. The data are mean ± SE percent maximal (CPAF) response at time = 0 from at least four pooled samples at each time point. KBP (D) or KBM (E) cells were incubated with various concentrations of PAF, 100 nM of the phorbol ester TPA, or lipid extracts from blood taken 1 h following sham- versus CS-exposed mice [as in (C)]. After 6 h, the supernatants were removed, and IL-8 was measured by ELISA. The data are mean ± SD IL-8 protein from at least three pooled samples. Statistically significant (*$p < 0.05$; ***$p < 0.0001$) differences from vehicle-treated CS PAF-R agonist formation measurements.
agonists. As shown in Fig. 1C, the maximum amounts of PAF-R agonistic activity (as a percentage of 1 μM CPAF response) were generated when the blood was drawn immediately after completing 5 h of CS (Time 0) and were substantially reduced after 60 min and were at baseline levels by 240 min following CS exposure. Of interest, approximately similar levels of PAF agonists were measured in WT and PAF-R-deficient mice, indicating that the PAF-R was not necessary for CS-mediated generation of PAF-R agonists (data not shown). In addition to the receptor-mediated intracellular calcium mobilization studies discussed above, we also used a second biochemical assay to verify that CS induces the production of PAF-R agonists. Several studies including ours have demonstrated that activation of the epidermal PAF-R via PAF-R agonists results in the release of IL-8 (34, 37). Consistent with these findings, we demonstrate that lipid extracts derived from CS-treated induced a greater increase in IL-8 secretion in KBP cells in comparison with sham-treated mice. However, blood derived from CS-treated mice did not result in IL-8 release in PAF-R-negative KMB cells (Fig. 1E). Taken together, our data indicate that PAF-R agonists are indeed produced in response to CS exposure.

Pharmacologic manipulation of CS-generated systemic PAF-R agonist formation

Our previous studies demonstrated that the generation of Ox-GPC PAF-R agonists by the pro-oxidative stressor UVB radiation can be blocked by antioxidants or the PAF-metabolizing enzyme serum PAF-AH (28, 35). Moreover, PAF-induced systemic immunosuppression is mediated via downstream COX-2/PG signaling (24, 25). Finally, several studies have implicated nicotine, a major constituent of cigarettes, as a mediator in CS-induced modulation of T cells’ responsiveness and increase risk of human cancers (5–7, 13). We therefore determined the effects of antioxidants, PAF-AH, the COX-2 inhibitor NS-398, and low-nicotine cigarettes on CS-mediated generation of PAF-R agonists. To that end, WT mice were fed either with a vitamin C–enriched diet (10 g/kg) and supplemented with NAC (5 mM) in drinking water for 10 d or with a regular diet prior to CS exposure. This antioxidant regimen has been shown to successfully block UVB-mediated augmentation of experimental melanoma growth, a process that is dependent on Ox-GPC PAF-R agonists (29). In separate experiments, WT mice were pretreated either with PAF-AH (5 mg/kg) or with the COX-2 antagonist NS-398 (200 ng) injected i.p., followed by exposing them to cigarette smoke for 5 h. Again, blood was drawn immediately following CS exposure, and lipid extracts were tested for the presence of PAF-R agonists by the KBP Ca2+ mobilization assay. As shown in Fig. 2, pretreatment with the PAF and Ox-GPC–metabolizing enzyme PAF-AH blocked CS-mediated generation of PAF-R agonists. Moreover, the antioxidant-enriched diet also ameliorated CS generation of PAF-R agonists, suggesting the importance of oxidized PAF-R agonists in this process (Fig. 2). These data are in agreement with studies by Lehr et al. (30) demonstrating that antioxidants can block lipid PAF-R agonists generated by CS exposure in hamsters. In contrast, pretreatment with the COX-2 inhibitor NS-398 had no effect on CS-mediated generation of PAF-R agonists. To define the role of nicotine in CS generation of PAF-R agonistic activity, WT mice were exposed to low-nicotine (<10% of usual content) instead of standard reference cigarettes. As shown in Fig. 2, exposure to low-nicotine cigarettes generated similar levels of PAF-R agonists as standard cigarettes. Collectively, these data suggest the importance of oxidative stress rather than nicotine in mediating the CS generation of PAF-R agonists.

Effect of CS-mediated generation of PAF-R agonists on CHS in WT and Ptafr−/− mice

Given our findings demonstrating that CS exposure generates systemic PAF-R agonists as well as previous studies demonstrating that these potent lipid mediators can suppress host immunity (24–27), our next experiments assessed the effect of CS-generated PAF-R agonists on CHS reactions. To that end, mice were exposed to CS for 5 h/d for 5 d, followed by sensitization of the back skin with DNFb 3 d later, and finally the elicitation of the CHS response by reapplying DNFb to one ear 9 d after the sensitizing the mice to DNFb and vehicle to the contralateral ear. The elicitation reactions were assessed by measuring the differences in ear thickness between DNFb- and vehicle-treated ears 24 h after the application of the eliciting agent (as a measure of inflammation). In other groups, treatment with metabolically stable PAF-R agonist CPAF (250 ng i.p.), histamine (200 μg s.c.), or 0.1% DMSO vehicle (i.p.) were given 5 d before sensitization. As shown in Fig. 3, there was no difference in the CHS reactions to DNFb between WT and Ptafr−/− mice. Smoking exposure inhibits CHS to DNFb in WT but not PAF-R−/− mice. Groups of six to eight WT or PAF-R-deficient female mice were exposed to 5 d of 5 h/d CS, followed 3 d later by treating back skin with DNFb (sensitization). Nine days later, ear thickness was measured, then one ear was treated with DNFb, and the other with vehicle and ear thickness (as a measure of inflammation) was measured 24 h later. In other groups, treatment with CPAF (250 ng i.p.), histamine (200 μg s.c.), or 0.1% DMSO vehicle given 5 d before sensitization. The data are mean ± SE differences in ear thickness measurements. *p < 0.05, statistically significant differences from vehicle-treated mice.
PAF-R–positive WT and Ptafr−/− mice in sham control groups. However, CS exposure inhibited CHS reactions to DNPB selectively in WT mice and not in Ptafr−/− mice. Similarly, i.p. injection of the CPAF, used as a positive control, resulted in an inhibition of CHS reactions only in WT mice. Intradermal injections of histamine, which suppresses systemic CHS responses independently of the PAF-R (38, 39), induces systemic immunosuppression in both WT and Ptafr−/− mice. These findings correlate with previous studies demonstrating that PAF-R agonists generated via UVB mediate systemic immunosuppression only in WT and not in Ptafr−/− mice (26, 27). These data also implicate the importance of PAF-R agonists generated via CS exposure in mediating systemic immunosuppression.

Effect of antioxidants on CS-mediated inhibition of CHS in WT mice

The next studies were designed to assess whether CS-generated PAF-R agonistic activity could be suppressed by systemic antioxidants in vivo using the CHS model. To achieve this, mice were fed either standard diet or a diet enriched with antioxidants (vitamin C and NAC) for 10 d before CS exposure and then were maintained on this diet during the experimental period. As above, mice were exposed to CS for 5 h/d for 5 d or were exposed to ambient air as sham controls and CHS to DNPB was assessed by measuring the elicitation reaction (ear thickness). We observed that CS-mediated inhibition of CHS reactions was observed in WT mice on a regular diet but was blocked in mice treated with systemic antioxidants (Fig. 4). These studies indicate that CS-exposure generates PAF-R agonists via a process involving ROS. These findings are consistent with our previous studies demonstrating that vitamin C blocks the generation of UVB-mediated PAF-R agonists and restored the inhibition of CHS reactions in WT mice (28).

Effect of PAF-AH and COX-2 antagonists on CS-mediated inhibition of CHS in WT mice

Given our findings that PAF-AH treatment blocks CS-mediated generation of PAF-R agonists and supporting evidence that PAF-AH prevented the inhibition of CHS by UVB (28), we next planned to determine whether exogenous PAF-AH can block the inhibition of CHS mediated by CS exposure. To that end, WT mice were pretreated with recombinant PAF-AH 2 d prior to CS exposure and the effect of CS on CHS reactions was assessed. We observed that PAF-AH treatment did not affect CHS responses in sham-treated mice yet blocked the CS-induced inhibition of CHS (Fig. 5). Of interest, previous studies have demonstrated that PAF-AH does not block the effects of the metabolically stable PAF-R agonist CPAF on CHS reactions (28).

Previous studies have shown that COX-2 metabolites are downstream mediators of PAF in UVB-mediated inhibition of CHS (24). Hence, we tested the hypothesis that COX-2 could be involved in CS-mediated inhibition of CHS reactions. For this, WT mice were pretreated with COX-2 antagonists NS-398 and SC-236 2 d prior to 5 h/d for 5 d of CS exposure, and the effect of CS on CHS reactions was similarly assessed. As shown in Fig. 5, blocking COX-2 by two pharmacologically distinct COX-2 enzymatic antagonists prevented CS-mediated inhibition of CHS to DNPB.

Involvement of Tregs in CS-mediated inhibition of CHS

Several studies have implicated CD4+CD25+ Tregs as mediators of UVB-induced local and systemic immunosuppression (40–42). In addition, our studies have demonstrated that Tregs are important in mediating UVB/PAF-R agonist-induced systemic immunosuppression and the augmentation of the growth of experimental murine melanoma (29). We have also shown that the depletion of Tregs by neutralizing Abs against CD25 abrogates UVB-mediated increase in Foxp3 expression, the master regulator of Tregs, in lymph nodes as measured by quantitative PCR, and these findings correlated very well with flow cytometry analysis (29). This depletion of Tregs attenuated UVB-mediated enhanced melanoma tumor growth (29). On the basis of these observations, we hypothesized that Tregs could be mediating CS-induced systemic immunosuppression. To answer this question, we used a Foxp3 reporter mouse model (Foxp3EGFP mice) that expresses EGFP under the control of the Foxp3 promoter (33). We first exposed Foxp3EGFP mice to 5 h/d of CS for two alternate days or left them unsmoked for sham control and isolated lymph nodes and quantified the expression of Foxp3 by performing quantitative PCR for EGFP, a surrogate marker for Tregs, after normalization against the T cell gene Cd3. We observed a significant increase in the expression of EGFP by CS exposure compared with their sham controls (Fig. 6A). We next defined whether this increase in the expression of EGFP by CS is dependent on PAF-R signaling.
this, we crossed Foxp3EGFP mice with Ptafr2/2 mice to generate Foxp3EGFP mice on PAF-R–deficient background (Foxp3EGFP-Ptafr2/2) and exposed them to CS and quantified Foxp3 expression from the lymph nodes as described previously. Unlike the WT Foxp3EGFP mice, we observed that CS exposure did not result in the enhanced expression of EGFP in Foxp3EGFP-Ptafr2/2 mice (Fig. 6A). These studies indicate a systemic increase in Tregs following CS exposure occurs in a PAF-R–dependent manner.

Interestingly, we also observed statistically (p < 0.05) increased expression of the Treg-associated immunosuppressive cytokine TGF-β in lymph nodes by CS in a PAF-R–dependent manner (data not shown).

Given our findings that CS exposure increased lymph node Foxp3 expression in a PAF-R–dependent fashion, coupled with our previous studies demonstrating by flow cytometry increased numbers of Foxp3-expressing T cells in lymph nodes following systemic CPAF treatment (29), we next tested whether depletion of Tregs by anti-CD25 Abs could abrogate CS-mediated inhibition of CHS reactions. To that end, Foxp3 EGFP mice were injected either with anti-CD25 Abs (IgG and IgM) or isotype control Abs and exposed to or without CS for 5 h/d for 3 d before removal of lymph nodes for RNA extraction and cDNA synthesis. The expression of EGFP was analyzed by quantitative RT-PCR and normalized against CD3. The data represent mean ± SD. **p < 0.01, statistically significant difference from sham-treated mice. (B) Depleting Abs against CD25 block expression of EGFP-positive Tregs. Foxp3 EGFP mice were injected with either isotype control Abs (IgG and IgM) or depletion Abs against CD25 (PC61.5.3 and 7D4). The expression of EGFP (marker for Foxp3-Tregs) in lymph nodes of these mice was then evaluated on the 10th day using EGFP as surrogate by FACS. (C) Treatment with anti-CD25 inhibits CS- and CPAF-mediated inhibition of CHS. WT mice (groups of six to eight) were injected either with isotype control Abs against IgG and IgM or depleting Abs against two different clones of anti-CD25 (PC61.5.3 and 7D4) (1 mg each) 2 d before beginning standard sham or CS (5 h/d × 5 d) exposure. Three days later, mice were then sensitized to DNFB, followed 9 d later by elicitation as outlined in Fig. 3. The data are mean ± SE differences in ear thickness measurements. *p < 0.05, statistically significant differences from sham-treated mice.

Discussion
Because CS is one of the most important immunosuppressive environmental exposures and is a well-known risk factor for lung cancer induction, it is important to understand the mechanisms involved. The present studies describe a previously unappreciated mechanism by which CS exposure can result in systemic immunosuppression. These data support the model that Ox-GPC PAF-R agonists produced in response to ROS from CS can exert systemic immunosuppressive effects in a process involving COX-2 metabolites and Tregs. Moreover, we present evidence that this novel immunomodulatory pathway is susceptible to pharmacologic inhibition.

Oxidation of esterified fatty acyl residues introduces oxy functions, rearranges bonds, and fragments carbon-carbon bonds by β-scission that generate a myriad of phospholipid reaction products including PAF-R agonists (19–22, 35, 36). In contrast to the tightly controlled enzymatic pathways for PAF biosynthesis, high levels of Ox-GPC PAF-R agonists can be produced non-enzymatically. In this regard, cellular membranes serve as the source of oxidized phospholipids and are thus the source of CS-mediated PAF formation. Although these studies did not address the exact cellular source for the CS-generated PAF-R agonists,
this is likely to be the lung epithelium in a process similar to skin keratinocytes and UVB.

Previous findings indicated that CS treatment of both hamsters and humans resulted in the generation of a PAF-R agonistic activity in a process inhibited by the antioxidant vitamin C (30, 40). The present studies confirm that CS exposure generates PAF-R agonists in murine blood. That antioxidants ameliorated the CS-mediated production of PAF agonists and that low-nicotine CS exposure generated equal levels of these novel lipids as the standard cigarettes all fit with the notion that ROS, not nicotine, mediates this response.

Exogenous pro-oxidative stressors ranging from aromatic hydrocarbons to UVB have been shown to inhibit contact hypersensitivity via PAF-R signaling (18, 19). Consistent with the ability of CS exposure to generate PAF-R agonists, CS induces systemic immunosuppression in a PAF-R–dependent process blocked by antioxidants. Production of PAF-R agonists begins a cascade of events leading to systemic immunosuppression. The cytokines that appear to be critical for the immunosuppression include IL-10– and COX-2–generated eicosanoids (24–27). Tregs also are implicated in PAF-R–dependent systemic immunosuppression (29). The ability of a Treg-depleting strategy successfully used to block UVB-mediated immunosuppression (29) to similarly block CS-mediated inhibition of CHS confirms Treg involvement. Of interest, use of a Treg-depleting strategy has been reported to modestly enhance CHS reactions (41, 42). As depicted in Fig. 6, our neutralizing Ab protocol did not result in an enhancement of baseline CHS reactions, although this strategy did deplete Tregs by ∼50%. It is possible that the level of Treg depletion in our study was not enough to exert this enhancement.

The model that has emerged indicates that mast cells are also crucial mediators of PAF-induced systemic immunosuppression. Mast cell–deficient mice do not undergo systemic immunosuppression in response to PAF agonist generators such as UVB or JP-8 jet fuel (38, 39, 43). In response to these agents, mast cells traffic to lymph nodes in a CXCR4-dependent manner in that a CXCR4 antagonist blocks not only UVB/PAF-R–mediated mast cell migration from skin to lymph nodes but also the systemic immunosuppression (39). Elegant studies transplanting mast cells into mast cell–deficient mice have indicated that mast cells appear to be the source for eicosanoids, which mediate the systemic immunosuppression (39).

PAF-R agonists have been reported to be measured in the blood from human volunteers exposed to CS (40), suggesting that this process could be generalized to humans. Indeed, CS-mediated systemic immunosuppression has been documented in humans (7, 10, 44). Of interest, a large study of subjects undergoing hepatitis B vaccination indicates that smokers were more likely to experience vaccine failure than nonsmokers (45). Although the current studies focused on the ability of CS to inhibit the sensitzation of a neoantigen in a PAF-R–dependent manner, our previous studies have demonstrated that systemic CPAF exposure can also inhibit elicitation reactions to a sensitized Ag (25). Thus, the CS generation of PAF agonists could have a profound effect on immunity. The findings from the present studies implicating Ox-GPC PAF-R agonist involvement in CS-mediated systemic immunosuppression suggests that strategies such as antioxidants and COX-2 inhibitors could potentially have clinical use in augmenting vaccine efficiency in smokers.

The major enzyme for PAF/Ox-GPC degradation is serum PAF-AH (PLA2G7) (reviewed in Ref. 46). PAF-AH deficiencies have been described, including a genetic loss of function mutation found to be homozygous in ∼4% of Japanese individuals (47). In addition, acquired PAF-AH deficiencies have been reported in disorders such as lupus erythematosus (46–48). Acute CS exposure has also been reported to inactivate PAF-AH (49). However, CS also has been demonstrated to increase serum PAF-AH levels (50). The present studies indicate that PAF-AH could play an important role in protecting against CS-mediated systemic immunosuppression via the inactivation of PAF-R agonists. Hence, there appears to be considerable variability in the ability of individuals to metabolize PAF/Ox-GPC generated by CS because of genetic/environmental influences, which could have clinical significance.

In summary, the present studies provide the first evidence, to our knowledge, that PAF-R signaling plays an important role in CS-mediated immunosuppression in a murine model of CHS. Inasmuch as this process involves the pro-oxidative qualities of CS rather than nicotine, it is possible that other smoke exposures including pollution could exert similar effects on the immune system. That these CS-mediated effects are neutralized by relatively simple and safe measures (e.g., antioxidants) could provide the impetus for future studies to define the clinical significance of this novel pathway in humans.

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Disclosures

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References


