Malondialdehyde–Acetaldehyde-Adducted Surfactant Protein Alters Macrophage Functions Through Scavenger Receptor A

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Background: Reactive aldehydes such as acetaldehyde and malondialdehyde generated as a result of alcohol metabolism and cigarette smoke exposure lead to the formation of malondialdehyde–acetaldehyde-adducted proteins (MAA adducts). These aldehydes can adduct to different proteins such as bovine serum albumin and surfactant protein A or surfactant protein D (SPD). Macrophages play an important role in innate immunity, but the effect of MAA adducts on macrophage function has not yet been examined. Because macrophage scavenger receptor A (SRA; CD204) mediates the uptake of modified proteins, we hypothesized that the effects of MAA-modified proteins on macrophage function are primarily mediated through SRA.

Methods: We tested this hypothesis by exposing SPD-MAA to macrophages and measuring functions. SPD-MAA treatment significantly stimulated pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) release in the macrophage cell line, RAW 264.7.

Results: A significant reduction in phagocytosis of zymosan particles was also observed. SPD-MAA stimulated a significant dose-dependent increase in TNF-α and interleukin (IL)-6 release from peritoneal macrophages (PMs) of wild-type (WT) mice. But significantly less TNF-α and IL-6 were released from PMs of SRA−/− mice. We observed a significant reduction in phagocytosis of zymosan particles in PMs from WT mice treated with SPD-MAA. No further SPD-MAA-induced reduction was seen in PMs from SRA−/− mice. SPD-MAA treatment significantly increased SRA mRNA expression, but had no effect on surface receptor protein expression. Protein kinase C alpha inhibitor and NF-κB inhibitor significantly reduced pro-inflammatory cytokine release in response to SPD-MAA.

Conclusions: In conclusion, our data demonstrate that SRA is important for MAA-adducted protein-mediated effect on macrophage functions.

Key Words: Alcohol, Cigarette Smoke, Adduct, Macrophage, Scavenger Receptor A.

ALCOHOL INTOXICATION COMPROMISES host defenses against bacterial infections as it leads to suppression of selected functions of the immune system (Nelson et al., 1989). Individuals with alcohol use disorders (AUDs) are more susceptible to bacterial pneumonia than those without AUDs (Cook, 1998). In addition, persons with AUDs are 2 to 3 times more likely to smoke cigarettes (Bobo and Husten, 2000; Miller and Gold, 1998) leading to more frequent (John et al., 2003) and higher rates of cigarette smoking among AUDs than in the general population (Narahashi et al., 2001).

Macrophages are often called first-responder phagocytes, ingesting and killing inhaled bacteria (Fujiwara and Kobayashi, 2005; Yang et al., 2014). They exhibit different anatomical and functional features depending upon their tissue location (Wynn et al., 2013). Macrophages exhibit an inflammatory phenotype and secrete pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 and IL-1β, all of which participate in the activation of antimicrobial mechanisms (Parihar et al., 2010). Macrophages also produce reactive oxygen and nitrogen intermediates such as nitric oxide and superoxide as a defense mechanism against microorganisms (Murray and Wynn, 2011). Because macrophages play an important role in antimicrobial defense as well as to initiate, maintain, and resolve inflammation (Fujiwara and Kobayashi, 2005), any compromise in antimicrobial defense could lead to increased susceptibility to infection and unchecked inflammation resulting in cellular and tissue damage.

Because both acute and chronic alcohol consumption lowers the ability of phagocytes to clear diverse pathogens, alcohol consumption often leads to lower/compromised innate and acquired immune response (Asplund et al., 2013).

Short- and long-term alcohol intake has also been shown to reduce phagocytic function of macrophages, which might play a role in increasing the risk of infection (Bagasra et al., 1988; Castro et al., 1993). Alcohol consumption, both acute and chronic, alters pro-inflammatory cytokine production. In acute or moderate alcohol consumption, the release of pro-inflammatory cytokines TNF-alpha, IL-6, and IL-1 from monocytes is decreased. This could also contribute to compromised immunity, as these cytokines are important for defense against pathogens (Mandrekar et al., 2009). In contrast to this, chronic alcohol consumption leads to increased pro-inflammatory cytokine release, which could result in uncontrolled inflammation (Mandrekar et al., 2009; Szabo, 1997).

Reactive aldehydes such as acetaldehyde and malondialdehyde are formed during alcohol metabolism and oxidative stress in response to excessive alcohol consumption (Sapkota et al., 2014; Tuma and Casey, 2003). These aldehydes, once formed, react together covalently with proteins and generate malondialdehyde–acetaldehyde-adducted proteins (MAA adducts), which possess immunogenic and pro-inflammatory properties (Tuma, 2002). In addition to alcohol, these reactive aldehydes could also form as a result of cigarette smoking and bind to protein and other molecules (McCaskill et al., 2011). During co-exposure to alcohol and cigarette smoke, high concentrations of acetaldehyde and malondialdehyde are generated resulting in the formation of MAA adducts (McCaskill et al., 2011; Wyatt et al., 2001). In lung, MAA covalently binds to surfactant protein D (SPD) secreted by type II epithelial cells to form an adducted protein called SPD-MAA (McCaskill et al., 2011).

Scavenger receptors are pattern recognition receptors expressed on the surface of macrophages (Peiser et al., 2002). Among them, macrophage scavenger receptor A (SRA; CD204) has been the most widely studied (Haworth et al., 1997). SRA has the ability to bind and mediate the cellular uptake of modified low-density lipoprotein (LDLs) (Fitzgerald et al., 2000). In addition to modified LDL, this receptor binds to many different ligands such as maleylated bovine serum albumin (BSA), polyribonucleotides, polysaccharides, and aldehyde-modified proteins (Berger et al., 2014; Duryee et al., 2005; de Winther et al., 2000).

MAA-adducted proteins have been shown to be ligands for SRA on liver cells and bronchial epithelial cells (Berger et al., 2014; Duryee et al., 2005). While the effects on hepatic stellate (Kharbanda et al., 2001), liver endothelial and Kupffer cells (Duryee et al., 2004), and airway epithelial cells (Berger et al., 2014) have been reported, the effects of MAA on major macrophage functions and the role of SRA on such effects have not been evaluated. In addition, SPD-MAA, a biologically relevant protein adduct, has never been used in studies of macrophage functions. We, therefore, hypothesized that SPD-MAA, a protein adduct relevant to aldehyde formation in smokers who drink alcohol, modulates macrophage functions primarily via SRA, one of the major receptor for MAA.

MATERIALS AND METHODS

Materials

Protein kinase C (PKC) beta inhibitor LY316976 was purchased from Bio-Techne (Minneapolis, MN); PKC delta inhibitor Rottlerin and PKC alpha inhibitor Gö 6976 were purchased from Millipore (Billerica, MA). PKC zeta inhibitor myristoylated PKC zeta inhibitory peptide and PKC epsilon inhibitor Ro 31-8220 were purchased from Enzo Life Science (Farmingdale, NY). Phorbol myristate acetate (PMA), nitro blue tetrazolium (NBT), fucoidan, fetuin, and parthenolide were purchased from Sigma-Aldrich (St. Louis, MO). Lipopolysaccharide was purchased from Fisher Scientific (Pittsburgh, PA). Rat Anti-mouse SRA (2F8) was purchased from AbD Serotec (Raleigh, NC), and isotype negative control was purchased from Biolegend (San Diego, CA).

Cell Culture and Reagents

RAW 264.7 macrophages were purchased from American Type Cell Culture (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% penicillin/streptomycin and maintained at 37°C in a humidified CO_2 incubator. Resident peritoneal macrophages (PMs) were isolated from C57BL/6 wild-type (WT) mice (Charles River, Wilmington, MA) and SR-A knockout mice bred from homozygous SRA deficient mice ($-/-$) (B6.Cg-$Msr1^{tm1Csk}$/J; Jackson Laboratory, Bar Harbor, ME) as described previously (Zhang et al., 2008). All experimental animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center. Briefly, PMs were isolated by adherence to 24-well, flat-bottom, tissue culture-treated polystyrene plastic plates in which 1×10^6 peritoneal exudate cells were seeded per well and allowed to adhere for 2 hours after which any nonadherent cells were washed away. At this point, the cells were 90% macrophages as confirmed by Diff-Quik™ Stain (Siemens Health Care Diagnostic Inc, Newark, DE). Isolated PMs were used for selective experiments.

Malondialdehyde–Acetaldehyde Synthesis

Human SPD adducted to MAA (SPD-MAA) was prepared as previously reported (Wyatt et al., 2012). Briefly, approximately 1 to 1.5 mg/ml of SPD was incubated with 1.0 mM acetaldehyde and 1.0 mM MDA in pyrogen-free phosphate buffered saline (PBS). The pH was brought to 7.4 and maintained at 37°C for 72 hours. At the end of incubation, the reaction mixture was exhaustively dialyzed against pyrogen-free PBS for 24 hours at 4°C. The endotoxin level in the MAA-SPD was measured by limulus assay and was below the limit of detection.

Pro-Inflammatory Cytokine Release (TNF-α and IL-6)

To identify the role of SPD-MAA on pro-inflammatory cytokine release, RAW 264.7 and resident PMs were treated with different concentrations of SPD-MAA (10 to 200 μg/ml) for different times (6 to 48 hours). The supernatant was then collected and stored at $-80°C$ for later analysis. To block SRA, RAW 264.7 macrophages were preincubated with fucoidan (50 μg/ml) for 72 hours or anti-SRA (10 μg/ml) for 24 hours followed by the addition of SPD-MAA for another 6 hours.

PKC Isoform Inhibition

To identify the role of PKC on MAA adduct-mediated pro-inflammatory cytokine release, RAW 264.7 macrophages were preincubated with different isoform-specific inhibitors to PKC beta (LY316976), PKC delta (Rottlerin), PKC zeta (myristoylated PKC zeta inhibitory peptide), PKC alpha (Gö 6976), and PKC epsilon

(Ro 31-8220) at 1 μM concentration for 1 hour followed by the addition of 200 μg/ml of SPD-MAA for another 6 hours. Resident PMs were incubated with PKC alpha isoform inhibitor, Gö 6976 (1 μM) for 1 hour followed by SPD-MAA treatment for 6 hours. After 6 hours, supernatant was collected and later analyzed for cytokine levels.

NF-κB Inhibition

To identify the role of NF-κB in MAA adduct-mediated pro-inflammatory cytokine release, macrophages were preincubated with 1 μM parthenolide for 1 hour followed by 200 μg/ml SPD-MAA treatment for another 6 hours. After 6 hours, supernatant was collected and later analyzed for pro-inflammatory cytokines.

ELISA

TNF-α and IL-6 levels in the supernatant were measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Phagocytosis

Phagocytosis of zymosan particles was assayed using a commercially available kit (Cell Biolabs, San Diego, CA). First, the macrophages were treated with 200 μg/ml SPD-MAA for 3 hours followed by the addition of zymosan particles (5×10^6) for 0 to 60 minutes and later analyzed for phagocytosis of the zymosan by measuring the optical density at 430 nm according to the manufacturer's instructions. For PMs, the macrophages were treated with 200 μg/ml SPD-MAA for 3 hours followed by the addition of zymosan particles (5×10^6) for 30 minutes.

Superoxide Ion Production

PMA-Stimulated Superoxide Ion Production. Superoxide ion production was assayed using a previously established NBT method (Sim Choi et al., 2006). RAW 264.7 macrophages were pretreated with 200 μg/ml SPD-MAA for 24 hours after which PMA (300 ng/ml) in NBT solution (1 mg/ml) was added to the macrophages. After 1 hour, macrophages were washed with PBS (pH 7.4) twice and later fixed with 100% methanol. The plate was then allowed to air-dry for 10 minutes. After the complete removal of methanol, 120 μl of 2 M potassium hydroxide was added followed by the addition of 140 μl of 100% dimethylsulfoxide and incubated at room temperature for 10 minutes. After 10 minutes, 200 μl of the solution was transferred to another 96-well plate and optical density was measured at 630 nm (BioTek Instruments, Inc, Winooski, VT).

Opsonized Zymosan-Stimulated Superoxide Ion Production. Opsonized zymosan was prepared using a previously established method (Suhonen et al., 2000). First 10 mg of zymosan A (Sigma-Aldrich) was opsonized with 1 ml of normal mouse serum (Invitrogen, Frederick, MD) by incubating at 37°C for 30 minutes followed by washing with PBS (pH 7.4) twice. RAW 264.7 macrophages were pretreated with 200 μg/ml SPD-MAA for 24 hours after which opsonized zymosan (1 mg/ml) in NBT solution (1 mg/ml) was added to the macrophages. After 30 minutes, macrophages were washed with PBS and superoxide ion production was measured as described above.

Nitrite Production. Nitrite production was assayed by measuring nitrite (a stable degradation product of nitric oxide) in the supernatant of cultured RAW 264.7 macrophage using Griess reagent. Briefly, RAW 264.7 macrophages were pretreated with 200 μg/ml of SPD-MAA for 3 hours followed by treatment with 0.3 mg/ml zymosan (Sigma-Aldrich) in Hams F-12 nutrient medium containing 10% FBS. After 24 hours, supernatant was collected and nitrite level was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI).

Real-Time Quantitative RT-PCR. RAW 264.7 macrophages were treated with SPD-MAA (200 μg/ml) for (1 to 24 hours), and later, total RNA was isolated from the macrophages using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity of RNA was checked by measurement of optical density (260/280) ratio using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized using 100 ng of template RNA and a TaqMan reverse transcription kit (Applied Biosystems, Austin, TX). Real-time PCRs were prepared using TaqMan Master Mix (Applied Biosystems) and primers and probe for SRA (Applied Biosystems; Mm 00446214_m1). Ribosomal (18S) RNA was used as an endogenous control. PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Threshold values were normalized to the expression of ribosomal RNA. Real-time PCR results are expressed as the fold change increase in comparison with media control.

SRA Surface Expression. RAW 264.7 macrophages were treated with SPD-MAA (200 μg/ml) for (1 to 24 hours). After treatment, macrophages were washed with ice-cold PBS followed by fixation with 3.7% paraformaldehyde for 10 minutes at 4°C. After fixation, macrophages were washed with PBS followed by incubation with rat anti-SRA labeled with FITC and matched isotype control antibody at 4°C for 1 hour. After incubation, macrophages were washed with PBS 3 times and the samples were analyzed by flow cytometry. Flow cytometry analysis was performed with FACS Calibur available in the UNMC Flow Cytometry Core (BD Biosciences, San Jose, CA).

Statistics

All data were analyzed using GraphPad Prism (version 5.00 for Windows; GraphPad Software, San Diego CA) and represented as mean $\pm$ SE. Data were analyzed for statistical significance using Student's *t*-test and both 1-way and 2-way analyses of variance employing Tukey's multiple comparison posttest corrections. A *p*-value less than 0.05 was regarded as statistically significant.

RESULTS

Cell Viability

Cell viability was assessed by trypan blue exclusion method as described previously (Reissis et al., 2013). No cell death was observed for different concentrations of SPD-MAA over a period of 6 to 48 hours. Also, the PKC alpha inhibitor, NF-κB inhibitor, fucoidan (a ligand for SRA), and anti-SRA did not affect cell viability (data not shown).

SPD-MAA Exposure Induces Pro-Inflammatory Cytokine TNF-α Release

To determine whether exposure to SPD-MAA induces pro-inflammatory cytokine TNF-α release, RAW 264.7 macrophages were exposed to 200 μg/ml of SPD-MAA for different time points (6 to 48 hours). RAW 264.7 macrophages produced TNF-α in response to SPD-MAA in a time-dependent manner. A significant increase ($p < 0.0001$)

in TNF-α release was observed as early as 6 hours in comparison with media control (Fig. 1*A*). Additionally, a dose-dependent increase in TNF-α release was observed in response to SPD-MAA. TNF-α release was significantly increased ($p < 0.001$) following exposure to 100 to 200 μg/ml of SPD-MAA at 6 hours (Fig. 1*B*). No significant increase in TNF-α release was observed when exposed to nonadducted lung SPD (Fig. 1*B*). These data demonstrate that SPD-MAA induces TNF-α release from RAW 264.7

macrophages in both a time- and concentration-dependent manner. Similarly, in PMs isolated from WT mice, we observed a significant dose-dependent ($p < 0.0001$) increase in TNF-α release at 6 hours when treated with 200 μg/ml of SPD-MAA (Fig. 1*C*). No such response was observed in PMs from SRA−/− mice (Fig. 1*C*). In addition, significantly less TNF-α release was observed at 100 μg/ml ($p < 0.05$) and 200 μg/ml ($p < 0.0001$) of SPD-MAA treatment in SRA−/− mice in comparison with WT mice
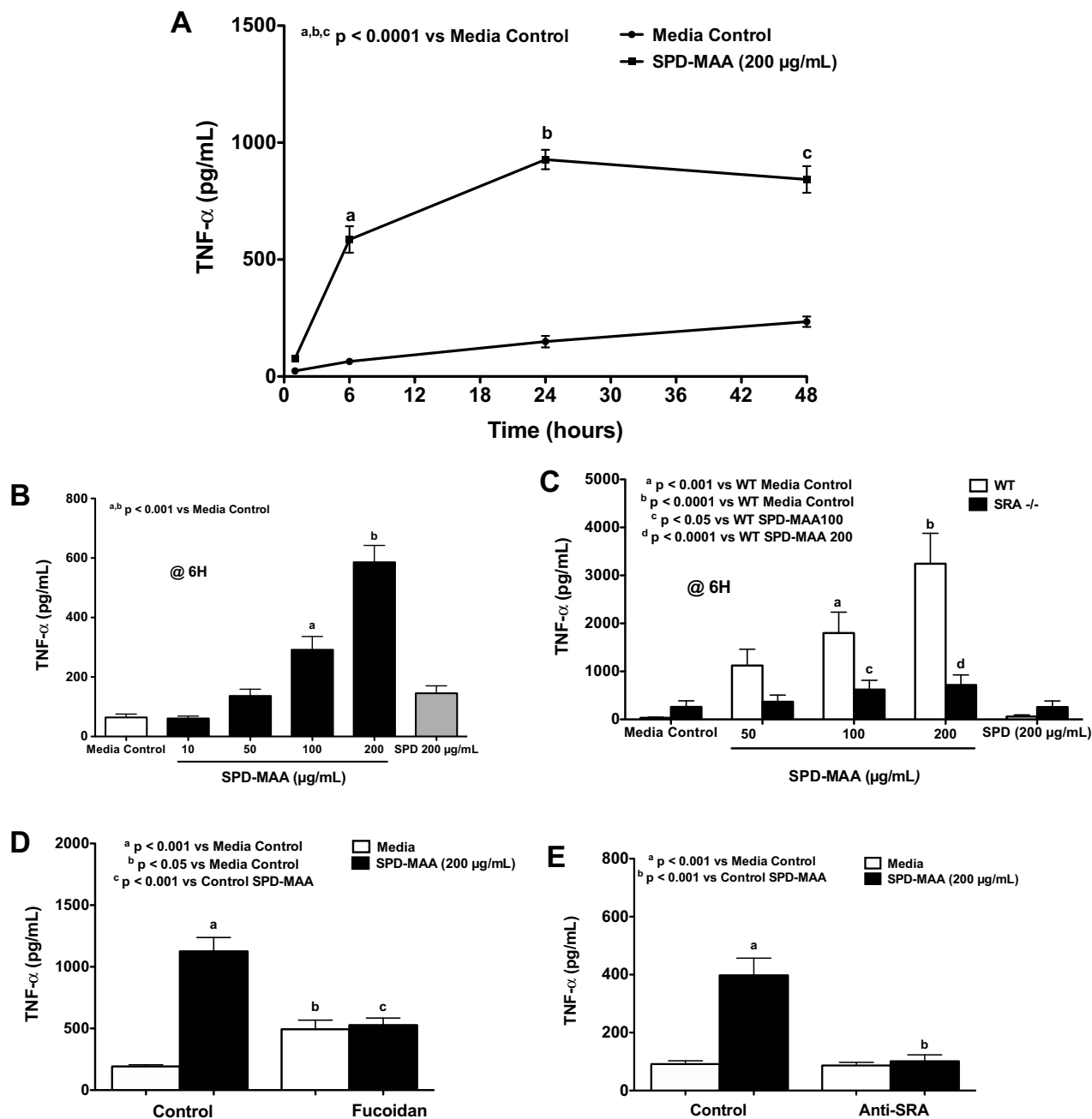
Fig. 1. Effect of SPD-MAA on pro-inflammatory cytokine TNF-α release. TNF-α release from RAW 264.7 macrophages was measured after treatment with 200 μg/ml SPD-MAA for 6, 24, and 48 hours (**A**). After treatment, supernatant was collected and TNF-α level measured by ELISA. SPD-MAA induced TNF-α release from RAW 264.7 (**B**) and wild-type peritoneal macrophages (**C**) in concentration-dependent manner at 6 hours. No such effect was seen in SRA−/− mice (**C**). RAW 264.7 macrophages were pretreated with 50 μg/ml of fucoidan (**D**) for 72 hours or 10 μg/ml of anti-SRA (**E**) for 24 hours followed by treatment of SPD-MAA for 6 hours. Values are the mean ± SEM of 3 independent experiments.

(Fig. 1*C*). Pretreatment with fucoidan, a ligand for SRA, significantly reduced ($p < 0.001$) SPD-MAA-induced TNF-α release in RAW 264.7 macrophages (Fig. 1*D*). No reduction in SPD-MAA-stimulated TNF-α release was observed when macrophages were treated with fetuin, a glycoprotein negative control for fucoidan (data not shown). Treatment with SRA blocking antibody (2F8) significantly reduced ($p < 0.001$) SPD-MAA-stimulated TNF-α release (Fig. 1*E*). No reduction in SPD-MAA-stimulated TNF-α release was observed when macrophages were treated with isotype-matched negative control, IgG (data not shown).

SPD-MAA Exposure Induces Pro-Inflammatory Cytokine IL-6 Release

To determine whether exposure to SPD-MAA induces pro-inflammatory cytokine IL-6 release, PMs from both WT and SRA−/− mice were exposed to different doses of SPD-MAA for 6 hours. A significant increase in IL-6 was detected at the highest concentration of SPD-MAA (200 μg/ml) ($p < 0.0001$) (Fig. 2). No such increase was observed in PMs from SRA−/− mice. We also observed a significant attenuation of IL-6 release at 200 μg/ml of SPD-MAA ($p < 0.0001$) treatment in PMs from SRA−/− in comparison with WT mice (Fig. 2). No time- or dose-dependent increase in IL-6 was observed when RAW 264.7 macrophages were exposed to SPD-MAA (200 μg/ml) for up to 24 hours (data not shown).
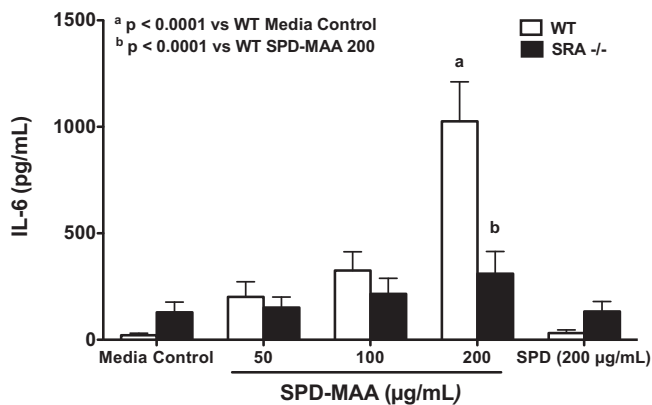
Effect of SPD-MAA on Phagocytosis of Zymosan Particles

To determine the role of SPD-MAA on phagocytic function, RAW 264.7 macrophages were incubated with 200 μg/ml SPD-MAA for 3 hours followed by the addition of zymosan particles for 0 to 60 minutes. Treatment with SPD-MAA significantly decreased the phagocytosis of zymosan
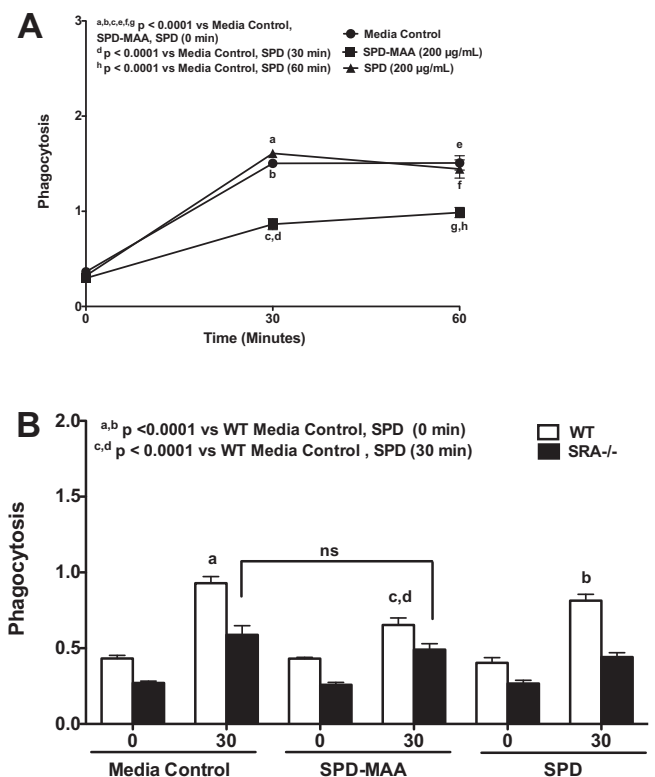
particles at 30 minutes ($p < 0.0001$) and 60 minutes ($p < 0.0001$) in comparison with their respective media control (Fig. 3*A*). No reduction was observed when macrophages were treated with nonadducted SPD only (Fig. 3*A*). A similar result was observed in PMs from WT mice treated with 200 μg/ml SPD-MAA for 3 hours. In PMs, a significant increase in phagocytosis of zymosan at 30 minutes was observed in both media control ($p < 0.0001$) and SPD ($p < 0.0001$) treatment groups when compared to 0 minutes (Fig. 3*B*). When treated with SPD-MAA, a significant decrease in phagocytosis ($p < 0.0001$) was observed at 30 minutes when compared to media control and nonadducted SPD treatments (Fig. 3*B*). The different letters (a, b) are used to indicate the significance from their respective treatment group at 0 minutes (Fig. 3*B*).

Effect of SPD-MAA on Superoxide Ion and Nitrite Release

To determine the role of SPD-MAA on superoxide ion and nitrite ion release, RAW 264.7 macrophages were preincubated with 200 μg/ml SPD-MAA for 24 hours followed by
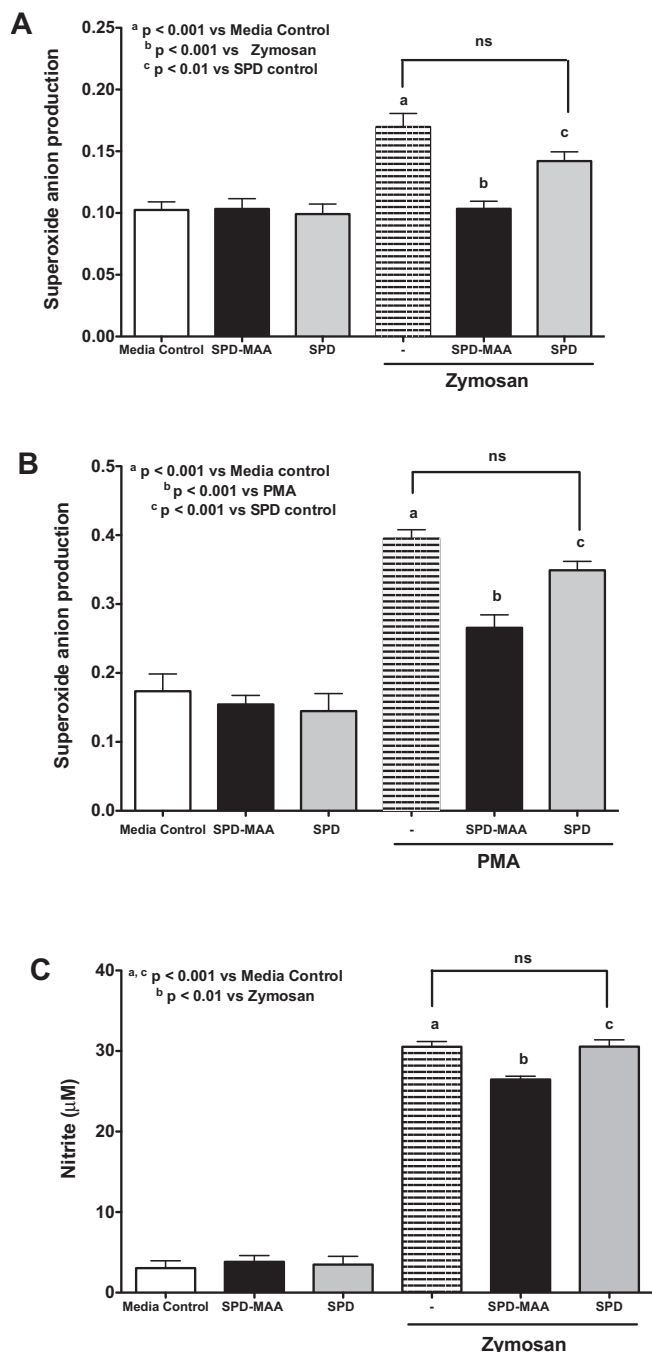

Fig. 3. Effect of SPD-MAA on phagocytosis of zymosan particles. RAW 264.7 macrophages and peritoneal macrophages (PMs) were treated with 200 μg/ml of SPD-MAA for 3 hours followed by the addition of zymosan particles for (0 to 60) minutes. Phagocytosis of the particles was detected as described in Materials and Methods section. SPD-MAA treatment significantly reduced the phagocytosis of particles by RAW 264.7 macrophage (**A**) and PMs (**B**) from wild-type (WT) mice (white bars). No such effect was seen in PMs from SRA−/− mice (**B**; black bars). Values represent mean ± SEM of 3 independent experiments. The different letters (a, b) are used to indicate the significance from their respective treatment group at 0 minutes.

Fig. 2. Effect of SPD-MAA on pro-inflammatory cytokine IL-6 release. Peritoneal macrophages (PMs) from wild-type (WT) and SRA−/− mice were treated 200 μg/ml SPD-MAA for 6 hours. After treatment, supernatant media were collected and IL-6 levels measured by ELISA. SPD-MAA induced IL-6 release from PMs from WT mice at highest concentration at 6 hours. No such effect was seen on PMs from SRA−/− mice. Values represent mean ± SEM of 3 independent experiments.

the addition of PMA (300 ng/ml) for 1 hour or opsonized zymosan for 30 minutes. SPD-MAA significantly reduced superoxide ion release from RAW 264.7 macrophages in response to zymosan ($p < 0.001$; Fig. 4A). A similar result was observed in response to PMA ($p < 0.001$; Fig. 4B).
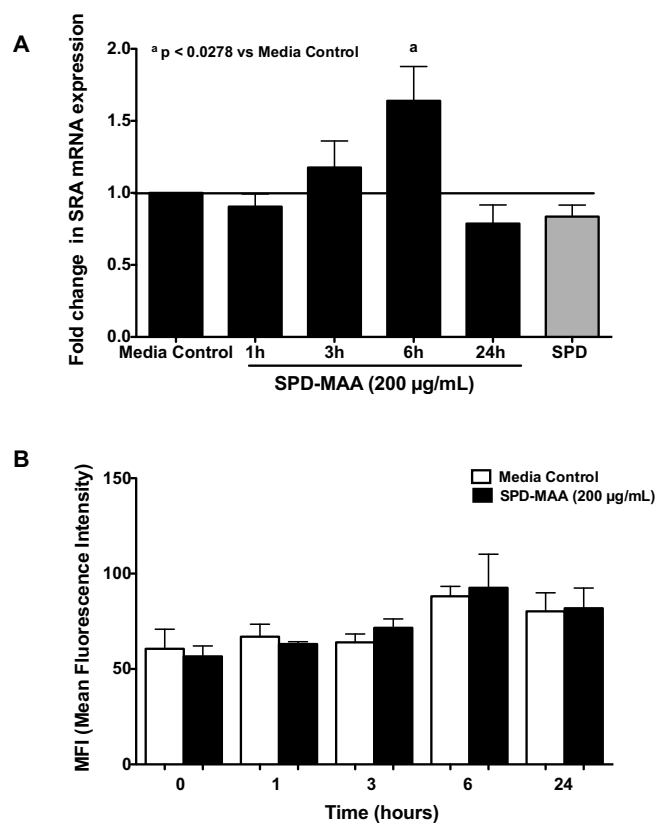
Pretreatment with nonadducted SPD had no such effect (Fig. 4A,B). To measure nitrite release, RAW 264.7 macrophages were pretreated with 200 μg/ml of SPD-MAA for 3 hours followed by the addition of zymosan (0.3 mg/ml) for another 24 hours. SPD-MAA significantly reduced ($p < 0.01$) nitrite release in response to zymosan (Fig. 4C). No reduction in nitrite release was observed when exposed to equal concentration of SPD (Fig. 4C).

Effect of SPD-MAA on SRA Expression

To determine the effect of SPD-MAA on SRA message expression, RAW 264.7 macrophages were treated with 200 μg/ml SPD-MAA for 1, 3, 6, and 24 hours and later analyzed for SRA mRNA expression. A significant up-regulation of SRA mRNA expression was observed at 6 hours ($p < 0.0278$; Fig. 5A). At 24 hours, however, SRA mRNA expression returned to baseline. No such effect on SR-A mRNA was seen when RAW 264.7 cells were treated with SPD for 6 hours (Fig. 5A).

To determine the effect of SPD-MAA on surface expression of SRA, RAW 264.7 macrophages were treated with 200 μg/ml SPD-MAA for 0 to 24 hours and analyzed for

Fig. 4. Effect of SPD-MAA on nitrite and superoxide ion release. RAW 264.7 macrophages were pretreated with 200 μg/ml SPD-MAA for 24 hours followed by treatment with PMA (300 ng/ml) for 1 hour (**A**) or opsonized zymosan (1 mg/ml) for 30 minutes (**B**). After treatment time, superoxide ion was measured using NBT method. For nitrite release, RAW 264.7 macrophages were pretreated with 200 μg/ml SPD-MAA for 3 hours followed by treatment with zymosan (0.3 mg/ml) for another 24 hours. After 24 hours, supernatant was collected and nitrite level measured (**C**). Values represent mean ± SEM of 3 independent experiments.
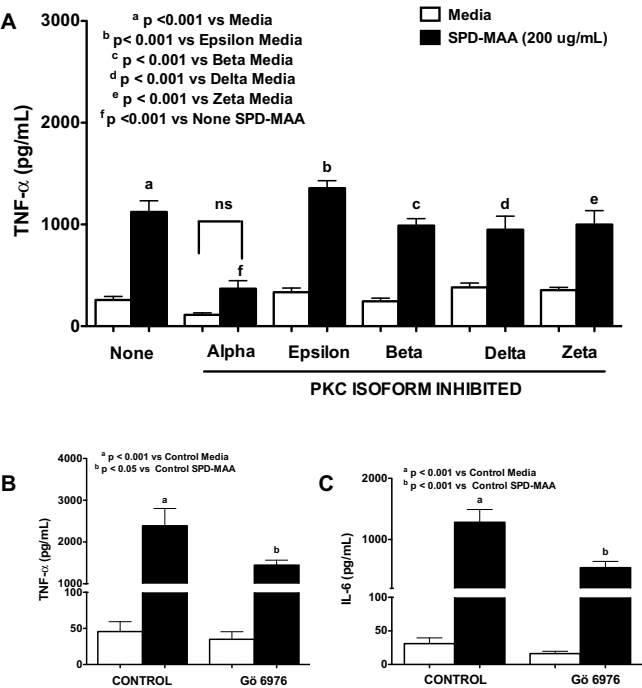

Fig. 5. Effect of SPD-MAA on SR-A expression. RAW 264.7 macrophages were treated with SPD-MAA for (1 to 24 hours) and later analyzed for SRA message (**A**) expression by RT-PCR. Results represent fold change in SRA message expression from media control. RAW 264.7 macrophages treated with SPD-MAA (0 to 24 hours) and later analyzed for SRA surface receptor expression by flow cytometry (**B**). Values represent mean ± SEM of 3 independent experiments.

surface receptor expression by flow cytometry. No significant change in SRA surface expression was detected at 1, 3, 6, or 24 hours (Fig. 5*B*). No rapid transient change in SRA expression was observed at 0 to 30 minutes (data not shown).

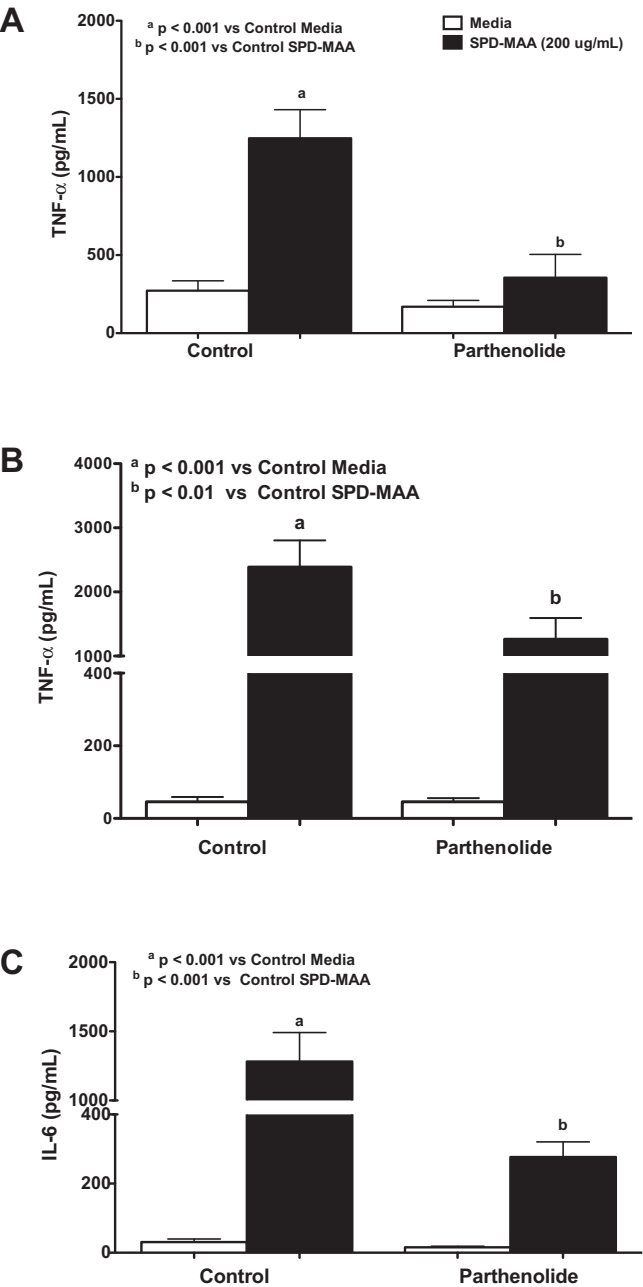### *MAA-Adducted Protein Binds to SRA and Causes PKC Activation*

To identify the role of PKC on SPD-MAA-induced pro-inflammatory cytokine release, RAW 264.7 macrophages were incubated with different PKC isoform inhibitors at 1 μM concentration for 1 hour before addition of 200 μg/ml SPD-MAA for 6 hours. PKC alpha inhibitor Gö 6976 pretreatment significantly decreased TNF-α release from RAW 264.7 macrophages in response to SPD-MAA ($p < 0.001$; Fig. 6*A*). No other PKC inhibitors affected TNF-α release (Fig 6*A*). A similar result was observed when PMs from WT mice were pretreated with PKC alpha inhibitor Gö 6976 for 1 hour ($p < 0.05$) (Fig. 6*B*). In addition to TNF-α, pretreatment with Gö 6976 also significantly decreased IL-6 release from PMs macrophages from WT mice in response to SPD-MAA at 6 hours ($p < 0.001$) (Fig. 6*C*).

MAA-Adducted Protein Binds to SRA and Causes NF-κB Activation

To identify the role of transcription factor NF-κB on SPD-MAA-induced pro-inflammatory cytokine release, RAW 264.7 and PMs were incubated with the NF-κB inhibitor parthenolide at 1 μM concentration for 1 hour before addition of 200 μg/ml SPD-MAA for 6 hours. Parthenolide

Fig. 6. Effect of PKC isoform inhibitors on SPD-MAA-stimulated TNF-α and IL-6 release. RAW 264.7 macrophages (**A**) were pretreated with different PKC isoform inhibitors at 1 μM concentration for 1 hour before addition of 200 μg/ml SPD-MAA for 6 hours (**A**). PMs from WT mice (**B**, **C**) were pretreated with PKC alpha inhibitor Gö 6976 (1 μM) for 1 hour and later treated with 200 μg/ml SPD-MAA or SPD for 6 hours. After treatment, supernatant was collected and TNF-α (**A**, **B**) or IL-6 (**C**) levels were measured by ELISA. Values are the mean ± SEM of 3 to 6 independent experiments.

Fig. 7. Effect of NF-κB inhibitor on SPD-MAA-stimulated TNF-α and IL-6 release. RAW 264.7 macrophages (**A**) were incubated with NF-κB inhibitor (parthenolide) at 1 μM concentration for 1 hour before addition of 200 μg/ml SPD-MAA for 6 hours (**A**). PMs from WT mice were pretreated parthenolide (1 μM) for 1 hour and later treated with 200 μg/ml SPD-MAA for 6 hours SPD (**B**, **C**). After treatment, supernatant was collected and TNF-α (**A**, **B**) and IL-6 (**C**) levels were measured by ELISA. Values are the mean ± SEM of 3 independent experiments.
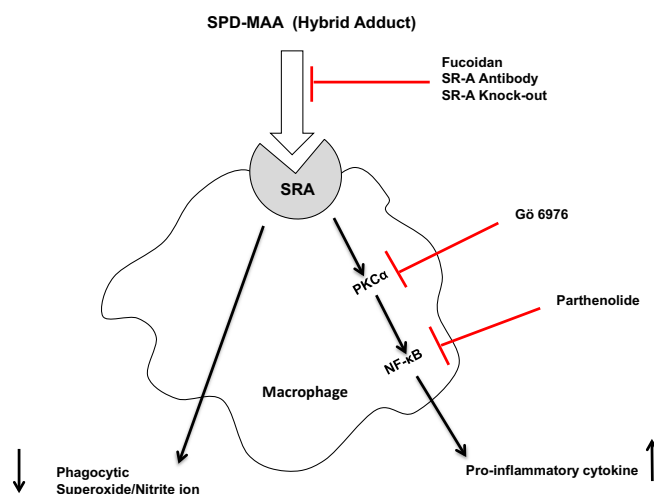
Fig. 8. Proposed model. Hybrid adduct SPD-MAA in the lung binds to SRA expressed on the macrophage surface and modulates macrophage functions. SPD-MAA exposure decreases phagocytosis, superoxide ion, and nitrite release as well as increases pro-inflammatory cytokines TNF-α and IL-6 release. Using SRA competing ligand fucoidan, SRA blocking antibody or knocking-out SRA gene diminishes these modulations. PKC alpha inhibitor Gö 6976 and NF-κB inhibitor parthenolide inhibited pro-inflammatory cytokines release from macrophage in response to SPD-MAA.

at the concentration used had no cytotoxic effect and did not induce apoptosis and oxidative stress (Tiuman et al., 2005; Wen et al., 2002). A significant reduction in TNF-α release was observed in both RAW 264.7 macrophages ($p < 0.001$; Fig. 7A) and PMs ($p < 0.01$; Fig. 7B). In addition to TNF-α, pretreatment with NF-κB inhibitor, parthenolide, significantly reduced IL-6 release from PMs at 6 hours in response to SPD-MAA ($p < 0.001$; Fig. 7C).

DISCUSSION

It is well established that macrophages play an important role in immunity. Therefore, any alterations in macrophage function can lead to inflammation, injury, and infection. Acetaldehyde and malondialdehyde-derived adducts have been shown to induce the release of pro-inflammatory cytokines and chemokines by Kupffer, endothelial, and stellate cells of the liver (Duryee et al., 2004; Kharbanda et al., 2001). In this study, we demonstrated that MAA adduct affects macrophage functions (Fig. 8). Our study shows that SPD-MAA, an MAA adduct to SPD, activates both RAW 264.7 and PMs to release pro-inflammatory cytokines TNF-α and IL-6. In addition to this, our study also shows that MAA adduct affects phagocytic function as well as superoxide ion and nitrite ion release, which were not studied previously. Our study also showed that PKC alpha and NF-κB may be important for MAA-stimulated TNF-α and IL-6 release.

Our data extend the concept that MAA adducts have a pro-inflammatory effect. MAA adducts have been shown to induce pro-inflammatory cytokine TNF-α in a purified rat

heart endothelial cell culture (Hill et al., 1998) and stimulate TNF-α, monocyte chemo-attractant protein-1, and macrophage inflammatory protein-2 in liver endothelial cells (Thiele et al., 2004). Interestingly, our study showed higher levels of TNF-α release from PMs than from RAW 264.7 macrophages. Unlike TNF-α, only very high concentrations of SPD-MAA stimulated IL-6 release from PMs. Differences in both cytokine expression and response between primary macrophages and immortalized macrophage cell lines could explain this result (Chamberlain et al., 2009).

Our data suggest a role for PKC alpha, a calcium-dependent classical PKC isoform and transcription factor NF-κB, in pro-inflammatory cytokine release in response to SPD-MAA from RAW 264.7 and PMs. Other PKC isoform-specific inhibitors failed to inhibit TNF-α release. This is in contrast to our previous finding in epithelial cells (Berger et al., 2014) where PKC epsilon was involved in the MAA-mediated pro-inflammatory cytokine release. This could be due to difference in cell type involved and cytokine measured (Kupffer cells). In our study, PKC alpha inhibitor does not totally inhibit TNF-alpha and IL-6 release when compared to untreated cells. This may suggest other signaling pathways such as p38/JNK involved in addition to PKC alpha as suggested by others (Ben et al., 2013; Hsu et al., 2001; Nikolic et al., 2011).

In our study, the dose of MAA used is higher than required for airway cells (100 μg/ml) (Berger et al., 2014) and the amount detected (500 ng/ml) (McCaskill et al., 2011) in lung of mice after exposure to alcohol and cigarette smoke for 8 weeks. But, this is the amount detected only after 8-week exposure, and people abuse alcohol and smoke cigarettes for years. Therefore, the amount of adduct formed in their lung could be much higher than detected in the mice.

Our study also reports that SPD-MAA significantly reduces the phagocytic function of macrophages in RAW 264.7 and PMs. A significant reduction in superoxide ion and nitrite ion release was observed when RAW 264.7 macrophages were exposed to SPD-MAA. Alcohol has been previously reported to reduce phagocytic function, antimicrobial activity, superoxide ion, and nitric oxide release from macrophages (Andrade et al., 2008; Castro et al., 1993; D'Souza et al., 1996; Libon et al., 1993; Satapathy and Shrivastava, 2012; Szabo, 1999). Our findings may suggest MAA adduct formation could be one of the mechanisms through which alcohol may exert such actions.

Our study shows that SPD-MAA mediates effects in both RAW 264.7 and PMs through SRA. Only a few previous studies have examined a role for scavenger receptor in MAA-mediated effects in mice and bronchial epithelial cells (Thiele et al., 2001). Our study further identified a role for SRA in SPD-MAA-mediated pro-inflammatory cytokine release in macrophages. TNF-α and IL-6 release was significantly reduced in SRA$-/-$ PMs in comparison with cells from WT mice. Pretreatment with fucoidan (a ligand that competes with SPD-MAA for scavenger receptor) or SRA blocking antibody significantly reduced SPD-MAA induced

TNF-α release from RAW 264.7 macrophages. These results suggest that the SPD-MAA adduct most likely binds to SRA and signals the release of cytokines in both RAW 264.7 and PMs. In addition, MAA-mediated reduction in phagocytic function was also SRA dependent. Our results from PMs from SRA−/− mice imply that SRA is important for MAA adduct binding resulting in the modulation of zymosan phagocytosis.

We also report that exposure to SPD-MAA induced SRA mRNA expression in RAW 264.7 macrophages. Although SRA mRNA expression is increased, no change in surface expression of SRA at any time point tested was observed. This finding is in contrast to our previous finding in airway cells where a transient decrease in SRA surface expression was observed after short-term exposure to MAA (Berger et al., 2014). This could be due to difference in the cell type involved. Also SPD-MAA being a ligand for SRA may induce gene expression and subsequent receptor secretion. But due to rapid binding of MAA to the newly expressed receptors, no change in surface receptor expression could be observed even though new SRA receptors are secreted (Murphy et al., 2005).

A major limitation of our study is that our results are limited to RAW 264.7 and PMs. Because SPD is a lung defense protein shown to be the target of MAA adduction, primary lung macrophages would have been ideal to further confirm SPD-MAA effects observed in RAW 264.7 cells and PMs, but they were difficult to obtain in sufficient viable numbers (500,000 cells per well) to perform the extensive studies reported in the current study. In addition, a large number of mice (30 to 50) were required to obtain enough cells to conduct a single experiment. It is important to note that MAA adduct formation can involve a wide variety of target proteins and the MAA moiety has itself been shown to be inflammatory and immunogenic (Wyatt et al., 2012). So several previous studies have utilized nonbiologically relevant proteins such as BSA for lung and liver studies (Kharbanda et al., 2001; Thiele et al., 2004; Wyatt et al., 2001, 2012). Therefore, all studies were carried out in RAW 264.7 and PMs. To overcome the above-mentioned limitation, future in vivo studies using WT and SRA−/− mice are necessary to further confirm results observed in both macrophages and airway epithelial cells. This will also justify the role of SRA in SPD-MAA-mediated lung effects.

To summarize, our current study demonstrates that MAA-adducted proteins modulate certain macrophage inflammatory and effector functions in RAW 264.7 and PMs. Such modulations may involve secretion of pro-inflammatory cytokines such as TNF-α and IL-6 as well as compromised phagocytic and superoxide and nitrite ion release. Additionally, our study also emphasizes the functional role of SRA in mediating the effects of SPD-MAA because in the absence of SRA, MAA failed to reduce macrophage function. Our data also suggest that PKC alpha and NF-κB play roles in MAA adduct-stimulated pro-inflammatory cytokine release by these macrophages.

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