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Malondialdehyde-Acetaldehyde Adduct Formation Decreases Immunoglobulin A Transport across Airway Epithelium in Smokers Who Abuse Alcohol

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Alcohol misuse and smoking are risk factors for pneumonia, yet the impact of combined cigarette smoke and alcohol on pneumonia remains understudied. Smokers who misuse alcohol form lung malondialdehyde—acetaldehyde (MAA) protein adducts and have decreased levels of anti-MAA secretory IgA (sIgA). Transforming growth factor-β (TGF-β) down-regulates polymeric Ig receptor (pIgR) on mucosal epithelium, resulting in decreased sIgA transcytosis to the mucosa. It is hypothesized that MAA-adducted lung protein increases TGF-β, preventing expression of epithelial cell pIgR and decreasing sIgA. Cigarette smoke and alcohol co-exposure on sIgA and TGF-β in human bronchoalveolar lavage fluid and in mice instilled with MAA-adducted surfactant protein D (SPD-MAA) were studied herein. Human bronchial epithelial cells (HBECs) and mouse tracheal epithelial cells were treated with SPD-MAA and sIgA and TGF-β was measured. Decreased sIgA and increased TGF-β were observed in bronchoalveolar lavage from combined alcohol and smoking groups in humans and mice. CD204 (MAA receptor) knockout mice showed no changes in sIgA. SPD-MAA decreased pIgR in HBECs. Conversely, SPD-MAA stimulated TGF-β release in both HBECs and mouse tracheal epithelial cells, but not in CD204 knockout mice. SPD-MAA stimulated TGF-β in alveolar macrophage cells. These data show that MAA-adducted surfactant protein stimulates lung epithelial cell TGF-β, down-regulates pIgR, and decreases sIgA transcytosis. These data provide a mechanism for the decreased levels of sIgA observed in smokers who misuse alcohol. *(Am J Pathol 2021, 191: 1732—1742; https://doi.org/10.1016/j.ajpath.2021.06.007)*

Alcohol misuse has long been known to negatively affect innate lung defense against respiratory pathogens because individuals with alcohol use disorder (AUD) have an increased risk for pneumonia.[1,2] Sustained alcohol exposure in cell and animal models had identified numerous alterations to lung immunity at the level of mucociliary clearance, cytokine responses, and macrophage function.[3] Coincidentally, AUD is associated with cigarette smoking, and cigarette smokers also have an increased risk for pneumonia.[4] Cigarette smoking causes chronic obstructive pulmonary disease (COPD), the third leading disease cause

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of death.[5] Both cigarette smoking and alcohol misuse are implicated in the etiology of COPD, and COPD is a significant predisposing factor to pneumococcal pneumonia.[6] Although reasons for these associations likely are multifactorial, lungs from patients with COPD are deficient in secretory immunoglobulin A (sIgA), an important antimicrobial mucosal agent that can attenuate the risk of pulmonary infection.[7]

Mucosal production of sIgA represents an important aspect of innate lung defense.[8] Circulating monomeric and polymeric IgA antibodies bind to the polymeric Ig receptor (pIgR) basally located on lung epithelial cells, where they are internalized and processed into a dimeric form, and then released as antimicrobial sIgA to the mucosal surface.[9] sIgA prevents bacterial and viral ligands from binding to epithelial cells. IgA also has an established relationship with COPD[10] because lungs of patients with COPD have decreased levels of sIgA.[11] This likely contributes to the decreased antimicrobial defense and increased pathogen-induced exacerbations[12] observed in COPD patients. The COPD lung also has increased transforming growth factor β (TGF-β).[13] Importantly, TGF-β has been shown to decrease airway epithelial IgA processing through the down-regulation of pIgR.[14]

Pathogenesis of alcohol and cigarette smoke co-exposure have been examined in a cohort of individuals (nonsmokers and smokers) with and without AUD.[15] Individuals with alcohol and cigarette co-exposure showed significantly decreased levels of lung sIgA compared with individuals with only cigarette smoking, or individuals with AUD who did not smoke. At 1 week of alcohol cessation, the AUD smoker group showed increases in sIgA. In addition, the highest levels of malondialdehyde−acetaldehyde (MAA)-adducted protein was detected in lungs of smokers with AUD. MAA adducts have been implicated in altering several aspects of airway function such as proinflammatory cytokine release, ciliary beat frequency, and epithelial wound repair.[16] A highly stable covalent modification, MAA has been shown to adduct to surfactant protein D (SPD) in the lung.[17] MAA-adducted proteins bind to scavenger receptor A1 (also known as CD204) on the surface of both airway epithelial cells[18] and macrophages.[15] Because CD204 expression has been associated with an activated and profibrotic M2 macrophage phenotype,[19] significant up-regulation of CD204 has been reported in COPD patients,[20,21] a disease associated with decreased sIgA.

Because the lungs represent a unique target for MAA adduct formation in smokers who drink alcohol, the mechanism of smoke- and alcohol-generated MAA-adducted protein on the reduction of lung sIgA was investigated. It was hypothesized that SPD adducted to MAA (SPD-MAA) would decrease transcytosis of IgA across bronchial epithelium by increasing TGF-β, which in turn decreases expression of epithelial cell pIgR and sIgA in lung. To test this hypothesis, sIgA, pIgR, and TGF-β were measured using bronchoalveolar lavage (BAL) samples from human and mouse, as well as *in vitro* cell models of transcytosis.

Materials and Methods

AUD Bronchoalveolar Lavage Samples

Human BAL samples were obtained through the Colorado Pulmonary Alcohol Research Collaborative. The Institutional Review Board at the University of Colorado Anschutz Medical Campus and the University of Nebraska Medical Center approved this study. Previously obtained de-identified biospecimens from participants were selected to establish four defined groups consisting of nonsmoking non-AUD, non-AUD smokers, nonsmoking AUD, and smokers with AUD, as carefully demographically detailed in previous investigations.[16,22] Aliquots of BAL were processed[22] and delivered to the University of Nebraska Medical Center as previously described.[16]

Mouse Exposure Model

Mouse Model of Cigarette Smoke and Alcohol Co-Exposure

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Both wild-type C57BL/6J mice and scavenger receptor A (CD204) knockout (KO) mice obtained from Jackson Laboratory (Bar Harbor, ME) were used. Four exposure groups were generated, consisting of sham-treated mice, cigarette smoke−exposed mice, alcohol-fed mice, and the combination of smoke- and alcohol-treated mice as previously described in detail.[19,20] BAL was collected and processed as described,[19] and trachea were removed for the production of primary epithelial cells in culture.[21]

Mouse Model of MAA Adduct Nasal Instillation

SPD was purified from pulmonary alveolar proteinosis lung lavage fluid and proteins were adducted with malondialdehyde and acetaldehyde to generate SPD-MAA as described.[17] As a control, bovine serum albumin (BSA) was MAA-adducted in the same manner and used in transcytosis assays. Purified proteins were sterile-filtered and delivered into the lungs of mice using a nasal instillation protocol previously described.[17]

Cell Culture and Treatments

Primary mouse tracheal epithelial cells (MTECs) were cultured for 4 weeks at an air−liquid interface (ALI) as described.[23] Human bronchial epithelial cells (HBECs) were obtained from donor lungs with a minimum of three different donor source cell lines used.[24] The murine alveolar macrophage cell line (MH-S; American Type Culture Collection, Manassas, VA) was cultured as described.[25]

Cell cultures were treated with media, SPD, SPD-MAA, BSA, or MAA-adducted BSA for 4 to 48 hours at various concentrations (1 to 50 μg/mL) on both apical and basal ALI

compartments with 0.1 mg/mL dimeric IgA (Athens Research and Technology, Athens, GA) on the basolateral surface. Media were collected for subsequent protein analysis.

To control for any treatment injury leading to artifactual results, transepithelial electrical resistance measurements using electric cell impedance sensing (Applied Biosciences, Troy, NY) were made before and after all treatment conditions to rule out loss of barrier function in the cell monolayer.[26] Cell viability was maintained under all conditions (>95% viable) as determined by a lactate dehydrogenase kit (Sigma, St. Louis, MO).

Enzyme-Linked Immunosorbent Assay

Both human and mouse BAL and ALI samples were collected and stored at −80°C for protein analyses using enzyme-linked immunosorbent assay (ELISA).

ELISA for sIgA

Donor HBECs were expanded on porous Transwell inserts and subsequently differentiated into a pseudostratified mucociliary epithelium. ALI cultures were treated for 24 hours in both apical and basal compartments with 0.6 mL MAA-adducted protein with or without interferon-γ (IFN-γ), an inflammatory stimulus as a positive control,[27] an equal amount of phosphate-buffered saline diluted in the culture media as a negative control, and nonadducted SPD as a carrier protein control. Commercially available polymeric IgA (pIgA) was placed in the basal compartment at 0.1 mg/mL and incubated for an additional 24 hours. Inserts were washed on the apical side at 48 hours to collect transported IgA. sIgA was measured by ELISA as previously described.[11]

ELISA for MAA Adducts

MAA-adducted proteins were assayed in human BAL samples by indirect competitive ELISA as previously described.[20]

ELISA for TGF-β

TGF-β1 and TGF-β2 were measured by sandwich ELISA in BAL and cell culture samples as previously described.[14]

Quantitative RT-PCR for pIgR mRNA

ALI cells were harvested for RNA isolation after the various treatment conditions. As previously described,[11] total RNA from HBECs was extracted using the RNAqueous-Micro Kit (Applied Biosystems/Ambion, Austin, TX), according to the manufacturer's protocol. Total RNA from ALI-cultured cells was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA), according to the manufacturer's specifications. Primer sequences were as follows: pIgR forward: 5′-CTCTCTGGAGGACCACCGT-3′, reverse: 5′-CAGCCGTGACATTCCCTG-3′; hypoxanthine phosphoribosyltransferase 1 (HPRT) forward 5′-TGCTCGAGATGTGATGAAGGAG-3′, reverse 5′-TGATGTAATCCAGCAGGTCAGC-3′.

Immunolocalization of pIgR

Primary HBECs were grown on ALI and pretreated for 24 hours with 50 μg/mL control SPD or SPD-MAA. pIgA then was placed in the basal media for an additional 24 hours. Media were removed and cells were fixed and permeabilized in a blocking-permeabilization solution (0.05 mol/L Tris, 0.02% sodium azide, 0.5% Triton X-100 (Sigma), and 1% BSA) for 30 minutes. Cells were stained for pIgR (R&D Systems, Minneapolis, MN), acetylated α-tubulin (anti-rabbit; Santa Cruz (Dallas, TX): 6 to 11-B1), and cell nuclei (DAPI; Thermo Fisher, Waltham, MA) following a previously described method.[28] The fluorochromes used were Alexa 488 and 540. Cell monolayers were imaged by confocal microscopy using a Zeiss LSM 510 confocal laser-scanning microscope (Göettingen, Germany), using an argon/krypton laser (488 nm/568 nm/647 nm) provided by the University of Nebraska Medical Center Confocal Microscopy Core.

Statistical Analysis

Data are presented as means ± SEM. To detect significant changes between groups, a one-way analysis of variance was used and a *post hoc* test (Tukey or nonparametric Mann-Whitney) was performed to account for multiple comparisons if the P value was <0.05. For all analyses, Prism software version 8.31 (GraphPad, La Jolla, CA) was used.

Results

Lung sIgA Levels Are Reduced in Smokers with AUD

Total sIgA levels were measured using BAL fluid samples from a cohort of well-characterized individuals with and without cigarette smoking and alcohol use histories. The highest levels of sIgA detected in BAL were observed in the nonsmoking, non-AUD control group. A small but significant reduction in BAL sIgA was observed in both non-AUD smokers ($P < 0.05$) as well as nonsmoking AUD subjects ($P < 0.05$) compared with the aforementioned control group. However, a significantly large ($P < 0.0001$) decrease in BAL sIgA was measured in the AUD smoking group compared with the other three groups (Figure 1A). Likewise, the formation of smoke- and alcohol-generated MAA-adducted protein was significantly present ($P < 0.0001$) only in the AUD smoking group compared with the other three groups (Figure 1B).

Lung sIgA Levels Are Reduced in a Mouse Model of Smoke+Alcohol Co-Exposure

A mouse model of cigarette smoke and alcohol co-exposure was developed to minimize the exposomal variables present

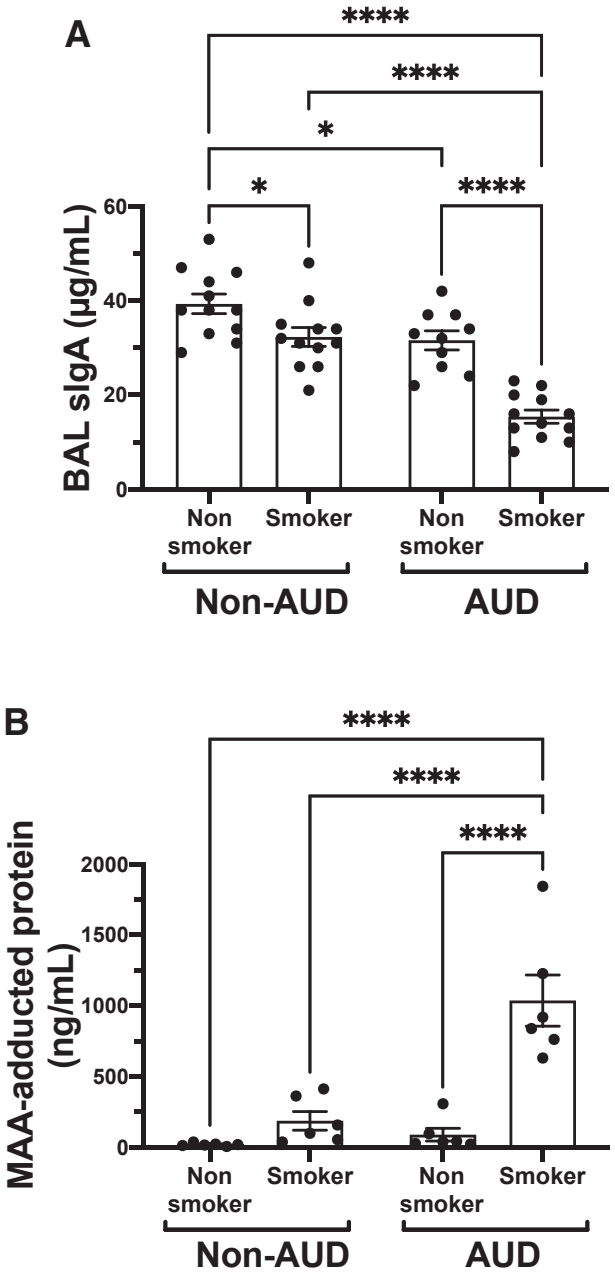

Figure 1 Lung secretory IgA (sIgA) and malondialdehyde—acetaldehyde (MAA) in smokers with alcohol use disorder (AUD). Bronchoalveolar lavage (BAL) fluid from nonsmoker non-AUD, smoker non-AUD, AUD nonsmoker, and AUD smoker subjects measured for sIgA (**A**) or MAA-adducted protein (**B**) by enzyme-linked immunosorbent assay. Values are presented as means ± SEM. $n = 12$ for sIgA (**A**); $n = 6$ for MAA (**B**). $^{*}P < 0.05$, $^{****}P < 0.0001$ (one-way analysis of variance with Tukey *post hoc* multiple comparisons).

in human subjects.[19] BAL samples were collected from smoke+alcohol co-exposed mice as well as individual smoke or alcohol mice to measure sIgA levels. Both smoke-treated ($P < 0.0001$) and alcohol-fed ($P < 0.0001$) mice showed significantly decreased levels of lung sIgA compared with sham-exposed control mice exposed only to room air and water (Figure 2A). Importantly, the smoke+alcohol co-exposure mice further showed significant reductions in sIgA compared with either smoke ($P < 0.05$) or alcohol ($P < 0.0001$) single-exposure groups. Because only co-exposure to smoke+alcohol results in the formation of large amounts of MAA-adducted protein in mouse lung,[20] sterile MAA-adducted lung SPD was instilled repetitively into mouse lungs over a 3-week period and the effect on lung sIgA levels was evaluated. Nasal instillation of wild-type mice with 50 µg/mL SPD-MAA resulted in a significant ($P < 0.01$) reduction in BAL sIgA levels compared with mice instilled with sterile saline (Figure 2B). As a control, non-adducted SPD produced no significant reduction in lung sIgA levels after repetitive nasal instillation of mice. Because CD204 [scavenger receptor A (SRA)] is a receptor for MAA-adducted protein, the same nasal instillations also were performed using CD204 KO mice. Interestingly, although CD204 KO mice showed relatively diminished sIgA levels in BAL compared with wild type, no significant differences in sIgA levels were detected in SPD- or SPD-MAA—instilled mice lacking scavenger receptor A.

MAA-Adducted Protein Decreases Lung Epithelial Cell Transcytosis of sIgA

An *in vitro* ALI cell culture model of airway epithelial cell IgA transcytosis was used to better define epithelial cell sIgA responses to MAA-adducted protein. MTECs were isolated and cultured until confluent monolayers were established. Upon addition of 5 ng/mL pIgA to the basal media compartment only, control-media-only—treated MTECs processed and released maximal amounts of sIgA by 48 hours, while 50 µg/mL MAA-treated MTECs showed a significantly ($P < 0.05$) reduced level of pIgA transcytosis (Figure 3A). Treatment with either MAA-adducted SPD or MAA-adducted BSA reduced pIgA transcytosis regardless of the target adducted protein, while nonadducted SPD or BSA altered transcytosis of pIgA. As a positive control, 200 U/mL IFN-γ stimulated a significant increase in transcytosis of pIgA in MTEC ALI cultures over baseline media (Supplemental Figure S1). Similar to that seen with BAL sIgA levels, SPD-MAA treatment did not result in reduced sIgA in MTECs from scavenger receptor A KO mice compared with those cells cultured from wild-type mice (Figure 3B). None of the cell treatment conditions resulted in a loss of viability (not shown) or transepithelial electrical resistance (Supplemental Figure S2). Importantly, the same reduction in sIgA in response to SPD-MAA was observed in primary HBEC ALI cultures ($n = 3$ normal donors) (Figure 3C).

MAA-Adducted Protein Decreases Airway Epithelial Cell pIgR Expression

The mechanism for MAA-adducted protein-induced reduction of sIgA release was investigated in primary HBECs.

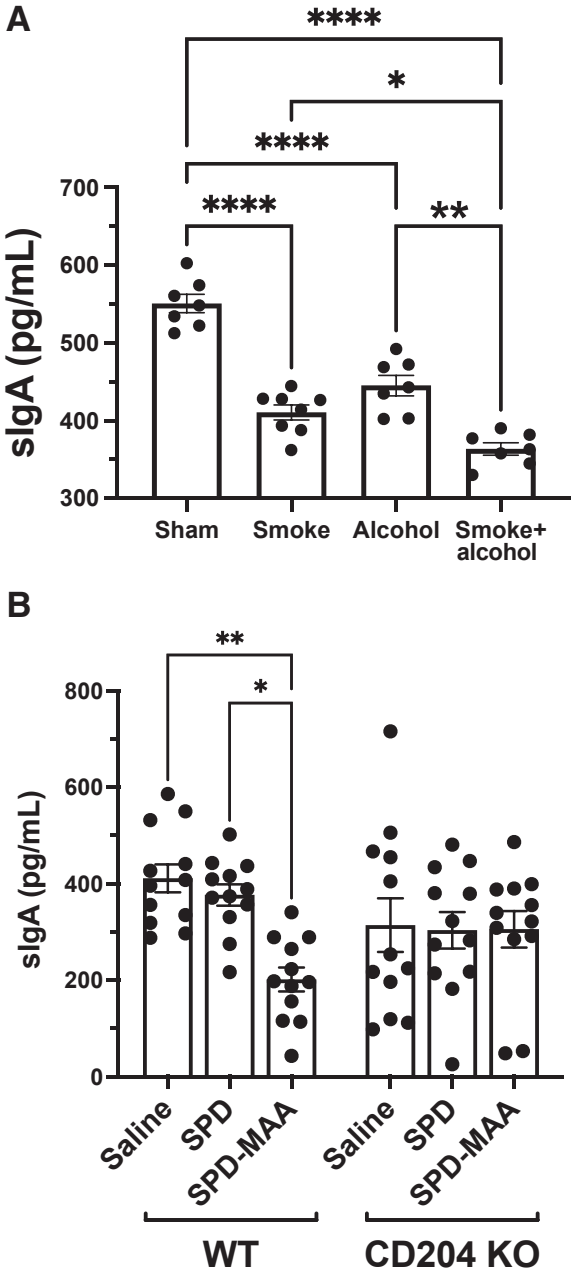

Figure 2 **A:** Lung secretory IgA (sIgA) in mice exposed to cigarette smoke, alcohol, and malondialdehyde—acetaldehyde (MAA)-adducted protein. Mouse bronchoalveolar lavage (BAL) fluid representing handling control (Sham), cigarette smoke exposure (Smoke), *ad libitum* alcohol-fed (Alcohol), or co-exposure to both smoke and alcohol (Smoke+alcohol) for 8 weeks and assayed for sIgA. **B:** Wild-type (WT) or scavenger receptor A knock out (CD204 KO) mice nasally instilled for 3 weeks with either sterile phosphate-buffered saline (Saline), 50 μg/mL surfactant protein D (SPD), or 50 μg/mL MAA-adducted surfactant protein D (SPD-MAA) and BAL assayed for sIgA. Values are presented as means ± SEM. $n = 7$ (**A**); $n = 12$ (**B**). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$ (one-way analysis of variance with Tukey *post hoc* multiple comparisons).

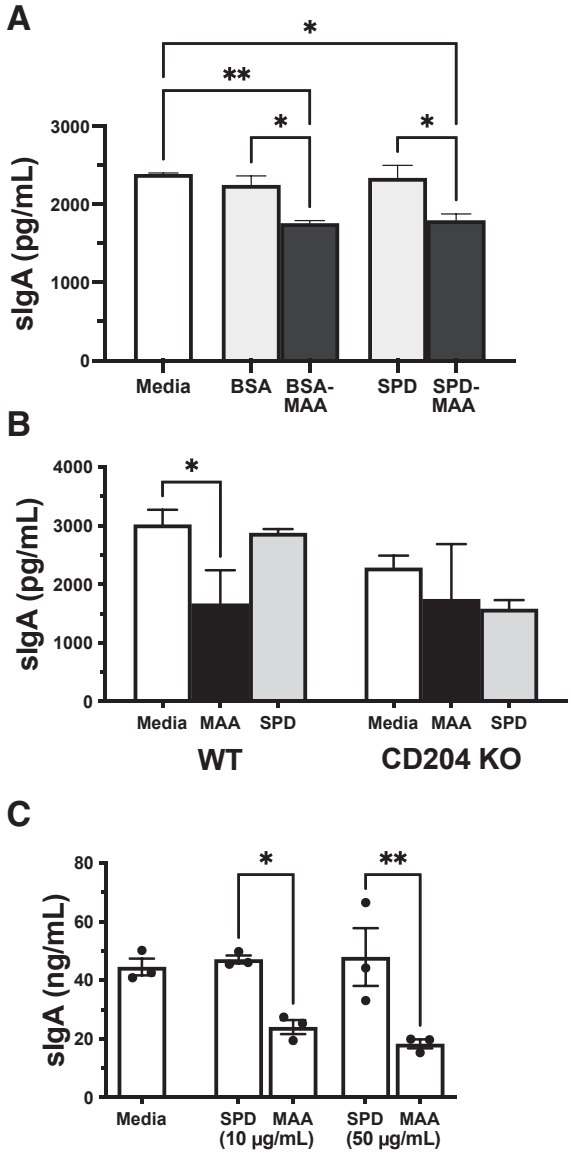

Figure 3 Secretory IgA (sIgA) transcytosis in airway epithelial cells treated with malondialdehyde—acetaldehyde (MAA)-adducted protein. Primary mouse tracheal epithelial cells (MTECs) (**A** and **B**) and human bronchial epithelial cells (HBECs) (**C**) grown on an air—liquid interface were pretreated with MAA-adducted protein in both basal and apical compartments for 24 hours followed by the addition of dimeric IgA in the basal compartment and apical sIgA release measured by enzyme-linked immunosorbent assay. Cells were treated with control media, nonadducted bovine serum albumin (BSA), MAA-adducted BSA (BSA-MAA), nonadducted surfactant protein D (SPD), or MAA-adducted SPD (SPD-MAA) (**A**). MTECs cultured from wild-type (WT) or scavenger receptor A knock out (CD204 KO) mice were treated with media, MAA-adducted SPD (MAA), or nonadducted SPD (SPD) (**B**). HBEC cultures were treated with 10 or 50 μg/mL of MAA-adducted SPD (MAA), or nonadducted SPD (SPD) (**C**). Values are presented as means ± SEM. $n = 3$ donors (**A**—**C**). $^{*}P < 0.05$, $^{**}P < 0.01$ (one-way analysis of variance with Tukey *post hoc* multiple comparisons).

HBECs ($n = 3$ normal donors) cultured on ALI were treated for 48 hours with either media, 200 U/mL IFN-γ, 50 μg/mL SPD-MAA, or both IFN-γ+MAA and pIgR mRNA measured by quantitative RT–PCR. Compared with control media-treated cells, SPD-MAA decreased significantly ($P < 0.01$) while IFN-γ increased pIgR mRNA expression (Figure 4A). Co-treatment with SPD-MAA also significantly reduced ($P < 0.05$) the IFN-γ—stimulated increase in

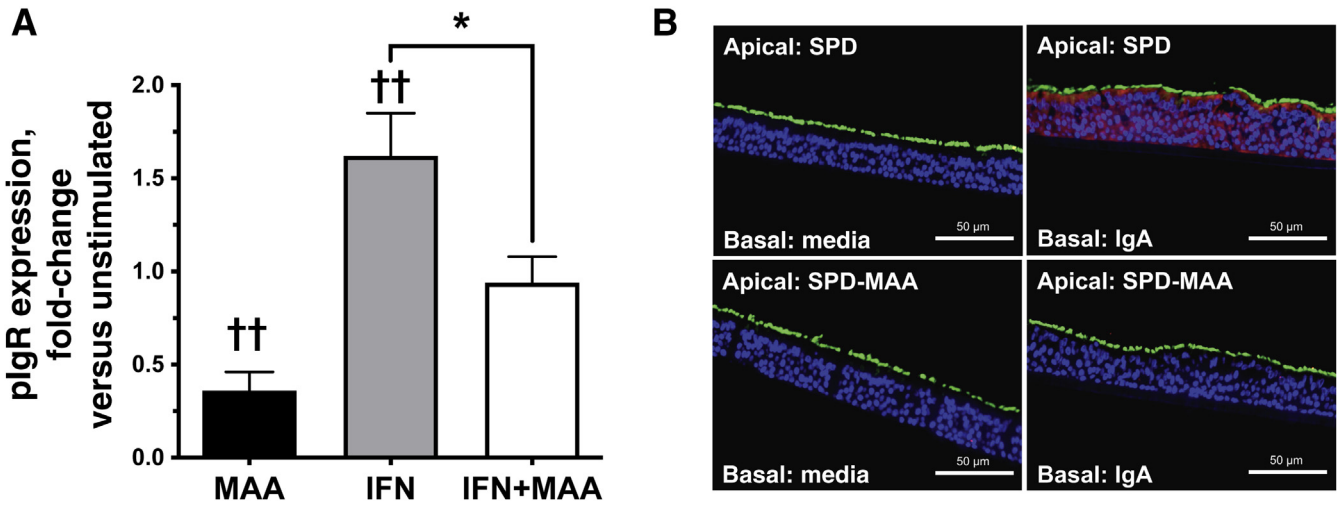

Figure 4 Polymeric Ig receptor (pIgR) expression in malondialdehyde—acetaldehyde (MAA)-adducted protein-treated airway epithelial cells. **A:** Primary human bronchial epithelial cells (HBECs) grown on an air—liquid interface and pretreated for 24 hours with 50 μg/mL MAA-adducted SPD (MAA), 200 U/mL interferon-γ (IFN; positive control), or both IFN and MAA in the apical and basal compartments. Cells were assayed for pIgR mRNA. Values are presented as means ± SEM. **B:** Representative confocal images from primary HBECs grown on an air—liquid interface and pretreated for 24 hours with 50 μg/mL control SPD or SPD-MAA. pIgA then was placed in the basal media for 24 hours and cells were stained for pIgR. pIgR is shown in red, cilia is shown in green, and nuclei are shown in blue. pIgR up-regulation was detected in response to IgA in the presence of SPD, but not when cells were pretreated with SPD-MAA. $n = 3$ donors (**A**). $^{*}P < 0.05$ (one-way analysis of variance with Tukey *post hoc* multiple comparisons); $^{\dagger\dagger}P < 0.01$ versus untreated control cells (one-way analysis of variance with Tukey *post hoc* multiple comparisons). Scale bars = 50 μm. SPD, surfactant protein D.

pIgR mRNA. Protein expression and localization then were determined by confocal microscopy of pIgR in fully differentiated HBECs cultured on ALI. HBECs were pretreated apically for 24 hours with 50 μg/mL SPD or SPD-MAA, followed by the basal addition of 5 ng/mL pIgA for an additional 24 hours. Up-regulation of pIgR was detected in response to basal IgA in SPD-treated HBECs, but not in SPD-MAA—treated cells (Figure 4B). HBECs cultured for 48 hours in the absence of pIgA in the basal compartment showed little protein staining for pIgR.

Lung TGF-β Levels Are Increased in Smokers with AUD

TGF-β has been shown to decrease pIgR in airway epithelium.[14] BAL from a smoking and alcohol use cohort to measure TGF-β was examined. The highest levels of TGF-β detected in BAL were found in the smoking AUD group (Figure 5A). Smaller, but significantly increased ($P < 0.05$), levels of TGF-β also were observed in both non-AUD smokers as well as nonsmoking AUD subjects compared with the control nonsmoking, non-AUD group.

MAA-Adducted Protein Increases Lung Epithelial Cell TGF-β

To further investigate the mechanism of MAA-adducted protein suppression of pIgR, TGF-β production after MAA exposure in primary HBECs was measured. SPD-MAA (10 to 50 μg/mL at 48 hours) treatment produced a dose-dependent increase ($P < 0.001$) in TGF-β release (Figure 5B). Identical treatment with nonadducted SPD produced no change in TGF-β compared with control media-treated HBECs (data not shown). Similarly, SPD-MAA stimulated a significant release ($P < 0.0001$) of TGF-β in MTECs, although fourfold to fivefold less than that observed in HBECs (Figure 5C). Under the same treatment conditions, but in MTECs from CD204 KO mice, SPD-MAA did not increase TGF-β release significantly. Levels of TGF-β after media and nonadducted SPD treatments were increased in CD204 KO MTECs, but were not significantly different from wild-type MTECs.

MAA-Adducted Protein Increases Lung Macrophage TGF-β

Because lung macrophages produce greater amounts of TGF-β than epithelial cells and MAA-adducted protein binds to macrophages,[29] the effect of SPD-MAA on TGF-β release was examined using MH-S cells, a mouse lung macrophage cell line. SPD-MAA, but not nonadducted SPD, dose-dependently increased the release of TGF-β maximally by 48 hours (Figure 6A) at a treatment dose of 50 μg/mL (Figure 6B).

Discussion

Stable, hybrid MAA adducts are highly immunogenic and contribute to a robust antibody response.[30] An increased quantity of IgA specific to MAA in the serum of AUD smokers has been reported, in parallel with decreased levels

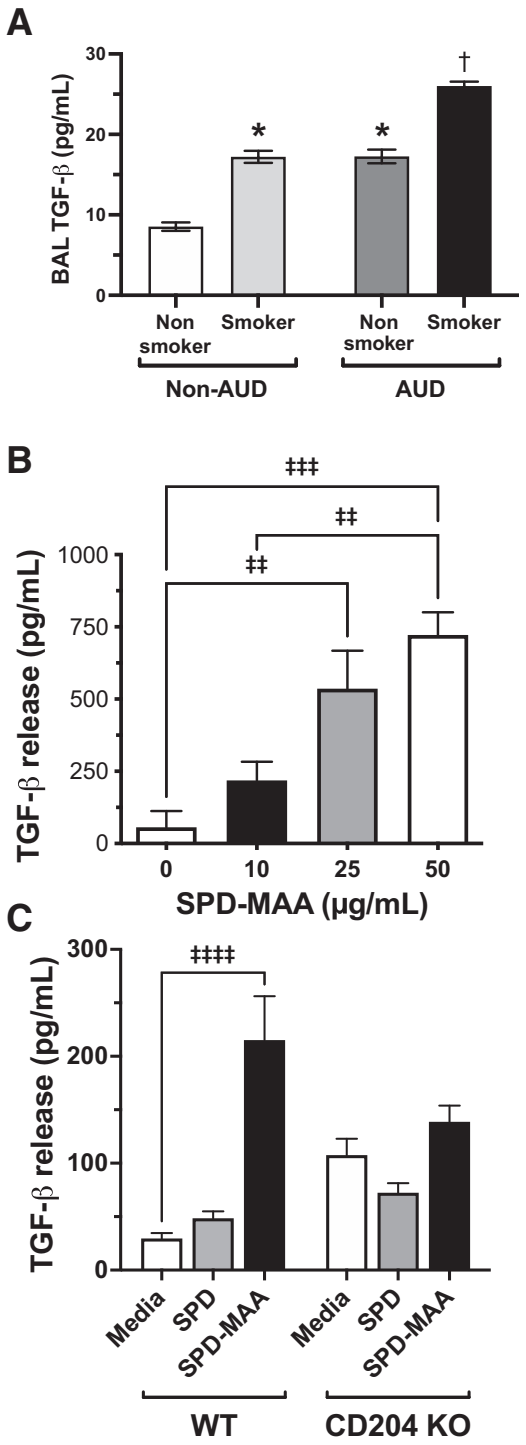

Figure 5 Transforming growth factor-β (TGF-β) in smokers with alcohol use disorder (AUD). **A:** Bronchoalveolar lavage (BAL) fluid from nonsmoker non-AUD, smoker non-AUD, AUD nonsmoker, and AUD smoker subjects measured for TGF-β by enzyme-linked immunosorbent assay. Values are presented as the means ± SEM. **B:** Primary human bronchial epithelial cells (HBECs) were treated with 10 to 50 μg/mL malondialdehyde–acetaldehyde–adducted surfactant protein D (SPD-MAA) for 48 hours and supernatants were assayed for TGF-β. Mouse tracheal epithelial cells (MTECs) cultured from wild-type (WT) or scavenger receptor A knock-out (CD204 KO) mice were treated with media, MAA-adducted SPD (MAA), or nonadducted SPD (SPD) (**C**). $n = 12$ (**A**). $^{*}P < 0.05$ versus non-AUD nonsmoker; $^{\dagger}P < 0.05$ versus non-AUD smoker and AUD nonsmoker (one-way analysis of variance with Tukey *post hoc* multiple comparisons); $^{\ddagger\ddagger}P < 0.01$, $^{\ddagger\ddagger\ddagger}P < 0.001$, and $^{\ddagger\ddagger\ddagger\ddagger}P < 0.0001$.

of secreted MAA IgA in BAL from these same individuals.[15] A synergistic *in vivo* effect of cigarette smoke and alcohol feeding on the production of MAA-adducted protein (<100 ng/mL smoke or alcohol alone versus >300 ng/mL smoke+alcohol) is shown in the lungs of mice.[20] Similar to the mouse model of MAA formation, wherein significant levels of MAA were observed only in the smoke and alcohol co-exposure group,[20] MAA-adducted protein was most abundant in BAL from the AUD smoker group in the current investigation. Although both non-AUD smokers and AUD nonsmokers showed significant reductions in sIgA compared with the nonsmoking non-AUD control group, more striking decreases in total sIgA levels in BAL from AUD smokers was observed along with the greatest quantity of total MAA-adducted protein compared with either single-exposure group. The findings extend previous observations that cigarette smoking decreases lung sIgA[31] by showing an additional impact of alcohol misuse. Recently, sIgA was reported to be highly effective at binding to the S-protein of severe acute respiratory syndrome coronavirus 2 and neutralizing binding to the angiotensin-converting enzyme 2 receptor in airway epithelium using a humanized mouse model of IgA expression,[10] suggesting a role for sIgA in innate coronavirus disease-2019 defense.

The finding of reduced sIgA using an established mouse model of controlled cigarette smoke and alcohol exposure[19] was recapitulated, whereby combination exposure resulted in further sIgA reduction compared with individual treatments. This alcohol feeding model uses the established *ad libitum* Meadows-Cook diet that already has shown an alcohol-mediated reduction in IFN-γ, a stimulator of IgA transcytosis.[32] To determine if co-exposure to alcohol and smoke reduces sIgA in response to MAA-adducted protein formation, the previously characterized mouse model of purified MAA-adducted protein nasal instillation was used to deliver SPD-MAA into the lung airways.[17] Repetitive doses of SPD-MAA (15 doses over 3 weeks) resulted in a significant reduction in mouse sIgA, but the same non-adducted SPD had no such effect, suggesting that alcohol and cigarette smoke co-exposure–derived MAA adduct formation represents one etiology of sIgA reduction. Because it was established previously that MAA-adducted protein binds to scavenger receptor A (CD204) in the lung,[18] sIgA transcytosis in response to MAA using the CD204 KO mouse was evaluated. No significant sIgA reduction in SPD-MAA from control saline or SPD instilled mice was detected. Of note, levels of sIgA were quantitatively lower in the CD204 KO mice, which may explain previous reports of a decreased injury response in these mice.[15,33]

An established model of transcytosis was used to investigate the mechanism of MAA suppression of sIgA release.[14] This model takes advantage of airway epithelial cells cultured on a permeabilized membrane to form a polarized ALI that can bind, internalize, and process dimeric

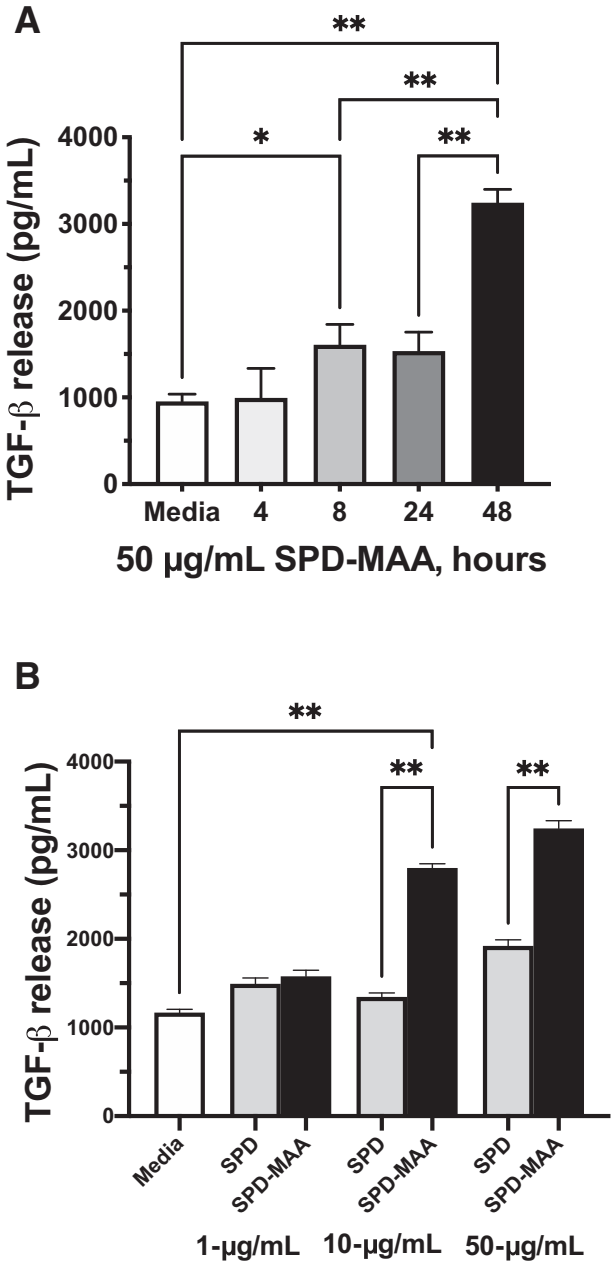

Figure 6 Transforming growth factor-β (TGF-β) release from malondialdehyde—acetaldehyde (MAA)-adducted protein-treated alveolar macrophage cell line. Time course (**A**) and dose response (**B**) in an alveolar macrophage cell line (MH-S) treated with MAA-adducted surfactant protein and TGF-β measured by enzyme-linked immunosorbent assay. Values are presented as means ± SEM. $*P < 0.05$, $**P < 0.0001$ (one-way analysis of variance with Tukey *post hoc* multiple comparisons).

IgA placed in the basal compartment, and release sIgA into the apical compartment. This model was used to recapitulate the *in vivo* findings that SPD-MAA reduces sIgA release, in experiments with primary mouse tracheal as well as human bronchial epithelial cells, and dissect specific transcytosis pathway responses to MAA. Consistent with the established role of pIgR in transcytosis,[9] a MAA-induced down-regulation in mRNA and protein expression was observed in isolated epithelial cells treated in culture for 48 hours. Consistent with Gohy et al,[14] it was found that SPD-MAA increases both MTEC and HBEC TGF-β, a mediator known to decrease IgA secretion via pIgR down-regulation. Previous studies support that TGF-β levels are increased in the BAL fluid of patients with ventilator-associated pneumonia[34] and in the BAL fluid of experimental animal models of chronic alcohol use,[35] however, until now, no study has shown that TGF-β levels are increased in the BAL fluid of patients with AUD. MAA-stimulated production of TGF-β was diminished in the CD204 KO mouse. Because the macrophage is a significant source for lung TGF-β, SPD-MAA was shown to stimulate significant release of TGF-β from a mouse lung macrophage cell line, suggesting an additional source of increased TGF-β for transcytosis suppression. These data support a role for MAA in the TGF-β—induced reduction of pIgR in the suppression of IgA transport across lung epithelium.

Smoking in the context of AUD is extremely common; however, downstream pathologic effects from combined exposures have been poorly described in the literature. Consistent with reported TGF-β reduction in the BAL of smokers,[36] decreased TGF-β was detected in the non-AUD smoking group. However, the work is novel in its observations of enhanced TGF-β production with decreased sIgA in AUD smokers. The current study unraveled a novel mechanism for such TGF-β production in response to the reactive aldehydes generated from smoke and alcohol. Although such aldehydes can be derived from either tobacco pyrolysis or alcohol metabolism, the additive impact of combination use most certainly produces the unique formation of the MAA adduct that was not observed to form in the same concentrations from only smoke or only alcohol. The combination of smoke and alcohol is an additive effect that is required for the production of this bioactive aldehyde adduct. Recently, the up-regulation of the *PIGR* gene was identified in AUD active smokers.[37] This supports the impact of combined smoke and alcohol on the pIgR pathway and suggests a potential feedback response to the observed reduction of transcytosed sIgA in the AUD smoker group. The study further suggests that MAA decreases pIgR expression to attain this effect.

MAA-adducted protein is likely not the only mediator of alcohol-associated dysfunctional IgA responses in lung. Existing roles for IL-17, IL-13, and IFN-γ in alcohol modulation of sIgA have also been reported. T-helper 17 cell production of IL-17 greatly stimulates pIgR.[38] Trevejo-Nunez et al[39] showed that alcohol decreases IL-17. A role for MAA-CD204 in down-regulating IL-17 needs to be explored. In addition, asthma and pIgR down-regulation via TGF-β[40] establishes a role for IL-13 because exogenous IL-13 significantly inhibited secretory component release and pIgR expression. Increased serum IL-13 has been observed in AUD.[41] Alcohol blunts IFN-γ release in mouse lung,[42] although this does not appear to be the mechanism in MH-S

macrophages. Chronic alcohol suppresses T-cell–derived IFN-γ in alcohol-fed mice.[43] Spontaneous (basal) release of tumor necrosis factor α by alveolar macrophages was the same in AUD and non-AUD humans. Chronic alcohol consumption significantly suppresses lipopolysaccharide-stimulated alveolar macrophage production of tumor necrosis factor α. Because tumor necrosis factor α production is an important element in host defense, this may explain, in part, the susceptibility of chronic alcohol abusers to a variety of infections.[44]

The observations presented herein can have significant relevance to public health. Alcohol is an independent risk factor for COPD,[45,46] and a large number of cigarette smokers misuse alcohol. COPD patients have decreased sIgA, an important antimicrobial mucosal agent against microbial infections.[7,10] Patients with COPD have decreased levels of sIgA,[11] and COPD lung has increased TGF-β levels.[13] In addition to macrophages, the neutrophil is implicated in this TGF-β response in COPD.[47] Future examination of paracrine neutrophil and macrophage responses to MAA should be explored in both healthy and COPD lungs. In addition, it has been proposed that COPD causes the reprogramming of bronchial epithelium in pIgR down-regulation.[48] Furthermore, alcohol misuse has long been associated with a significant increase in lung infections. Alcohol consumption dose-dependently increases viral infections,[49] and furthermore increases the risk of community-acquired infections.[50] Given that infection plays such a major role in the life history of smoking alcoholic patients, these results translate beyond COPD.

Although the mouse and *in vitro* models corroborate findings in the human AUD cohort, limitations exist in the models that preclude conclusively understanding the contributing role for alcohol and smoking co-exposure in COPD. Barrier function was the same in all exposure groups before and after treatment, suggesting that a monolayer leak was not a contributing factor to changes in sIgA transcytosis. Vectoral transport integrity was maintained because no sIgA was detected in the basal compartment of ALI and no loss of viability occurred. However, differences in the total detectable amounts of sIgA in humans versus mice were noted even though overall responses were consistent. Human sIgA levels were robust and detectable whereas TGF-β levels were lower; the reverse was evident in mouse sIgA. In MTECs, treatment with MAA-adducted protein resulted in lower sIgA transcytosis from basal to apical. In mice exposed to cigarette smoke and fed alcohol, a significant decrease in BAL sIgA was observed compared with smoke or alcohol individual exposure. These data suggest that the combination of alcohol drinking and cigarette smoking may negatively impact sIgA-mediated lung innate defense. Although the antimicrobial nature of sIgA is established, the complexities of innate defense are comprised of multiple tissue injury aspects of drinking and smoking. The specific contribution of smoke- and alcohol-mediated sIgA suppression of transcytosis cannot be exactly determined owing to the complexities involved in regulating sIgA in the context of such functions as mucociliary transport, phagocytosis, and defensins, which collectively impact innate defense. Therefore, the focus of future studies should be determining the functional significance of such a compromise to mucosal immunity and should be characterized by bacterial clearance assays as a cumulative consequence of this multifaceted injury.

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2021.06.007*.

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