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Hyaluronan Mediates Ozone-induced Airway Hyperresponsiveness in Mice*

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Ozone is a common urban environmental air pollutant and significantly contributes to hospitalizations for respiratory illness. The mechanisms, which regulate ozone-induced bronchoconstriction, remain poorly understood. Hyaluronan was recently shown to play a central role in the response to noninfectious lung injury. Therefore, we hypothesized that hyaluronan contributes to airway hyperreactivity (AHR) after exposure to ambient ozone. Using an established model of ozone-induced airways disease, we characterized the role of hyaluronan in airway hyperresponsiveness. The role of hyaluronan in response to ozone was determined by using therapeutic blockade, genetically modified animals, and direct challenge to hyaluronan. Ozone-exposed mice demonstrate enhanced AHR associated with elevated hyaluronan levels in the lavage fluid. Mice deficient in either CD44 (the major receptor for hyaluronan) or inter-α-trypsin inhibitor (molecule that facilitates hyaluronan binding) show similar elevations in hyaluronan but are protected from ozone-induced AHR. Mice pretreated with hyaluronan-binding peptide are protected from the development of ozone-induced AHR. Overexpression of hyaluronan enhances the airway response to ozone. Intratracheal instillation of endotoxin-free low molecular weight hyaluronan induces AHR dependent on CD44, whereas instillation of high molecular weight hyaluronan protects against ozone-induced AHR. In conclusion, we demonstrate that hyaluronan mediates ozone-induced AHR, which is dependent on the fragment size and both CD44 and inter-α-trypsin inhibitor. These data support the conclusion that pulmonary matrix can contribute to the development of airway hyperresponsiveness.

Ozone is a commonly encountered urban air pollutant that significantly contributes to increased morbidity (1–4) and can lead to a significant economic burden. It has been estimated that each year inhalation of ambient ozone contributes to 800 premature deaths, 4,500 hospital admissions, 900,000 school absences, and more than 1 million restricted activity days with an estimated $5 billion annual economic burden (5). Population-based studies suggest that for each 10 ppb increase in 1-h daily maximum level of ozone there is an increase in mortality risk of 0.39–0.87%, especially in individuals with pre-existing respiratory disease (2, 3, 6, 7). However, the biological mechanisms, which regulate the response to ambient ozone exposure, remain poorly understood.

Hyaluronan is an abundant extracellular matrix component, which has been recently shown to play a significant role in the response to noninfectious lung injury. Short fragment hyaluronan (sHA)[2] is released in the lung after sterile injury such as bleomycin instillation (8) or high tidal volume ventilation (9) and can modify the tissue response to injury. Furthermore, hyaluronan has been identified in airway secretions from asthmatics (10), and high molecular weight hyaluronan can attenuate the bronchoconstrictive response in exercise-induced asthma (11). We therefore hypothesized that hyaluronan may contribute to the biological response to airway injury after exposure to ozone.

In this study, we provide evidence that hyaluronan mediates ozone-induced AHR. Mice exposed to ozone demonstrate elevated lung lavage fluid levels of hyaluronan, which is of low molecular weight. We observed that animals deficient in either CD44 (a hyaluronan surface receptor) or inter-α-trypsin inhibitor (IaI, which facilitates hyaluronan binding) are protected from ozone-induced AHR. Overexpression of hyaluronan by airway epithelia enhances ozone-induced AHR. Furthermore, pretreatment of mice with hyaluronan-binding peptide or high molecular weight hyaluronan protects animals from the development of ozone-induced AHR. Direct instillation of low molecular weight, but not high molecular weight, hyaluronan alone induces AHR. Our observations support a critical role for short fragment hyaluronan in the development of airway hyperresponsiveness after exposure to ozone.

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[2] The abbreviations used are: sHA, short fragment hyaluronan; AHR, airway hyperreactivity; HABP, hyaluronan-binding protein; HA, hyaluronan; HAS2, hyaluronan synthase 2; IaI, inter-α-trypsin inhibitor; SBP, or scrambled binding protein; HMW-HA, high molecular weight hyaluronan; TK, tissue kallikrein.

EXPERIMENTAL PROCEDURES

Mice—CD44-deficient mice were provided by T. Mak and backcrossed onto C57BL/6J for >10 generations (12). Bikunin/IaI-deficient mice were backcrossed onto C57BL6J for >10 generations (13). CC10-HAS2 transgenic animals were backcrossed onto C57BL/6J background for >5 generations (14). Experimental groups consisted of 10 male 6–8-week-old mice unless otherwise stated. Experimental protocols were approved by the Animal Care Committee at either the NIEHS, National Institutes of Health, or Duke University.

Exposure Protocol—C57BL/6J, $CD44^{-/-}$, $IaI^{-/-}$, or HAS2 transgenic overexpressing mice were exposed to either Hepa-filtered air or ozone. Animals were housed in cages with low endotoxin bedding and given water and chow *ad libitum*. Ozone exposures were 2 ppm for 3 h. Our selection of ozone concentration levels in the mouse is based on similar biological response observed in human exposure studies and published deposition fraction data for O_3 in rodent models (15). Exposures were performed in a Hinner-style chamber. Air at 20–22 °C and 50–60% relative humidity was supplied at 20 exchanges/h. Ozone was generated by directing 100% O_2 through a UV light generator and mixed with air supply to the chamber. Chamber ozone concentration was monitored continuously with a UV light photometer (1003AH, Dasibi, Glendale, CA). In some experiments, C57BL/6 mice were given 10 mg/kg subcutaneously hyaluronan-binding protein (HABP) or scrambled binding protein control (SBP) (16) 1 h prior to exposure. In other experiments, $IaI^{-/-}$ mice were injected intraperitoneally with 1 ml of 0.5 mg/ml IaI (ProThera, East Providence, RI) or 1 ml of 1 mg/ml urinary trypsin inhibitor/bikunin (GenScript, Piscataway, NJ) 1 h prior to ozone exposure.

HA Challenge—Sterile, endotoxin-free (0.00008 endotoxin units/ml) high molecular weight hyaluronan (HMW-HA) (Healon, AMO, Santa Ana, CA) was reconstituted at 0.5 mg/ml in 0.02 M acetate, 0.15 M sodium chloride, pH 6.0. For the production of low molecular weight hyaluronan (sHA), hyaluronan was sonicated on ice. Sizes were confirmed by gel electrophoresis (17). In some experiments, 50 μl of HMW-HA, sHA, or vehicle were instilled oropharyngeally into isoflurane-anesthetized mice, and AHR was measured invasively 2–4 h later. In other experiments, 50 μl of HMW-HA or vehicle was instilled 1 h before and 23 h after acute ozone exposure, and AHR was measured invasively 24 h after ozone exposure.

Airway Physiology—Twenty four hours after beginning the exposure, tracheas of anesthetized mice (pentobarbital sodium, 60 mg/kg intraperitoneally) were surgically dissected and intubated, and mice were paralyzed (pancuronium bromide, 0.08 μg/kg intravenously) and ventilated with a computer-controlled small animal ventilator (FlexiVent, SCIREQ, Montreal, Canada) at a tidal volume of 7.5 ml/kg and a positive end-expiratory pressure of 3 cm of H_2O. Forced oscillation was used for measurements of respiratory mechanics. Briefly, airway pressure and tidal volume data were generated by the application of a 2-s sine wave volume perturbation with 0.2-ml amplitude and 2.5 Hz frequency. Following base-line resistance measurements, mice were challenged with methacholine aerosol (DeVilbiss ultrasonic) at 0, 10, 25, and 100 mg/ml; between aerosol doses the lung was hyperinflated with total lung capacity breath to return resistance to base-line levels. Total lung resistance measurements were averaged at each dose and graphed (R_T cm H_2O/ml/s) along with the initial base-line measurement.

Whole Lung Lavage—Whole lung lavage was performed as described previously (18). Supernatants were stored at −70 °C.

Hyaluronan Measurements—Lavage hyaluronan levels were performed with a commercially available enzyme-linked immunosorbent assay (Echelon, San Jose, CA) as per the manufacturer's instructions. For electrophoresis, lavage fluid was incubated 1:1 (v/v) with protease (Sigma), at 37 °C for 24 h, then boiled for 10 min, quenched on ice, and centrifuged, and the supernatant was concentrated 10× in a vacuum centrifuge. Concentrates were run on a 0.5% agarose gel, together with Healon HA, sonicated Healon HA, and hyaluronan ladders (Hyalose, Oklahoma City, OK), stained overnight with 0.005% Stains-All (Sigma) in 50% ethanol, and then de-stained in distilled water and photographed.

Immunohistochemistry—Formalin-fixed lungs were sectioned in 5-μm-thick sections and stained with phycoerythrin-Cy5-conjugated anti-mouse CD44 (Pharmingen) and biotinylated hyaluronan-binding protein (HABP) (Seikagaku Corp., Associates of Cape Cod, Falmouth, MA). A secondary streptavidin-Alexa 488 fluorochrome (Invitrogen) was used to detect hyaluronan.

Statistics—Data are expressed as mean ± S.E. Significant differences between groups were identified by analysis of variance and the Student's *t* test unless otherwise stated. A two-tailed *p* value of <0.05 was considered statistically significant.

RESULTS

Ozone Exposure Increases Hyaluronan Concentration in Mouse Lung Lavage Fluid—We examined the hypothesis that hyaluronan modifies the response to inhaled ozone. First, C57BL/6 mice, CD44-deficient mice, and IaI-deficient mice were exposed to 2 ppm ozone for 3 h. The level of airway injury as measured by lavage protein was similar in all ozone-exposed groups and increased when compared with filtered air-exposed (control) mice (Fig. 1*A*). Exposure to ozone increased the levels of soluble hyaluronan in bronchial alveolar lavage fluid of all strains of mice (Fig. 1*B*). Consistent with the role of CD44 and IaI in clearance of free hyaluronan, we observed enhanced levels of HA in $CD44^{-/-}$ and $IaI^{-/-}$ when compared with wild-type mice. Soluble hyaluronan in the lavage fluid was of lower molecular mass, averaging about 100–200 kDa (sHA) (Fig. 1*C*). After ozone exposure, the hyaluronan receptor CD44 was detected on both the airway epithelia and alveolar macrophages by fluorescent microscopy. Hyaluronan was primarily visible in the subepithelial space, where there was increased hyaluronan deposition after ozone exposure (Fig. 2). Hyaluronan was particularly visible around subepithelial myocytes (Fig. 3), whereas CD44 and hyaluronan colocalized on alveolar macrophages (Fig. 4). Cumulatively, these observations suggest that ozone exposure can release hyaluronan in both the lavage fluid and subepithelial space, where different cell types could bind hyaluronan and mediate hyaluronan-induced effects.

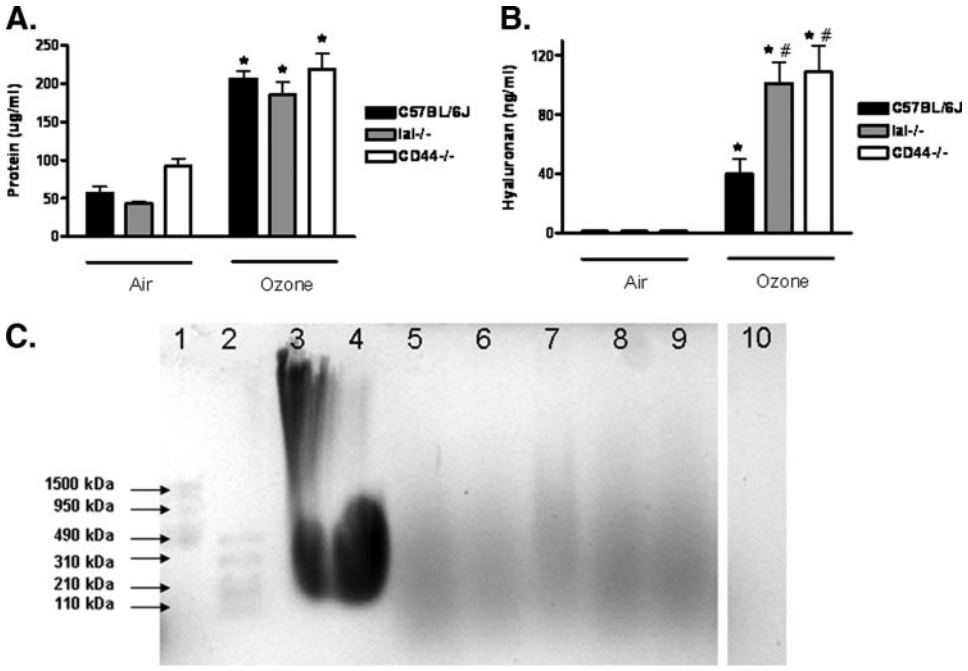

FIGURE 1. **Ozone and hyaluronan in BALF.** *A,* total lung lavage protein 24 h after either air- or ozone-exposed mice of all strains. *B,* lung lavage fluid hyaluronan level 24 h after either air or ozone exposure. *C,* agar gel, electrophoresis of concentrated lung lavage fluid hyaluronan, visualized with staining with Stains-All (Sigma). *Lane 1,* high molecular weight hyaluronan ladder. *Lane 2,* low molecular weight hyaluronan ladder. *Lane 3,* high molecular weight hyaluronan (Healon). *Lane 4,* sonicated Healon. *Lane 5,* C57BL/6, ozone-exposed. *Lane 6,* Iai wild-type ozone-exposed. *Lane 7,* Iai-deficient, ozone-exposed. *Lane 8,* CD44-deficient, ozone-exposed. *Lane 9,* HAS2 transgenic, ozone-exposed. *Lane 10,* representative free air exposed lavage for all strains (*, $p < 0.001$, air *versus* ozone; #, $p < 0.01$ compared with C57BL/6/ozone).

HA Recognition Is Required for the Development of AHR after Ozone Exposure—To determine the role of known receptors of hyaluronan in the biologic response to ozone, we first characterized CD44-deficient mice. We found that these mice were protected from ozone-induced airway hyperresponsiveness, when compared with C57BL/6J mice (Fig. 5*A*). Furthermore, we examined the role of IaI, which facilitates hyaluronan-dependent signaling (19). Consistent with our findings in $CD44^{-/-}$, $IaI^{-/-}$ animals were also protected from the physiologic response to ambient ozone (Fig. 5*A*). IaI consists of two heavy chains, which can bind hyaluronan, and a light chain called urinary trypsin inhibitor/bikunin, which is an anti-inflammatory protease inhibitor, but does not bind hyaluronan (20). We therefore tested whether the hyaluronan-binding component of IaI was necessary to mediate ozone-induced AHR. Intraperitoneal injection of IaI but not urinary trypsin inhibitor/bikunin into IaI-deficient mice reconstituted the physiologic response to ozone (Fig. 5*B*). This observation supports an essential role of IaI in the biologic response to ozone and provides further evidence for the role of hyaluronan in mediating ozone-induced AHR. These observations demonstrate that the biological response to ozone is, in part, dependent on both CD44 and IaI. Each of these genes is known to be involved in the cellular recognition of hyaluronan, further supporting the role of this molecule in the functional response to ozone.

HA Binding Attenuates Ozone-induced Airway Hyperresponsiveness—To directly determine the role of hyaluronan in airway hyperresponsiveness to ozone, C57BL/6J animals were treated with HABP before ozone exposure. This reagent has been used previously to bind HA and attenuate the biological effects of this molecule (16). Immunohistochemical staining demonstrated that HABP significantly attenuated CD44-hyaluronan binding on alveolar macrophages after ozone exposure, compared with scrambled control peptide or saline (Fig. 6). There was no difference in the degree of lung injury as quantified by lung lavage protein levels (Fig. 7*A*). Treatment with either HABP or SBP had no effect on base-line AHR (Fig. 7*B*). We observed that neutralization of hyaluronan with HABP significantly attenuated ozone-induced AHR similar to base line, when compared with either SBP or untreated mice (Fig. 7*C*). These observations further support the role of HA in the biological response to ozone.

Overexpression of Hyaluronan Enhances Ozone-induced AHR—To determine the role of increased levels of hyaluronan in the response to ozone, transgenic mice, which overexpress hyaluronan synthase 2 by airway epithelia resulting in enhanced production of high molecular weight HA (14), were exposed to ozone. Despite increased epithelial expression of hyaluronan, we observed a similar degree of lung injury as quantified by lung lavage protein levels (Fig. 8*A*). After exposure to ozone, transgenic animals demonstrate a more robust and increased level of soluble hyaluronan when compared with littermates (Fig. 8*B*). There was no difference in AHR observed between transgene-negative and transgene-positive animals with filtered air exposure. After ozone exposure, transgene-negative littermate mice demonstrated the expected enhanced response to methacholine after exposure to ozone. However, transgene-positive animals demonstrate an exaggerated AHR response after ozone challenge (Fig. 8*C*). Cumulatively, these observations suggest that elevated levels of high molecular weight HA alone are not sufficient to cause AHR and further support that ozone-induced modifications of HA are required to induce AHR.

Short Fragment HA Is Sufficient to Induce Airway Hyperresponsiveness—CD44 and IaI can each bind to hyaluronan, and recent evidence supports that sHA mediates noninfectious lung injury (14). To specifically address the role of sHA in airway hyperresponsiveness, naive mice were directly challenged by oropharyngeal aspiration of hyaluronan in a proof-of-principle experiment. We sonicated endotoxin-free high molecular weight HA to create sHA of similar size as hyaluronan in lung lavage fluid after ozone exposure (Fig. 1*C*). Instillation of sHA, but not HMW-HA, into naive C57BL/6J mice induced AHR when compared with vehicle (Fig. 9*A*). The

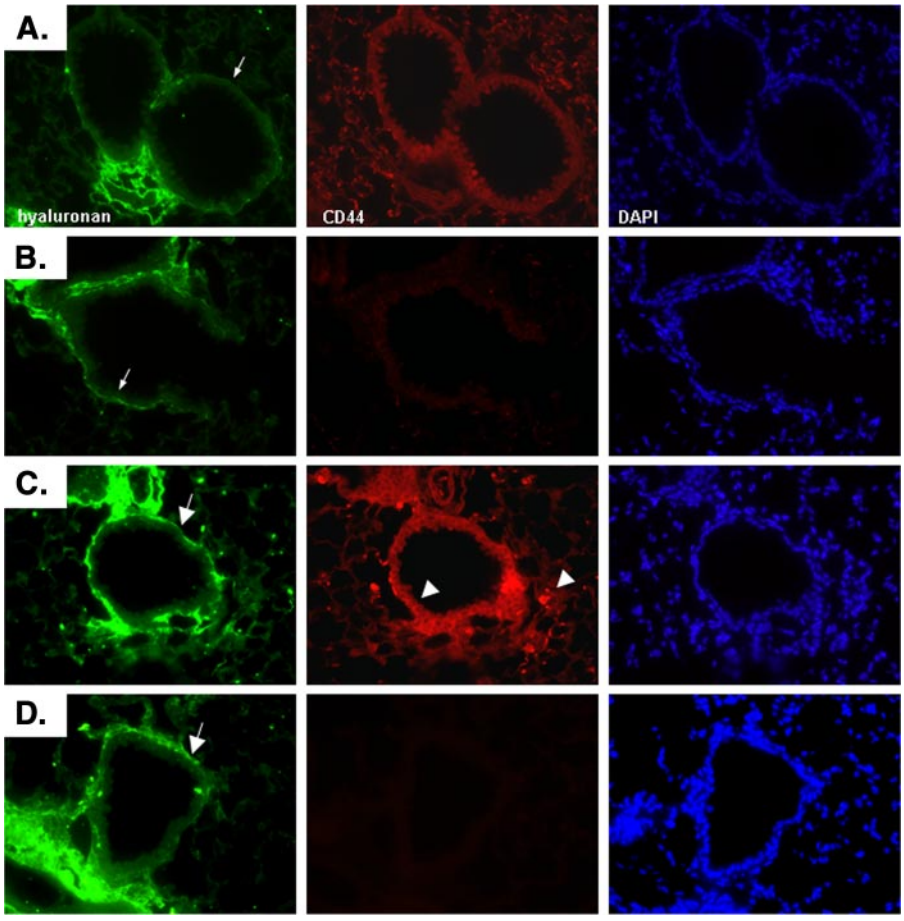

FIGURE 2. **Hyaluronan and CD44 immunohistochemistry after air or ozone exposure.** Expression and localization of hyaluronan (*green*) and CD44 (*red*) were identified immunohistochemically in naive (*A* and *B*) and ozone-exposed (*C* and *D*) mouse lungs. *A,* C57BL/6, air-exposed; *B,* CD44$^{-/-}$, air-exposed; *C,* C57BL/6, ozone-exposed; *D,* CD44$^{-/-}$, ozone-exposed. Hyaluronan is faintly visible in the subepithelial space in air-exposed mice (*small arrows*) but is more visible after ozone exposure (*big arrows*). CD44 (*red*) is localized in bronchial epithelial cells and macrophages (*arrowheads*). ×400 magnification.

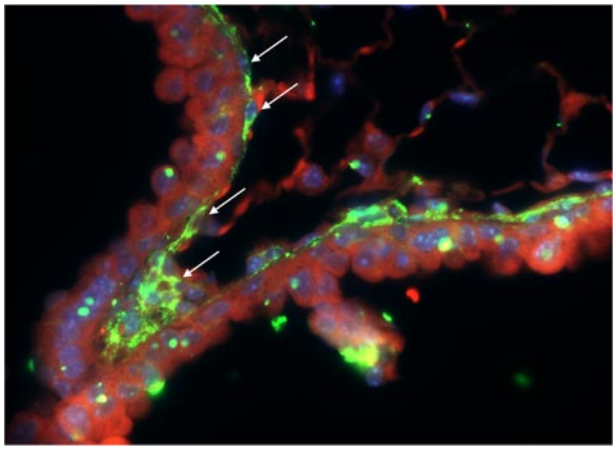

FIGURE 3. **Airway staining of hyaluronan and CD44 after ozone exposure.** In a higher magnification merged image, hyaluronan (*green*) is found adjacent to the basal membrane below bronchial epithelia (*red*) as well as surrounding subepithelial myocytes (*small arrows*). ×600 magnification.

response to sHA is dose-dependent. We demonstrate increased response to high dose sHA (3.5 mg/ml or 87.5 μg) when compared with low dose sHA (0.5 mg/ml or 25 μg) (Fig. 9*B*). Furthermore, the surface receptor CD44 was necessary for this hyaluronan-dependent AHR (Fig. 9*C*). These data indicate that sHA can, in part, mimic ozone-induced AHR. Interestingly, we did not observe significant changes in total cells or cell composition in the lung lavage fluid after sHA instillation (not shown), suggesting that the sHA effect on AHR may not require the presence of recruited inflammatory cells. To further corroborate this finding, we treated mice with HMW-HA before and after ozone challenge, because HMW-HA can competitively inhibit sHA effects (21). We observed significant attenuation of AHR in mice treated with HMW-HA concomitantly with ozone exposure (Fig. 9*D*). Cumulatively, these findings demonstrate that sHA can induce airway hyperresponsiveness and that HMW-HA has a protective role in ozone-induced AHR, supporting that hyaluronan size is an important factor in ozone-induced AHR.

Inflammatory Cell Migration into the Lungs Is Dependent on Hyaluronan Binding through CD44 and IaI—CD44 and IaI can either negatively or positively modify cellular inflammation in the lung depending on the severity of lung injury, as well as the environmental stimuli. Inflammation in the lung has also been associated with the severity of AHR. It was therefore essential to characterize the severity of alveolar inflammation after exposure to ozone in CD44$^{-/-}$ and IaI$^{-/-}$ mice. For both CD44-deficient and IaI-deficient mice, decreased total cell counts in whole lung lavage fluid were observed at 24 h after exposure (the time of maximum AHR) when compared with C57BL/6 mice. Macrophages accounted for most of the observed differences. Recruitment of inflammatory cells was rescued in IaI$^{-/-}$ mice through pre-injection of IaI but not UTI/bikunin (Fig. 10*A*). This finding is consistent with the hypothesis of CD44-IaI-mediated inflammatory cell migration that is dependent on hyaluronan binding. Both CD44 and IaI have been described to mediate cell binding to hyaluronan (19), and CD44 plays a role in endothelial adhesion of monocytes (22). In our hyaluronan-binding experiments, we observed a significant decrease on inflammatory cells (mainly macrophages) in the lavage fluid of HABP-treated mice but not SBP-treated mice (Fig. 10*B*). By contrast, we found a decrease in inflammatory cells in ozone-treated HAS2 transgene-positive mice, compared with ozone-treated controls (Fig. 10*C*). Finally, we did not observe a significant change in total cells or any cell type in the alveolar compartment after exposure to either high or low dose sHA when compared with vehicle (data not shown).

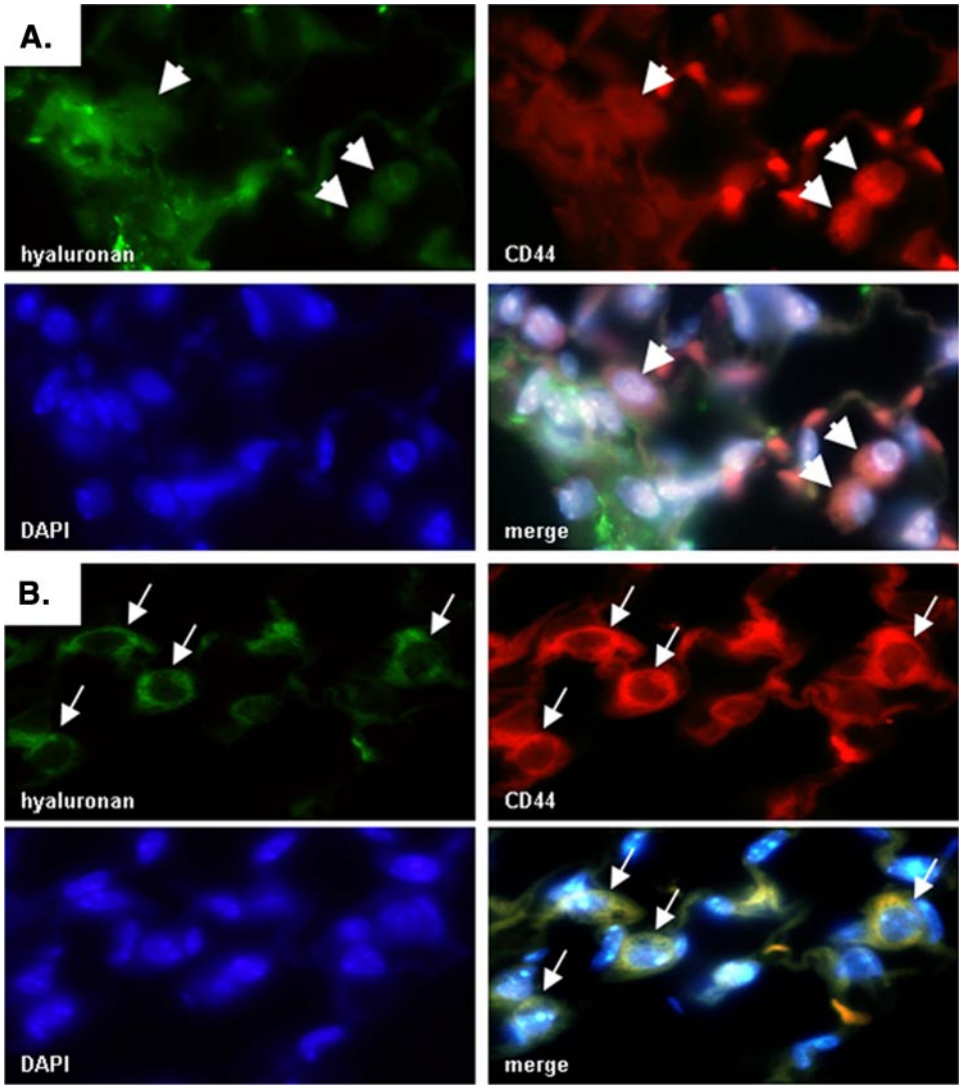

FIGURE 4. **Macrophage staining of hyaluronan and CD44 after ozone exposure.** C57BL/6J mice were exposed to either air or ozone and evaluated by immunohistochemistry for cellular distribution of CD44 and hyaluronan. *A,* after air exposure, alveolar macrophages (*arrowheads*) stain positive for CD44 (*red*) but not for hyaluronan (*green*). *B,* after ozone exposure, hyaluronan (*green*) and CD44 (*red*) colocalize on alveolar macrophages (*arrows*), yielding an *orange* appearance on merged image. ×600 magnification.

These observations support an important role of CD44 and IaI in recruitment of inflammatory cells to the lung after exposure to ozone, but also suggest that the role of hyaluronan in airway hyperreactivity and cell recruitment is complex.

DISCUSSION

In this study, we demonstrate that hyaluronan mediates ozone-induced AHR via binding to IaI and CD44. Furthermore, we demonstrate that hyaluronan size is important for its biological action in ozone-induced AHR. Specifically, HMW-HA instillation protected mice from the effects of ozone, whereas direct instillation of sHA in naive mice induced AHR. Finally, we show that CD44 and IaI are important for cellular lung infiltration after ozone exposure. These results support a novel role for hyaluronan in the pathogenesis of reactive airway disease and provide a mechanistic explanation for ozone-induced airway hyperresponsiveness.

Hyaluronan exists as a high molecular compound in extracellular matrix, with molecular masses exceeding 1000 kDa. HMW-HA is generally thought to be protective and was shown to attenuate elastase-induced AHR in a large-animal model (23). However, tissue injury can lead to release of low molecular weight hyaluronan (sHA), which acts as a pro-inflammatory mediator after bleomycin lung injury (8) or ventilator-associated lung injury (9). Hyaluronan receptors such as CD44 are located on cell types that can regulate the inflammatory response in the lungs, such as airway epithelia (24) and alveolar macrophages (25), implying that these cells have the potential to bind hyaluronan and mediate hyaluronan-induced effects. *In vitro* studies support that ozone and sunlight can result in fragmentation of hyaluronan (26), as observed in our murine model. Additionally, IaI is required for optimal binding of CD44 to hyaluronan (19), possibly through changing the conformation of hyaluronan. We demonstrate that the presence of both IaI and CD44 is required for ozone-induced AHR. We speculate that IaI can act as an extracellular mediator of hyaluronan binding to its cell-bound receptor CD44. Furthermore, it is known that another molecule, TSG-6 (tumor necrosis factor-associated gene 6), is necessary for transfer of hyaluronan onto IaI heavy chains (27, 28). We would therefore speculate that TSG-6 deficiency would also lead to a similar phenotype as we have observed in our experiments.

We demonstrate that the biological effects of hyaluronan in regulation of AHR are dependent on molecular weight. Specifically, HMW-HA attenuates ozone-induced AHR, whereas sHA induces AHR. These observations suggest that different sizes of hyaluronan may compete for receptor binding resulting in divergent physiological response. Because hyaluronan oligomers of as few as six disaccharide molecules can be recognized by CD44, and extracellular hyaluronan can reach a size of well over 1000 kDa, there is an immense size range of potential hyaluronan fragments that may compete for CD44 binding. Hyaluronan is a molecule that is not known to undergo modification such as glycosylation or sulfation after its synthesis, and therefore size, location, and concentration of hyaluronan may be the most important modifiers of activity. The biologic significance and biochemical specificity of the antagonistic actions of high and low molecular weight hyaluronan remain unclear. However, it is biologically plausible that HMW-HA is present

in the naive lung and that only after fragmentation by oxidation into sHA or *de novo* formation of sHA will there be a biologically active hyaluronan form. It is interesting that our HAS2 transgenic mice did not exhibit any change in their base-line AHR; however, they had increased AHR after ozone exposure, associated with sHA in their lavage fluid. We were unable to consistently show a significant change in expression of hyaluronan synthases after ozone exposure in our mice. We cannot rule out a post-translational activation of hyaluronan synthases. However, our results suggest that ozone-induced AHR is at least partly mediated by sHA fragments, which result from ozone-induced fragmentation of pre-existing HMW-HA. We speculate that HMW-HA could be competing with sHA by sterically inhibiting approximation of coreceptors to CD44. Alternatively, it remains possible that CD44 signaling requires internalization of the receptor-ligand complex, which could also be dependent on hyaluronan size. Our findings are consistent with previous observations, which reported that HA can ameliorate elastase-induced bronchoconstriction by binding and inactivating tissue kallikrein (TK) (23). We were unable to detect significant TK activity in lung lavage at 24 h after ozone exposure, which may be due to the different exposure model (ozone *versus* elastase) and sampling time point (24 h *versus* 30 min). Therefore, we cannot exclude that the protective effect of HMW-HA is dependent on TK blockade. It was recently shown that the IaI light chain urinary trypsin inhibitor/bikunin, but not the full IaI molecule, is most important for the TK inhibition in human airways (29). In our hands IaI, but not bikunin, mediates AHR. It is therefore possible that the full IaI molecule

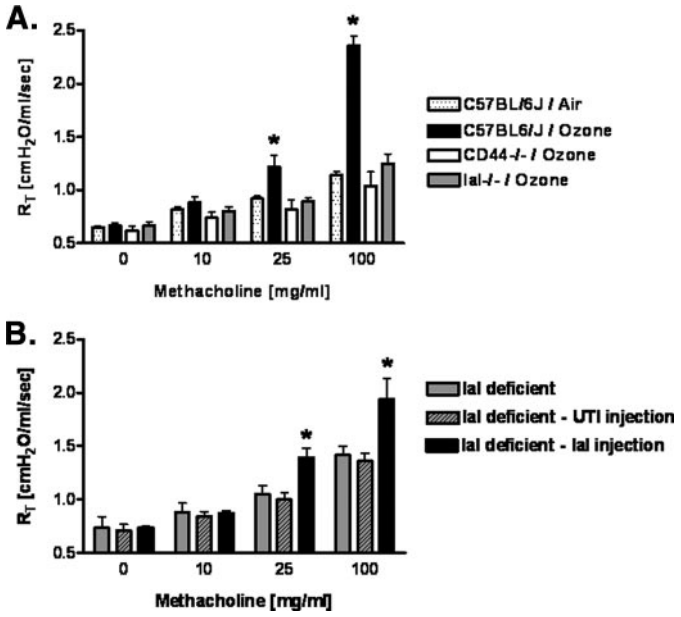

FIGURE 5. **Ozone-induced airway hyperresponsiveness.** *A,* CD44 and IaI are essential for the development of ozone-induced AHR (*, $p < 0.01$ compared with CD44- and IaI-deficient). *B,* IaI but not UTI/bikunin injection reconstitutes AHR in IaI-deficient mice (*, $p < 0.05$ compared with other groups).

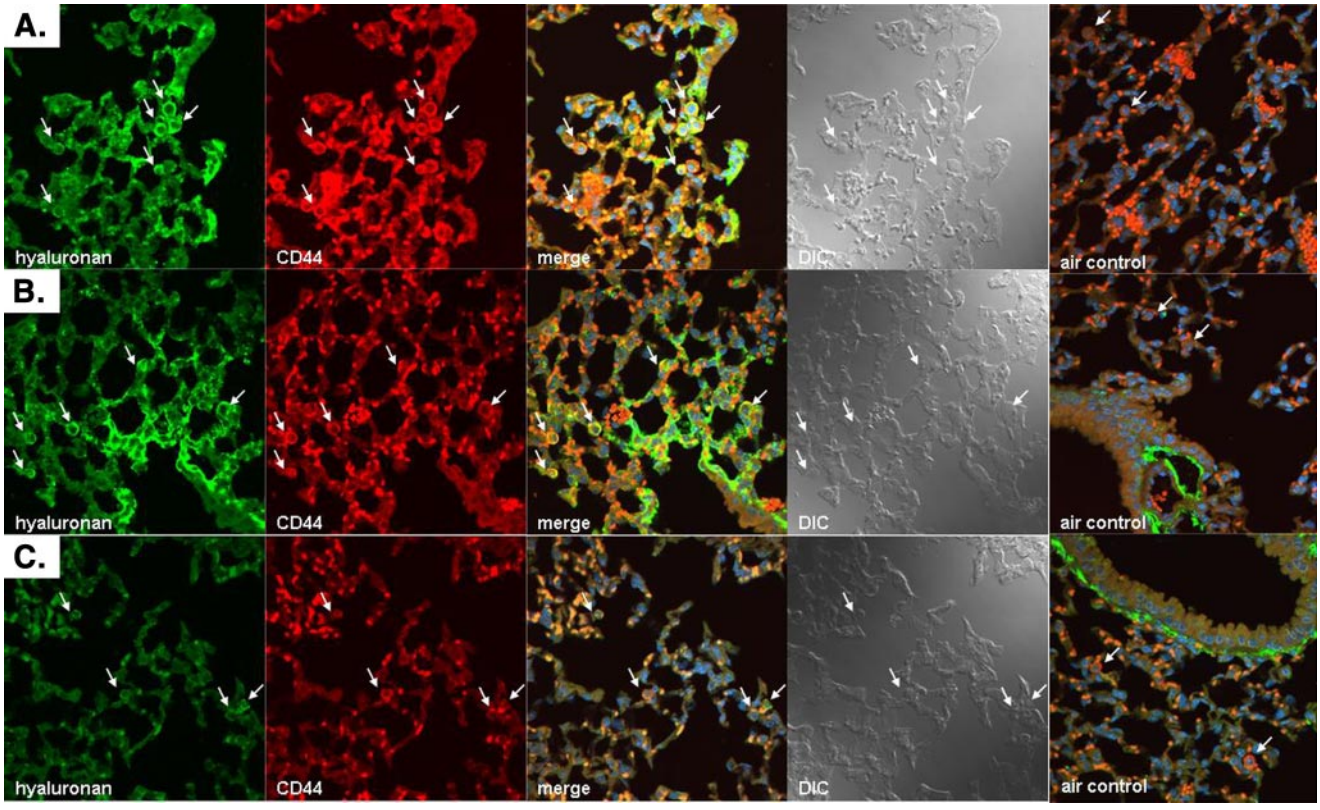

FIGURE 6. **HABP effect on CD44-hyaluronan binding after ozone exposure.** Confocal immunohistochemistry shows staining for hyaluronan (*green, far left panels*), CD44 (*red, left middle panels*), or merged images (*right middle panels*), and differential interference contrast images (*right panels*). *A,* saline-pretreated mouse lungs demonstrate significant hyaluronan and CD44 colocalization (*yellow* in merged image). *B,* scrambled peptide pretreated mouse lungs also show significant hyaluronan staining and colocalization with CD44. *C,* HABP-pretreated mouse lungs demonstrate significantly decreased hyaluronan staining on macrophages. ×630 magnification. There is no difference in hyaluronan or CD44 staining in respective air controls (*far right panels*).

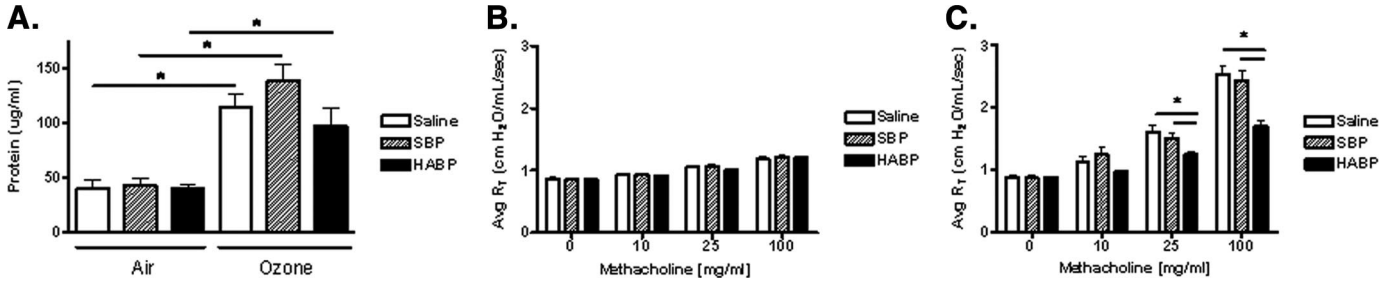

FIGURE 7. **Blockade of hyaluronan attenuates ozone-induced AHR.** *A,* treatment with vehicle, SBP, or hyaluronan-binding peptide does not alter ozone-induced increases in total protein in the lung lavage. *B,* treatment with vehicle, SBP, or HABP does not affect AHR in air-exposed animals. *C,* hyaluronan-binding protein, but not scrambled protein, significantly decreased AHR after ozone exposure (HABP *versus* other groups *, $p < 0.05$).

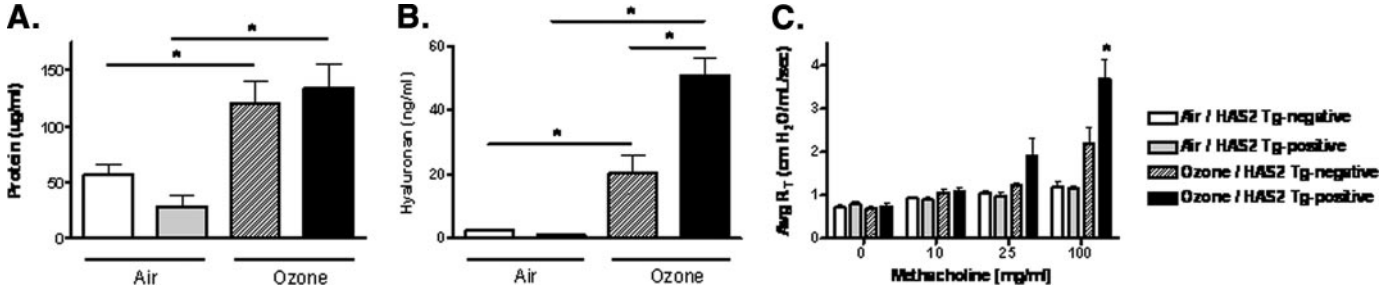

FIGURE 8. **Overexpression of hyaluronan enhances ozone-induced AHR.** *A,* animals, which overexpress HAS2 in airway epithelia, have similar levels of total protein in the lung lavage after exposure to ozone. *B,* both strains of mice have increased levels of soluble hyaluronan after exposure to ozone when compared with air exposure. HAS2 transgenic mice have significantly more soluble hyaluronan when compared with littermate controls (*, $p < 0.01$). *C,* HAS2 overexpressing animals are no different from littermate controls after exposure to filtered air but have enhanced AHR response after exposure to ozone (*, $p < 0.01$ compared with all other groups).

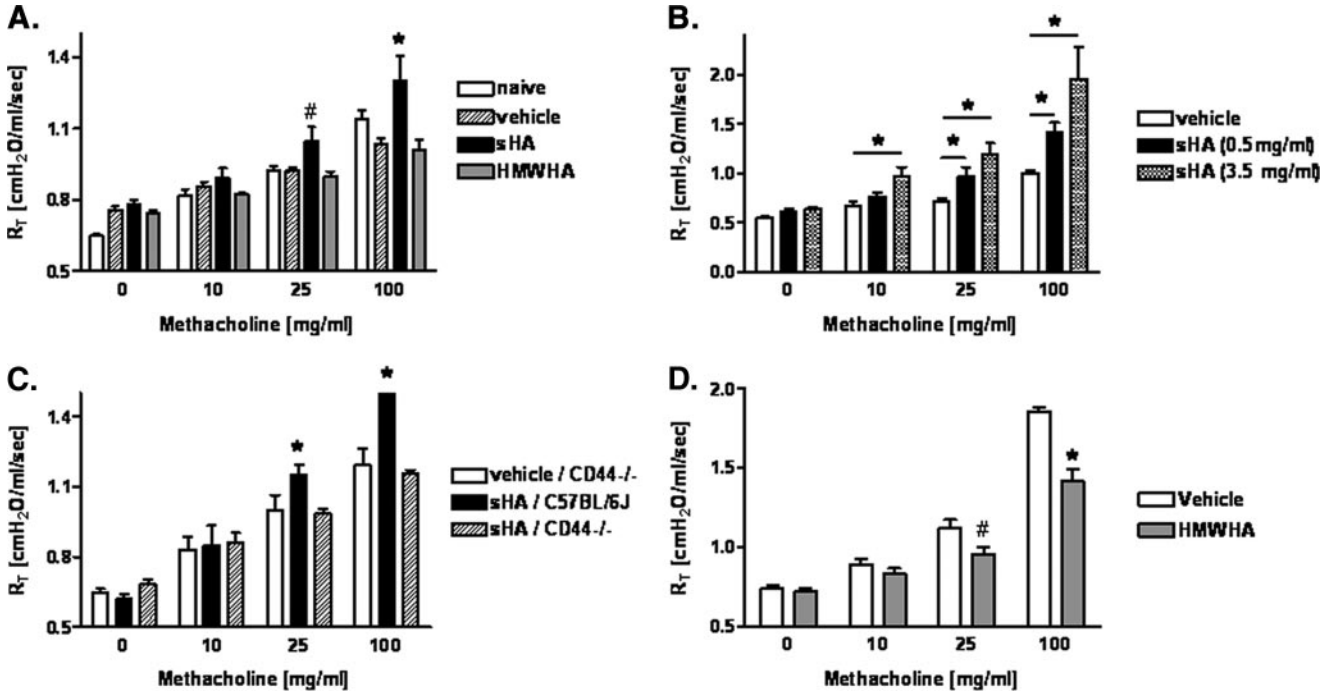

FIGURE 9. **Airway hyperresponsiveness in mice after intratracheal instillation of hyaluronan.** *A,* sHA but not HMW-HA or vehicle induces AHR in naive C57BL/6 mice (*, $p < 0.05$ compared with other groups; #, $p < 0.05$ compared with HMW-HA). *B,* response to sHA is dose-dependent. Both low dose (25 μg, 0.5 mg/ml) and high dose (87.5 μg, 3.5 mg/ml) sHA induce AHR. *C,* CD44-deficient mice are resistant to sHA-induced AHR compared with C57BL/5 mice (*, $p < 0.05$, sHA treated C57 *versus* sHA treated $CD44^{-/-}$). *D,* instillation of HMW-HA but not vehicle before and after ozone exposure to ozone significantly ameliorates AHR (*, $p < 0.01$ vehicle *versus* HMW-HA; #, $p < 0.05$, vehicle *versus* HMW-HA).

and its light chain bikunin have opposing effects on ozone-induced AHR. Further study is needed to fully clarify this effect.

An additional key finding of this study was that CD44 and IaI were required for cellular infiltration into injured lungs after ozone exposure. The synergistic action of CD44 and IaI in the cell attachment to hyaluronan substratum has been recently shown (19). The literature on the role of CD44 on inflammatory cell accumulation appears somewhat contradictory, with some studies showing that absence of CD44 leads to increased cellularity in inflammatory sites (8, 30) and other studies showing a

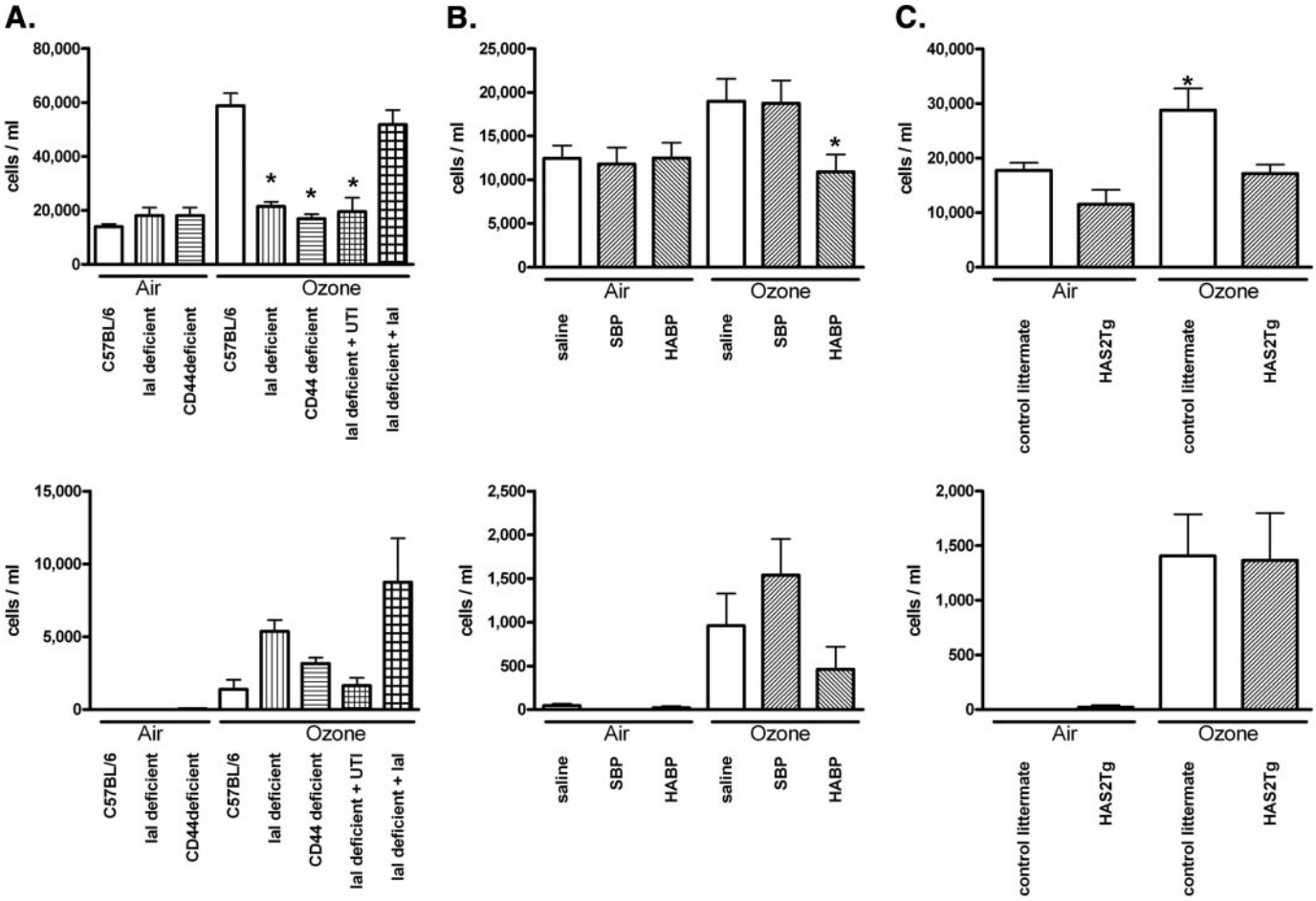

FIGURE 10. **Lung lavage cells in ozone-treated mice.** *A,* 24 h after ozone exposure Ial (*vertical stripes*) and CD44 (*horizontal stripes*) deficiency leads to significantly decreased numbers of inflammatory cells in the lung lavage fluid, which are mostly macrophages (*top panel*), with a few neutrophils (*bottom panel*). Injection of Ial-deficient mice with Ial (*bold cross-stripes*) but not equimolar bikunin (*fine cross-stripes*) reconstitutes the C57BL/6 phenotype. (*, $p < 0.001$ compared with C57BL/6 and Ial-deficient + Ial, Bonferroni multiple comparisons testing.) *B,* instillation of HABP (*right hatched*) but not scrambled SBP (*left hatched*) reduces lavage cells, which are mostly macrophages (*top panel*) with a few neutrophils (*bottom panel*) (*, $p < 0.01$ compared with saline and SBP-treated). *C,* HAS2 transgene-positive animals (*hatched*) have decreased lung lavage cells after ozone exposure compared with control littermates. The difference is because of macrophages (*top panel*) (*, $p < 0.05$ compared with HAS2 transgenic ozone-exposed).

moderate decrease (31). We speculate that the crucial factor may be vascular integrity and shear stress. In the setting of severe tissue injury, such as bleomycin-induced lung injury or bacterial pneumonia, the breakdown of vascular integrity with sequestration of blood in the inflamed tissues likely renders cell adhesion processes redundant for cellular diapedesis. Therefore during severe tissue injury, CD44 is important for extracellular matrix absorption and debris removal, and indeed when CD44 is absent in these models, cellularity increases within sites of injury because cells are unable to clear hyaluronan. Also, within tissues served by low vascular flow, such as occurs in venules, the absence of CD44 does not affect neutrophil rolling and adhesion on the endothelium (31). However, within tissues of relatively high vascular flow, *i.e.* the pulmonary capillaries, CD44/IaI/hyaluronan binding appears to be an essential component for inflammation and cellular infiltration responses to a moderate inflammatory stimulus induced by ozone exposure. Whether this cellular infiltration is necessary for the bronchoconstrictive effect of hyaluronan remains unclear. We did show that hyaluronan binding by HABP reduces both AHR and inflammatory cell infiltration after ozone inhalation. However, we did not observe a change in lavage cellularity after sHA instillation or after HMW-HA instillation in ozone-exposed mice to explain their respective effects on AHR, and we demonstrate that HAS2 transgenic mice have increased AHR but decreased cells in the lavage fluid. Prior research has shown that epithelial production of HMW-HA by means of transgenic expression of HAS2 confers a protective effect on epithelia after sterile lung injury (14). This may, in part, explain the decrease in inflammatory cells after ozone exposure in these mice. On the other hand, hyaluronan is largely localized in the subepithelial area (Figs. 2 and 3) in direct apposition to both epithelia and smooth muscle cells, both of which have CD44 receptors. It is therefore possible that sHA release after ozone exposure has a direct effect on airway smooth muscle or indirectly by induction of pro-inflammatory cytokines known to regulate ozone-induced AHR (for review see Ref. 32). Therefore, our data suggest that hyaluronan-mediated AHR after ozone exposure is at least in part independent of inflammatory cell influx. The specific cell types and effector molecules, which contribute to this phenotype, will be an area of future investigation.

In summary, we demonstrate a central role of hyaluronan in ozone-induced AHR. Exposure to ozone results in elevated levels of short fragment hyaluronan. The biological response to ozone is dependent on molecules known to bind hyaluronan, including CD44 and IaI. We demonstrate hyaluronan has divergent functional consequences within the lung dependent on the molecular weight. Specifically, short fragments of hyaluronan contribute to AHR, and high molecular weight hyaluronan attenuates ozone-induced AHR. This is the first study demonstrating short fragments of hyaluronan contribute to AHR, which is mediated through interaction with CD44 and IaI. Our observations provide insight into the pathogenesis of environmental airways disease. In conclusion, these data define a novel role for pulmonary matrix in lung pathophysiology and identify pharmacologic modification of hyaluronan as a potential target for treatment of reactive airways disease.

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