High-mobility group box 1 promotes extracellular matrix synthesis and wound repair in human bronchial epithelial cells

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1Department of Physiology and Pathophysiology, University of Manitoba, Winnipeg, Manitoba, Canada; 2Department of Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada; 3Section of Thoracic Surgery, University of Manitoba, Winnipeg, Manitoba, Canada; and 4Biology of Breathing Group, Manitoba Institute of Child Health, Winnipeg, Manitoba, Canada

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Ojo OO, Ryu MH, Jha A, Unruh H, Halayko AJ. High-mobility group box 1 promotes extracellular matrix synthesis and wound repair in human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 309: L1354–L1366, 2015. First published October 2, 2015; doi:10.1152/ajplung.00054.2015.—High mobility group box 1 (HMGB1) is a damage-associated molecular pattern (DAMP) protein that binds Toll-like receptors (e.g., TLR4) and the receptor for advanced glycation end products (RAGE). The direct effects of HMGB1 on airway structural cells are not fully known. As epithelial cell responses are fundamental drivers of asthma, including abnormal repair-restitution linked to changes in extracellular matrix (ECM) synthesis, we tested the hypothesis that HMGB1 promotes bronchial epithelial cell wound repair via TLR4 and/or RAGE signaling that regulates ECM (fibronectin and the γ2-chain of laminin-5) and integrin protein abundance. To assess impact of HMGB1 we used molecular and pharmacological inhibitors of RAGE or TLR4 signaling in scratch wound, immunofluorescence, and immunoblotting assays to assess wound repair, ECM synthesis, and phosphorylation of intracellular signaling. HMGB1 increased wound closure, and this effect was attenuated by blocking RAGE and TLR4 signaling. HMGB1-induced fibronectin and laminin-5 (γ2 chain) was diminished by blocking TLR4 and/or blunting TLR4 signaling. Similarly, induction of α3-integrin receptor for fibronectin and laminin-5 was also diminished by blocking TLR4 signaling and RAGE. Lastly, rapid and/or sustained phosphorylation of SMAD2, ERK1/2, and JNK signaling mediated HMGB1-induced wound closure. Our findings suggest a role for HMGB1 in human airway epithelial cell repair and restitution via multiple pathways mediated by TLR4 and RAGE that underpin increased ECM synthesis and modulation of cell-matrix adhesion.

extracellular matrix; damage-associated molecular pattern protein; DAMP; Toll-like receptor 4; TLR4; receptor for advanced glycation end products; RAGE; asthma; COPD

EXACERBATIONS OF ASTHMA or chronic obstructive pulmonary disease (COPD) can be triggered by allergens, pollutants, and viral infection (19, 44, 71). These triggers can cause bronchial epithelium damage, resulting in the activation of wound healing processes to restore integrity of the bronchial epithelial cell layer. Epithelial wound healing occurs in three phases: inflammatory, repair, and, remodeling (1, 32). Immune cells are recruited in the inflammatory phase, facilitated by epithelial cells that secrete growth factors such as epidermal growth factor (EGF) and extracellular matrix (ECM) proteins, including collagen, fibronectin, and laminins. ECM deposition creates a substrate to enable leukocyte attachment and migration of structural cells, including epithelial cells (45, 63). During the repair phase epithelial cell-cell cadherin contacts are lost, permitting the initiation of wound closure involving a select repertoire of integrins and ECM proteins, including laminins and fibronectin, to create a scaffold to support wound closure (24, 33). Laminin-5 is increased at sites of epithelial injury in COPD (34) and accumulates in the airways of allergic models of asthma (10). During the remodeling phase of wound healing, epithelial cells fully reestablish cell-cell contact and cell-matrix contacts to complete wound closure.

Alarmins are a class of secreted proinflammatory mediators that are typically intracellular but can be released frequently through nonconventional pathways by stressed and damaged cells in response to different triggers, including inflammation (5, 11). They are part of a larger family of damage-associated molecular pattern molecules (DAMPs) that include S100 proteins, purine metabolites, DNA, and the proinflammatory protein high-mobility group box 1 (HMGB1). HMGB1 is of interest with respect to its release by activated immune and structural cells and downstream modulation of inflammation associated with sepsis (76), arthritis (59), asthma, and COPD (11, 78). In unstressed cells endogenous HMGB1 resides in the nucleus as a DNA binding protein, stabilizing nucleosomes and modulating both DNA-associated protein assembly and gene transcription (56). Cell stress can trigger passive or active release of HMGB1 by lung immune cells, bronchial and alveolar type II epithelial cells, fibroblasts, and airway smooth muscle (ASM) cells (2, 14, 25). Extracellular HMGB1 induces the release of ECM proteins (17), can modulate integrin function (6), promotes airway remodeling (36), and contributes to bleomycin-induced lung fibrosis in mice (22).

Studies of immune cells indicate that the effects of HMGB1 are mediated by Toll-like receptors (TLR), in particular TLR2 and TLR4 (47, 77), and by the multiligand receptor for advanced glycation end products (RAGE) (28). TLR2, TLR4, and RAGE are implicated in orchestrating inflammation leading to tissue remodeling in obstructive or inflammatory airway disease and fibrotic lung disease (4, 65). These receptors activate multiple intracellular signaling pathways, including the mitogen-activated protein kinases (MAPK), ERK1/2, JNK, and p38 MAPK, that are linked to wound repair responses (61). Despite apparent links between HMGB1 and TLR2, TLR4, and/or RAGE with inflammatory and fibrotic responses in lung disease, understanding of the factors that modulate their expression and the role of HMGB1 in bronchial airway epithelial cell wound repair is limited.

In this study we tested the hypothesis that HMGB1 promotes human bronchial epithelial cell wound repair in vitro via
receptor-mediated signaling mechanisms that regulate ECM protein synthesis and integrin expression. We analyzed the effects of exogenous HMGB1 on epithelial repair using a scratch-wound assay, as well as its effects on ECM protein and integrin expression. Furthermore, we dissected TLR4- and RAGE-mediated effects, including downstream intracellular signaling. Our data indicate that HMGB1 promotes ECM protein and integrin expression and that this underpins bronchial epithelial cell wound healing. HMGB1-induced effects appear to be mediated by RAGE- and/or TLR4-associated intracellular signaling pathways. Our findings reveal an important role for HMGB1 in airway epithelial repair and restitution, implicating this as an important contribution to both inflammatory and tissue remodeling in obstructive airway diseases such as asthma and COPD.

METHODS

Cell culture and reagents. Primary human bronchial epithelial cells (Lonza Group) were cultured in bronchial epithelium growth medium (BEGM) and used between passages 2–4. These cells were used to validate signaling and functional responses and corroborated initial observations in the transformed bronchial epithelial cell line BEAS-2B. For initial observations in BEAS-2B cell lines, cells were cultured in LHC-9 media (Invitrogen, Ontario, Canada) and grown on tissue culture dishes precoated with collagen (3 mg/ml), fibronectin (0.2 mg/ml), and bovine serum albumin (BSA; 1 mg/ml).

Where indicated, recombinant HMGB1 (0.3–30 ng/ml; HMGBio-Tech, Milan, Italy), the TLR4 signaling inhibitor CLI-095 (Invivogen), the TGF-β receptor 1 inhibitor (ALK4, 5, and 7) SB505124, the ERK1/2 inhibitor U0126, the extracellular HMGB1 inhibitor glycyrrhizin, or the JNK inhibitor SB600125 (Sigma) was added to culture media. Antibodies used included rabbit anti-human fibronectin and mouse anti-human RAGE (Abcam); rabbit anti-human E-cadherin, anti-human phospho-SMAD2, anti-human total SMAD2/3, anti-human phospho-JNK, and rabbit anti-phospho-ERK; rabbit anti-phospho-SMAD2; rabbit anti-phospho-ERK; 1:300 for rabbit anti-laminin-5 (γ-chain), mouse anti-α3-integrin; 1:2,000 for mouse anti-SNAIL: 1:4,000 for mouse anti-GAPDH; and 1:20,000 for rabbit anti-β-actin. Thereafter, membranes were washed with PBS and blocked in PBS containing 1% BSA before recombinant HMGB1 exposure.

Cell stimulation. Bronchial epithelial cells were cultured until 60–70% confluent and then stimulated with recombinant HMGB1 (0.3–30 ng/ml) for up to 72 h. For inhibition or cell signaling assays, cultures were preincubated with CLI-095 (1 μM) or neutralizing RAGE antibody (10 μg/ml) for 2 h, glycyrrhizin (100 μM) for 4 h, or U0126 (10 μM), SB600125 (10 μM), or SB505124 (5 μM) for 1 h before recombinant HMGB1 exposure.

Scratch wound assay. Bronchial epithelial cells were grown to a confluent monolayer and a single vertical scratch wound was made using a 200-μl pipette tip across the surface. The cells were washed twice with prewarmed PBS and then incubated in fresh culture media with or without HMGB1 and specific inhibitors for up to 72 h. Time-lapse cell culture images were captured using a phase contrast microscope (Olympus CK40, Ontario, Canada) equipped with a CCD camera (Olympus DP10). Wound closure rate was determined using ImageJ software. JPEG microscope images (×4) were inverted to reveal wound edges and then using an automated “find edges” and LUT macro to generate a wound outline image. The size of the wound area was measured in each image using a macro tool as previously described (https://www2.le.ac.uk/colleges/medbiopsych/facilities-and-services/cbs/lite/aff/tips-and-tricks-1/wound-healing-assay). For each condition three to four individual experiments were performed in duplicate (1 scratch-wound per well). Wound closure at each time point was normalized as mean percentage of the total wound area that was measured immediately after wounding (t = 0).

Semiquantitative RT-PCR. RNA samples were extracted using RNeasy Mini kit (Qiagen, Ontario, Canada) and reverse transcribed using the QuantiTect reverse transcription kit (Qiagen). PCR amplification was conducted using the Taq polymerase kit with specific primers under the following conditions: initial denaturation of 94°C for 5 min, 40 cycles of denaturation, annealing, and product extension of 94°C 15 s, 58°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 min before being resolved using gel electrophoresis. Blots were inverted and band density quantified by ImageJ software. Primers were as follows: TLR4 (F-AGGCGAAAGGTGATGTGTTGTTG, R-ACGTGCGAGTCGACACATTCTCAT); GAPDH (F-AGCAATGCCTGTCCACACAAA, R-AGACTGTGGATGGCCCGCCCGG); and RAGE (F-GATCTCCGCCTCCACCTCTCCTCAGG, R-CAGGCTCCTCCTTCTCTCCTCGTTTCG).

Immunoblotting. Total protein was resolved by SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes as described previously (18). Membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and 5% milk for 1 h at room temperature and then incubated overnight at 4°C with primary antibody diluted in TBST solution containing 5% milk. Antibody dilutions used were as follows: 1:1,000 for rabbit anti-fibronectin, rabbit anti-E-cadherin, rabbit anti-phospho-SMAD2, rabbit anti-phospho-JNK, and rabbit anti-phospho-ERK; 1:300 for rabbit anti-laminin-5 (γ-chain), mouse anti-α3-integrin; 1:2,000 for mouse anti-SNAIL: 1:4,000 for mouse anti-GAPDH; and 1:20,000 for rabbit anti-β-actin. Thereafter, membranes were washed in TBST and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse Ig, 1:3,000; anti-rabbit Ig, 1:5,000; Sigma) for 1 h at room temperature. After a final wash with TBST, blots were developed by using an enhanced chemiluminescence (ECL) reagent kit (Amersham Biosciences) and chemiluminescence was captured on Kodak Biomax film (Sigma). For quantification, blots were scanned using an Epson Perfection 3170 photo scanner (Epson, Ontario, Canada) and the size and intensity of individual bands was measured using ImageJ software.

Immunofluorescence. Cluster plates (24-well) used for scratch assay were fixed with 4% formaldehyde (wt/vol) in phosphate-buffered saline (PBS; 10 min, room temperature) and permeabilized in PBS containing 0.3% Triton-X-100 (vol/vol) for 10 min, room temperature. Cells were washed with PBS and blocked in PBS containing 1% BSA (wt/vol), 5% undiluted donkey serum (vol/vol), and 0.1% Triton-X-100 (vol/vol) for 1 h. Subsequently, cells were washed with PBS and incubated with rabbit anti-fibronectin or mouse anti-α3-integrin (dilution 1:200 in PBS buffer; 4°C, overnight). After being washed with PBS, cells were incubated with appropriate secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or cyanine 3 dye (Cy3) (dilution 1:100 in PBS buffer; 4°C, overnight). Images were observed and analyzed using an Olympus IX70 epi-fluorescence microscope (Olympus) and images were captured by CCD camera (QImaging RETIGA-SRV, British Columbia, Canada).

Chronic allergen challenge. As we have described (40), under inhaled anaesthesia (isoflurane) 8-wk-old Balb/c mice were exposed intranasally to 2.5 g/l of saline containing purified house dust mite (HDM) whole body extract (2.5 μg/μl HDM–protein extract in saline) (Greer Laboratories) for 5 consecutive days over 2 consecutive wk initially and followed by HDM exposure three times a week on alternative days for an additional 3 wk. All studies were performed using animals and protocols approved by the Animal Ethics Committee of the University of Manitoba.

Immunohistochemistry: human and murine lung tissue. Peripheral human lung tissue specimens from macroscopically healthy tissue areas were obtained from COPD subjects undergoing lung resection surgery for cancer (University of Manitoba REB H2002:150). All subjects gave informed consent and subjects were grouped according
to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification using predicted forced expiratory volume in 1 s (FEV₁) and FEV₁ to forced vital capacity (FVC) ratio (Table 1). Murine lungs were fixed in 4% formalin and processed as previously described (40). For histology, segmental sections were deparaffinised in xylene and rehydrated in graded solutions of ethanol and subjected to epitope unmasking in citrate buffer (0.1 M citric acid and 0.1 M sodium citrate, pH 6.0) for 20 min at 90°C followed by cooling at room temperature for 20 min. Thereafter, sections were blocked in TBS solution containing 10% mouse serum (vol/vol) and 1% BSA (wt/vol) for 2 h at room temperature and then incubated overnight at 4°C with rabbit anti-HMGB1 (1:50, TBS containing 1% BSA). Following a series of washes, tissue specimens were next incubated for 1 h with HRP-conjugated secondary antibody (1:100) in TBS containing 1% BSA (wt/vol) at room temperature prior to undergoing diaminobenzidine (DAB) staining using a peroxidase assay DAB substrate kit. Specimens were counter-stained with hematoxylin and then mounted on glass slides using Permount Mounting media (Fischer Scientific). For image capture we used a conventional bright field light microscope and images shown are representative of the overall experimental results.

Statistical analysis. Differences between groups was assessed using one-way ANOVA with Newman-Keuls post hoc test or using Student’s t-test. Immunohistochemical experiments in human lung tissue are representative of four COPD and non-COPD donors and for murine lung tissue three mice were used. Three-to-five (n = 3–5) independent experiments were completed using BEAS-2B cell lines and for primary human bronchial epithelial cells three to seven (n = 3–7) donors were used. All values are presented as means ± SE and significance was taken as P < 0.05.

RESULTS

Bronchial epithelial cells express HMGB1, TLR4, and RAGE. To justify in vitro studies on the impact of extracellular HMGB1 on bronchial epithelial cells, we confirmed that HMGB1 is present in the lungs of human COPD donors and murine models of airway disease. We used immunohistochemistry to assess HMGB1 presence in formalin-fixed lung tissue specimens from human COPD and non-COPD donors, including naive and HDM challenged mice. Consistent with reports measuring HMGB1 in sputum of COPD and non-COPD donors (78), we observe more intense staining in COPD lung tissue sections compared with basal levels in non-COPD lung tissue (Fig. 1A). In lung tissue sections from HDM-challenged mice, cells in the interstitium clearly expressed HMGB1, and strong labeling of HMGB1 is also present in structural cells of the interstitium compared with naive control mice (Fig. 1B). We also assessed immunolabeling of RAGE in tissue specimens from COPD donors, and this was localized to the interstitium with some diffuse staining in the epithelium compared with non-COPD (Fig. 1A). Similar observations were also confirmed in tissue sections from HDM challenged mice compared with naive control mice. We also confirmed elevated expression of RAGE in murine lung tissue from allergen challenged compared with naive control mice (Fig. 1C).

Using semiquantitative PCR we assessed the effects of HMGB1 exposure (24 h) on TLR4 and RAGE abundance in the human bronchial epithelial cells. Constitutive TLR4 mRNA was readily detectable in primary bronchial epithelial cells and BEAS-2B cells (latter not shown) and its abundance did not appear to be affected by HMGB1 exposure. RAGE mRNA was increased with HMGB1 exposure (Fig. 1, D and E) and we further show the same is true for RAGE protein abundance in primary human bronchial epithelial cells (Fig. 1F) by demonstrating increased protein abundance (1.8-fold) following HMGB1 exposure for 24 h.

HMGB1 induces expression of ECM proteins involved in wound repair. To model the wound healing-relevant dependence of bronchial epithelial cells to exogenous HMGB1 we treated transformed human bronchial epithelial cell line cultures with various concentrations of HMGB1 from 0.3 to 30 ng/ml (24 h) and employed immunoblotting to monitor the abundance of the tight junction protein E-cadherin. Indicative of a loss of cell-cell contacts, a response required for the onset of epithelial cell migration, we observed HMGB1-induced depletion of E-cadherin, with robust loss of protein in cultures exposed to 3 ng/ml HMGB1 (Fig. 2A). On this basis we used 3 ng/ml HMGB1 in subsequent studies in primary human bronchial epithelial cells to assess epithelial wound repair and cell signaling.

We also assessed HMGB1-treated human bronchial epithelial cell line cultures for changes in expression of ECM proteins and their requisite receptors, as these are critical for cell attachment and migration during wound repair. Expression of ECM proteins occurs at different times depending on the stimuli and culture conditions; therefore, we looked at expression at different time points. In epithelial cells, the γ2-chain of laminin-5 has been shown to be induced by 3 h (37), α3-integrin at 8 h (69), and fibronectin expression as early as 4 h (54) by various stimuli. Using immunoblotting we observed expression of fibronectin, the γ2-chain of laminin-5, and the fibronectin receptor-associated α5-integrin in human bronchial epithelial cell line cultures in response to HMGB1 exposure (Fig. 2, B–D). It is important to note that expression of ECM receptor integrin α5 occurs after induced expression of ECM substrates, fibronectin, and γ2-chain of laminin-5.

HMGB1 promotes wound closure in bronchial epithelial cells. We measured the impact of HMGB1 on primary human bronchial epithelial monolayer wound repair in vitro using a scratch wound healing assay over a 72-h period (Fig. 3, A and B). HMGB1 exposure enhanced scratch wound closure by almost 160% compared with control cultures in the absence of HMGB1 (control wound closure = 12.5 ± 2.8% vs. HMGB1-treated wound closure = 31.3 ± 3.8%; P < 0.05).

TLR4 and RAGE mediate HMGB1-induced bronchial epithelial cell wound repair. To dissect the signaling pathways that mediate wound repair in HMGB1-treated cells, we suppressed TLR4 and RAGE signaling using CLI-095 (a pharmacological small molecule inhibitor of intracellular adaptor of TLR4 signaling) or neutralizing antibodies for RAGE, respectively. As a comparator for the effects of selective receptor

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Table 1. Clinical characteristics of COPD and non-COPD donors

<table>
<thead>
<tr>
<th>COPD Donors</th>
<th>Non-COPD Donors</th>
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<tr>
<td>Age, yr</td>
<td>72 (68–79)</td>
</tr>
<tr>
<td>Smoking history, pack yr/quit yr</td>
<td>50.7/26.5*</td>
</tr>
<tr>
<td>FEV₁, %predicted</td>
<td>70 (56–78)</td>
</tr>
<tr>
<td>FEV₁/FVC, %</td>
<td>67.7 (65–70)</td>
</tr>
<tr>
<td>GOLD classification</td>
<td>Moderate (stage II)</td>
</tr>
</tbody>
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Data are expressed as means with ranges in parentheses, COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease. *One current smoker.
blockade, we also assessed the impact of glycyrrhizin, an aggregate inhibitor of extracellular HMGB1. As a positive control we compared the magnitude of HMGB1-induced wound closure to the effects of TGF-β1 (Fig. 4A).

These experiments confirmed a direct effect for HMGB1 to induce bronchial epithelial cell wound closure, as in the presence of glycyrrhizin wound closure in HMGB1-treated cultures was only 4.9 ± 0.8%, a response 84% less than that observed in HMGB1-treated cultures. Moreover, with glycyrrhizin wound closure was 60% less that than of control cultures, suggesting a role, in part, for endogenous HMGB1 in scratch wound closure and basal responses. The magnitude of wound closure in cultures treated with TLR4 or RAGE inhibitors (10.3 ± 1.7 and 11.7 ± 2.9%, respectively) was markedly blunted compared with HMGB1-exposed cultures and was similar to that measured in control cultures. Thus both TLR4 and RAGE appear to be required for the augmented wound closure induced by HMGB1. However, inhibition of only one receptor is sufficient to block this effect, suggesting that cross talk may be required to fully orchestrate the response to HMGB1.
Fig. 2. HMGB1 mediates expression of extracellular matrix (ECM) proteins involved in wound repair. A: BEAS-2B cells were treated in the presence or absence of HMGB1 (0.3–30 ng/ml; 24 h) and cell lysates (10 μg) analyzed by immunoblotting using E-cadherin and GAPDH antibodies to assess protein expression levels. B: BEAS-2B were treated in the presence or absence of HMGB1 (3 ng/ml) for up to 180 min and cell lysates (10 μg) were run on 12% gels and analyzed by immunoblotting using γ2-chain laminin-5 antibody to assess protein expression levels. C: BEAS-2B cells treated with HMGB1 (3 ng/ml) for up to 360 min were run on 10% gels and probed for fibronectin protein expression by immunoblotting. D: cell lysates (10 μg) from untreated (control) and HMGB1-treated BEAS-2B cells (3 ng/ml; up to 24 h) were run on 10% gels and probed for α3-integrin expression by immunoblotting. GAPDH served as loading control and blots are representative images of 3 independent experiments. *P < 0.05, **P < 0.01, ###P < 0.001 vs. control.
Fig. 3. HMGB1 promotes bronchial epithelial cell wound healing. A: primary human bronchial epithelial cell wound repair was assessed in the presence or absence of HMGB1 (3 ng/ml; 72 h) in a scratch-wound healing assay. Wound closure was analyzed using an edge-smoothing tool in ImageJ. B: composite phase contrast image of scratch-wound at 0 and 72 h under a light microscope. Values represent means ± SE of experiments in 4 donors. ###P < 0.001 vs. control.

In complementary experiments we performed immunostaining for fibronectin and α3-integrin in HMGB1-treated scratch wound cultures in the presence and absence of TLR4 inhibitor CLI-095 or neutralizing antibody for RAGE (Fig. 4B). Abundant fibronectin and α3-integrin immunoreactivity was evident in HMGB1-treated cultures. As a positive control we have used TGF-β-treated cultures. TGF-β upregulates αs/β1 (fibronectin-specific) and αs/β1 (collagen-specific) but not the less selective integrin αs-chain that can bind laminin, collagen, and fibronectin (35). Therefore, we have used these TGF-β-treated cells as positive controls for induction of wound healing and fibronectin expression.

TLR4 and RAGE mediate HMGB1-induced ECM protein synthesis. We measured HMGB1-induced ECM synthesis and further assessed the effects of TLR4 or RAGE inhibition with CLI-095 or blocking antibody, respectively. Immunoblot and densitometry analysis of cell lysates obtained 24 h after HMGB1 exposure revealed that blockade of TLR4 or RAGE markedly attenuated fibronectin protein accumulation by 42 and 50%, respectively (Fig. 5A). We confirmed the specificity for the effects of exogenous HMGB1 in a complementary experiment in which we cotreated cell cultures with glycyrhrizin and observed that fibronectin accumulation was abolished. As α3-integrin is a subunit for the fibronectin-selective integrin, αsβ1, we monitored its abundance, confirming a glycyrhrizin-sensitive induction by HMGB1 and revealing a requirement for RAGE or TLR4, as both CLI-095 and neutralizing antibody for RAGE abrogated the effect of HMGB1 exposure (Fig. 5B). Last, we also assessed whether HMGB1 exposure induced synthesis of the γ2-chain of laminin-5, as it accumulates at sites of epithelial damage (33). Immunoblot analysis showed that exposure to exogenous HMGB1 for 24 h induced both a precursor form of laminin γ2-chain (Fig. 5C, top band) and the processed, mature form of the γ2-chain (Fig. 5C, bottom band). HMGB1-induced accumulation of both forms of laminin γ2-chain was abrogated by cotreatment with the TLR4 inhibitor CLI-095, neutralizing RAGE antibody, and glycyrhrizin (Fig. 5C).

MAPK and SMAD2 mediate HMGB1-induced wound repair in bronchial epithelial cells. To elucidate the downstream pathways that mediate the effects of exogenous HMGB1 on wound closure we performed immunoblotting to profile changes in effector protein phosphorylation status after HMGB1 exposure in vitro. We observed an increase in phosphorylation of the MAPKs, ERK1/2, and JNK, 24 h after HMGB1 exposure (Fig. 6A). This appeared to be a direct receptor-mediated response to exogenous HMGB1 as TLR4 and RAGE blockade, and inhibition of extracellular HMGB1 with glycyrhrizin, prevented phosphorylation of ERK1/2 and JNK. During the wound repair process, in addition to a loss of E-cadherin, markers of epithelial mesenchymal transition (EMT) are also expressed in epithelial cell cultures (39, 42). To monitor the induction of EMT, we measured the impact of HMGB1 exposure on abundance of SNAI1, a zinc-finger transcription factor that represses E-cadherin that is considered a key trigger for EMT. Immunoblot analysis of cell lysates obtained 72 h after HMGB1 exposure revealed that SNAI1 abundance increased in a concentration dependent manner (Fig. 6B, top). We also surveyed SMAD2 expression and phosphorylation as its activation has also been linked to EMT and wound repair (30). We found that exogenous HMGB1 was sufficient to induce rapid and sustained (3 h) phosphorylation of SMAD2, the proximal regulatory effector of canonical TGF-β1 signal transduction. (Fig. 6B, bottom).

We next tested whether HMGB1-induced intracellular signal pathways were required to mediate the augmentation of epithelial cell wound closure that we observed in HMGB1-treated cultures. Employing our scratch wound closure assay we measured the impact of treating cultures with selective pharmacological inhibitors of MEK (the immediate upstream activator of ERK1/2) (U0126), JNK (SB600125), or TGF-β receptor 1 isoforms ALK4, ALK5, and ALK7 (SB505124; Fig. 6C). We observed that inhibition of each of these signaling effectors was sufficient to limit wound closure to only 4.2 ± 1.2, 5.8 ± 2.0, and 7.9 ± 2.0%, respectively. This fully abrogated HMGB1-induced wound closure (P < 0.05) and may even have limited wound closure compared with control cultures in the absence of exogenous HMGB1, although this was not statistically significant.

DISCUSSION

This study builds on observations from murine models of repeated allergic challenge and human airway specimens from asthmatic and COPD donors that demonstrate epithelial damage and dysregulated epithelial repair (31, 43, 48). The complex integration of inflammation, tissue damage, and tissue repair underpins the pathogenesis of airway diseases; however, full understanding of the factors that modulate these processes
remains elusive. Here we have investigated the roles and mechanisms of HMGB1, an alarmin that has been associated with lung inflammation and fibrosis, in the control of wound repair in human bronchial epithelial cells. Similar to other studies, we confirm that TLR4 (mRNA) and RAGE (mRNA and protein) is expressed in primary human bronchial epithelial cells (58). We also confirm the upregulation of HMGB1 in the airway epithelium in human COPD lung tissue and murine allergic model of asthma. Although our study was not specifically designed to directly mimic the in vivo condition, we used in vitro models to provide the necessary data required to proceed with future in vivo studies using models to mimic human pathophysiology. These observations in lung tissue serve as an important rationale for investigating the role of extracellular HMGB1 in wound healing. To extrapolate any in vitro effect of extracellular HMGB1 with observations in lung tissue requires specific longitudinal pathological studies to track/correlate epithelia damage and HMGB1 at time of allergen exposure over the course of disease.

We also demonstrate that extracellular HMGB1 promotes the wound repair process in cultured human bronchial epithelial cells in vitro. Although extracellular HMGB1 differentially induces RAGE and TLR4 expression, for the first time we show that both TLR4 and RAGE signaling mediate HMGB1-induced wound repair involving bronchial epithelial cells, concomitantly promoting the expression of the γ2-chain of laminin-5, fibronectin, and the α3-integrin subunit of fibronectin-selective receptors. Our study also reveals that ERK1/2 and JNK are required for TLR4- and RAGE-mediated wound closure in vitro, and we provide evidence that HMGB1 induces markers for EMT, including loss of E-cadherin, accumulation of SNAIL, and induction of canonical TGF-β1-receptor-mediated signaling involving SMAD2. Collectively, this study uncovers a direct role for HMGB1 in airway epithelial wound repair and generates mechanistic understanding that will enable better understanding of airways disease pathogenesis and heterogeneity in response to clinical therapies.

Extracellular HMGB1 is a proinflammatory member of the DAMP family molecules that are released from stressed or damaged cells (5). As such, HMGB1 has been identified as an initiating and perpetuating factor for cellular responses associated with stress and injury, including that of repetitive exposure to allergens and viral infection (59, 62). Epithelial shedding (43, 48) and its repair and restoration, involving EMT (3, 12, 74), are pathogenic features of allergic asthma and COPD. We show in human lung tissue from COPD donors that HMGB1 localizes to the airway epithelium and parenchyma regions and that there is strikingly robust labeling for HMGB1. This reveals that there are multiple cellular sources for HMGB1 in the inflamed airway that can contribute to microenvironments with disparate levels of the secreted alarmin. This is of relevance because whereas elevated systemic HMGB1 drives sepsis (76) and chronic inflammatory diseases such as

Fig. 4. HMGB1-TLR4/RAGE axis mediates bronchial epithelial cell wound repair. A: primary human bronchial epithelial cell wound repair was assessed in the presence or absence of HMGB1 (3 ng/ml) with TLR4 inhibitor (CLI-095, 1 μM), neutralizing RAGE antibody (10 μg/ml), combined TLR4 inhibitor and neutralizing RAGE antibody, or glycyrrhizin (100 μM) pretreatment at 72 h in a scratch-wound healing assay. TGF-β1 was used as positive control. Wound closure was analyzed using an edge smoothing tool in ImageJ. B: primary human bronchial epithelial cells from scratch-wound assay were probed with antibodies for fibronectin (FN) and α3-integrin (α3) by immunofluorescence using FITC and Cy3 conjugated secondary antibodies respectively. Hoechst staining (DNA) of cell nuclei is shown in blue. Values represent means ± SE of experiments in 5–7 donor cells. ***P < 0.01, ###P < 0.001 vs. control; ***P < 0.001 vs. HMGB1.
rheumatoid arthritis (59), nanomolar concentrations of HMGB1 in skeletal muscle promotes tissue repair and regeneration (52). Our experiments assessing responses of human bronchial epithelial cells to HMGB1 indicate that loss of E-cadherin only at exogenous concentrations of 3 ng/ml or higher. In rat alveolar epithelial cells HMGB1 levels have been measured and found to be around 8–10 ng/ml following six linear scratch wounds (49). We selected the loss of E-cadherin protein as a relevant marker because of its role as a cell-cell adhesion protein and the requirement for its downregulation during epithelial cell migration (39, 42).

Based on these findings we used a single effective concentration of HMGB1 (3 ng/ml) for experiments that investigated wound repair and the receptor-mediated pathways that underpin the effects of exogenous HMGB1. In addition, we have focused on the role of extracellular HMGB1 and used a pharmacological tool that “mops up” extracellular HMGB1. We acknowledge that combined use of pharmacological tools alongside techniques such as siRNA can address the lack of specificity, off target effects, and secondary effects that both drugs and molecular tools carry. Although silencing RNA could provide some information, its use in our study answers a different question. Silencing endogenous HMGB1 addresses the protein’s intracellular role in the nucleus as well as possible autocrine effects as a DAMP.

We are specifically investigating whether and how cells respond to extracellular HMGB1 in the process of wound healing, thus silencing endogenous HMGB1 would confound our experimental design.

As HMGB1 has been linked to cell migration (75) and with tissue repair and regeneration (55), we investigated the effects of exogenous HMGB1 on human airway epithelial wound closure using an in vitro scratch wound assay. Scratch wound healing in vitro occurs through migration of epithelial cells from the monolayer into the wound area and in part from proliferation of cells at the leading edge (46, 70). Our experiments were not designed to dissect the relative contribution of migration vs. proliferation in HMGB1-exposed cultures, but HMGB1 does not appear to induce proliferation of lung epithelial cells in scratch wound assays (49), whereas several reports describe the capacity for HMGB1 to induce the migration of a number of different cell types (60, 75). We show that HMGB1 significantly promotes wound closure to a degree that is equivalent to that achieved with TGF-β1, which can induce EMT (7), an effect that promotes epithelial repair (53). Similar to the known effects of TGF-β1 on epithelial repair, we also reveal that SNAIL, a trigger of EMT and transcriptional repressor of E-cadherin, is also expressed following HMGB1 exposure. These similarities between HMGB1 and TGF-β1 on wound closure suggest that changes associated with EMT occur as part of the repair process upon HMGB1 exposure.

To gain mechanistic understanding of HMGB1 effects, we complemented our assessment of cell and monolayer function with a survey of fibronectin, the γ2-chain of laminin-5, and α3-integrin, which are known to play important roles in substrate formation and its interaction with epithelial cells to direct the repair phase of wound healing (24, 33). Consistent with the wound repair-promoting effects of HMGB1 we observed a concomitant induction of γ2-chain of laminin-5, fibronectin, and α3-integrin synthesis by human airway epithelial cells.

A unique feature of our study is experiments that demonstrate TLR4 and RAGE mediate wound closure, ECM protein
and receptor expression, and intracellular signaling induced by HMGB1 in human airway epithelial cells. Our findings are consistent with a report describing a role for TLR4- and RAGE-dependent signaling in promoting the rate of wound closure in rat alveolar type-II epithelial cells (49). Our data reveal that inhibition of only one receptor is sufficient to abrogate responses to HMGB1, indicating that there may be need for cooperation between both receptors or cross talk that is required for the functional outcomes we assessed. Evidence for cooperative effects of TLR4 and RAGE have been reported for studies investigating synergy of HMGB1-LPS complexes; these indicate that the inhibition of TLR4 or RAGE signaling is sufficient to attenuate the synergistic effect of HMGB1-LPS complexes on cytokine release (23, 51). Further compelling evidence for TLR4 and RAGE receptor interaction has been demonstrated by Sakaguchi et al. (57), using ligands that bind both receptors and those that bind TLR4 or RAGE alone. They reported that intracellular signaling domains of TLR4 and RAGE share adaptor proteins, including Toll-interleukin-1 receptor adaptor protein (TIRAP) and myeloid differentiation primary response gene 88 (MYD88), to transduce signals to downstream MAPKs. They further confirmed that downstream MAPK signaling induced by HMGB1-LPS complexes are diminished when function-blocking mutated cytoplasmic domains of RAGE or TLR4 are expressed. These findings are highly consistent with our current observations, as we show that inhibition of either TLR4 or RAGE is sufficient to diminish HMGB1-induced signaling, ECM protein or receptor expression, and wound closure. Homeostatic control of ECM synthesis and wound healing is tightly regulated, and repetitive damage and release of HMGB1 may “break” this control leading to excessive ECM deposition and fibrosis. Profibrotic responses have been attributed to exogenous HMGB1 including TLR4 and/or RAGE signaling. For example He et al. (22) demonstrated that whereas markers of EMT are induced upon HMGB1 exposure by alveolar epithelial cells from wild-type mice, this response was significantly abolished in cells from RAGE deficient mice treated with HMGB1.

**Fig. 6.** MAPK and SMAD-2 signaling mediate HMGB1-induced wound repair in bronchial epithelial cells. **A:** cell lysates (10 μg) of primary human bronchial epithelial cells in the presence or absence of HMGB1 (3 ng/ml) with or without TLR4 inhibitor (CLI-095; 1 μM), neutralizing RAGE antibody (10 μg/ml), or glycyr rhizin (100 μM) pretreatment were run on 12% gels and analyzed by immunoblotting for phospho-ERK and phospho-JNK expression. **B:** SNAIL (72 h; top) and phospho-SMAD2/total SMAD2/3/GAPDH (0–180 min; bottom) protein expression in primary human bronchial epithelial cells in the presence or absence of HMGB1 (3 ng/ml) were run on 12% gels and analyzed by immunoblotting. **C:** primary human bronchial epithelial cell wound healing was assessed in the presence or absence of HMGB1 (3 ng/ml) with 10 μM ERK inhibitor (U0126), 10 μM JNK inhibitor (SP600125), or 5 μM TGF-β receptor 1 ALK4, ALK5, and ALK7 inhibitor (SB505124) pretreatment for 1 h at 72 h in a scratch-wound healing assay. Wound closure was analyzed using an edge smoothing tool in ImageJ. Values represent means ± SE of experiments in 3–7 donors. ###P < 0.001 vs. control; **P < 0.01, ***P < 0.001 vs. HMGB1.
TLR4 ligand, uric acid (which is also a DAMP), recapitulates lung fibrosis and repair in a bleomycin lung injury model (16). In allergic asthma, abrogation of HMGB1 also reduced indices of airway remodelling (36).

Few reports have focused on the role of the HMGB1-TLR4 axis in human lung epithelial cells (8, 47, 77). Despite the limited number of studies on HMGB1-TLR4 axis in epithelial wound repair, it is known that TLR4 plays an important role in tissue repair and remodelling. For example, hyaluronan, a TLR4 ligand expressed on the surface of lung alveolar epithelial cells, is a key mediator of tissue repair and remodelling that helps support maintenance of alveolar epithelial cell integrity (26). A study by Pittet et al. (49) in alveolar epithelial cells showed that TLR4- and RAGE-dependent wound closure in response to HMGB1 occurs through a TGF-β-linked pathway. More studies are needed to fully dissect HMGB1-TLR4 axis in lung fibrosis, but based on the limited evidence available, and the new data uncovered in our current study, it appears that TLR4 signaling may serve as a self-limiting pathway between tissue repair and fibrosis.

A number of studies show that RAGE mediates signaling that is important for HMGB1-induced wound and tissue repair and regenerative processes (52, 55). One study observed that lungs of RAGE-deficient mice challenged with bleomycin and alveolar epithelial cells from RAGE-deficient mice treated with bleomycin were both protected against bleomycin-induced fibrosis (13). A recent study also revealed that HMGB1 release by airway epithelial cells contributes to pathophysiologic hallmarks of allergic asthma via mechanisms that involve RAGE- and TLR4-mediated signaling (68). In the context of COPD and tissue repair, a study by Stogsdill et al. (64) reported that conditional overexpression of RAGE in adult murine alveolar epithelia resulted in altered matrix turnover. To our knowledge, our study and those by Pittet et al. (49) are unique in demonstrating a role for RAGE in mediating HMGB1-induced intracellular signaling and wound healing in airway epithelial cells.

A diverse repertoire of downstream signaling pathways modulate wound repair in various tissues, (9, 66), and some are associated with TLR4- or RAGE-mediated cell responses (21, 50, 73). RAGE signaling can induce ECM synthesis through ERK1/2-dependent pathways associated with wound healing and fibrosis, but in contrast to our studies, this study did not uncover the induction of JNK phosphorylation (72). Further evidence in support of our observations demonstrated that a truncated intracellular domain of RAGE altered cell migration and adhesion and impaired RAGE-induced phosphorylation of ERK1/2, p38 and JNK (27). Canonical TLR4 activation recruits intracellular adaptor proteins MYD88 and TIRAP inducing interferon-β (TRIF) downstream of the TLR4 complex (15). In our studies we used CLI-095 to block the intracellular binding site for these adaptor proteins to blunt TLR4-mediated signaling (41). In human airway epithelial cells, TLR4 ligands such as lipopolysaccharide (LPS) and cigarette smoke extract induce ERK1/2 and JNK activation in airway epithelial cells (20, 67). We demonstrated that ERK1/2 and JNK activation occurred downstream of both TLR4 and RAGE and that inhibition of both MAPKs significantly reduced HMGB1-induced wound closure. Our data support a clear role for ERK and JNK, and perhaps the signaling differences reported in the literature may be specific to cell type or tissue localization.

Our study is the first in human airway epithelial cells to show that HMGB1 induces phosphorylation of SMAD2 and that HMGB1-induced wound closure was abolished by TGF-β receptor 1 inhibition. Taken together, this demonstrates a link between RAGE and/or TLR4 receptors with SMAD2 signaling. Few studies have established a direct link between HMGB1 and SMAD2 in the lung. Li et al. (38) demonstrated that RAGE-induced SMAD2 phosphorylation in TGF-β receptor I and II mutant cells was abolished by MAPK inhibitors, suggesting that MAPKs may mediate cross talk between HMGB1 receptor (RAGE or TLR4) and TGF-β-receptor-mediated effects. A similar study on fibrosis also reported that HMGB1, via RAGE, can concomitantly induce ECM protein synthesis, and phosphorylation of ERK1/2 and SMAD2, an effect that appears to be independent of TLR4 signaling (29).

A direct role for TLR4 signaling and SMAD2 activation is unclear, but evidence from a recent study demonstrated that the TLR4 ligand LPS sensitizes lung fibroblasts to the effects of TGF-β, augmenting canonical SMAD signaling and TGF-β-induced ECM protein synthesis in lung fibroblasts (4). Taken together, these studies support our observations that link exogenous HMGB1 with TGF-β signaling, including SMAD2 and the EMT transcription marker SNAIL. This contributes to wound repair; however, additional experiments are still needed to fully dissect the molecular mechanisms associated with

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Fig. 7. Schematic representation of HMGB1-induced wound repair in bronchial epithelial cells. Following airway injury or damage, HMGB1 is released and interacts with its receptor RAGE and TLR4 (1). This receptor mediated effect is linked to activation of downstream signaling MAPKs (ERK1/2 and JNK; 2), the ECM proteins α3 intergrin, fibronectin, and γ2 chain laminin-5 (3); and wound repair in bronchial epithelial cells (4). The HMGB1-induced wound repair occurs through canonical TGF-β receptor 1 signaling (possible through SMAD2) including ERK1/2 and JNK signaling.
HMGB1-induced TGF-β-SMAD2 signaling in human airway epithelia cells.

In summary, we show that HMGB1 promotes human airway epithelial wound repair. This is important as it is well known that wound repair is essential to reconstitute and restore the needed integrity of the airway epithelial layer following damage or injury and failure to do so contributes to the pathogenesis of obstructive airway diseases. A minor limitation of our study is the use of monolayer cultures rather than air-liquid interface (ALI) differentiated human airway epithelial cells. Mechanical scratch wound in ALI differentiated cultures proved technically challenging to study the effects of extracellular HMGB1 on wound healing, and perhaps using a nonmechanical wound method for cell detachment such as high air pressure or NaOH in ALI cultures may provide a more feasible approach. Nonetheless, our study reveals that HMGB1 signaling axis involves TLR4 and RAGE and a coordinated induction of ERK1/2 and JNK that underpins epithelial wound closure through mechanism that involves loss of epithelial cell-cell contact, induction of the synthesis of ECM proteins (fibronectin and laminin-5), and receptors for ECM. Moreover, our data suggest that HMGB1 promotes canonical TGF-β1-receptor-mediated signaling that also contributes to wound repair (Fig. 7). Airway exposure to irritants or allergens can stress or damage cells in the airway leading to HMGB1 release. Our study suggests that HMGB1 promotes wound repair and implies the need for future investigation using imaging modalities to study and longitudinally track the impact of HMGB1 on wound repair hallmarks in vivo. Furthermore, our findings are important in the context of understanding the effectiveness of therapies for obstructive airway diseases, as approaches that have broad suppressive effects on inflammation may negatively impact on the HMGB1 prerepair mechanism. Follow-up studies using primary bronchial epithelial cells from diseases (COPD or asthma) donors from this current work are also important to provide a tie-in with disease context.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

O.O.O. conception and design of research; O.O.O. and A.J. performed experiments; O.O.O. and M.H.R. analyzed data; O.O.O. interpreted results of experiments; O.O.O. prepared figures; O.O.O. drafted manuscript; H.U. and A.J.H. approved final version of manuscript; A.J.H. edited and revised manuscript.

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