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ARTICLES

Regulation of lung injury and repair by Toll-like receptors and hyaluronan

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Mechanisms that regulate inflammation and repair after acute lung injury are incompletely understood. The extracellular matrix glycosaminoglycan hyaluronan is produced after tissue injury and impaired clearance results in unremitting inflammation. Here we report that hyaluronan degradation products require MyD88 and both Toll-like receptor (TLR)4 and TLR2 *in vitro* and *in vivo* to initiate inflammatory responses in acute lung injury. Hyaluronan fragments isolated from serum of individuals with acute lung injury stimulated macrophage chemokine production in a TLR4- and TLR2-dependent manner. *Myd88*$^{-/-}$ and *Tlr4*$^{-/-}$*Tlr2*$^{-/-}$ mice showed impaired transepithelial migration of inflammatory cells but decreased survival and enhanced epithelial cell apoptosis after lung injury. Lung epithelial cell–specific overexpression of high-molecular-mass hyaluronan was protective against acute lung injury. Furthermore, epithelial cell–surface hyaluronan was protective against apoptosis, in part, through TLR-dependent basal activation of NF-κB. Hyaluronan-TLR2 and hyaluronan-TLR4 interactions provide signals that initiate inflammatory responses, maintain epithelial cell integrity and promote recovery from acute lung injury.

Successful repair of tissue injury requires a coordinated host response to limit the extent of structural cell damage. A hallmark of tissue injury is increased turnover of extracellular matrix (ECM). Failure to remove ECM degradation products from the site of tissue injury results in the host succumbing to unremitting inflammation[1]. Hyaluronan, a major component of ECM, is a nonsulfated glycosaminoglycan composed of repeating polymeric disaccharides D-glucuronic acid and *N*-acetyl glucosamine. Under physiologic conditions hyaluronan exists as a high-molecular-weight polymer ($>10^6$ Da) and undergoes dynamic regulation, resulting in accumulation of lower-molecular-weight species after tissue injury and inflammation[1,2]. Clearance of hyaluronan fragments is crucial to resolving lung inflammation and is dependent on the hyaluronan receptor CD44 (ref. 3), expressed on hematopoietic cells[1]. But in the absence of CD44, expression of genes involved in inflammation persists, suggesting that signaling pathways distinct from hyaluronan-CD44 regulate macrophage responses to hyaluronan fragments[1]. The polymeric structure of hyaluronan and the recent report suggesting that hyaluronan oligomers can signal through TLR4 in dendritic cells[4] and endothelial cells[5] led us to investigate the potential role of TLRs in noninfectious lung injury, after which matrix turnover is an important regulator of the inflammatory response[4,6]. In this study, we found that hyaluronan recognition by the host requires both TLR2 and TLR4, and this interaction regulates both the innate inflammatory response as well as epithelial cell integrity that is crucial for recovery from acute lung injury.

RESULTS

Hyaluronan stimulates chemokines through TLR2 and TLR4

It became apparent from studies of lung injury in CD44-null mice that although CD44 is essential for regulating hyaluronan turnover, it is not required for expression of chemokines by macrophages *in vivo*[3]. To further investigate the mechanisms regulating macrophage effector functions in response to hyaluronan degradation products, we generated hyaluronan fragments of defined size and used them to stimulate wild-type and CD44-null macrophages *in vitro*. We found that 135 kDa hyaluronan fragments induced chemokine MIP-2 expression in CD44-deficient peritoneal macrophages (**Fig. 1a**) and bone marrow–derived macrophages (data not shown). Because hyaluronan is a repeating disaccharide structure with features of 'pathogen-associated molecular patterns,' we investigated whether hyaluronan fragments could signal through a TLR-mediated pathway. Elicited peritoneal macrophages from mice deficient in the TLR adaptor protein MyD88 were treated with purified hyaluronan fragments. Hyaluronan-induced expression of *Cxcl2* mRNA (which encodes MIP-2) was abolished in *Myd88*$^{-/-}$ macrophages (**Fig. 1b**). We then examined the role of TLRs in hyaluronan fragment–induced expression of chemokines in macrophages. Expression of *Cxcl2* mRNA was reduced,

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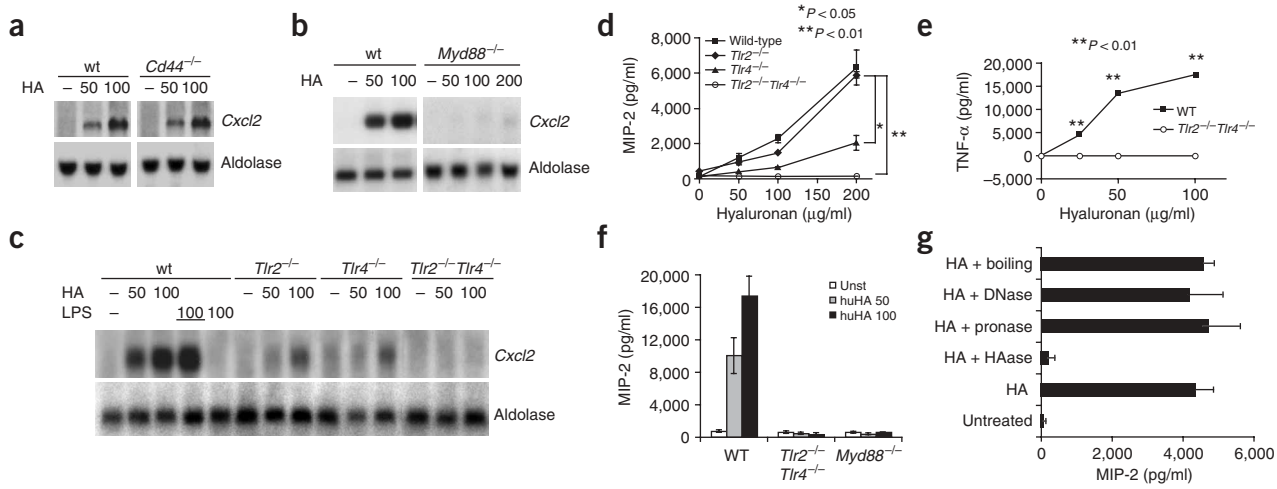

Figure 1 Hyaluronan fragments stimulate chemokine expression through both TLR4 and TLR2. (**a**) *Cxcl2* mRNA expression by peritoneal macrophages from *Cd44*$^{-/-}$ and wild-type (wt) mice treated with 135 kDa hyaluronan fragments (in μg/ml) in the presence of polymyxin B for 4 h, detected by northern analysis. (**b**) *Cxcl2* mRNA expression by peritoneal macrophages from wild-type or *Myd88*$^{-/-}$ mice treated with 135 kDa hyaluronan fragments (in μg/ml) detected by northern analysis. (**c**) *Cxcl2* mRNA expression by peritoneal macrophages from wild-type, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ or *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice treated with 135 kDa hyaluronan fragments or lipopolysaccharide (100 ng/ml) in the presence or absence (underlined) of polymyxin B, detected by northern analysis. (**d**) MIP-2 protein expression by peritoneal macrophages from wild-type, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ or *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice treated with 135 kDa hyaluronan fragments ($n = 5$). (**e**) TNF-α expression by peritoneal macrophages from wild-type or *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice treated with 135 kDa hyaluronan ($n = 5$). (**f**) MIP-2 protein expression by peritoneal macrophages from wild-type, *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ or *Myd88*$^{-/-}$ treated with 50 or 100 μg/ml of human hyaluronan fragments (huHA) from the serum of individuals with acute respiratory distress syndrome ($n = 4$). Unst, unstimulated. (**g**) 135 kDa hyaluronan (HA, 100 μg/ml) alone or after digestion with hyaluronidase (HAase), pronase or deoxyribonuclease I (DNAse) were used to stimulate peritoneal macrophages. MIP-2 levels in 24-h conditioned media were measured ($n = 4$).

but remained present in both TLR2- and TLR4-deficient macrophages (**Fig. 1c**). *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$ mice were crossed to generate TLR2 and TLR4 double-knockout mice (*Tlr2*$^{-/-}$*Tlr4*$^{-/-}$). Hyaluronan fragment–induced expression of protein and mRNA encoding chemokines and cytokines was completely abolished in *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ peritoneal macrophages (**Fig. 1c–e** and **Supplementary Fig. 1** online). In contrast, targeted deletion of the genes encoding TLR1, TLR3, TLR5 and TLR9 had no effect on hyaluronan fragment–induced expression of chemokines (**Supplementary Fig. 2** online). To determine whether these findings were relevant to acute lung injury in humans, we then examined circulating hyaluronan fragments produced *in vivo* and purified from serum of individuals with acute lung injury. The human hyaluronan degradation products were of similar molecular mass (peak, 200 kDa) to the *in vitro*–generated hyaluronan degradation products and stimulated chemokine MIP-2, MIP-1α and KC production in wild-type but not *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ peritoneal macrophages (**Fig. 1f** and **Supplementary Fig. 3** online).

We performed experiments on numerous controls to rule out the potential effects of contaminating endotoxin, protein or DNA on the hyaluronan-mediated induction of chemokine expression (**Supplementary Fig. 4** online). Notably, the induction of chemokine expression was abolished when the hyaluronan fragments generated *in vitro* or purified from human serum were exhaustively digested with

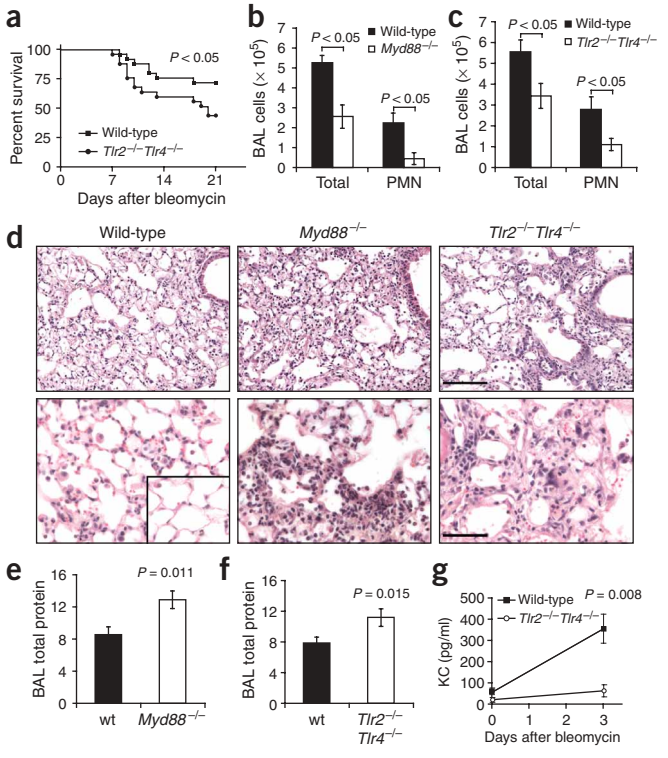

Figure 2 Impaired lung inflammatory cell recruitment but increased lung injury in the absence of TLR2 and TLR4. (**a**) Wild-type and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice ($n = 25$) were subjected to intratracheal bleomycin treatment. Percentages of surviving mice were plotted over a 21-d period. $P < 0.05$ by the log-rank test. (**b**,**c**) Total BAL cells and polymorphonuclear neutrophils (PMN) were counted from wild-type, *Myd88*$^{-/-}$ (**b**) or *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice (**c**) 7 d after bleomycin treatment. (**d**) H&E staining of lung tissue sections from wild-type, *Myd88*$^{-/-}$ and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice 7 d after bleomycin treatment. Scale bars in upper panels, 100 μm; in lower panels, 50 μm. Insert shows relatively normal alveolar structures. (**e**,**f**) Total protein concentrations (ng/ml) of BAL were measured from *Myd88*$^{-/-}$ (**e**) or *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice (**f**) 7 d after bleomycin treatment. (**g**) KC protein was measured in BAL from wild-type and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice 3 d after bleomycin-induced lung injury.

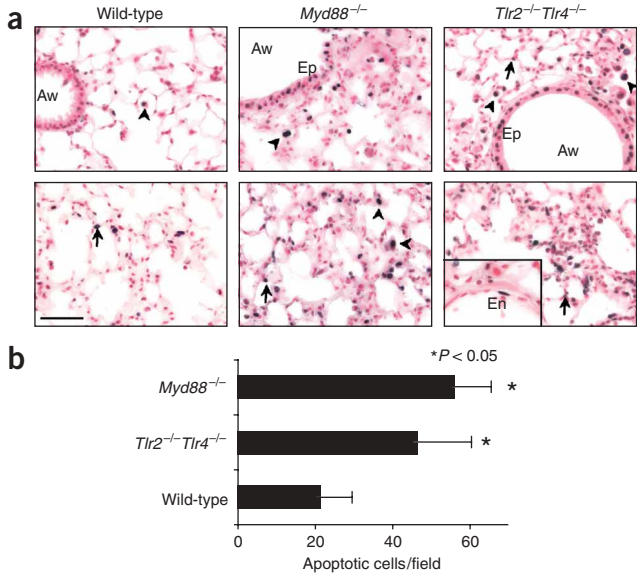

Figure 3 TLRs and hyaluronan regulate lung cell apoptosis. TUNEL staining (**a**) and quantification (**b**) of apoptosis from lung tissue sections of wild-type, *Myd88*$^{-/-}$ and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice 5 d after bleomycin injury. Apoptosis of airway epithelial cells, inflammatory cells and some alveolar epithelial cells is more apparent. Occasionally endothelial cell apoptosis can be seen (insert in *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$). Scale bar, 50 μm. Arrowheads indicate inflammatory cells. Aw, airway; Ep, airway epithelial cells; En, endothelial cells; arrows, alveolar epithelial cells. Quantification of TUNEL-positive staining cells was performed by counting cells in 40 fields of each slide at original magnification of ×200 (n = 5–7).

testicular hyaluronidase (**Fig. 1g**), but unaffected by pronase, deoxyribonuclease 1, polymyxin B or boiling treatment (**Supplementary Fig. 4** online).

Role of TLR2 and TLR4 in noninfectious lung injury

We then examined the role of TLRs in lung inflammation and repair *in vivo* using the bleomycin model of acute lung injury[7]. Notably, we found that *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice were more susceptible than control mice to bleomycin-induced lung injury (**Fig. 2a**). Examination of the accumulation of inflammatory cells in the alveolar space showed a decrease in total cells, specifically neutrophils, in the bronchoalveolar lavage (BAL) fluid from both *Myd88*$^{-/-}$ and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice after lung injury (**Fig. 2b**,**c**). But lung tissue sections from the MyD88- or TLR-deficient mice showed evidence of enhanced injury with thickened interstitium, increased inflammatory cell accumulation within the interstitium (**Fig. 2d**) and increased protein accumulation in the BAL fluid relative to wild-type littermate controls (**Fig. 2e**,**f**).

To confirm the histological observation, we quantified inflammatory cells in lung tissue 5 d after bleomycin-induced lung injury. We found that there was no difference in total lung inflammatory cells between wild-type and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice, although substantially fewer neutrophils were present in the lungs of *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice (**Supplementary Fig. 5** online). We then examined the expression of the neutrophil chemotactic factor KC[8–10] after lung injury and found that *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice produced significantly less KC in the alveolar space than did wild-type controls (**Fig. 2g**). We found that bleomycin directly induced production of KC by purified lung epithelial cells from wild-type mice *in vitro*, whereas epithelial cells from *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice did not produce KC (**Supplementary Fig. 6** online). These data suggest that not only can KC be produced by macrophages in response to hyaluronan fragments, but also by pulmonary epithelial cells in direct response to bleomycin. Both of these responses are TLR dependent.

To determine whether TLRs have a generalized protective effect against noninfectious lung injury, we examined the role of deficiency of TLR2 and TLR4 in hyperoxia-induced lung injury. Mice exposed to >98% O_2 undergo pathophysiological changes characterized by endothelial and epithelial injury and enhanced alveolar capillary protein leak, similar to those seen in acute lung injury or adult respiratory distress syndrome[11,12]. We observed that *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice were more sensitive to hyperoxia. Survival time was reduced by nearly half in *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice (**Supplementary Fig. 7** online).

Role of TLRs in epithelial cell integrity

A crucial determinant of repair of lung injury is the extent of alveolar epithelial cell injury and apoptosis. Inhibitors of apoptosis have been shown to be protective against acute lung injury[13,14]. We examined apoptosis by TUNEL staining in lung sections of wild-type, *Myd88*$^{-/-}$ and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice after bleomycin-induced lung injury (**Fig. 3**). *Myd88*$^{-/-}$ and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice showed significant increases in TUNEL-positive cells when compared with wild-type mice at day 5 after injury (**Fig. 3**). The distribution of TUNEL staining is consistent

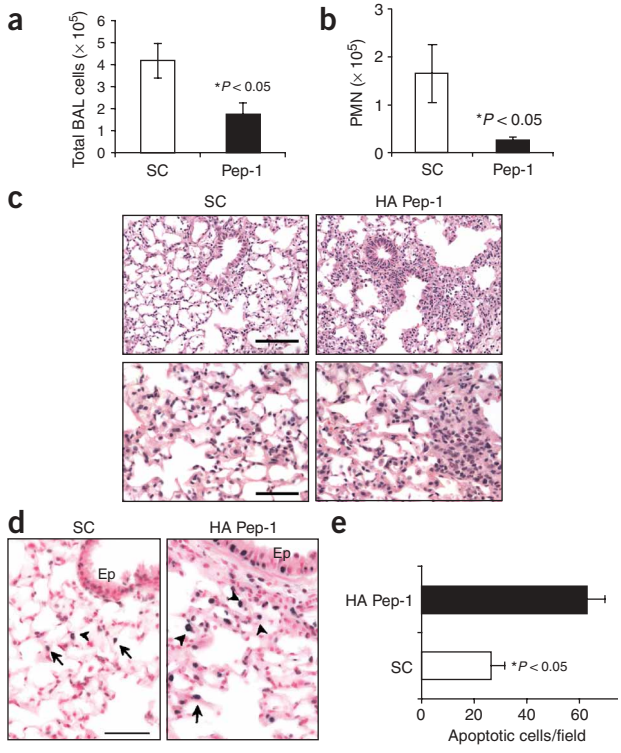

Figure 4 Blockade of hyaluronan function *in vivo* impairs lung inflammatory cell recruitment but worsens lung injury. HA Pep-1 or scrambled peptide (SC) was administrated intraperitoneally 2 h before and 3 and 5 d after bleomycin treatment. Total BAL cells (**a**) and polymorphonuclear neutrophils (**b**) were counted 7 d after bleomycin treatment (n = 4). (**c**) H&E staining of lung tissue sections of Pep-1– or SC-treated mice 7 d after bleomycin treatment (n = 6). Scale bars in upper panels, 100 μm; in lower panels, 50 μm. (**d**) TUNEL staining and (**e**) quantification of apoptosis from lung tissue sections of Pep-1– or SC-treated mice 5 d after bleomycin injury. Arrowheads indicate inflammatory cells. Ep, airway epithelial cells; arrows, alveolar epithelial cells. Scale bar, 50 μm.

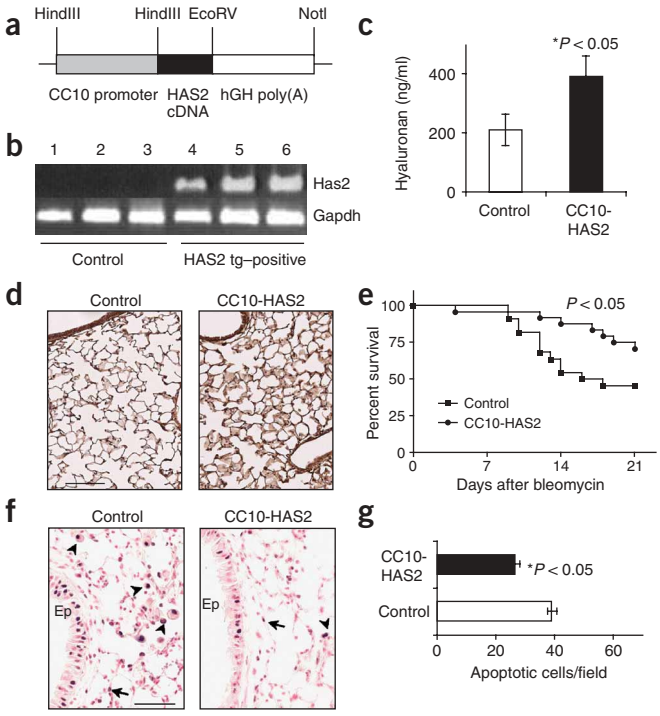

Figure 5 Overexpression of high-molecular-mass hyaluronan ameliorates lung injury in CC10-HAS2 transgenic mice. (**a**) The construct used to generate of CC10-HAS2 transgenic mice is shown. (**b**) The lung-specific expression of *Has2* mRNA in transgene-positive mice (lanes 4–6) was assessed by RT-PCR with specific primers spanning the transgene. (**c**) Hyaluronan levels in BAL fluid of unchallenged CC10-HAS2 transgenic mice and littermate control ($n = 5$). (**d**) Lung sections from 8-week-old CC10-HAS2 transgenic mice and littermate control mice stained for hyaluronan. Scale bar, 50 μm. (**e**) CC10-HAS2 transgenic mice have improved survival after high-dose (7.5 U/kg) bleomycin treatment compared with littermate controls ($n = 23$). $P < 0.05$ by the log-rank test. (**f**) Decreased lung cell apoptosis in CC10-HAS2 transgenic mice on day 5 after bleomycin treatment (7.5 U/kg). Arrowheads indicate inflammatory cells. Ep, airway epithelial cells; arrows, alveolar epithelial cells. Scale bar, 50 μm. (**g**) Quantification of TUNEL-positive cells from lung tissue sections CC10-HAS2 and littermate control ($n = 5$).

with both lung epithelial cells and inflammatory cells undergoing apoptosis (**Fig. 3a**).

Blocking hyaluronan interactions with cognate receptors

We sought to determine whether interaction of hyaluronan fragments with TLRs regulated lung injury and repair *in vivo*. To block interactions between hyaluronan and cells, we used the hyaluronan-blocking peptide Pep-1, which has been shown to function effectively *in vivo* in inhibiting hyaluronan-dependent leukocyte recruitment[15]. Systemic administration of Pep-1 but not scrambled peptide generated a phenotype that was notably similar to the TLR2-TLR4– and MyD88-deficient state after acute lung injury. Pep-1 treatment resulted in a decrease in inflammatory cell recruitment to the alveolar space during bleomycin-induced lung injury (**Fig. 4a**,**b**). Examination of tissue sections showed histologic evidence of acute lung injury in the perivascular space with thickened interstitium that was consistent with enhanced lung injury (**Fig. 4c**). But despite the impaired transepithelial migration of neutrophils, the Pep-1–treated wild-type mice showed increased mortality (**Supplementary Fig. 8** online). We examined TUNEL staining in wild-type mice treated with Pep-1 or scrambled peptide after bleomycin-induced lung injury. Pep-1–treated mice showed significant increases in TUNEL-positive cells compared with wild-type mice with scrambled peptide in a manner that was notably similar to that observed in the *Myd88*$^{-/-}$ and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice after bleomycin treatment (**Fig. 4d**,**e**).

Overexpression of high-molecular-mass hyaluronan

To more directly examine the role of hyaluronan in regulating the repair of lung injury *in vivo*, we generated transgenic mice that constitutively express hyaluronan synthase 2 (HAS2, encoded by *Has2*)[16] under the direction of the lung-specific Clara cell promoter of the *Ugb* gene (which encodes CC10)[17] (CC10-HAS2; **Fig. 5a**,**b**). CC10-HAS2 mice produced increased concentrations of high-molecular-mass hyaluronan ($>10^6$ Da, data not shown) in BAL fluid compared to littermate control mice (**Fig. 5c**), and hyaluronan staining showed increased hyaluronan content in the alveolar epithelium and interstitium of CC10-HAS2 mice (**Fig. 5d**). When we challenged the CC10-HAS2 transgenic mice with high-dose intratracheal bleomycin treatment (7.5 U/kg), they showed improved survival relative to transgene-negative control mice (**Fig. 5e**). The improved survival correlated with a decrease in lung epithelial TUNEL-positive cells at day 5 after lung injury (**Fig. 5f**,**g**).

Epithelial hyaluronan protects against apoptosis through NF-κB

To directly examine the role of hyaluronan in regulating epithelial cell integrity, we isolated primary lung epithelial cells[18] from TLR-deficient mice and wild-type controls. We found that bleomycin induced apoptosis in alveolar epithelial cells *in vitro* (**Fig. 6**) as well as *in vivo*. This increase in apoptosis was prevented by the exogenous administration of high-molecular-mass hyaluronan. Primary epithelial cells from *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice were found to have a significant increase in spontaneous apoptosis relative to wild-type (**Fig. 6a**). The absolute magnitude of the apoptotic response to bleomycin was also significantly greater. Notably, the protective effect of high-molecular-mass hyaluronan was lost in epithelial cells from *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice. Similar results were found with the primary lung epithelial cells from *Myd88*$^{-/-}$ mice (**Fig. 6a**).

Activation of NF-κB has been shown to protect cells against proapoptotic stimuli[19–22]. We examined NF-κB activation in both wild-type and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ primary lung epithelial cells. Wild-type lung epithelial cells showed basal NF-κB–DNA binding activity that is further increased by bleomycin treatment (**Fig. 6b**). In contrast, *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ epithelial cells were found to have markedly diminished basal NF-κB–DNA binding activity that did not increase with bleomycin treatment. Furthermore, high-molecular-mass hyaluronan decreased the activation of NF-κB, and the NF-κB inhibitor Bay 11-7082 (refs. 23,24) inhibited basal NF-κB activation (**Fig. 6b**). To further define the role of epithelial cell hyaluronan, we examined cell-surface expression of hyaluronan and made the unexpected finding that *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ epithelial cells express considerably less hyaluronan on the cell surface (**Fig. 6c**). Treatment of wild-type lung epithelial cells with testicular hyaluronidase reduced both cell-surface expression of hyaluronan (**Fig. 6d**) and basal NF-κB–DNA binding activity (**Fig. 6e**). We then examined the relationship between NF-κB activation and epithelial cell apoptosis. The NF-κB inhibitor BAY 11-7082 increased basal epithelial cell apoptosis and augmented bleomycin-induced apoptosis (**Fig. 6f**). High-molecular-mass hyaluronan inhibited bleomycin-induced apoptosis. Finally, hyaluronidase treatment increased basal apoptosis and potentiated bleomycin-induced epithelial cell apoptosis (**Fig. 6g**,**h**). These data

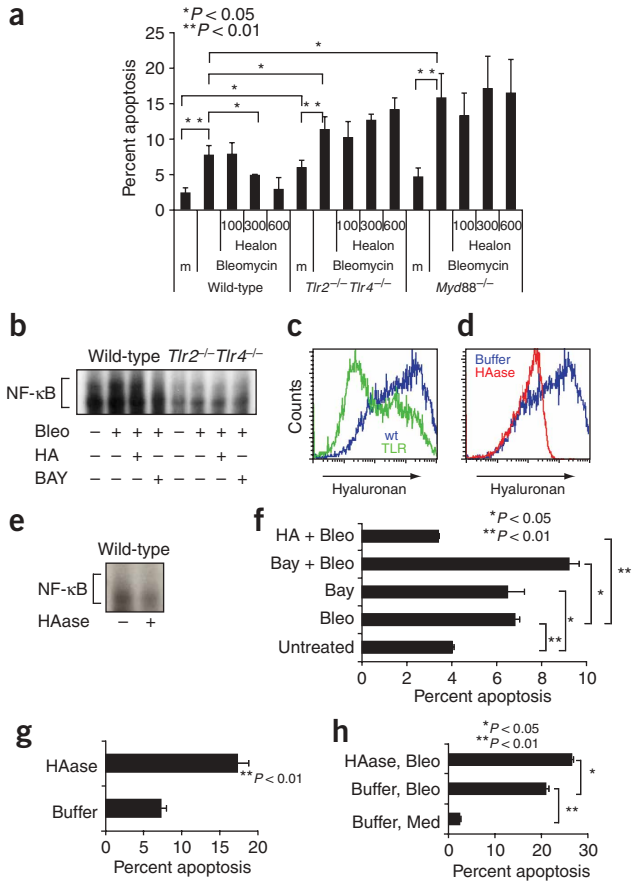

Figure 6 Cell-surface hyaluronan protects epithelial cells from apoptosis through NF-κB. (**a**) Apoptosis was induced in the purified primary lung epithelial cells from wild-type, *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ or *Myd88*$^{-/-}$ mice by bleomycin treatment for 24 h, or in the presence of high-molecular-mass hyaluronan (Healon, in μg/ml). m, medium only. Percentages of Annexin V–positive cells were present ($n = 5$–8). (**b**) Lung epithelial cells from wild-type or *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$mice were treated with bleomycin (Bleo) or in the combination with high-molecular-mass hyaluronan (HA) or Bay 11-8072 (Bay). NF-κB–DNA binding activity was assessed with electrophoresis mobility shift assay. (**c**) Hyaluronan expression on epithelial cell surface was compared between wild-type and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ by biotinylated hyaluronan-binding protein staining via flow cytometry. (**d**) Wild-type epithelial cells were treated with hyaluronidase (HAase). Hyaluronan expression on cell surface was analyzed. (**e**) NF-κB–DNA binding activity was assessed in wild-type epithelial cells treated with hyaluronidase. (**f**) Lung epithelial cells from wild-type mice were treated with bleomycin (Bleo), or in combination with high-molecular-mass hyaluronan (Healon) or Bay 11-8072 (Bay). Percentages of Annexin V–positive cells were present ($n = 4$). (**g**) Wild-type lung epithelial cells were treated with hyaluronidase for 1 h. Percentages of Annexin V–positive cells were present. (**h**) Wild-type lung epithelial cells were treated with hyaluronidase for 1 h, and followed by bleomycin treatment overnight. Percentages of Annexin V–positive cells were present ($n = 4$). Med, medium only.

suggest that epithelial cell-surface hyaluronan promotes basal NF-κB activation in a TLR-dependent manner, and this activation has a protective effect against injury.

DISCUSSION

We have explored the role of hyaluronan and TLRs in regulating lung inflammation after noninfectious lung injury. Our *in vitro* studies support a previously unrecognized role for the combination of TLR2 and TLR4 in mediating the induction of chemokine gene expression by hyaluronan degradation products in macrophages. We have previously shown that hyaluronan fragments induce a variety of genes involved in inflammation in macrophages from C3H/HeJ mice, which are defective in TLR4 signaling[25,26]. In this study, we generated a new preparation of purified hyaluronan degradation products and provided a number of controls to exclude contaminating substances as essential for the biological effect. Further support for a role of hyaluronan degradation products in mediating expression of genes involved in inflammation by macrophages is provided by the purified hyaluronan from serum of individuals with acute lung injury. The human hyaluronan fragments from individuals with acute lung injury stimulated peritoneal macrophages to produce chemokines in a TLR2- and TLR4-dependent manner. A recent study provided evidence that hyaluronan oligomers of 12–16 disaccharides stimulated cytokine production by dendritic cells[4]. The level of TNF-α production was much higher in macrophages with the larger polymer[25,26] compared to the oligosaccharide treatment of dendritic cells[4]. We cannot exclude oligosaccharides as a subset of hyaluronan polymers contributing to the effect in macrophages observed here, but the greater magnitude of the response suggests that the mechanism may not be identical to that in dendritic cells and is consistent with a synergistic effect of TLR4 and TLR2.

We anticipated that *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ mice would be protected from bleomycin-induced lung injury because of an impaired ability of macrophages to respond to hyaluronan degradation products. We did observe an inhibition of neutrophil recruitment to the alveolar space and reduced KC production in BAL from *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ mice. Despite the impaired recruitment of neutrophils to the alveolar space, both the *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ mice showed decreased survival after acute lung injury. Both alveolar macrophages and epithelial cells seem to contribute KC to the inflammatory milieu. Deficiency of TLR2 and TLR4 resulted in an inadequate chemokine gradient and impaired neutrophil transepithelial migration.

As lung epithelial cell repair is essential for host survival in acute lung injury[27,28], we explored the possibility that hyaluronan-TLR interactions may have a role in regulating lung epithelial cell responses to acute injury. We found that both *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ mice showed substantially increased TUNEL-positive staining of lung epithelial cells after bleomycin treatment. Lung epithelial cells have recently been shown to express TLR2 and TLR4 (ref. 29). Although lung epithelial cells may express TLR2 and TLR4 to recognize pathogens, these data support a previously unrecognized role for TLR2 and TLR4 in regulating epithelial cell survival after acute lung injury.

Recent evidence has identified a protective role for TLR2 and TLR4 in a noninfectious model of colitis[30]. This protective effect of TLR2 and TLR4 required the presence of commensal bacteria[30]. Our data also show a protective effect of TLR2 and TLR4 in a model of noninfectious tissue injury, but there are no commensal bacteria present in the distal lung and the protective effect seems to be generated by the interaction of endogenous matrix with TLR2 and TLR4. In addition, our data also show that the presence of TLRs protects against hyperoxia-induced lung injury, another model of noninfectious lung injury. These data are, to our knowledge, the first to suggest a role for TLRs in maintaining structural cell integrity in the context of tissue injury.

We were able to provide further evidence for a requirement for both hyaluronan and TLRs in regulating tissue injury and repair. Inhibition of hyaluronan binding recapitulated the phenotype observed in the *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ mice after lung injury. Upregulation of

native high-molecular-mass hyaluronan production in lung epithelial cells protected against acute lung injury, reduced epithelial cell apoptosis and promoted survival. High-molecular-mass hyaluronan reduced bleomycin-induced apoptosis in alveolar epithelial cells from wild-type mice, and the protective effect was observed in an hyaluronan dose–dependent manner, and was dependent on TLR4, TLR2 and MyD88. We provided evidence to support a role for hyaluronan-TLR interactions in promoting basal NF-κB activation as an important component of the protective mechanism against epithelial cell injury. Deficiency of TLR2 and TLR4 results in both decreased cell-surface expression of hyaluronan and basal NF-κB activation. Removal of cell-surface hyaluronan from wild-type epithelial cells enhanced both basal and bleomycin-induced epithelial cell apoptosis.

These data suggest two major functions for endogenous polymeric matrix fragment–TLR interactions in tissue injury and repair. Soluble hyaluronan degradation products generated during noninfectious lung injury can stimulate macrophages to produce chemokines that recruit neutrophils to the site of injury. The observation that purified hyaluronan fragments from serum of individuals with acute lung injury stimulates macrophages to produce neutrophil chemotactic factors suggests that circulating hyaluronan fragments could contribute to unremitting inflammation and multiorgan failure in sepsis. In addition, these data also support a previously unrecognized role for native cell-surface high-molecular-mass hyaluronan and TLRs in limiting the extent of lung epithelial cell injury by inhibiting apoptosis and promoting the repair of parenchymal cell injury. Our data suggest that exogenous high-molecular-mass hyaluronan mimics endogenous cell-surface hyaluronan. Soluble hyaluronan fragments may displace endogenous hyaluronan and impair epithelial repair processes. We propose that endogenous matrix and the TLR2-TLR4-MyD88 pathway serve two crucial functions in host defense against noninfectious lung injury: (i) soluble hyaluronan degradation products generated after tissue injury stimulate macrophages to produce inflammatory mediators as part of the innate immune response, and (ii) endogenous native high-molecular-mass hyaluronan provides a protective signal to epithelial cells that promotes survival and repair. Altering the balance of hyaluronan in favor of high-molecular-mass forms could favor recovery from acute lung injury.

METHODS

Chemicals and reagents. We prepared hyaluronan fragments (M_w = 135 kDa) from *Streptococcus* hyaluronan (M_w ~ 1,500 kDa) by digestion with concentrated HCl[31]. The hyaluronan preparation contained no protein and low endotoxin level (<40 pg/mg hyaluronan) by a Limulus Amebocyte Lysate assay (BioWhittaker). Clinical-grade high-molecular-mass hyaluronan, Healon, was from Kabi Pharmacia. The *E. coli* 011:B4 LPS were from Sigma. We prepared human hyaluronan fragments (M_w = 200 kDa) by fractionation from serum of the individuals with adult respiratory distress syndrome[32]. We obtained samples after informed consent and the protocol was approved by the Institutional Review Board of the Massachusetts General Hospital. All treatments with hyaluronan were in the presence of 10 μg/ml polymyxin B (Calbiochem) to exclude the effects of any contaminating lipopolysaccharide on experimental conditions.

Hyaluronan digestions. We digested hyaluronan fragments (M_w = 135 kDa) with hyaluronidase, pronase and deoxyribonuclease I, and we then boiled all samples for 10 min. We brought samples to neutral pH before treating peritoneal macrophages (**Supplementary Methods** online).

Mice. *Myd88*$^{-/-}$ mice[33] and TLR1-, TLR2-, TLR3-, TLR4-, TLR5 and TLR9-deficient mice have been described[33–38]. We crossed *Myd88*$^{-/-}$ mice onto the C57Bl/6J background for nine generations, and *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice[34] for six generations before use. *Cd44*$^{-/-}$ mice were previously described[1]. We generated CC10-HAS2 transgenic mice by cloning the mouse *Has2* cDNA downstream of the rat *Ugb* promoter. We backcrossed transgenic mice onto C57Bl/6 background for more than six generations before use. Background C57Bl/6 mice were from Jackson Laboratories. We housed all mice and cared for them in a pathogen-free facility at Yale University, and all animal experiments were approved by the Institutional Animal Care and Use Committee at Yale University (**Supplementary Methods** online).

Isolation of peritoneal macrophages. We isolated peritoneal macrophages as described previously[39]. We performed all hyaluronan treatments in the presence of polymyxin B for 4 h for RNA analysis, and for 24 h for chemokine protein determination in conditioned media.

ELISA. We measured levels of chemokines and cytokines in the BAL fluid or conditioned media of hyaluronan-treated peritoneal macrophages with commercial ELISA kits (R & D Systems).

mRNA analysis. We extracted total RNA using TRIzol Reagent (Invitrogen). We performed northern analysis as described previously[1]. We always included aldolase to show equal RNA loading.

Bleomycin administration and bronchoalveolar lavage. Intratracheal bleomycin administration, collection of BAL fluid and cytospin preparation of BAL cells were done as described previously[1].

Synthesis of Pep-1 and control peptide. Pep-1 (GAHWQFNALTVR) and scrambled control peptide (WRHGFALTAVNQ) both with an amidated GGGS linker[15] were synthesized by Sigma-Genosys. We administered the peptides at a dose of 0.8 mg/mouse intraperitoneally 2 h before and repeated on days 3 and 5 after bleomycin treatment.

Histology. We performed histologic analysis of lung sections with conventional hematoxylin and eosin staining.

TUNEL staining. We determined apoptosis with conventional TUNEL assay as described previously[1]. We quantified TUNEL-positive staining cells by counting cells in 40 fields of each slide at an original magnification of ×200.

Hyaluronan quantification and staining. We measured hyaluronan contents in BAL fluid with a hyaluronan-specific enzyme-linked immunosorbent assay as described[1]. We stained lung sections with biotinylated hyaluronan-binding protein as described previously[1].

Apoptosis of primary lung epithelial cells. We isolated primary lung epithelial cells and maintained them as previously described[18]. We treated the cells with bleomycin at a concentration of 250 μg/ml or in the combination with hyaluronan for 24 h. We subjected the cells to apoptosis analysis by staining cells with FITC-conjugated Annexin V (BD Pharmingen) and propidium iodide, and then analyzed them on a FACScalibur (BD Bioscience).

Digestion of hyaluronan on epithelial cells. We treated confluent epithelial cells with testicular hyaluronidase in 300 μl digestion buffer (1 M sodium acetate, pH 5.5, in PBS) at 37 °C for 1 h. After wash, we collected the cells for analysis or treated them with bleomycin for further experiments.

Electrophoretic mobility shift assay. We isolated nuclear extracts from cultured lung epithelial cells. We performed electrophoretic mobility shift assays with NF-κB consensus oligonucleotide 5′-AGTTGAGGGGACTTTCC CAGGC-3′ as described previously[40].

Flow cytometric analysis of hyaluronan on cell surface. We added biotinylated hyaluronan-binding protein[1] to 5 × 10^5 cultured lung epithelial cells and incubated them at 4 °C for 20 min. We then incubated PE-conjugated streptavidin for another 20 min. After wash, we analyzed hyaluronan on cell surface on a FACSCalibur (**Supplementary Methods** online).

Statistical analysis. We assessed differences in measured variables between genetically altered mice and the control group using the Student *t*-test. Data are expressed as the mean ± s.e.m. where applicable. Statistical difference was

accepted at $P < 0.05$. Statistic significance of survival curves was analyzed with the log-rank test. Statistical difference was accepted at $P < 0.05$.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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