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TLR4 Is a Negative Regulator in Noninfectious Lung Inflammation

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Low m.w. hyaluronan (LMW HA) has been shown to elicit the expression of proinflammatory cytokines and chemokines in various cells in vitro. However, the effects of this molecule in vivo are unknown. In this study, we report that intratracheal administration of LMW HA (200 kDa) causes inflammation in mouse lung. A lack of TLR4 is associated with even stronger inflammatory response in the lung as shown by increased neutrophil counts and elevated cytokine and chemokine concentrations. We also demonstrate that TLR4 anti-inflammatory signaling is dependent upon a MyD88-independent pathway. TLR4-mediated IL-1R antagonist production plays a negative regulatory role in LMW HA (200 kDa) induced lung inflammation. These data provide a molecular level explanation for the function of TLR4 in LMW HA (200 kDa)-induced lung inflammation, as inhibition of the β form of pro–IL-1 promotes an anti-inflammatory response. The Journal of Immunology, 2010, 184: 5308–5314.

The innate immune response is commonly mediated through activation of members of the Toll/IL-1R (TIR) superfamily. TLRs respond to different microbial products and endogenous agonists, and the signaling pathways activated are very similar to those generated by the proinflammatory cytokine IL-1. IL-1R and TLRs are highly homologous in their cytosolic portions, possessing a common intracellular TIR domain involved in the initiation of signaling (1).

TLR4 plays a key role in the recognition of Gram-negative bacterial components (LPS). TLR4 responds not only to exogenous canonical ligand (LPS), but also to other endogenous agonists generated by injured cells and tissues (2), including oligosaccharide hyaluronan (3, 4). Endogenous danger signals initiate an immune response in the absence of infection. LPS activates two distinct intracellular signaling pathways via TLR4. The two signaling pathways involve different combinations of adaptor proteins. The TLR4–MyD88–dependent pathway involves adaptor proteins MyD88 and MyDR8 adaptor-like/TIR domain-containing adaptor protein. TLR4 also signals via the TIR domain-containing adaptor-inducing IFN-β–related adaptor molecule (TRAM) and TIR domain-containing adaptor-inducing IFN-β (TRIF) adaptor proteins. TRAM and TRIF are critical for production of type 1 IFN through IFN regulatory factor 3 (IRF3) activation as well as late activation of NF-κB (5). However, the TRAM–TRIF pathway can be selectively activated depending on the nature of the ligands. The envelope glycoprotein G of the vesicular stomatitis virus has been shown to trigger a third signaling pathway downstream from TLR4 (6). The third pathway does not activate NF-κB. It is fully dependent upon the adaptor TRAM, only partially dependent upon TRIF, and independent of MyD88 adaptor-like and MyD88, leading to type 1 IFN (IFN-α/β) production (6).

IL-1 is a central proinflammatory cytokine among the cytokines/chemokines involved in acute and chronic inflammation (7). IL-1 is known to elicit multiple biologic effects. IL-1 can increase gene expression for CSFs and can increase the expression of ICAM-1 and VCAM-1 on endothelial cells, resulting in neutrophil accumulation in the airways (7). Injection of IL-1 into rodent tracheas results in neutrophil infiltration as well as acute alveolar leakage (8). IL-1 plays a predominant role in bleomycin-induced lung acute inflammation and chronic fibrosis. Bleomycin-induced pulmonary inflammation and fibrosis in mice requires the β form of pro–IL-1 (IL-1β) and IL-1R signaling through MyD88. IL-1β induced by bleomycin can upregulate the production of TNF-α, IL-6, and matrix metalloproteinase-9 (9). IL-1β production and IL-1R activation are important in another noninfectious uric acid-triggered pulmonary inflammation (10). IL-1 receptor antagonist (IL-1RA) is a specific receptor antagonist in the IL-1 family. IL-1RA is structurally related to IL-1 but specifically blocks the binding of IL-1 to cell-surface receptors without itself activating target cells (7).

High m.w. hyaluronan (HA) is an important component of the lung interstitium and functions to maintain lung structural integrity. High m.w. HA is broken down into low m.w. (LMW) HA fragments upon tissue injury (11). The bronchoalveolar lavage fluid (BALF) of patients with acute respiratory distress syndrome (ARDS) contains a high percentage of LMW HA, whereas only high m.w. HA is found in BALF of non-ARDS patients and normal controls (12, 13). LMW HAs act as an endogenous danger signal to activate innate inflammatory responses via various signaling pathways (3, 4, 12, 14, 15), but the effects of TLR4 in LMW HA (200 kDa)-induced lung injury in vivo are unclear.
We now report that TLR4 negatively regulates LMW HA (200 kDa)-induced lung inflammation.

Materials and Methods

**Mice and model**

C57BL/6, C3H/HeJ, and C3H/HeOuJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TLR4<sup>−/−</sup> (16), MyD88<sup>−/−</sup> (17), IRAF<sup>−/−</sup> (18), and TRAM<sup>−/−</sup>TRIF<sup>−/−</sup> mice were generated by crossing TRIF<sup>−/−</sup> (19) with TRAM<sup>−/−</sup> (20). Routinely, mice 6–8 wk of age were used for experiments. All mice used in this report were housed in a pathogen-free rodent barrier facility. All animal experiments were approved in advance by the Massachusetts General Hospital Center for Comparative Medicine (Boston, MA). LMW HA (200 kDa, 65 mg/kg) was delivered directly to the tracheas with a microsprayer (Penne-Century, Wyndmoor, PA). A total of 300 μg/mouse IL-1RA (Anakira, Engen, Cheserbrook, PA) or TNF inhibitor (etanercept, Amgen) was injected s.c. 30 min before intratracheal administration of LMW HA (200 kDa).

**LMW HA digestion**

LMW HA (200 kDa, 32 mg/ml) were digested with hyaluronidase (from bovine testes, 3200 U/ml; Sigma-Aldrich, St. Louis, MO) at 37°C overnight, pronase (from Streptomyces griseus, 320 U/ml; Calbiochem, San Diego, CA) at 55°C for 1 h, DNaase I (from bovine pancreas, 100 mg/ml; Roche Applied Science, Indianapolis, IN) at 37°C for 1 h, and all samples were then boiled for 10 min. The samples were brought to neutral pH before treating mice. The treated LMW HA (65 mg/kg) was delivered directly to the tracheas with a microsprayer.

**Pep-1 and control peptide**

HA-blocking peptide Pep-1 (GAHWQFNALTVR) and scrambled control (C3H/HeOuJ) (23). We found that neutrophil counts in BALFs were significantly increased 24 h after LMW HA (200 kDa) treatment.

**BALF**

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge needle. BALF was obtained by instilling and collecting four 0.5-ml volumes of cold PBS through the incised trachea. A total of 1.8 ml lavage fluid was retrieved per mouse. Each BALF sample was centrifuged, and the supernatants were stored at −80°C until use. Total cell numbers in BALF were counted from each sample in a hemocytometer. BALF neutrophil counts were determined on cytospin preparations stained with a Diff-Quick staining kit (IMEB, San Marcos, CA).

**ELISA**

IL-1β, IL-1α, IL-6, TNF-α, MIP-2, and keratinocyte cell-derived chemokine (KC) in BALF were determined using mouse ELISA assay kits (R&D Systems, Minneapolis, MN). Albumin and IgM levels in BALF were measured using a mouse albumin ELISA kit (ALPCO, Salem, NH) and a mouse IgM ELISA kit (Bethyl Laboratories, Montgomery, TX), respectively.

**Isolation of BALF neutrophils and culture**

BALFs were harvested 24 h after the intratracheal administration of LMW HA (200 kDa). BALF neutrophils were prepared using the neutrophil isolation kit (Miltenyi Biotec, Auburn, CA). The RPMI 1640 medium containing 10% FBS and penicillin/streptomycin was used for cell culture. Neutrophils were plated in 96-well plates at 1 × 10<sup>5</sup> cells/well in 100 μl medium. Cells were cultured in the medium alone or stimulated with LMW HA (100 μg/ml) for 12 h, and the cytokine produced in the supernatants was determined by ELISA.

**Myeloperoxidase assay**

After BALFs were performed, whole-lung homogenates were measured by a mouse myeloperoxidase (MPO) ELISA kit (Cell Sciences, Canton, MA), according to the manufacturer’s protocol.

**Lung injury score**

Severity of lung inflammation was semiquantitatively assessed according to methods previously described (22). Quantal assessment of injury was performed in blinded fashion by grading four histological findings: alveolar septae, alveolar hemorrhage, intra-alveolar fibrin, and intra-alveolar infiltrates. Lungs were fixed overnight in 10% buffered formalin. The fixed lung sections were dehydrated, cleared, and embedded in paraffin by conventional H&E staining. A minimum of five fields were examined for each section.

Statistics

All data are given as means ± SEM. For all experiments were analyzed by unpaired Student t test (two groups) or ANOVA (>2 groups) using the Statview 6.0 software program (SAS Institute, Cary, NC). Comparisons between groups and tests of interactions were made assuming a two-factor analysis, with the interaction term testing each main effect and with the residual error testing the interaction. All comparisons were made using Fisher’s least significant difference procedure, so that multiple comparisons were made at the 0.05 level only if the overall F test from the ANOVA was significant at p < 0.05.

Results

**TLR4-deficient mice develop severe lung injury**

To investigate the role of TLR4 in LMW HA (200 kDa)-induced lung injury in vivo, we administered highly pure LMW HA (200 kDa, 65 mg/kg) to the tracheas of TLR4<sup>−/−</sup>, wild-type (WT) (C57BL/6J) mice (Fig. 1A). Surprisingly, we found that deletion of TLR4 did not ameliorate the course of lung inflammation, but resulted in severe lung inflammation and injury. Twenty-four hours after LMW HA (200 kDa) administration, TLR4<sup>−/−</sup> mice demonstrated decreased spontaneous movement and an increase in huddling and ruffled fur. In contrast, WT mice were healthy in appearance. Pathological observation revealed that the lungs had a hemorrhagic appearance (Fig. 1B). Histological examination of the lungs from LMW HA (200 kDa)-treated TLR4<sup>−/−</sup> mice revealed severe inflammatory infiltrates in pulmonary alveoli (Fig. 1C). neutrophils, and RBCs in the BALF (Fig. 1D). Quantal scoring of the severity of histological lung injury showed that the lung injury score was significantly higher in TLR4<sup>−/−</sup> mice than in WT mice (Fig. 1E).

Neutrophil counts in BALF were significantly increased 24 h after LMW HA (200 kDa) administration (Fig. 1F). Next, we examined whether the number of interstitial neutrophils were increased. Neutrophil infiltration into the lung tissue is quantified by measuring MPO activity. TLR4<sup>−/−</sup> mice had a significant increase in MPO activity compared with WT (Fig. 1G). Taken together, these data demonstrate a dramatic infiltration of neutrophils into the interstitial and intra-alveolar compartment in the lungs of TLR4<sup>−/−</sup> mice after LMW HA (200 kDa).

Data reported thus far were generated in studies conducted with C57BL/6J background (TLR4-deficient) mice. To determine whether the observed inflammation is strain dependent, additional studies were performed using TLR4<sup>−/−</sup> mutant (C3H/HeJ) mice and their respective control (C3H/HeOuJ) (23). We found that neutrophil counts in BALFs did not differ between the two strains of TLR4-deficient/mutant mice (Fig. 1H), indicating that mouse strain does not influence the observed phenotypes. Next, we evaluated neutrophil kinetics during inflammation induced by LMW HA administration. We found that a single intratracheal administration of LMW HA (200 kDa)-induced lung injury is a model of acute pulmonary inflammation in mice (Fig. 1I, U).

These effects could not be attributed to any trace amounts of endotoxin coadministered with the LMW HA (200 kDa), as TLR4<sup>−/−</sup> mice are resistant to endotoxin (23). To further define LMW HA (200 kDa) as the only source to drive the lung inflammatory response.
FIGURE 1. TLR4 deficiency is associated with exacerbated LMW HA-induced lung injury. A, The elution profile of a LMW HA (200 kDa), prepared by gamma-irradiation of bacterially fermented HA, using SEC/TriSEC 302 detectors. The eluted peaks were Gaussian and did not exhibit tailing, shoulders, or extraneous peaks (red, refractive index; green, right-angle light scattering; black, low-angle light scattering; blue, differential pressure viscometer). B, Twenty-four hours after intratracheal administration of LMW HA (200 kDa, 65 mg/kg), pathological observation of the lung. C, Lung sections were stained with H&E, original magnification ×200. D, Diff-Quick–stained cytospins of bronchoalveolar lavage neutrophils, original magnification ×400. The BALF dilution in TLR4−/− plus LMW HA of D was 1/4. Arrows indicate RBCs. E, Lung injury score. F, Total cell and neutrophil counts were performed on BALF. G, After BALF was performed, the MPO of whole lung homogenates was measured. H, BALF neutrophil influx in two strains of TLR4-deficient/mutant mice (C57BL/6J, C3H/HeJ) and respective controls (C57BL/6J, C3H/HeOuJ). After 1, 3, and 7 d following LMW HA, neutrophils in BALFs were quantified (I), and MPO in lung tissues was analyzed (J). B–D, Representative data from multiple mice (n = 4–6 per group) are shown. Data represent mean ± SEM from three independent experiments (n = 4 mice per group). *p < 0.05.
TLR4 limits pulmonary inflammation through the MyD88-independent pathway

TLR4 signaling can be segregated into two distinct pathways: one leading to activation of the MyD88-dependent arm and the other leading to the MyD88-independent (TRAM/TRIF-mediated) arm (25). Both of these cascades lead to distinct outcomes: the former giving rise to expression of proinflammatory genes, such as TNF-α and IL-1 (26), whereas the latter gives rise to IRF3-mediated expression of type 1 IFNs (19, 25). To characterize TLR4 intracellular signaling pathways involved in LMW HA (200 kDa)-induced inflammation, we respectively administrated LMW HA (200 kDa) to TLR4−/−, MyD88−/−, TRAM–TRIF double−/−, and IRF3−/− mice. Histological examination of the lungs from LMW HA-treated TLR4−/−, TRAM–TRIF double−/−, and IRF3−/− mice revealed obvious severe inflammatory infiltrates in the alveolar space within 24 h after LMW HA (200 kDa) administration. In contrast, MyD88−/− mice did not show a significant change in neutrophil accumulation (Fig. 4A–C). Furthermore, TLR4−/−, TRAM–TRIF double−/−, and IRF3−/− mice all showed higher levels of IL-1β and lower levels of IL-1RA compared with WT mice (Fig. 4D, 4E). Thus, our data suggest that the protective role of TLR4 is dependent on TRAM/TRIF and IRF3 in LMW HA (200 kDa)-induced inflammation.

Lack of TLR4 shifts IL-1RA/IL-1β ratio toward a proinflammatory phenotype

IL-1 has been implicated as a central mediator of tissue damage and destruction in a number of diseases (7). To determine whether enhanced pulmonary inflammation in TLR4−/− mice was due to elevated IL-1β, we blocked IL-1β in vivo. TLR4−/− mice were treated with rIL-1RA (anakinra, Amgen) to block IL-1R activation. Pretreatment with IL-1RA protected TLR4−/− mice against LWM HA (200 kDa)-induced neutrophil infiltration (Fig. 5A) and decreased BALF IL-1β, MIP-2, KC, TNF-α, or IL-6 levels (Fig. 5B–F). In comparison, inhibition of
The three major consequences of intratracheal LMW HA (200 kDa) administration are airway neutrophilia, production of proinflammatory mediators, and airway epithelial cell damage. However, a lack of TLR4, by nasal bleomycin instillation; however, these results disagree with the findings by Noble’s group (12), which show that bleomycin requires the TLR2 and TLR4 pathways by intratracheal bleomycin administration. The existence of multiple sizes of HA with a peak molecular size of 540 kDa (29), as induced by bleomycin, may have contributed to difficulties in determining what defines HA as active or inactive. LMW HAs are likely to use different signaling pathways to induce the release or expression of inflammatory mediators. For example, LMW HA at a wide range of molecular sizes with a peak molecular size of ~200 kDa activates the macrophage immune response via TLR2, not TLR4 (14). Oligosaccharide HA (4–16 kDa) induces dendritic cell activation and stimulates endothelial recognition of injury via TLR4 (3, 4). Soluble HA (LMW HA at an unknown range of sizes) induces the expression of TNF-α and IL-1β in TLR4 mutant (C3H/HeJ) bone marrow-derived macrophages (15). However, LMW HA (135 kDa) does not induce the expression of TNF-α in TLR2–TLR4 double-deficient peritoneal macrophages (12). The mechanisms behind how LMW HAs mediate distinct signals are a central unresolved issue. It is possible that HA size plays an important role in cell signaling.

The three major consequences of intratracheal LMW HA (200 kDa) administration are airway neutrophilia, production of proinflammatory mediators, and airway epithelial cell damage. However, a lack of TLR4 is associated with a stronger neutrophil recruitment in BALFs, leading to excessive lung inflammation and hyperpermeability. Lung inflammatory responses are significantly inhibited when TLR4 mutant (C3H/HeJ) bone marrow-derived macrophages (15). However, LMW HA (135 kDa) does not induce the expression of TNF-α in TLR2–TLR4 double-deficient peritoneal macrophages (12). The mechanisms behind how LMW HAs mediate distinct signals are a central unresolved issue. It is possible that HA size plays an important role in cell signaling.
TNF-α, IL-6, and IL-1β levels (Fig. 5). All four of these proteins have been linked to neutrophil accumulation (30). Administration of IL-1RA also prevents the marked epithelial cell damage seen following LMW HA (200 kDa) (S. Leu and H. Zhao, unpublished observation). Thus, IL-1β induced by LMW HA (200 kDa) is a central mediator in the production of a variety of inflammatory mediators and neutrophil accumulation in mouse lung. The inflammation induced by LMW HA (200 kDa) administration is not dependent on TNF-α signaling because pulmonary inflammation was not ameliorated by treatment with TNF-α inhibitor (Fig. 5). Our findings are consistent with the data obtained in vitro: soluble HA (LMW HA) stimulates human peripheral monocytes and rabbit peritoneal macrophages to release IL-1 (31). MyD88 is a major mediator of IL-1 signal transduction downstream of IL-1R (17). LMW HA (200 kDa) may induce pulmonary inflammation via MyD88.

IL-1 is involved in host defense against exogenous pathogens or endogenous danger signals, functioning as a primary sensor of exogenous or endogenous ligands and activating signaling pathways that stimulate immunological and inflammatory cells to synthesize cytokine mediators, which subsequently create inflammatory responses (7). However, this inflammatory response must be dampened to limit excessive injury, as its dysregulation can significantly worsen inflammation. Excess IL-1 has also been implicated in the pathophysiology of a variety of inflammatory and immune lung diseases, including asthma, pulmonary fibrosis, pulmonary hypertension, and...
granuloma formation (32). Thus, IL-1 signals must be tightly regulated to avoid detrimental and inappropriate inflammatory responses. The inflammatory response is a highly regulated process in which the balance between proinflammation and anti-inflammation is orchestrated to ultimately drive and resolve inflammation. We have found that TLR4 is one of these inhibiting components in LMW HA-induced lung inflammation. TLR4 plays an immunosuppressive role through the induction of the anti-inflammatory mediator IL-1RA. IL-1RA acts as a negative regulator of the IL-1β signal. Administration of IL-1RA into TLR4−/− mice can inhibit the production of neutrophil chemottractants and neutrophil accumulation in the lung, indicating that lung destruction in TLR4−/− mice is due to a local imbalance between IL-1β and IL-1RA. The present investigation suggests that treatment with IL-1RA may afford significant protection against LMW HA (200 kDa)-induced lung inflammation.

Our data indicate that TLR4 signaling plays an anti-inflammatory role in LMW HA (200 kDa)-induced acute lung inflammation. Although the lack of TLR4 leads to excessive lung inflammation in our model, inflammation is eventually cleared in TLR4−/− mice, indicating that other pathways are also involved in the anti-inflammatory response. CD44 is another important anti-inflammatory receptor and its ligation by HA has been shown to induce IL-10 and TGF-β (33). In our model, inflammation is eventually cleared in TLR4−/− mice, indicating that other pathways are also involved in the anti-inflammatory response. CD44 is another important anti-inflammatory gene in noninfectious bleomycin-induced lung injury (29). Immunosuppression experiments confirmed the physical association of TLR4 and CD44 (33). These data suggest that TLR4-CD44 works together as a brake in LMW HA (200 kDa)-induced lung inflammation in vivo. Thus, additional work is needed to understand whether CD44 can regulate TLR4 activation in our model.

The inhalation of a TLR4 agonist (LPS) results in pulmonary inflammation and injury in humans (34). Therefore, intervention of TLR4-mediated inflammation may be a potential approach to treat and prevent lung injury and a variety of associated diseases. Two TLR4 antagonists, ES564 and TAK-242, have progressed into clinical phase III studies for the treatment of Gram-negative sepsis (35). Infectious etiologies, including sepsis and pneumonia, are leading causes of acute lung injury and a variety of associated diseases. Two TLR4 antagonists, ES564 and TAK-242, have progressed into clinical phase III studies for the treatment of Gram-negative sepsis (35). Infectious etiologies, including sepsis and pneumonia, are leading causes of acute lung injury and ARDS (36). However, our data have demonstrated that HA degradation products result in severe lung injury in the absence of the TLR4 signaling. Because BALFs of patients with ARDS contain a high percentage of LMW forms of HA (12, 13), our data suggest that caution should be exercised in using TLR4 antagonists during acute lung injury/ARDS HA breakdown.

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Disclosures

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