

Role of hyaluronan and hyaluronan-binding proteins in human asthma

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Background: The characteristics of human asthma are chronic inflammation and airway remodeling. Hyaluronan, a major extracellular matrix component, accumulates during inflammatory lung diseases, including asthma. Hyaluronan fragments stimulate macrophages to produce inflammatory cytokines. We hypothesized that hyaluronan and its receptors would play a role in human asthma.

Objective: To investigate the role of hyaluronan and hyaluronan-binding proteins in human asthma.

Methods: Twenty-one subjects with asthma and 25 healthy control subjects underwent bronchoscopy with endobronchial biopsy and bronchoalveolar lavage. Fibroblasts were cultured, and hyaluronan and hyaluronan synthase expression was determined at baseline and after exposure to several mediators relevant to asthma pathobiology. The expression of hyaluronan-binding proteins CD44, TLR (Toll-like receptor)-2, and TLR4 on bronchoalveolar lavage macrophages was determined by flow cytometry. IL-8 production by macrophages in response to hyaluronan fragment stimulation was compared.

Results: Airway fibroblasts from patients with asthma produced significantly increased concentrations of lower-molecular-weight hyaluronan compared with those of normal fibroblasts. Hyaluronan synthase 2 mRNA was markedly increased in asthmatic fibroblasts. Asthmatic macrophages showed a decrease in cell surface CD44 expression and an increase in TLR2 and TLR4 expression. Macrophages from subjects with asthma showed an increase in responsiveness to low-molecular-weight hyaluronan stimulation, as demonstrated by increased IL-8 production.

Conclusion: Hyaluronan homeostasis is deranged in asthma, with increased production by fibroblasts and decreased CD44

expression on alveolar macrophages. Upregulation of TLR2 and TLR4 on macrophages with increased sensitivity to hyaluronan fragments suggests a novel proinflammatory mechanism by which persistence of hyaluronan fragments could contribute to chronic inflammation and airway remodeling in asthma. (*J Allergy Clin Immunol* 2011;■■■■:■■■■-■■■■.)

Key words: Asthma, hyaluronan, cytokines, fibroblasts, macrophages

Chronic inflammation and airway remodeling are important characteristics of human asthma. These include the infiltration of inflammatory cells and an abnormal accumulation of extracellular matrix (ECM) in the subepithelial basement membrane region and submucosa.^{1,2} Fibroblasts from patients with hyperresponsive airways have been shown to produce more total proteoglycans than cells from subjects with normoresponsive airways.³ Fibronectin matrix accumulates in asthma and may contribute to the progression of asthma by altering both the airway remodeling and the functional properties of cells of the airway wall.⁴ Therefore, increases in ECM degradation products may be associated with airway fibrosis and decline in lung function.^{1,2,5} Understanding the contribution of ECM accumulation to asthma pathogenesis may lead to new therapeutics for patients with asthma.

Hyaluronan is a major component of ECM.⁶ It exists as a high-molecular-weight polymer under normal physiological conditions and undergoes dynamic regulation resulting in accumulation of lower-molecular-weight species during tissue injury and inflammation.⁷⁻¹⁰ Clearance of hyaluronan degradation products is essential for inflammatory resolution and restoration of tissue integrity. Hyaluronan fragment clearance from sites of inflammation requires the major hyaluronan-binding protein, CD44, to be expressed on hematopoietic cells.⁷

Low-molecular-weight hyaluronan induces the expression of a variety of genes by inflammatory cells *in vitro*, including chemokines, cytokines, and growth factors.¹¹⁻¹³ However, low-molecular-weight hyaluronan signaling can be CD44-independent under some circumstances and use Toll-like receptor (TLR)-4 and TLR2.¹⁴⁻¹⁶ Growing evidence shows that TLR signaling plays an important role in innate immunity in asthma^{17,18} and is critical for T_H2 response in murine asthma models.¹⁹⁻²¹ In addition, TLR2 gene expression was upregulated in human airway epithelial cells isolated from subjects with asthma and infected with *Mycoplasma pneumoniae*.²²

Human lung fibroblasts are an important source of hyaluronan production.²³⁻²⁵ There are 3 hyaluronan synthase (HAS) enzyme isoforms: HAS1, HAS2, and HAS3.^{26,27} HAS2 is the major isoform expressed in human lung fibroblasts.²⁸ Multiple studies

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Abbreviations used

BAL:	Bronchoalveolar lavage
BALF:	Bronchoalveolar lavage fluid
bHABP:	Biotinylated hyaluronan-binding protein
ECM:	Extracellular matrix
HAS:	Hyaluronan synthase
HYAL:	Hyaluronidase
NOX:	Nicotinamide adenine dinucleotide phosphate oxidase
PMA:	Phorbol 12-myristate 13-acetate
ROS:	Reactive oxygen species
TLR:	Toll-like receptor

have shown that human fibroblasts produce hyaluronan *in vitro* in response to stimulation by cytokines and growth factors including IL-1 β , TNF- α , and TGF- β .^{25,28-31} Hyaluronan appears in low concentrations in bronchoalveolar lavage (BAL) fluid (BALF) from healthy individuals and is elevated in BALF of patients with asthma.³²⁻³⁴ The concentration of hyaluronan in BALF was found to correlate significantly with the severity of asthma.³⁴ However, the role of hyaluronan homeostasis in human asthma has not been thoroughly explored.

We hypothesized that increased hyaluronan accumulation in the lungs of patients with asthma contributes to chronic inflammation and airway remodeling through processes mediated by both fibroblasts and macrophages. In the current study, we isolated airway fibroblasts and alveolar macrophages from patients with asthma and healthy subjects. We have examined hyaluronan production by airway fibroblasts, cell surface expression of hyaluronan-binding proteins on alveolar macrophages, and inflammatory mediator production by alveolar macrophages in response to low-molecular-weight hyaluronan and LPS stimulation. Samples were obtained from a total of 21 patients with asthma and 25 healthy control subjects. Our results demonstrate an increase in hyaluronan production by airway fibroblasts and an imbalance in hyaluronan-binding protein expression on alveolar macrophages in asthma that favors reduced clearance of hyaluronan fragments and increased production of cytokines in response to hyaluronan fragments and LPS.

METHODS**Study population**

Subjects age 18 to 60 years were recruited by advertisement. Samples used for this study are from a total of 21 patients with asthma and 25 healthy control subjects. Not all studies were performed on all subjects; numbers studied in each experiment are denoted in the Results section. Subjects were of mild severity per National Asthma Education and Prevention Program criteria³⁵ and used no controller medications. Healthy subjects demonstrated normal lung function, had no history of asthma, and used no medications. All the subjects were never-smokers. The characteristics of subjects are shown in Table I. The study was approved by the Duke University Institutional Review Board. All individuals gave informed consent.

Bronchoscopy

Subjects with asthma and healthy controls underwent bronchoscopy with BAL as previously described.³⁶ Three hundred milliliters of warm, sterile saline in 60-mL aliquots was used for the BAL. Cells derived from the BAL fluid were pelleted by centrifugation at 1000 rpm for 10 minutes. BAL cells were cultured for cytokine production analysis or fixed in 3.7% formaldehyde for flow cytometry.

Hyaluronan staining and quantitation of human lung sections

Lung sections from biopsy tissues of patients with asthma and healthy subjects were stained with biotinylated hyaluronan-binding protein (bHABP; 4 μ g/mL; Associates of Cape Cod Inc, East Falmouth, Mass) for 1 hour, then developed by using a Vectastain-Elite-ABC kit (Vector Laboratories, Burlingame, Calif). The specificity of the staining was determined by preincubating tissue samples with 10 U/mL *Streptomyces* hyaluronidase (HYAL) at 37°C for 2 hours in a humidification chamber and then staining with bHABP.

For each subject, photomicrographs were randomly taken at $\times 40$ magnification, avoiding the regions in proximity of the edges of the biopsies. Quantitative assessment of hyaluronan staining in lung tissue was quantified by using ImageJ (version 1.44f; National Institutes of Health). A color deconvolution module was created to threshold automatically only the tissue expressing hyaluronan. The vectors for the color deconvolution module were calculated to omit any marginal staining that was not directly related to hyaluronan. Epithelial cells were excluded because there was no significant staining of hyaluronan in all subjects (healthy and asthma). For such calculation, multiple sampling of the stained area was performed to allow consistency across photomicrographs and sections. Similarly, the total tissue area of the biopsy was calculated but by using a different color deconvolution module to detect the total tissue area. Finally, the thresholded images generated by these modules were visually compared with the photomicrographs for accuracy. Separate Java macro scripts were developed for hyaluronan staining and for total tissue area and applied to all images for automatic data generation. The volume percentage of positive tissue was calculated by the ratio of the value of the reactive tissue to hyaluronan and the total volume of the tissue.

Human airway fibroblast cell culture

Fibroblasts were cultured from endobronchial biopsy tissues as previously described.³⁷ Briefly, biopsy specimens were rinsed and cut into small pieces and cultured in Dulbecco modified Eagle medium supplemented with FBS (10%), streptomycin (100 μ g/mL), penicillin (10,000 U/mL), and gentamicin (100 μ g/mL). The purity of fibroblasts was confirmed as shown previously.³⁷ Cells from passage 2 to passage 4 were used for experiments. Airway fibroblasts were seeded in 12-well plates with 50,000 cells per well. Once the cells reached confluence, they were cultured for 1 additional week. The cells were then incubated in fresh serum-free medium with and without 10 ng/mL IL-13 (10 ng/mL), TNF- α (50 ng/mL), or TGF- β (10 ng/mL) for 48 hours. Conditioned media were collected for hyaluronan measurement, and cells were harvested in Trizol reagent (Invitrogen, Carlsbad, Calif) for RNA isolation.

Hyaluronan content determination

Hyaluronan concentrations in culture medium of airway fibroblasts were measured with a competitive ELISA-like assay by using bHABP (Associates of Cape Cod Inc) as described previously.⁷ Briefly, samples and bHABP were incubated in a microtube for 1 hour. The sample-bHABP mixtures were added onto a hyaluronan-coated microtiter Covalink NH modules (Nunc, Rochester, NY). Bound bHABPs were measured with a colorimetric reaction. Sample concentrations were calculated from a standard curve that was generated by using hyaluronan standards of known concentration (ranging 0-2000 ng/mL).

mRNA analysis

RNA was extracted from airway fibroblasts by using Trizol Reagent (Invitrogen) following the manufacturer's instructions. For real-time PCR analysis, 0.5 μ g total RNA was used for reverse transcription with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, Calif). One microliter cDNA was subjected to real-time PCR by using Power SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7500 Detection system (Applied Biosystems). The specific primers were designed on the basis of on cDNA sequences deposited in the GenBank database: HAS1 (NM_001532) sense GAGGCTGGTACAACCAGAA, antisense TGTACAGCCACTCACGGAAG; HAS2 (NM_005328) sense GCCTCATCTGTGGAGATGGT, antisense TCCCAGAGGTCCACTAATGC; HAS3

TABLE I. Subject characteristics

Parameter	Asthma (21)	Controls (25)	P value
Gender (F/M)	12/9	18/7	.29
Age	27 ± 2	31 ± 2	.17
Ethnicity	1 A, 7 B, 12 W	1 A, 13 B, 11 W	.52
FEV ₁ (L)	3.69 ± 0.2	3.28 ± 0.2	.06
FEV ₁ (% predicted)	83 ± 4	103 ± 4	.0005
FVC (L)	4.4 ± 0.2	3.9 ± 0.2	.10
FEV ₁ /FVC	83 ± 2	86 ± 1	.23
PC ₂₀ (mg/mL)*	0.46 ± 0.10	>16	.0001

A, Asian; B, black; F, female; FVC, forced vital capacity; M, male; W, white.

*The provocative concentration of methacholine resulting in a 20% fall in FEV₁.

(AF234839) sense GGCATTATCAAGGCCACCTA, antisense GACACAGGAATGAGGCCAAT; HYAL1 (NM_007321) sense CATCCTGAACGTGAC CAGTG, antisense AGCCATCTGTGCCTGATCTT; HYAL2 (NM_033158) sense ACATTGACCACCTGCAGACA, antisense GTAGCCATATTCATTGT CATA; and NAPDH oxidase (NOX)-4 (NM_016931) sense AGATGT TGGGGCTAGGATTG, antisense TCTCCTGCTTGAACCTTCT. The primers were all cDNA-specific, not amplifying genomic DNA. The conditions for real-time PCR were as follows: 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, and 1 cycle at 25°C for 2 minutes. The relative expression level of each gene was determined against the glyceraldehyde 3-phosphate dehydrogenase level in the same sample: human glyceraldehyde 3-phosphate dehydrogenase sense AATGCATCCTGCACCACCA, antisense GTAGCCATATTCATTGT CATA.

Hyaluronan size determination

Fibroblasts from asthmatic lung and healthy controls were seeded in 100-mm dishes. Cells were cultured in serum-free medium for 1 week after reaching confluence and then changed to fresh serum-free medium. Forty-eight-hour media were collected for experiments. Medium collected from cultured lung fibroblasts was concentrated with centrifugal filter (10,000-d cut-off; Millipore, Billerica, Mass) and then digested with protease Pronase (100 U/mL, Pronase from *Streptomyces griseus*; Calbiochem) at 55°C for 2 hours, followed by inactivation of protease activity by boiling the samples at 100°C for 10 minutes. Concentrated samples along with known molecular mass hyaluronan standards (1300, 790, and 132 kd) were electrophoresed on a 0.5% agarose gel, stained with 0.005% Stain-All (Sigma, St Louis, Mo) overnight, and then destained in water for 2 days, and final destaining was completed by exposing the gel to amber light for 30 minutes. Hyaluronan-agarose gels were photographed on a Geliance 600 Imaging System (PerkinElmer Life Sciences, Waltham, Mass). The captured gel images were analyzed with ImageJ (National Institutes of Health, Bethesda, Md) to document hyaluronan bands on the gel. A standard curve was determined by using known molecular mass hyaluronan standards. Hyaluronan peaks in the samples were calculated against the standard curve.

Flow cytometry

Macrophages derived from bronchoalveolar lavage fluid of either control subjects or patients with asthma were fixed in 3.7% formaldehyde before staining for flow cytometry. Cells were incubated with specific antihuman CD44 (clone G44-26; BD Pharmingen, San Diego, Calif), TLR2 (clone TL2.1; eBioscience, San Diego, Calif), and TLR4 (clone HTA125; eBioscience) antibody in staining buffer containing 0.5% BSA and 0.02% sodium azide at 4°C for 30 minutes. Samples were washed with PBS. Flow cytometry was performed by using a FACScanto II flow cytometer (BD Immunocytometry Systems, San Jose, Calif) and analyzed by using FlowJo 8.7 software. The macrophage population was gated on the basis of forward and side scatter characteristics. Percentages of CD44, TLR2, and TLR4-staining positive cells were calculated by comparing to control IgG.

Macrophage culture and cytokine measurement

Macrophages derived from BAL fluid of patients with asthma and healthy subjects who underwent bronchoscopy with bronchoalveolar lavage were plated into 12-well plates in RPMI1640 (Invitrogen) medium containing 10% FBS with penicillin and streptomycin overnight to allow the cells attach to the plate. Cells were washed once and then treated in serum-free RPMI medium containing either 100 µg/mL hyaluronan (ICN Biomedicals, Aurora, Ohio) or 100 ng/mL LPS (Sigma). Twenty-four-hour conditioned media were collected, and human IL-8 in the medium was measured by using ELISA (R&D Systems, Minneapolis, Minn) following the manufacturer's instructions.

Anti-TLR neutralization of cytokine production

Macrophages derived from BAL fluid of patients with asthma were incubated with antihuman TLR2 (10 µg/mL, cat #maba2-hltr2; InvivoGen, San Diego, Calif) or mouse IgA2 isotype control (10 µg/mL, cat #maba2-ctrl; InvivoGen), or with antihuman TLR4 (clone HTA125; eBioscience) or mouse IgG isotype control (20 µg/mL) at 37°C for 1 hour. BAL macrophages were pretreated with CD44 antibody (clone 5F12 at 20 µg/mL; Fisher, Pittsburgh, Pa) and control mouse IgG on ice for 30 minutes. Thereafter cells were treated with low-molecular-weight hyaluronan 100 µg/mL or LPS 100 ng/mL in serum-free medium. Twenty-four-hour culture media were collected for measuring human IL-8 concentration.

Statistical analysis

All statistical analyses for comparison between subjects with asthma and control subjects were performed by using unpaired, 2-sided Student *t* tests with Prism 5 (GraphPad Software, La Jolla, Calif).

RESULTS

Hyaluronan accumulates in asthmatic lung tissue

To determine the role of hyaluronan in human asthma, we first assessed hyaluronan accumulation in human lung tissues. Lung sections from patients with asthma and healthy control subjects were stained by using a bHABP-based protocol. A significant increase in hyaluronan immunohistochemical staining was observed in bronchial sections of patients with asthma (Fig 1, D and E) compared with that of bronchial sections from healthy subjects (Fig 1, A and B). Quantitative analysis of hyaluronan stained lung sections from multiple patients with asthma and healthy controls showed a significant increase in hyaluronan staining in the asthma group (Fig 1, G). These data are consistent with previous reports that hyaluronan concentration is elevated in BALF of patients with asthma compared with healthy individuals.³²⁻³⁴

Human airway fibroblasts from patients with asthma produce increased hyaluronan relative to healthy control subjects

We first compared baseline hyaluronan production by airway fibroblasts cultured from endobronchial biopsies of patients with asthma and control subjects. Hyaluronan concentrations in culture medium of asthmatic fibroblasts were significantly increased compared with those of normal fibroblasts at baseline (Fig 2, A). We then sought to determine whether asthmatic airway fibroblasts produced hyaluronan in response to relevant stimuli. IL-13, TNF-α, or TGF-β was used to stimulate hyaluronan production. Baseline production of hyaluronan from asthmatic fibroblasts was markedly elevated, and when stimulated with cytokines, some further augmentation was observed; however, the fold-increase was less than that observed between asthmatic and normal

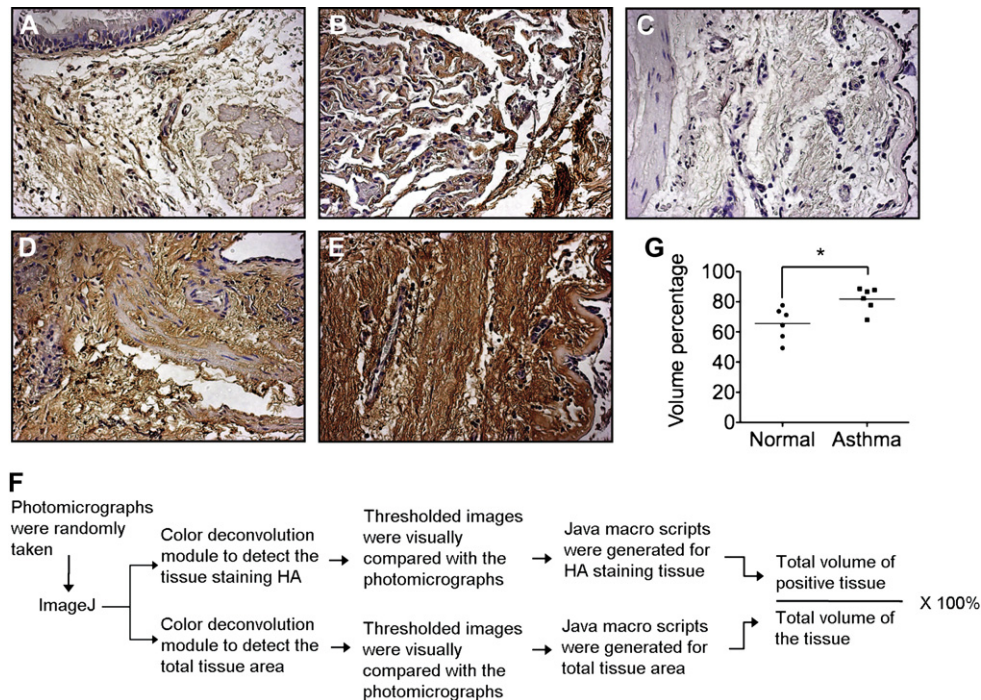


FIG 1. Sections of endobronchial biopsies from patients with asthma (FEV_1 : $95\% \pm 5\%$ predicted; $n = 6$) and healthy subjects (FEV_1 : $102\% \pm 5\%$ predicted; $n = 6$) were stained for hyaluronan (HA). **A** and **B**, Healthy. **D** and **E**, Asthmatic. **C**, Section from a patient with asthma was preincubated with HYAL. **F**, Flow chart to show quantitation of HA staining. **G**, Quantitation of HA staining of bronchial sections ($n = 6$ each; $*P < .05$).

fibroblasts at baseline (Fig 2, B). The key finding was that the constitutive production of hyaluronan by asthmatic airway fibroblasts was increased compared with normal fibroblasts.

Gene expression of hyaluronan synthase and HYAL isoforms in asthmatic airway fibroblasts

To delineate hyaluronan homeostasis in asthmatic fibroblasts, we measured gene expression of HAS isoforms and HYAL isoforms with quantitative PCR. We observed a dramatic increase in HAS2 gene expression in asthmatic fibroblasts compared with normal fibroblasts (Fig 3, A). HAS1 was too low to be detected after 40 cycles of PCR program and showed no difference between asthmatic and normal fibroblasts after performing secondary amplification (data not shown). There were no differences in HAS3 expression between asthmatic fibroblasts and fibroblasts from control subjects (Fig 3, B). Slight reductions in HYAL1 and HYAL2 gene expression in asthmatic fibroblasts were observed, but the differences did not reach statistical significance (Fig 3, C and D). These data suggest that the accumulation of hyaluronan in asthma may be a result of the elevated expression of HAS2 promoting synthesis of hyaluronan by asthmatic fibroblasts.

Asthmatic airway fibroblasts produce lower-molecular-weight hyaluronan species

Culture media of airway fibroblasts were concentrated and electrophoresed on agarose gels to determine the molecular mass distribution of hyaluronan produced by human airway fibroblasts. We found that fibroblasts from patients with asthma produced lower-molecular-weight hyaluronan fragments compared with

normal human airway fibroblasts (Fig 4, A and B; see this article's Fig E1, A-C, in the Online Repository at www.jacionline.org). These data suggested that hyaluronan produced by asthmatic fibroblasts contains lower-molecular-weight species in the size range that has been shown to stimulate cytokine production by macrophages *in vitro*.¹²

Studies have shown that reactive oxygen species (ROSs) induce depolymerization of hyaluronan,³⁸ and peroxynitrate is potent in generating hyaluronan fragments from high-molecular-weight precursors *in vitro*.^{39,40} Hyaluronan breakdown caused by ROSs in the lung further induces pathological responses.⁴¹⁻⁴³ ROSs are predominantly generated by the Nox/Dual oxidase family of proteins,⁴⁴ and NOX4 is the major isoform of NOX family that is expressed in human lung.⁴⁵ We explored the possibility that smaller-molecular-weight hyaluronan generated by asthmatic fibroblasts was a result of excessive production of ROSs. NOX4 expression in fibroblasts cultured from endobronchial biopsies of patients with asthma and healthy subjects was compared with real-time PCR. Our results indicated that NOX4 expression was significantly increased in asthmatic fibroblasts compared with fibroblasts from healthy subjects (Fig 4, C).

Cell-surface hyaluronan-binding protein expression on alveolar macrophages differs between subjects with asthma and healthy control subjects

Hyaluronan fragments stimulate macrophages to produce inflammatory cytokines through interaction with TLRs as well as CD44 on macrophages.^{12,14,46} CD44 expression on macrophages is required for hyaluronan clearance from inflamed tissues and promotes the resolution of inflammation in mice.^{7,27} To gain further insight into the role of hyaluronan homeostasis

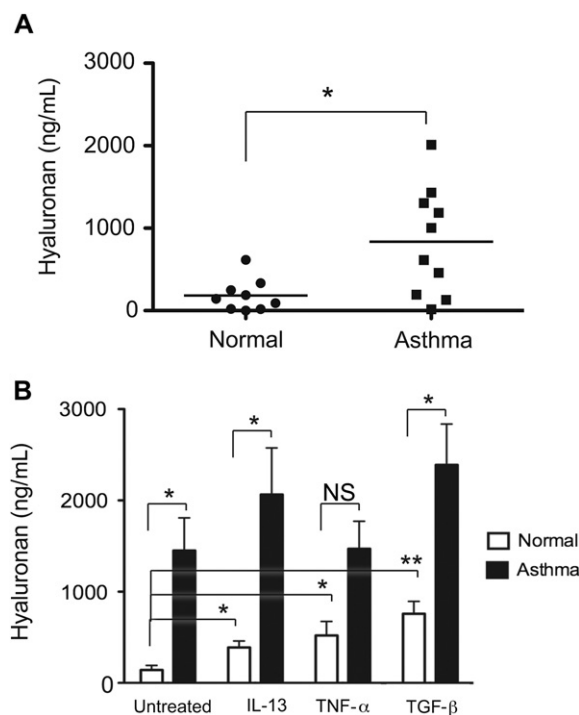


FIG 2. Asthmatic fibroblasts produce increased concentrations of hyaluronan. **A**, Hyaluronan levels in 48-hour culture media of untreated airway fibroblasts from patients with asthma and healthy controls (n = 9/10; *P < .05). **B**, Hyaluronan in 48-hour culture media of fibroblasts from patients with asthma and healthy controls treated with IL-13, TNF- α , or TGF- β (n = 5; *P < .05; **P < .01; NS, not significant). Within the asthma group, there was no significant difference comparing treatment versus no treatment.

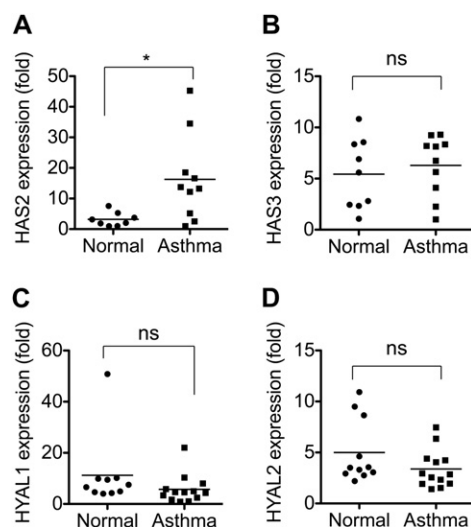


FIG 3. HAS and HYAL mRNA expression in fibroblasts from patients with asthma and healthy controls was compared by quantitative PCR. **A**, HAS2 (n = 8/10; *P < .05). **B**, HAS3 (n = 9/10; P = .563). **C**, HYAL1 (n = 10/13; P = .210). **D**, HYAL2 (n = 11/13; P = .129). NS, Not significant.

in asthma, we compared the expression of hyaluronan-binding proteins on the cell surface of alveolar macrophages derived from BALF of patients with asthma and control subjects by flow cytometry (Fig 5, A). Interestingly, we observed a significant decrease in CD44 expression coupled with an increase in

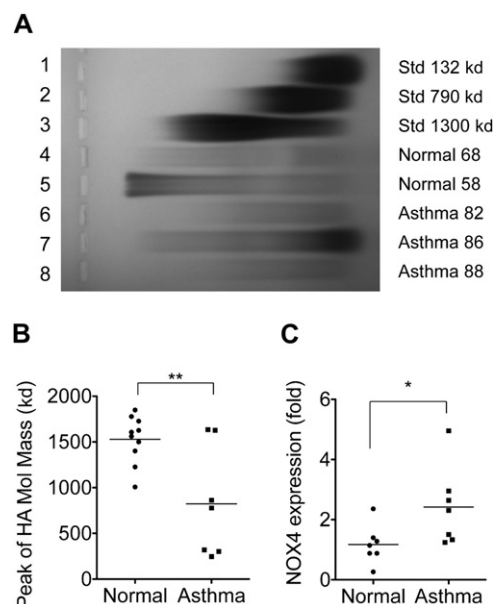


FIG 4. Molecular mass of hyaluronan (HA) produced by fibroblasts from patients with asthma and healthy controls. **A**, Size of HA in conditioned media of fibroblasts from subjects with asthma and healthy subjects. **Std**, Standard. **B**, Graph showing HA size represents 3 identical experiments with a total of 7 samples from asthmatic fibroblasts and 10 samples from normal fibroblasts (**P < .01). **Mol**, Molecular. **C**, NOX4 mRNA expression in fibroblasts from subjects with asthma and healthy subjects was determined with quantitative PCR (n = 7; *P < .05).

TLR2 and TLR4 expression on asthmatic alveolar macrophages (Fig 5, B and C). This dysregulation in the expression of hyaluronan-binding proteins on asthmatic alveolar macrophages has the potential to impair hyaluronan clearance and alter responsiveness to TLR ligands.

Cytokine production by asthmatic alveolar macrophages is altered in response to hyaluronan fragments and is TLR2-dependent and TLR4-dependent

To elucidate the functional consequences of the imbalance in hyaluronan-binding protein expression on alveolar macrophages from patients with asthma, we treated macrophages from BALF of patients with asthma and control subjects with low-molecular-weight hyaluronan and LPS and measured IL-8 production in culture medium. Untreated macrophages produced minimal cytokines. Asthmatic alveolar macrophages produced increased concentrations of IL-8 in response to hyaluronan fragment and LPS (Fig 6, A). Although hyaluronan weakly induced IL-10 release, there was no difference in IL-10 production between asthmatic and normal macrophages (see this article's Fig E2 in the Online Repository at www.jacionline.org).

Our previous study showed that hyaluronan signals through TLR2 and TLR4 in mouse macrophages.¹⁴ To determine whether hyaluronan signals in the same manner in human macrophages, we measured cytokine production with anti-TLR neutralization of human monocyte/macrophage cell line U937 cells and BAL macrophages. Asthmatic alveolar macrophages were treated with hyaluronan or LPS in the presence or absence of CD44, TLR2, or TLR4 antibodies. Our results showed that TLR2

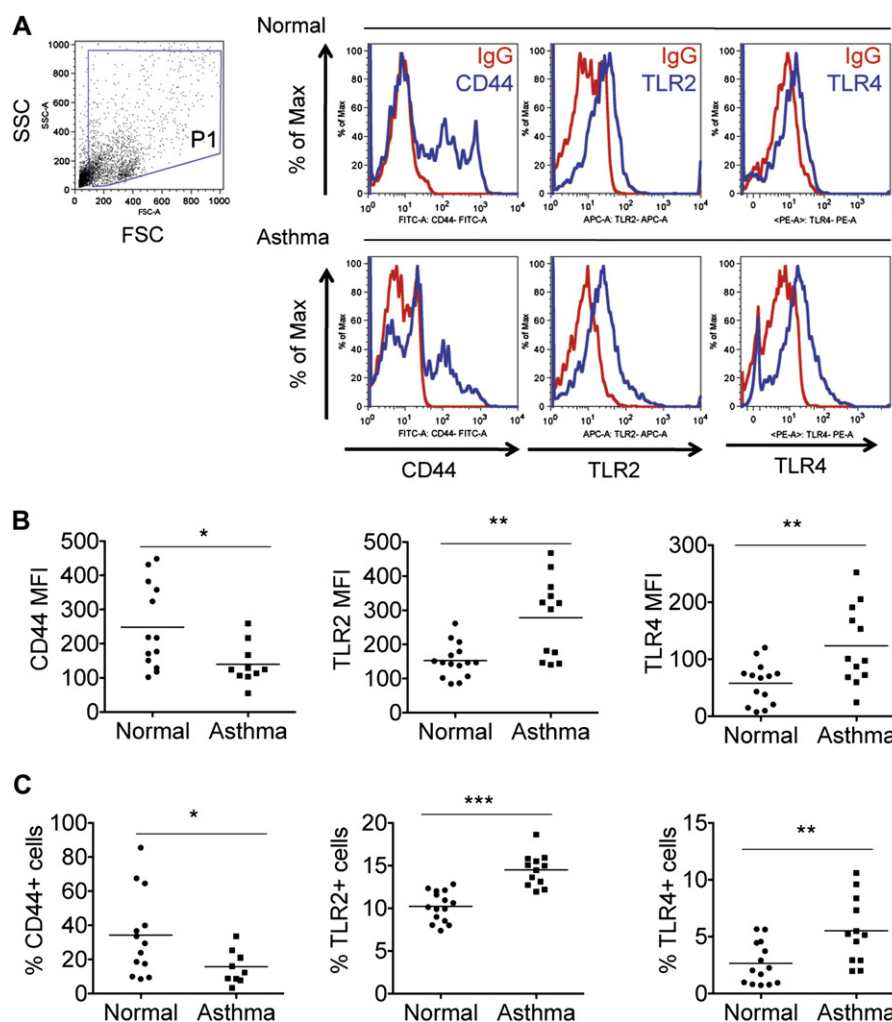


FIG 5. Flow-cytometric analysis of cell-surface hyaluronan-binding protein expression on alveolar macrophages from BAL of subjects with asthma and healthy subjects. **A**, Live macrophage population (P1) was gated on the basis of forward (FSC) and side scatters (SSC). Overlay of histograms of macrophages stained with specific antibodies to CD44, TLR2, or TLR4 (blue lines) and respective control IgG (red lines) is shown. The mean fluorescence intensity (MFI; **B**) and the percentages (**C**) of CD44, TLR2, and TLR4-staining positive cells were calculated by comparing with control IgG (CD44, n = 10/13; TLR2, n = 12/15; TLR4, n = 12/14; * $P < .05$, ** $P < .01$, *** $P < .0001$). Max, Maximum.

neutralizing antibody significantly decreased hyaluronan-induced IL-8 production by BAL macrophages (Fig 6, B). TLR4 neutralizing antibody decreased IL-8 production by BAL macrophages induced by both hyaluronan and LPS (Fig 6, C). Similarly, U937 cells were pretreated with phorbol 12-myristate 13-acetate (PMA), and then adherent macrophages were treated with hyaluronan or LPS in the presence or absence of TLR2 or TLR4 antibodies. Our results showed that both TLR2 and TLR4 antibodies significantly reduced hyaluronan-induced IL-8 production in U937 cells (see this article's Fig E3 in the Online Repository at www.jacionline.org). Interestingly, CD44 blockade increased LPS-induced IL-8 release by BAL macrophages (Fig 6, D). These data suggest that increased IL-8 production of asthmatic macrophages in response to hyaluronan stimulation is regulated in part by TLR signaling. The CD44 antibody data are consistent with our previous observation that alveolar macrophages from CD44-null mice produce an increased amount of inflammatory chemokines in response to LPS.⁴⁷

DISCUSSION

In this study, we have explored the roles of hyaluronan and hyaluronan-binding proteins in human asthma. Hyaluronan has been shown to accumulate in the BALF of patients with asthma and may correlate with persistence of asthma symptoms.^{33,34} Our data show that asthmatic airway fibroblasts produce constitutively higher concentrations of lower-molecular-weight hyaluronan with increased HAS2 gene expression compared with airway fibroblasts from healthy controls. In addition, alveolar macrophages from patients with asthma exhibit altered expression of hyaluronan-binding proteins compared with healthy subjects, with a decrease in cell-surface CD44 and increases in TLR2 and TLR4 expression. This translated into increased responsiveness of asthmatic alveolar macrophages to hyaluronan and LPS stimulation as demonstrated by increased IL-8 production. The reduction of CD44 on asthmatic alveolar macrophages could lead to impaired clearance of hyaluronan, which in turn could promote persistent inflammation and, potentially, asthma symptoms.

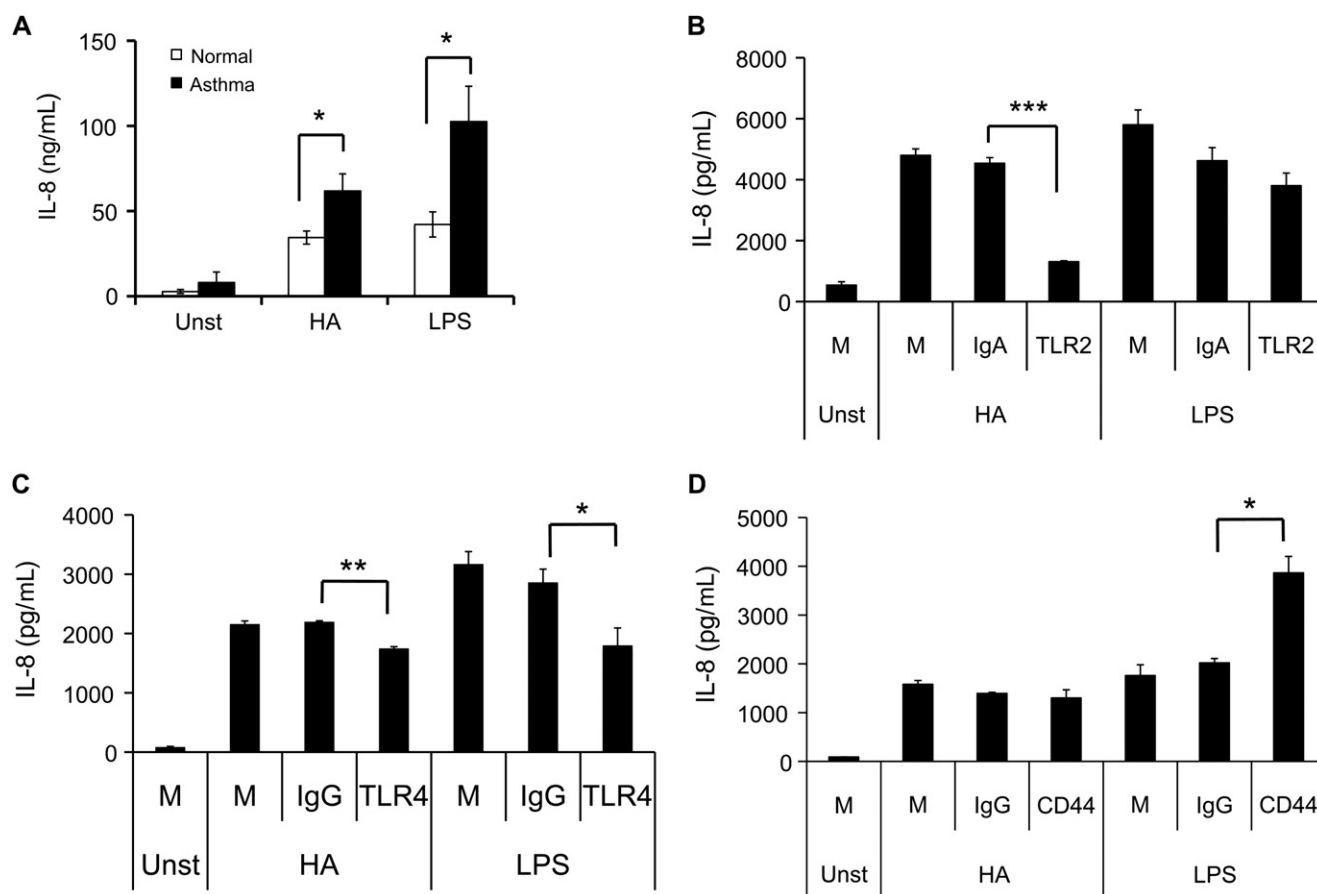


FIG 6. Cytokine production by alveolar macrophages from subjects with asthma and healthy subjects. **A**, IL-8 levels in 24-hour conditioned media from BAL alveolar macrophages from subjects with asthma and healthy subjects treated with either hyaluronan (HA) or LPS ($n = 8$; $*P < .05$). **B–D**, IL-8 levels in 24-hour conditioned media from BAL alveolar macrophages from patients with asthma treated with HA or LPS in the presence or absence of TLR2 (**B**), TLR4 (**C**), or CD44 (**D**) antibody pretreatment ($n = 3$; $*P < .05$, $**P < .01$, $***P < .001$). M, Medium only; Unst, unstimulated.

Our data show that asthmatic airway fibroblasts constitutively release more hyaluronan than normal fibroblasts. In addition, asthmatic fibroblasts release relatively lower-molecular-weight hyaluronan than normal fibroblasts. The significant increase in HAS2 expression suggests that asthmatic fibroblasts acquired an activated phenotype with increased hyaluronan production.⁴⁸ *In vitro* experiments with cultured fibroblasts or COS1 cells suggested that HAS1 and HAS3 generated hyaluronan with broad size distributions (molecular masses of 2×10^5 to 2×10^6 d), whereas HAS2 generated hyaluronan with a broad but extremely large size (average molecular mass of $>2 \times 10^6$ d).⁴⁹ Subsequent studies suggested that all 3 HAS enzymes drive the biosynthesis and release of high-molecular-mass hyaluronan (1×10^6 d).⁵⁰ Thus, elevated expression of HAS2 may be responsible for increased hyaluronan production. Hyaluronan-degrading enzymes HYAL1 and HYAL2 expression was not different between asthmatic and normal fibroblasts. Hyaluronidases usually hydrolyze the hexosaminidic $\beta(1-4)$ linkages in hyaluronan and release hyaluronan fragments. Although HYAL from *Streptococcus pneumoniae* hydrolyzes hyaluronan to release disaccharide D-glucuronic acid-N-acetyl-D-glucosamine, the vertebrate HYALs generate a various range of hyaluronan oligomers.⁵¹ A recent study reported that asthmatic airway smooth muscle cells showed reduced hyaluronan secretion with decreased HAS1 and

HAS2 expression and increased expression of HYAL1.⁵² Several reports have demonstrated that hyaluronan concentration is elevated in BALF of patients with asthma,^{32–34} and the concentration of hyaluronan in BALF was positively correlated with the severity of asthma.³⁴ Human lung fibroblasts are an important source of hyaluronan production^{23–25} in addition to other cell types such as epithelial cells.⁵³ Furthermore, fibroblasts isolated with our protocol are α -smooth muscle actin-negative.³⁷ The reduced hyaluronan release by airway smooth muscle cells suggested that smooth muscle cells do not contribute to the hyaluronan accumulation in BALF and lung tissue in patients with asthma.

Importantly, hyaluronan is susceptible to degradation by excessive ROSs during inflammation. Studies have shown that ROSs contribute to hyaluronan breakdown during lung injury^{41–43} and that human lung fibroblasts express NOX4.^{45,54} A recent report has shown that S-nitrosoglutathione reductase activity and inducible nitric oxide synthase were upregulated in BALF of patients with asthma.³⁶ Our results indicated that NOX4 expression was elevated in asthmatic fibroblasts and might contribute to the production of low-molecular-weight hyaluronan in culture medium of fibroblasts from patients with asthma. To our knowledge, we are the first to demonstrate increased NOX4 expression in asthmatic fibroblasts. Collectively, these data suggest that the increase in hyaluronan fragments in asthmatic fibroblasts is likely

a result of enhanced NOX4 expression and oxidative depolymerization rather than digestion by HYALs.

Toll-like receptors are a major component of the innate immune system and have been studied in asthma.¹⁷ Studies with murine asthma models have shown that TLR4^{18,20,21} and TLR2 are required for regulating T_H2 immune responses in antigen-sensitized mice.⁵⁵ TLRs are expressed by all effector cells of innate immunity including epithelial cells, mucosal mast cells, and dendritic cells⁵⁶ and are important for pathogen recognition. In this study, we have found that both TLR2 and TLR4 on human alveolar macrophages from patients with asthma are upregulated with functional consequences of enhanced cytokine production in response to ligand. Blockade of TLR4 inhibited hyaluronan fragment-stimulated expression of IL-8 in asthmatic alveolar macrophages. Future studies will examine the role of TLR2 as well as other inflammatory cells such as eosinophils. This observation has potential implications for the role of infection in the pathobiology of chronic asthma and provides some insights into the potential mechanisms to explain spirals of symptoms that develop in patients with asthma with airway infections.

Previous work from our group has shown that CD44 is required for hyaluronan clearance and the resolution of noninfectious lung inflammation in mice.⁷ We have also shown that CD44 is a negative regulator of TLR signaling. Alveolar macrophages from CD44-null mice produce an increased amount of inflammatory chemokines in response to LPS.⁴⁷ Loss of CD44 on alveolar macrophages from patients with asthma may not only impair hyaluronan clearance from asthmatic lungs but also enhance TLR signaling, thus promoting persistent inflammation.

Collectively, these data suggest that hyaluronan homeostasis is deranged in asthma and may provide some insights into connections among hyaluronan accumulation, macrophage function, and fibroblast activation in asthmatic lung. Asthmatic airway fibroblasts produce increased concentrations of low-molecular-weight hyaluronan. Decreased CD44 expression commensurate with increases in TLR2 and TLR4 on asthmatic macrophages may enhance the response to hyaluronan and TLR ligand LPS. Impaired hyaluronan clearance by decreased CD44 expression on macrophages leads to persistent inflammation in asthmatic lung. This previously unrecognized inflammatory regulatory pathway in asthma could lead to targeted approaches in selected patients experiencing severe and persistent asthma. Targeting the proinflammatory cycle involving fibroblasts, macrophages, and matrix turnover may present a novel therapeutic strategy in the treatment of asthma.

Key messages

- Hyaluronan homeostasis is deranged in asthma, with increased production by airway fibroblasts and decreased CD44 expression on alveolar macrophages that impairs hyaluronan fragment clearance.
- An imbalance in hyaluronan-binding protein expression on alveolar macrophages in asthma suggests a novel proinflammatory mechanism by which persistence of hyaluronan fragments could contribute to chronic inflammation and airway remodeling in asthma.

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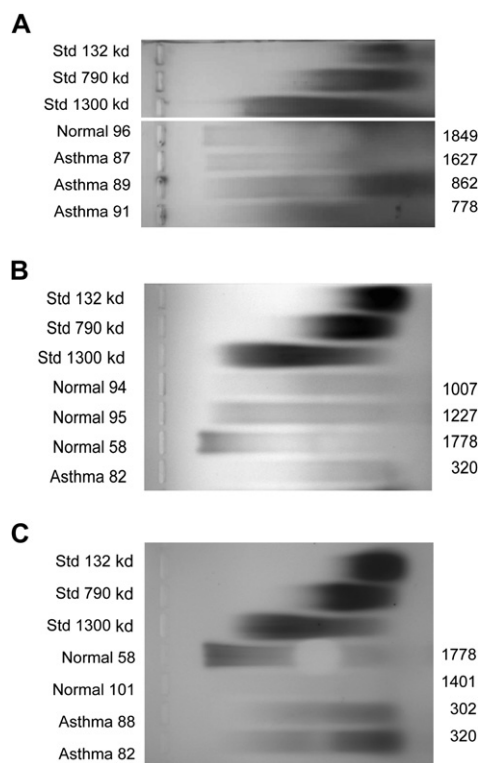


FIG E1. Molecular mass of hyaluronan from 48-hour conditioned media of fibroblasts from patients with asthma and healthy controls (**A–C**). Hyaluronan molecular mass peaks are shown on the *right*. *Std*, Standard.

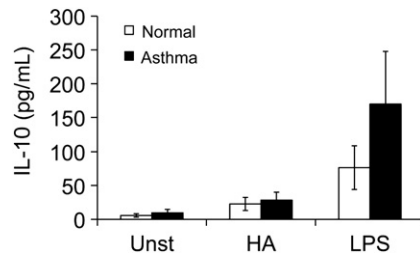


FIG E2. IL-10 levels in 24-hour conditioned media collected from BAL alveolar macrophages from subjects with asthma and healthy subjects treated with either hyaluronan (HA) or LPS ($n = 7-8$; $P > .05$ between healthy and asthma). *Unst*, Unstimulated.

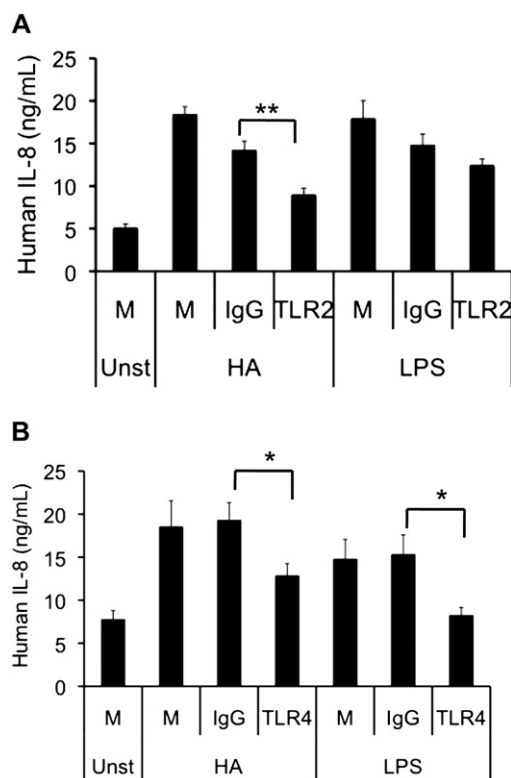


FIG E3. Cytokine production by macrophages is altered in response to hyaluronan (HA) fragments and is TLR2-dependent and TLR4-dependent. U937 cells were incubated with 100 nm PMA at 37°C for 24 hours. Adherent cells were then washed 3 times with PBS and incubated in complete media (without PMA) for an additional 24 hours before use for experiments. Cells were incubated with antihuman TLR2 (clone TL2.1) or mouse IgG_{2a} isotype control (20 μ g/mL) at room temperature for 30 minutes (**A**) or with antihuman TLR4 (clone HTA125) or mouse IgG_{2a} isotype control (20 μ g/mL) at 37°C for 1 hour (**B**). Thereafter cells were treated with low-molecular-weight HA 100 μ g/mL or LPS 100 ng/mL in serum-free medium. Twenty-four-hour culture media were collected for measuring human IL-8 concentration. All antibodies were from eBioscience ($n = 7-8$; * $P < .05$, ** $P < .01$). M, Medium only; Unst, unstimulated.