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Hyaluronan Fragments Act as an Endogenous Danger Signal by Engaging TLR2[1]

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Upon tissue injury, high m.w. hyaluronan (HA), a ubiquitously distributed extracellular matrix component, is broken down into lower m.w. (LMW) fragments, which in turn activate an innate immune response. In doing so, LMW HA acts as an endogenous danger signal alerting the immune system of a breach in tissue integrity. In this report, we demonstrate that LMW HA activates the innate immune response via TLR-2 in a MyD88-, IL-1R-associated kinase-, TNFR-associated factor-6-, protein kinase Cζ-, and NF-κB-dependent pathway. Furthermore, we show that intact high m.w. HA can inhibit TLR-2 signaling. Finally, we demonstrate that LMW HA can act as an adjuvant promoting Ag-specific T cell responses in vivo in wild-type but not TLR-2^{null} mice. ***The Journal of Immunology,*** **2006, 177: 1272–1281.**

Inherent to two signal models of immune activation is the fact that the Ags are not the critical determinant of whether recognition will lead to activation or tolerance, but rather molecules that have the ability to activate APCs. To account for these observations, Janeway posited that the immune system responds to pathogen-associated molecular patterns (PAMPS)[4] that are inherent to the make-up of infectious agents and distinguish them from host molecules (1). Indeed, PAMPS can account for immune responses against infectious agents. However, such molecules do not directly explain immune responses generated against transplanted organs and tumors. This fact led Fuchs and Matzinger (2–4) to propose a model in which the immune system is activated by danger signals. In their model, such signals could be derived from either the host or infectious agents. Although TLR engagement by infectious agents can activate the innate immune response, so too can the cellular products released during necrotic cell death. The precise nature of these intracellular-host-derived signals is emerging. Uric acid, a byproduct of nucleotide metabolism, is released upon necrotic cell death (5). Similarly, HMGB1 is emerging as an important inflammatory cytokine released upon necrotic cell death (6).

Hyaluronan (HA) is a negatively charged high m.w. (HMW) glycosaminoglycan, which is ubiquitously distributed in the extracellular matrix (7, 8). HA, a component of the basement membrane of normal lungs, joints, and vitreous fluid, functions in water homeostasis, plasma protein distribution and transportation, joint lubrication, and matrix structure (7, 8). The vast majority of HA is produced by fibroblasts and to a lesser degree by smooth muscle cells (7). In vivo, at sites of inflammation, the HMW HA (M_r $2–6 \times 10^6$) can be depolymerized to lower m.w. (LMW) (M_r 0.2×10^6) fragments via oxygen radicals and enzymatic degradation by hyaluronidase, β-glucuronidase, and hexosaminidase (8). Likewise, inflammatory cytokines, such as TNF-α, can stimulate pulmonary fibroblasts to produce increased amounts of HA fragments (9).

In its HMW form, HA is believed to play a homeostatic role. However, in the setting of tissue destruction, HMW HA is broken down into its lower m.w. components that possess the ability to induce inflammatory gene expression (10–15). Indeed, LMW HA fragments activate inflammatory gene expression in epithelial cells, endothelial cells, fibroblasts, dendritic cells (DCs), and macrophages (14, 16–19). Genes induced by LMW HA include members of the chemokine family (MIP-1α, MIP-1β, KC, RANTES, MCP-1, and IFN-inducible protein-10), cytokines (IL-8, IL-12, and TNF-α), as well as the matrix-modifying enzymes (murine metalloelastase, inducible NO synthase, and plasminogen activator inhibitor-1) (10, 11, 13, 14, 20). Furthermore, LMW HA fragments enhance T cell responses by activating and up-regulating costimulatory molecules on DCs (16, 21). The importance of HA in vivo is highlighted by the fact that not only is LMW HA associated with active inflammation but also lack of clearance of HA leads to enhanced inflammation-induced pathology (15, 22–28).

Although it is known that NF-κB is an important downstream mediator of LMW HA fragment activation, the precise receptor is somewhat controversial (14, 15, 29–33). On the basis of the ability of LMW HA to initiate inflammatory responses, we and others have hypothesized that LMW HA fragments were inducing activation via a TLR (15, 31–33). In this report, we identify TLR-2 as the HA fragment-activating receptor and demonstrate the ability of HMW HA to inhibit TLR-2 signaling. Furthermore, we show that LWM HA can act as an adjuvant, promoting the activation of Ag-specific T cells in vivo. These data support the concept that

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[4] Abbreviations used in this paper: PAMPS, pathogen-associated molecular patterns; HA, hyaluronan; HMW, high m.w.; LMW, low m.w.; PEC, peritoneal elicited macrophage; DC, dendritic cell; HKAL, heat-killed *A. laidlawii*; poly(I:C), polyinosinic-polycytidylic acid; PKC, protein kinase C; WT, wild type; IRAK, IL-1R-associated kinase; TRAF, TNFR-associated factor.

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LMW HA fragments act as endogenous extracellular danger signals and that HMW HA may act to temper inflammation by inhibiting TLR-2 signaling by HA and other TLR-2 agonists.

Materials and Methods

Cells, mice, and cell lines

The mouse alveolar macrophage cell line MH-S was purchased from the American Type Culture Collection and maintained per the manufacturer's guidelines (34). HEK-293 cells stably transfected with human TLR-2, TLR-2/6, TLR-3, or TLR-5 were purchased from InvivoGen and grown according to the manufacturer's guidelines. Thioglycollate-elicited peritoneal macrophages were lavaged from female C3H/HeJ LPS hyporesponsive mice, TLR4^{null}, CD44^{null}, IL-1R^{null}, IL-18R^{null}, OT-II TCR-transgenic (The Jackson Laboratory), MyD88^{null} (Akira and Golenbock) or TLR-2^{null} mice (M. Fenton and The Jackson Laboratory) 4 days after injection of 2 ml of sterile thioglycollate (Sigma-Aldrich). The cells were allowed to adhere overnight in RPMI 1640 supplemented with 10% heat-inactivated low-LPS FBS and 1% penicillin-streptomycin/1% glutamine before use. To exclude the effects of contaminating LPS on experimental conditions, cell stimulation was conducted in the presence of polymixin B 10 μg/ml (Calbiochem Novabiochem). All animal experiments were approved by the Johns Hopkins Committee on Animal Use, and experiments were conducted in accordance to their guidelines and regulations.

Chemicals and reagents

Purified LMW HA fragments from human umbilical cords (Calbiochem Novabiochem) are free of protein and other glycosaminoglycans with a peak m.w. of 200,000. Ultrapure HMW HA from chicken combs (Genzyme) is free of protein, other glycosaminoglycans, and LPS with a peak m.w. of 6,000,000. Heat-killed *Acholeplasma laidlawii* (HKAL), polyinosinic-polycytidylic acid (poly(I:C)), ultrapure LPS, FSL-1, Pam3CSK4, and flagellin were purchased from InvivoGen. Stock solutions of reagents were tested for LPS contamination using the *Limulus* amebocyte assay (Sigma-Aldrich).

Northern analysis of mRNA production

RNA was extracted from confluent cell monolayers using 4 M guanidine isothiocyanate and purified by centrifugation through a 5.7 M cesium chloride gradient for 12–18 h at 35,000 rpm as described in Ref. 11. Differences in RNA loading were documented by hybridizing blots with ^{32}P-labeled cDNA for aldolase. Blots were developed on a STORM PhosphorImager (Molecular Dynamics). Quantification of bands was determined by the PhosphorImager using a fixed area with the object average program for determining the background (ImageQuant; Molecular Dynamics) to account for interlane background variation.

Western analysis of protein secretion

Western blot analysis was performed as described (11). Briefly, 200 μg of macrophage conditioned media were fractionated by SDS-PAGE (10%), transferred to a nylon membrane, blocked and washed, incubated with the polyclonal phosphor-protein kinase C (PKC) or individual isoforms of PKC Abs at a dilution of 1:2500 (R&D Systems), and developed with a chemiluminescent system according to the manufacturer's instructions (Amersham).

Transient transfections

Transient transfections were performed using Lipofectamine 2000 (Invitrogen Life Technologies) in MH-S cells and lyovec (InvivoGen) in the H293 cells according to the manufacturer's guidelines (29). p-NIFTY NF-κB luciferase reporter construct was purchased form InvivoGen. PKC constructs were from Dr. J.-W. Soh (Columbia University, New York, NY), and the MyD88, IL-1R associated kinase (IRAK), and TNFR-associated factor-6 (TRAF6) dominant constructs were a gift from M. Arditi (Cedars-Sinai Medical Center, Los Angeles, CA). Luciferase expression was measured using a Dual Luciferase Kit (Promega) and Zylux femtomaster FB-12 luminometer.

ELISA for protein secretion

ELISAs for MIP-1α, IL-2, IFN-γ, and TNF-α were performed according to the manufacturer's guidelines (R&D Systems). Colorimetric changes were measured in an ELISA plate reader and analyzed with Microplate Manager III (Bio-Rad) software.

In vivo immunization and rechallenge

Splenocyte/lymphocyte preparations from C57BL/6 OT-II (TCR-transgenic-specific OVA peptide) mice were adoptively transferred into C57BL/6 wild-type (WT) and TLR-2 null hosts by i.v. injection ($\sim$5–6 $\times$ 10^6 clonotypic cells per mouse). The next day the mice received s.c. foot pad injections of 150 μg of OVA peptide with 150 μg of LMW HA or 150 μg of HMW HA. On day 3, draining lymph nodes were harvested, and 5 $\times$ 10^5 lymphocytes were rechallenged with 6 μg/ml OVA peptide and 5 $\times$ 10^5 syngeneic irradiated C57BL/6 splenocytes per well in 96-well U-bottom plates. After 48 h, supernatants were harvested and analyzed for IL-2 and IFN-γ by ELISA.

Statistics

Statistical analysis was performed between groups using an unpaired *t* test from GraphPad Software. A difference between groups of $p < 0.05$ was considered significant.

Results

LMW HA induces inflammatory gene expression

In its HMW form, HA is a major component of the extracellular matrix and plays an important role in maintaining tissue integrity and water homeostasis (7, 8). In the setting of tissue insults, when protective barriers are breached, HMW HA is broken down into lower m.w. species that have the ability to induce the up-regulation of inflammatory genes. Incubating thioglycollate elicited peritoneal macrophages (PEC) from LPS hyporesponsive C3H/HeJ mice with LMW HA fragments, but not HMW HA, results in the induction of MIP-1α mRNA as measured by Northern analysis (Fig. 1*a*). Furthermore, LMW HA has the ability to induce a diverse array of inflammatory chemokines, cytokines, and enzymes in primary macrophages (Fig. 1*b*). In addition to macrophages, LMW HA also has the ability to stimulate other cell types including DCs, epithelial cells, and endothelial cells (11–14, 16–19). Bone marrow-derived DCs were stimulated with LMW HA or LPS, and MIP-1α protein expression was determined by ELISA (Fig. 1*c*). DC-derived chemokine production in response to LMW HA was equivalent to that seen by LPS stimulation.

The expression profile of LMW HA fragment induced chemokines is similar to that induced by LPS. As such, it was critical for us to demonstrate that these results were not due to LPS contamination. First, these experiments were performed using macrophages derived from C3H/HeJ mice that are hyporesponsive to LPS. Second, these experiments were performed in the presence of 10 μg/ml of polymixin B, which inhibits LPS activation. Indeed, under these conditions LPS failed to induce inflammatory gene up-regulation (Fig. 1, *a* and *b*). Finally, we stimulated PECs derived from TLR-4^{null} mice. Whereas PEC from control mice responded to both LMW HA and LPS (these experiments were done in the absence of polymixin B in LPS-stimulated cells), PEC from TLR-4^{null} mice up-regulated MIP-1α mRNA expression in response to LMW HA but not LPS (Fig. 1*d*). These data further rule out a role for LPS contamination as an explanation for the ability of LMW HA to induce inflammatory gene expression and suggest that LMW HA is not signaling via TLR-4.

CD44, a cell surface molecule found on numerous cells of hematopoietic origin, has a high affinity for HA. HA-CD44 interactions have been implicated in cell migration and tumor metastasis as well as in the clearance of LMW HA from sites of inflammation (22, 35). In fact, CD44 has been proposed as the receptor mediating LMW HA-induced inflammatory gene expression (14). To evaluate the potential role of CD44 in HA fragment induced chemokine gene expression, we stimulated thioglycollate PEC from CD44^{null} and littermate control mice with LMW HA fragments (Fig. 1*e*). There was no difference between the expression of MIP-1α mRNA in the macrophages derived from the WT control

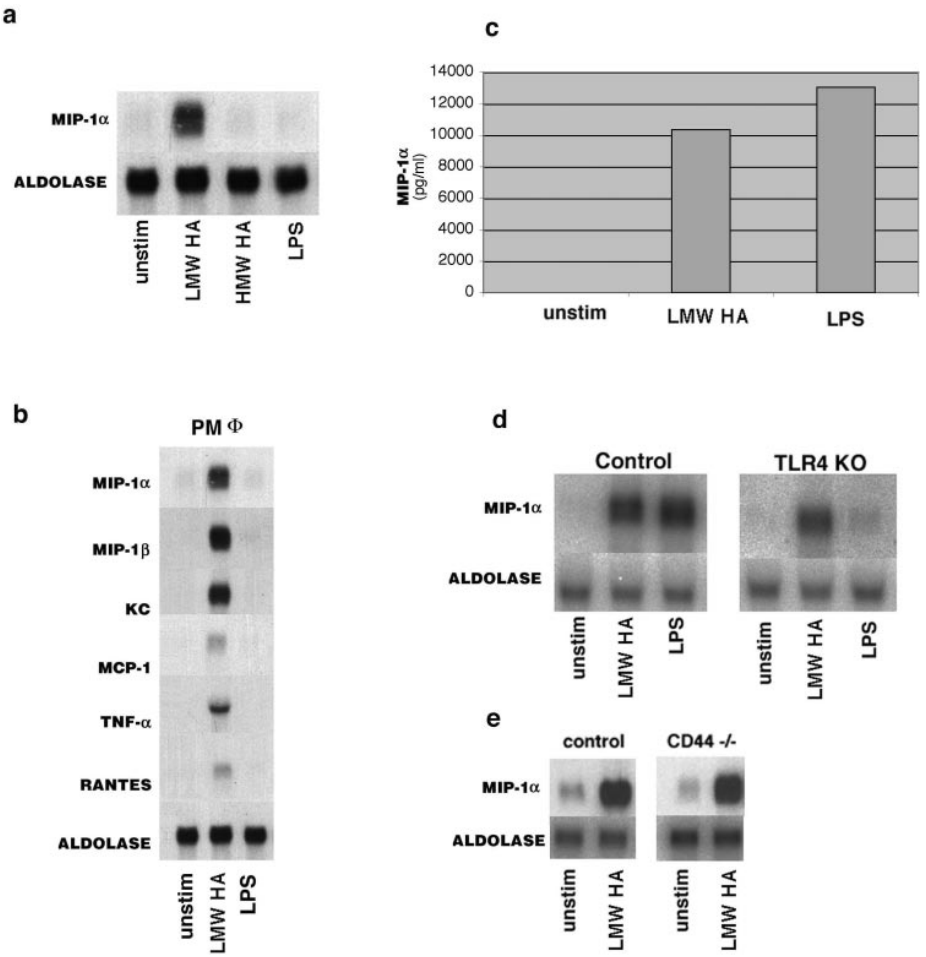

FIGURE 1. LMW HA induces MIP-1α RNA and protein expression in PEC and DCs. *a,* Northern analysis of mRNA derived from PEC from C3H/HeJ mice stimulated with LMW HA (100 μg/ml), HMW HA (100 μg/ml), or LPS (100 ng/ml) + polymixin B (10 μg/ml) for 6 h. *b,* Northern analysis of mRNA derived from PEC from C3H/HeJ mice stimulated with LMW HA (100 μg/ml) or LPS 100 ng/ml + polymixin B (10 μg/ml) for 6 h. *c,* ELISA of protein from DCs from C57BL/6 mice stimulated with LMW HA (100 μg/ml) or LPS 100 ng/ml for 24 h. *d,* Northern analysis of mRNA derived from PEC from control or TLR4^{null} mice stimulated with LMW HA (100 μg/ml) or LPS (100 ng/ml) for 6 h. *e,* Northern analysis of mRNA derived from PEC from control or CD44^{null} mice stimulated with LMW HA (100 μg/ml) or LPS (100 ng/ml) for 6 h. Data are representative of six experiments. Unstim, Unstimulated; KO, knockout; KC.

vs CD44^{null} mice. Thus, these data suggest that the ability of LMW HA to induce inflammatory gene expression is independent of CD44.

HA-induced gene expression is MYD88 dependent

While the ability of LMW HA to induce inflammatory gene expression was not due to TLR-4 engagement, the gene expression profile following HA activation was similar to that seen by activation of a number of the TLRs. This observation led us to further pursue the hypothesis that LMW HA might be signaling via a TLR. To test this hypothesis, we took advantage of the fact that the adaptor protein MyD88 plays an essential role in signaling for nearly all of the TLRs (36, 37). PEC from MyD88^{null} or WT control mice were stimulated with LMW HA or LPS as a control (Fig. 2*a*). As expected, LPS failed to induce MIP-1α mRNA expression in the macrophages derived from the MyD88^{null} mice while inducing robust level of MIP-1α in the macrophages derived from the WT controls. Similarly, LMW HA also failed to induce MIP-1α in the macrophages derived from the MyD88^{null} mice. These data suggest that the signal transduction pathways necessary for LMW HA-induced gene expression are MyD88 dependent.

TLR engagement leads to the activation of MyD88 followed by the activation of IRAK, TRAF6, and ultimately NF-κB (36, 37). Thus, we wanted to determine whether LMW HA-induced signaling also required IRAK and TRAF6. To this end, transient transfection experiments in MH-S cells, an alveolar macrophage cell line, with either empty vector or MyD88, IRAK, or TRAF6 dominant negative constructs and an NF-κB-driven luciferase reporter construct were performed. Although LMW HA induced NF-κB activation in the cells that were transfected with the empty vector, LMW HA-induced NF-κB activation was markedly reduced in the presence of MyD88, IRAK, and TRAF6 dominant negative constructs (Fig. 2*b*) (empty vector vs MyD88 dominant negative, $p = 0.0006$; empty vector vs IRAK dominant negative, $p < 0.0001$; empty vector vs TRAF6 dominant negative, $p < 0.0001$). Thus, LMW HA fragments signal via an MyD88-IRAK-TRAF6-dependent pathway for inflammatory gene induction.

LMW HA signaling requires PKC-ζ activation

TLR-induced signaling results in the downstream activation of PKC (36, 37). To further define the signaling pathways required for HA-induced gene expression, we investigated the ability of HA to induce PKC activation. MH-S cells were stimulated with LMW HA, and cytoplasmic extracts were harvested and evaluated for phosphorylation of PKC (Fig. 3*a*). Phospho-PKC, which is barely expressed in unstimulated cells, is increased by 15 min and remains elevated 2 h after LMW HA fragment stimulation. Additionally, Western blot analysis of the same extracts for the various isoforms of PKC demonstrates that MH-S cells express PKC-α, -β, -δ, -ε, and -ζ but not PKC-θ (Fig. 3*b*). To determine which isoform of PKC is necessary for LMW HA signal transduction, we performed transient transfection assays using dominant negative and WT constructs of PKC-δ, -ε, and -ζ along with an NF-κB-driven luciferase reporter construct. In these experiments, the DN

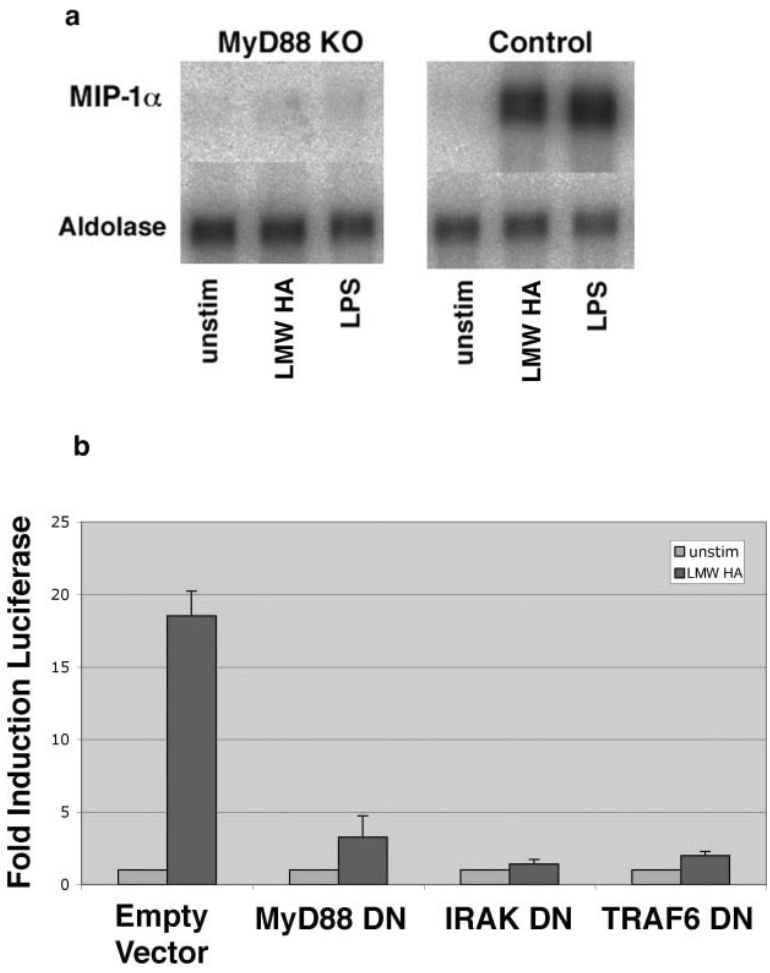

FIGURE 2. LMW HA induced gene expression is dependent on MyD88, IRAK, and TRAF6. *a,* Northern analysis of mRNA derived from PEC from control and MyD88^{null} mice stimulated with LMW HA (100 μg/ml) or LPS (100 ng/ml) for 6 h. *b,* Transient transfections of MH-S cells with an NF-κB luciferase reporter construct and MyD88, IRAK, and TRAF6 dominant-negative constructs overnight and then stimulated with LMW HA (100 μg/ml) for 24 h. Data are expressed as fold induction of luciferase over unstimulated (unstim) and are representative of *a* or the average of (*b*) five experiments. KO, Knockout.

PKC-ζ construct but not the DN PKC-δ or -ε isoforms inhibited LMW HA fragment induced reporter expression (Fig. 3*c*) (empty vector vs PKC-δ, $p = 0.3005$; empty vector vs PKC-ε, $p = 0.6112$; empty vector vs PKC-ζ, $p = 0.0015$). Thus, PKC isoform ζ, appears to be necessary in the LMW HA fragment signal transduction pathway. Overexpression of the WT forms of each construct did not have any effect on LMW HA-induced activation (data not shown). This is not surprising given that PKC regulation does not occur at the level of transcription but rather PKC activation. Thus, together these data suggest a role for PKC-ζ in LMW HA-induced inflammatory gene regulation.

LMW HA does not signal via the IL-1 or IL-18 receptors

Our data demonstrating the necessity for MyD88 in LMW HA signaling strongly suggested that HA was engaging a TLR. In addition to the TLRs, the MyD88 superfamily also consists of IL-1 and IL-18 receptors (38). In particular, IL-1 has been shown to be induced by LMW HA stimulation (39). Thus, it was possible that either LMW HA was signaling directly through the IL-1R or that LMW HA induced IL-1 production that in turn led to the up-regulation of the other inflammatory genes. To ensure that LMW HA was not signaling via the IL-1 or IL-18 receptors, we obtained PEC from IL-1R and IL-18R^{null} mice and stimulated them with LMW HA. When compared with PEC derived from WT mice, stimulation of PEC from IL-1R and IL-18R^{null} mice with LMW HA induced similar levels of MIP-1α protein by ELISA (Fig. 4). Thus, LMW HA-induced chemokine expression, although MyD88 dependent, is not mediated through the IL-1 or IL-18 receptors.

LMW HA signals via TLR-2

Next, we devised a strategy to determine which TLR was responsible for LMW HA signaling. HEK 293 embryonic kidney cells have very low levels of endogenous TLR expression and most importantly do not respond to LMW HA stimulation. Thus, HEK 293 cells, stably transfected with human TLR 2, 3, and 5, were transiently transfected with an NF-κB-driven luciferase reporter construct and then stimulated with LMW HA or various TLR agonists. LMW HA was able to induce NF-κB reporter activity in the cells that expressed TLR2 (Fig. 5*a*) (HEK 293-TLR2 unstimulated vs HA; $p = 0.0001$). Likewise, NF-κB was also activated when these cells were stimulated with the TLR-2 ligand HKAL (HEK 293-TLR2, unstimulated vs HKAL; $p = 0.0001$). On the other hand, poly(I:C) and flagellin failed to induce increased NF-κB activity in these cells. Poly(I:C) did, however, stimulate cells expressing its receptor TLR3 (Fig. 5*b*), whereas flagellin stimulated cells expressing TLR5 (Fig. 5*c*). Importantly, LMW HA did not up-regulate NF-κB activity in either HEK 293-TLR3 or HEK 293-TLR5 cells. These data support the hypothesis that TLR2 is the receptor for HA-induced inflammatory gene expression.

To further examine the role of TLR2 in LMW HA signaling, we obtained PEC from TLR-2^{null} mice. As expected, TLR2^{null} macrophages, when compared with control mice, do not respond to the TLR-2 agonist HKAL (Fig. 6*a*). On the other hand, both null and control cells show a robust and nearly identical response to LPS (Fig. 6*b*; LPS 1000 ng/ml, control vs TLR2$^{-/-}$ cells; $p = 0.5388$).

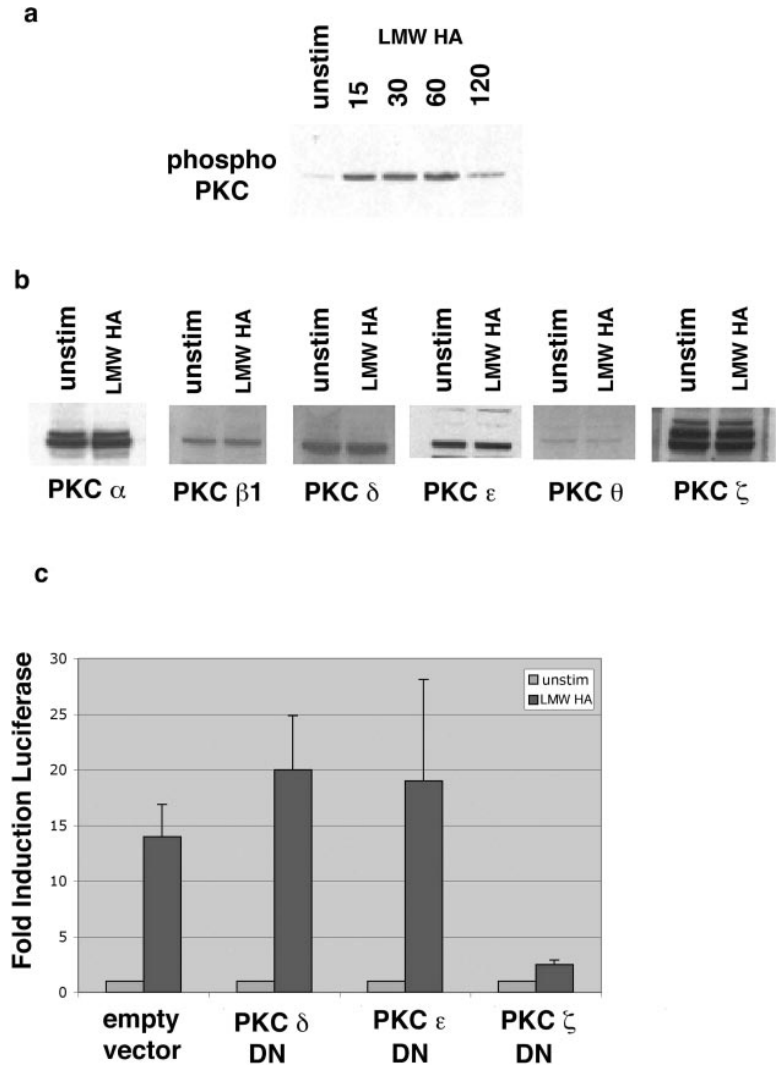

FIGURE 3. LMW HA requires PCK-ζ. *a,* Western blot for total phospho-PKC from cytoplasmic extracts from MH-S cells stimulated with LMW HA (100 μg/ml) for varying times. *b,* Western blot for different isoforms of PKC from cytoplasmic extracts from MH-S cells stimulated with LMW HA (100 μg/ml) for 1 h. *c,* Transiently transfections of MH-S cells with an NF-κB luciferase reporter construct and PKC-δ, -ε, or -ζ dominant-negative (DN) constructs overnight and then stimulated with LMW HA (100 μg/ml) for 24 h. Data are expressed as fold induction of luciferase over unstimulated and are representative of or the average of four identical experiments.

However, when PEC from TLR2^{null} mice were stimulated with LMW HA, MIP-1α production was nearly completely eliminated (Fig. 6*c*; HA 125, 250, 500, or 1000 μg/ml, control vs TLR2$^{-/-}$; $p = 0.0001$). Thus, these data using PEC from TLR^{null} mice support the data obtained using the TLR2-expressing HEK 293 cells that TLR2 is the LMW HA receptor.

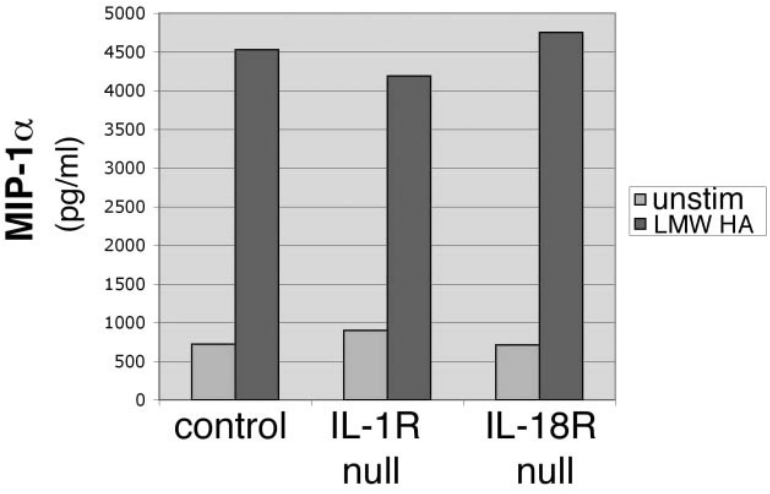

FIGURE 4. LMW HA-induced MIP-1α expression is independent of IL-1 and IL-18 receptors. ELISA of protein from PEC from IL-1 and IL-8 receptor null mice stimulated with LMW HA (100 μg/ml) for 18 h. Data are representative of three similar experiments. Unstim, Unstimulated.

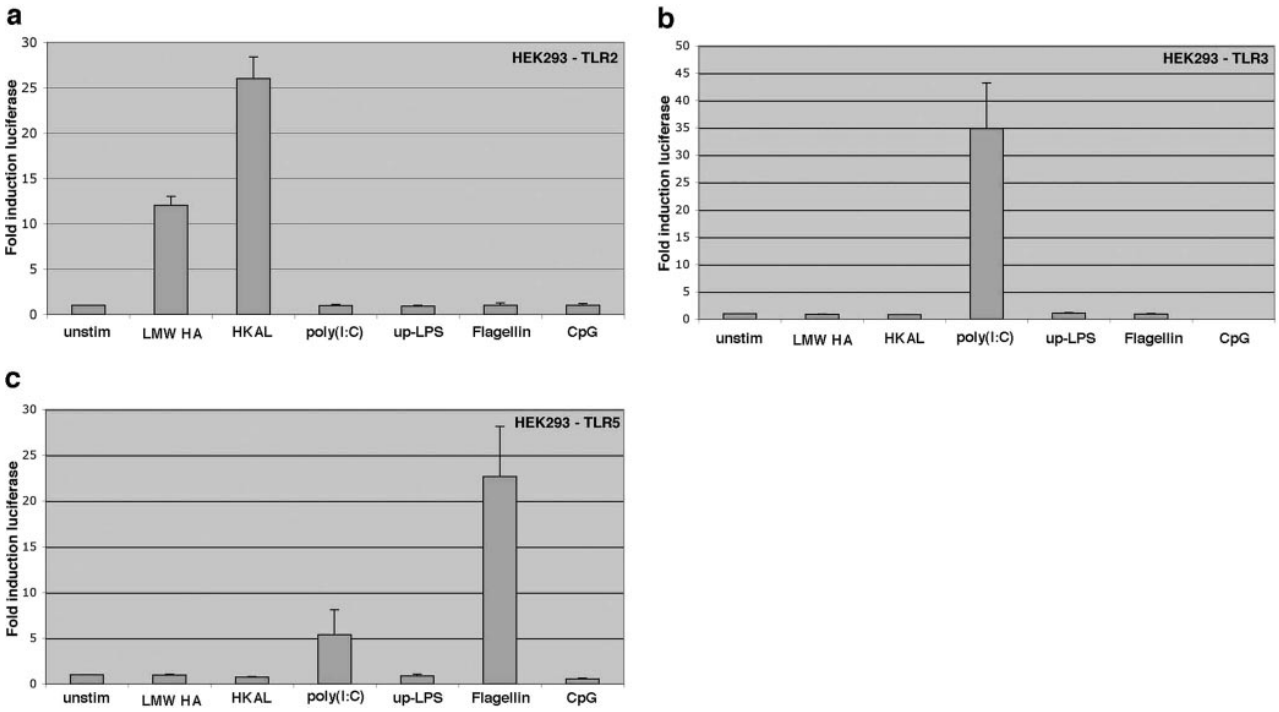

FIGURE 5. LMW HA-induced gene expression is dependent on TLR2 receptors. HEK-293 cells stably transfected with individual TLRs were transiently transfected with an NF-κB luciferase reporter construct overnight before stimulation with LMW HA (500 μg/ml), HKAL (100×10^8 cells/ml), poly(I:C) (10 ng/ml), ultrapure LPS (100 ng/ml), flagellin (100 ng/ml), and CpG (40 μg/ml) for 24 h. Data are the average of four identical experiments. Unstim, Unstimulated.

HMW HA inhibits TLR2 signaling

HMW HA can act as an inhibitor of LMW signaling (14). Preincubation of MH-S cells for 1 h with HMW HA (250 μg/ml) significantly blocked LMW HA-induced TNF-α expression by nearly 50% (Fig. 7; HA 25 μg/ml vs HA 25 μg/ml + HMW, 48% inhibition; $p = 0.0002$). The inhibition of LMW HA induced TNF-α by HMW HA also held true over a range of concentrations of LMW HA (data not shown; LMW HA 50 μg/ml vs HA 50 μg/ml + HMW, 44% inhibition, $p = 0.0012$; HA 100 μg/ml vs HA 100 μg/ml + HMW, 42% inhibition, $p = 0.0002$). The m.w. of HMW HA is 6×10^6 and the LMW is only 0.2×10^6; thus on a mol to mol basis, the ratio of agonist (LMW HA) to antagonist (HMW HA) favors the agonist. This may account for the incomplete inhibition. Increasing the molarity of the HMW HA results in a marked increase in viscosity, rendering it difficult to use. Nonetheless, the inhibition although not complete is consistent.

Inasmuch as LMW HA signals via TLR2, we tested the ability of HMW HA to block HKAL-induced activation. The HMW HA also blocked signaling by the TLR2-specific agonist HKAL up to nearly 40% over a range of doses of the TLR2 agonist HKAL (Fig. 7; HKAL 5×10^8 cells/ml vs HKAL 5×10^8 cells/ml + HMW, 39% inhibition; $p = 00059$). The inhibition of HKAL induced TNF-α by HMW HA also held true over a range of concentrations of HKAL (data not shown; HKAL 10×10^8 cells/ml vs HKAL 10×10^8 cells/ml + HMW, 35% inhibition, $p = 0.0060$; HKAL 25×10^8 cells/ml vs HKAL 25×10^8 cells/ml + HMW, 30% inhibition, $p = 0.0295$). Additionally, TLR-2-specific signaling by the synthetic lipoproteins FSL-1 and Pam3CSK4 were also inhibited by HMW HA (FSL 10 μg/ml vs FSL 10 μg/ml + HMW, 54% inhibition, $p = 0.001$; FSL 100 μg/ml vs FSL 100 μg/ml + HMW, 66% inhibition, $p = 0.022$; Pam3CSK4 200 μg/ml vs Pam3CSK4 200 μg/ml + HMW, 66% inhibition, $p = 0.0175$; Pam3CSK4 400 μg/ml vs Pam3CSK4 400 μg/ml + HMW, 71% inhibition, $p = 0.017$).

Importantly, the ability of HMW HA to inhibit signaling is TLR-2 specific in that the HMW HA did not inhibit signaling via the TLR-4 agonist LPS (Fig. 7; LPS 1 ng/ml vs LPS 1 ng/ml + HMW HA, 7% induction; $p = 0.8678$). HMW HA did not inhibit LPS-induced TNF-α over a range of concentrations of LPS (data not shown; LPS 10 ng/ml vs LPS 10 ng/ml + HMW HA, 10% induction, $p = 0.7003$; LPS 100 ng/ml vs LPS 100 ng/ml + HMW HA, 6% inhibition, $p = 0.5397$). Thus, not only do LMW fragments of the extracellular matrix component HA induce inflammatory gene expression via TLR2 ligation but also intact HMW HA can act as a competitive inhibitor of TLR2 signaling presumably dampening the inflammatory response to danger.

LMW HA acts an adjuvant in vivo in a TLR2-dependent manner

Although the ability of adjuvants to enhance T cell responses to Ags is well established, it has only recently been recognized that such agents contain potent activators of TLRs. Because we posit that LMW HA acts as an endogenous danger signal, we hypothesized that LMW HA could also act as an adjuvant. To this end, OT-II TCR-transgenic T cells specific for $OVA_{323–339}$ peptide were adoptively transferred into WT and $TLR2^{null}$ mice. The mice were then immunized with OVA mixed with either HMW HA or LMW HA. Lymphocytes from draining lymph nodes were harvested on day 3, rechallenged in vitro with OVA peptide + syngeneic APCs and IL-2, and IFN-γ were measured by ELISA. Vaccination with LMW HA as an adjuvant resulted in activated OT-II T cells which produced significantly more IL-2 and IFN-γ upon rechallenge when compared with the mice that were vaccinated with OVA + HMW HA (Fig. 8). Furthermore, the ability of LMW HA to act as an adjuvant is lost in the $TLR2^{null}$ mice, indicating

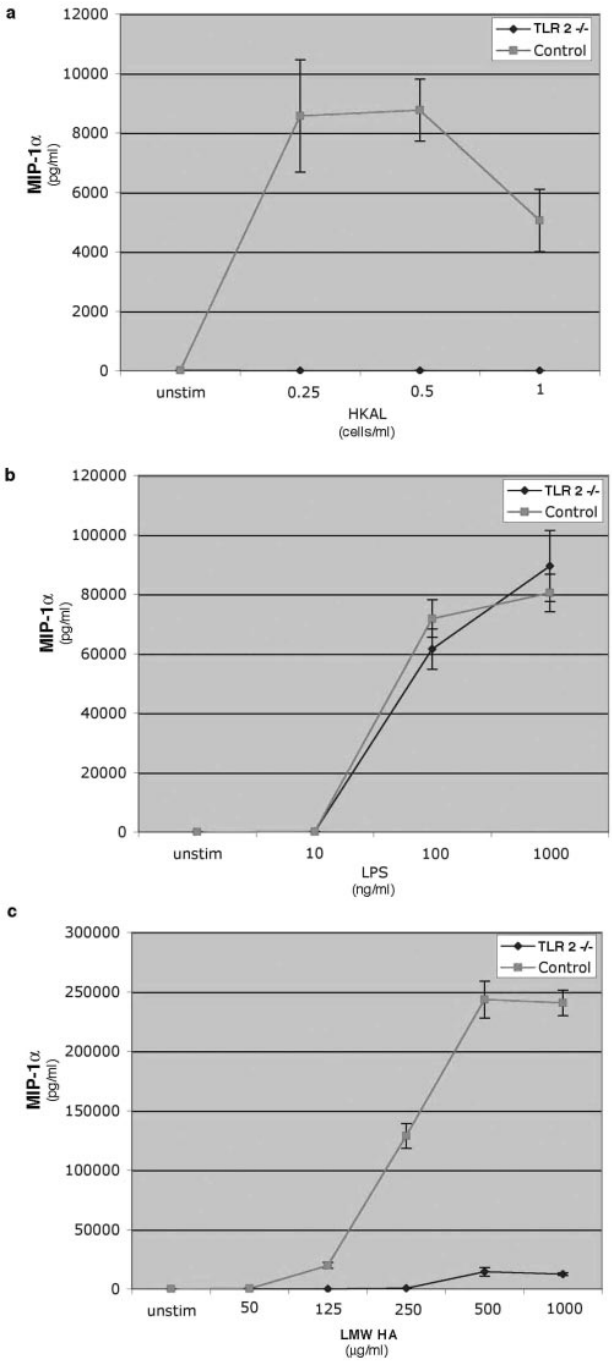

FIGURE 6. LMW HA-induced MIP-1α expression requires the TLR2 receptor. ELISA of MIP-1α protein expression from PEC from control or TLR2^{null} mice stimulated with varying doses of HKAL (*a*), LPS (*b*), or LMW HA (*c*) for 24 h. Data are the average of four identical experiments.

that this effect is TLR2 dependent. The facts that LMW HA but not HMW HA led to an enhanced T cell response to Ag and that this effect was absent in the TLR2^{null} mice strongly support the concept that LMW HA acts as a danger signal in vivo.

Discussion

In the setting of tissue destruction, HMW HA is broken down into LMW species which have the ability to promote inflammation by inducing the release of reactive oxygen species, cytokines, chemokines, and destructive enzymes and facilitating the recruitment of CD44$^+$ leukocytes (10, 11, 13, 14). Where as the HMW HA maintains homeostasis and potentially down-regulates inflammation, the generation of LMW HA may act as an endogenous danger signal, leading to the activation of both innate and acquired immunity. The fact that lack of clearance of LMW HA leads to excess damage whereas overexpression of HMW HA is protective in the noninfectious bleomycin-induced lung injury model supports this hypothesis (15, 22). The ability of LMW HA to act as an adjuvant and promote T cell activation in vivo further supports this view (Fig. 8). Whereas PAMPS alert the immune system to exogenous pathogens and molecules like uric acid and HMGB1 signal necrotic cell death, LMW HA heralds a breach of barriers and the destruction of tissue integrity (5, 6). In this way, LMW HA-induced TLR signaling might activate the immune response before the development of an established infection or necrotic cell death. If tissue destruction is due to or in conjunction with an infection then, the ability of LMW HA to activate APCs will enhance the generation of acquired immunity to pathogen-derived Ags.

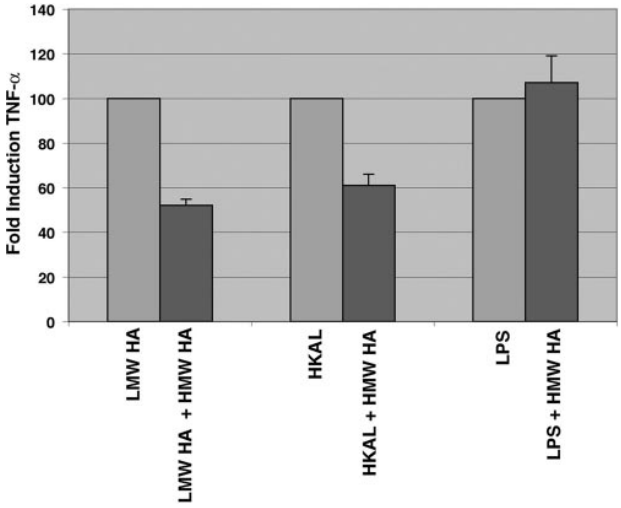

FIGURE 7. HMW HA specifically inhibits TLR2 signaling. ELISA of TNF-α protein expression in cultured cell supernatants from MH-S cells preincubated with HMW HA (250 μg/ml) for 1 h before stimulation with LMW HA (25 μg/ml), HKAL (5×10^8 cells/ml), or LPS (1 ng/ml) for 24 h. Data are the averages of five separate experiments.

Concomitant with the ability of LMW HA to enhance inflammation, it also directly activates DCs (16, 40). Inasmuch as DCs play a critical role in the initiation of the acquired immune response, LMW HA thus has the ability to influence the decision between T cell tolerance and activation. Indeed, LMW HA generated in the tissue can enter the lymphatics by binding to the lymphatic vessel endothelial HA receptor expressed on lymphatic endothelium, enter lymph nodes, and warn the immune system of a noxious insult (41). Furthermore, HA-stimulated DCs up-regulate costimulatory molecules and are potent activators of T cells (16, 40).

The TLRs have emerged as a critical link between activation of the innate immune response and the induction of acquired immunity (36, 37). Consistent with this paradigm, we have demonstrated that LMW HA signals via TLR-2. Previously, we and others have shown that LMW HA leads to NF-κB activation (20, 29, 30). In this report, we demonstrate that the activation occurs via TLR2 engagement in a MyD88-, IRAK-, TRAF6-, PKC-ζ-dependent manner. A role for PKC-ζ in LMW HA signaling is consistent with the findings of Fitzgerald et al. (42). However, in their model,

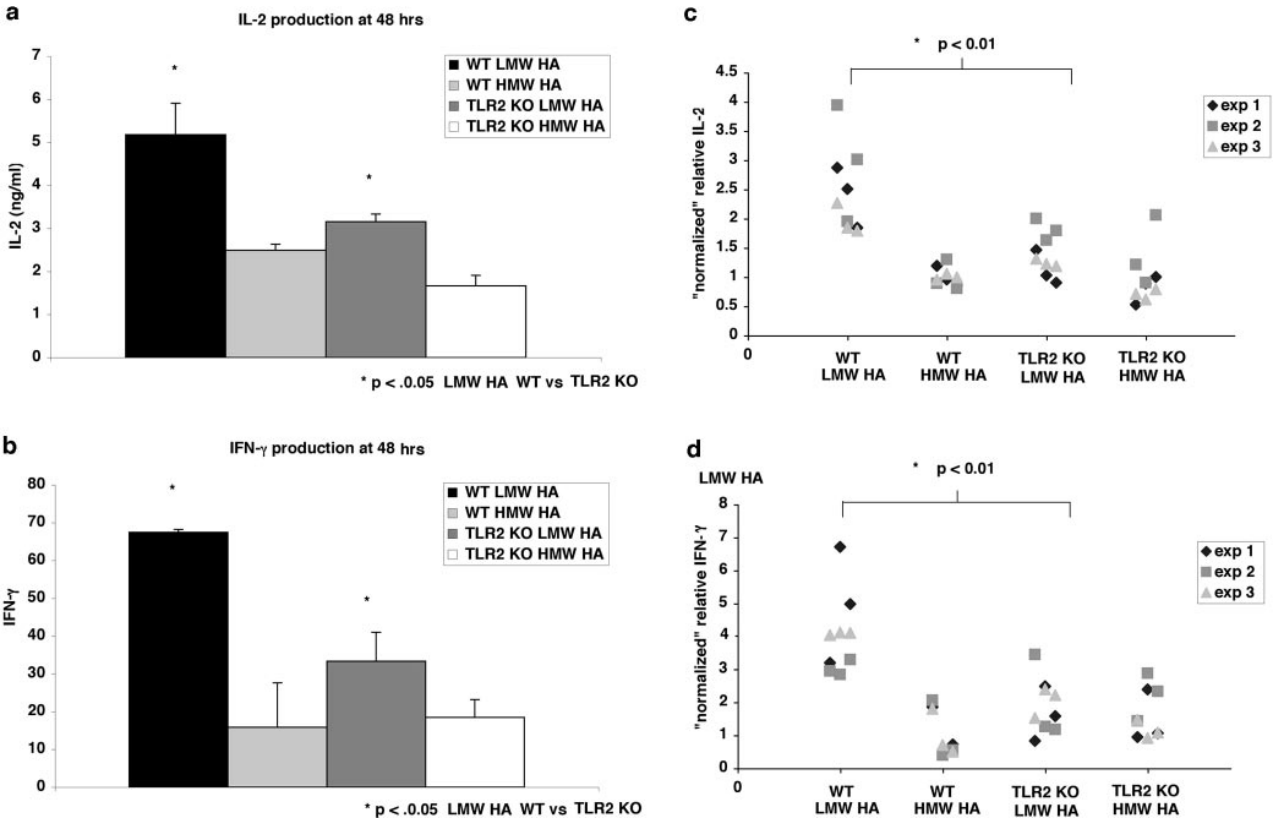

FIGURE 8. LMW HA act as an endogenous danger signal in vivo via TLR2 ligation. OT-II TCR-transgenic OVA-specific T cells were adoptively transferred into WT (C57BL/6) or TLR2^{null} mice that were subsequently treated with 150 μg of OVA peptide $\pm$ 150 μg of LMW HA or HMW HA. Lymphocytes were isolated on day 3 and rechallenged with OVA peptide. ELISA for IL-2 (*a*) or IFN-γ (*b*) for a representative experiment using three mice per condition. Composite data from three experiments where each point represents one mouse for IL-2 (*c*) or IFN-γ (*d*) induction normalized to fold induction over HMW HA WT response. KO, Knockout; unstim, unstimulated.

which used carcinoma cell lines, LMW HA signaling could be blocked by anti-CD44 Abs (42).

Lipoproteins and peptidoglycans are the best characterized ligands for TLR2 which is believed to play an important role in host defense against Gram-positive organisms, spirochetes, mycobacteria, protozoa, and fungi (43). Interestingly, for many ligands, TLR-2 signaling requires the formation of a heterodimer with TLR1 or TLR6 (43). In our studies, LMW HA induced NF-κB activation in cells that expressed TLR2 alone. Inasmuch as the HEK cells have very low levels of endogenous TLR-1 and TLR-6 expression, these data suggest that LMW HA-induced TLR2 signaling may recruit either TLR1 or TLR6 to the surface to act as a coreceptor for TLR2. Alternatively, it is possible that polymers of repeating disaccharide units that make up HA facilitate homodimer cross-linking of the TLR2 receptor.

The ability of noninfectious agents to stimulate via TLRs is not without precedent. Fibronectin, surfactant protein A, soluble heparan sulfate, β-defensin-2, and high mobility group box protein have been shown to signal via TLR4, where as heat shock proteins have been shown to engage TLR2 (44–49). Recently, however, the ability of endogenous ligands to stimulate via TLRs has come into question due to possible contamination with LPS and lipoproteins (50, 51). We do not believe our LMW HA fragment induction of inflammatory genes via TLR-2 is due to contaminants. The LMW HA fragments still retain their ability to signal despite purification with proteinase K, DNase and heat inactivation effectively removing protein, DNA and heat labile LPS contaminants (data not shown). Although we know that our LMW HA fragment preparation, derived from human umbilical cords, is contaminated with trace LPS (<10 ng/ml), the use of cells derived from C3H/HeJ and TLR4^{null} mice, in addition to the use of the LPS inhibitor polymixin B, strongly argues against this contaminant as mediating LMW HA-induced activation.

The precise mechanism by which LMW HA induces inflammatory gene expression has been somewhat controversial. For example, one group has proposed that oligosaccharides (6–16 disaccharides) derived from digestion of HMW HA signal via TLR4 (32, 33). This proposal is based on the observations that DCs and endothelial cells from C3H/HeJ mice were hyporesponsive to stimulation with their HA oligosaccharides (32, 33). The exact relationship between these disparate findings and ours that employ LMW HA fragments (M_r 0.2 $\times$ 10^6) is unclear. We have always performed our experiments using cells derived from LPS-hyporesponsive C3H/HeJ mice, and in this study we clearly demonstrate the ability of cells derived from TLR4^{null} mice to respond to LMW HA equal to that of mice with intact TLR4s (12).

More recently, another group has proposed that HA acts via both TLR2 and TLR4 (15). They base this claim on the fact that their LMW HA still induces gene expression in either TLR2 or TLR4^{null} cells but not TLR2/4$^{null/null}$ cells. In contrast, our data clearly demonstrate (Fig. 1, *a* and *b*) robust chemokine gene expression from C_3H/HeJ LPS-hyporesponsive mice by only LMW HA (not HMW HA or LPS). Similarly, Fig. 1*d* demonstrates that LMW HA equally induces MIP-1α in TLR4 knockout and WT macrophages. Interestingly, Feiber with Termeer have also published data consistent with our findings that demonstrate that in

murine embryonic fibroblasts oligosaccharide HA signals in a non-TLR4-dependent pathway (31). It is possible that different sizes of HA might signal differentially between TLR2 and TLR4. Alternatively, it is possible that Jiang et al. observed signaling in the TLR2^{null} cells from LPS contamination and that this effect was eliminated by knocking out TLR4.

Although HMW HA is constantly being turned over in the day to day maintenance of normal matrix, the HMW HA is rapidly degraded into very small, non-biologically active fragments which are quickly cleared by the liver (7, 8). However, elevated serum and tissue levels of LMW HA are found in situ in both acute and chronic inflammation, and levels of LMW HA are elevated in a number of autoimmune diseases (52–54). Normally, in the course of an immune response, inflammation is self-limiting, and the biologically active LMW HA fragments are removed as healing occurs. However, in states of ongoing inflammation and fibrosis, such as sarcoidosis, chronic bronchitis, and idiopathic pulmonary fibrosis, there is ongoing tissue destruction and remodeling, leading to the persistence of HA degradation products (52–54). Along these lines, CD44^{null} mice display an excessive inflammatory response to bleomycin-induced lung injury (22). Inasmuch as CD44 is a receptor for HA, this increase in disease susceptibility has been attributed to the inability of CD44^{null} macrophages to clear LMW HA (22).

Our data support the idea that a balance between HMW and LMW HA may control the activation of the innate immune response in situations of tissue damage and danger. Along these lines, the ability of HMW HA to inhibit signaling from non-HA TLR2 agonists raises the possibility of HMW HA as an endogenous inhibitor of inflammation, specifically by inhibiting TLR2 signaling. Interestingly, HMW HA has been used clinically to decrease inflammation both in inflammatory joint and lung diseases where one finds an accumulation of LMW HA fragments (55, 56). Our data suggest that the mechanism behind this protective effect may be by blocking TLR2 activation. On the other hand, LMW HA may in itself prove to be a useful vaccine adjuvant by promoting TLR2-induced activation of DCs.

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Disclosures

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