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Oligosaccharides of Hyaluronan Are Potent Activators of Dendritic Cells

Christian C. Termeer, Jörn Hennies, Ursula Voith, Thomas Ahrens, Johannes M. Weiss, Peter Prehm, and Jan C. Simon

The extracellular matrix component hyaluronan (HA) exists physiologically as a high m.w. polymer but is cleaved at sites of inflammation, where it will be contacted by dendritic cells (DC). To determine the effects of HA on DC, HA fragments of different size were established. Only small HA fragments of tetra- and hexasaccharide size (sHA), but not of intermediate size (m.w. 80,000–200,000) or high m.w. HA (m.w. 1,000,000–600,000) induced immunophenotypic maturation of human monocyte-derived DC (up-regulation of HLA-DR, B7-1/2, CD83, down-regulation of CD115). Likewise, only sHA increased DC production of the cytokines IL-1β, TNF-α, and IL-12 as well as their allostimulatory capacity. These effects were highly specific for sHA, because they were not induced by other glycosaminoglycans such as chondroitin sulfate or heparan sulfate or their fragmentation products. Interestingly, sHA-induced DC maturation does not involve the HA receptors CD44 or the receptor for hyaluronan-mediated motility, because DC from CD44-deficient mice and wild-type mice both responded similarly to sHA stimulation, whereas the receptor for hyaluronan-mediated motility is not detectable in DC. However, TNF-α is an essential mediator of sHA-induced DC maturation as shown by blocking studies with a soluble TNFR1. These findings suggest that during inflammation, interaction of DC with small HA fragments induces DC maturation.

Hyaluronan (HA) is a ubiquitous extracellular matrix (ECM) component, present at high concentrations in the skin, where it is synthesized primarily by dermal fibroblasts and by epidermal keratinocytes (1–3). In normal skin, HA exists as a high m.w. polymer (600,000–1,000,000) (HMW-HA) nonsulfated glycosaminoglycan (GAG) composed of repeating units of β-glucuronic acid- N-acetyl-β-glucosamine. Functional properties of HMW-HA are the maintenance and hydration of the cutaneous ECM, as well as the binding of various growth factors and smaller GAGs with specificity for cellular receptors, termed hyaladherins (reviewed in Refs. 4 and 5).

The physiological degradation of HMW-HA within the skin includes the uptake into keratinocytes, which is related to the high affinity HA receptor CD44 (6, 7) and intracellular fragmentation to intermediate sized fragments (INT-HA 300,000–60,000). Fragmented HA is released by keratinocytes, passes the basement membrane, and is liberated without significant further catabolism by dermal cells into lymphatic vessels (8). These fragments are degraded within skin-draining lymph nodes (9). Alternatively, uptake and catabolism of HA from the blood stream by liver endothelial cells have been described (10). Finally, during inflammation, platelet-derived chemotactic factors like fibrin stimulate the influx and activation of fibroblasts (11, 12). These cells directly degrade the surrounding ECM components by the secretion of hyaluronidase resulting in increased tissue concentrations of small HA fragments (sHA) (11, 12). Furthermore, cleavage of HA can be induced by reactive oxygen species released for example by granulocytes or in UV-irradiated skin, demonstrating that different proinflammatory stimuli can trigger unspecific degradation of HA (13, 14).

During cutaneous immune responses, APCs like DC infiltrate skin, where they encounter Ag. This involves both the influx of DC progenitors from the blood stream and the migration of Ag-laden DC residing within the epidermis, the Langerhans cell (LC), into the dermis (reviewed in Refs. 15 and 16). Subsequently, DC emigrate from the dermis into lymphatic vessels and enter the regional lymph nodes to elicit a specific T cell response by presentation of Ag in the context of MHC I or MHC II molecules (15). During this process DC become activated, which is associated with a distinct change in phenotype and function, termed DC-maturation (reviewed in Refs. 15 and 16). These maturational events can be reproduced during in vitro culture of DC from progenitor cells in GM-CSF and IL-4 containing medium by the addition of “maturation stimuli,” such as LPS, TNF-α, monocyte-conditioned medium (MCM) or CD40 ligation (17, 18). Mature human DC have a nonadherent dendritic phenotype, express selected surface markers like CD1a and CD83 (19), but lack the CSF-1 receptor CD115 (17, 18). These phenotypic changes, which also include high expression of MHC class I and class II as well as costimulatory molecules B7-1, B7-2, and CD40, result in an improved capacity to stimulate resting T cells. Additionally, DC maturation coincides with a loss of Ag uptake mechanisms (15, 16).

To date it remains unclear which stimuli are responsible for DC maturation during inflammatory processes in vivo. Previous studies suggested that epidermal keratinocytes on activation are a paracrine source of GM-CSF and TNF-α, factors needed for DC...
survival (20). However, these two cytokines alone are not sufficient to induce terminal DC maturation (18). We reasoned that during their migration into sites of cutaneous immune responses, DC come into close contact with HMW-HA, INT-HA, and sHA fragments, the latter being produced exclusively during inflammatory processes (4, 8). Thus, we became interested in the effects of HA fragments of different size on DC. Here, we report that sHA fragments but not INT-HA or HMW-HA potently and specifically induce maturation of human and murine DC.

Materials and Methods

Media and reagents

RPMI 1640 cell culture medium was supplemented with 1% penicillin-streptomycin, 1% l-glutamine (all Seromed, Berlin, Germany) and 1% human serum albumin (Bayer, Munich, Germany). All reagents, media, and buffers used in our assays were checked by Limulus amoebocyte lysate assays and contained <0.06 endotoxin U/ml of endotoxin in accordance with the European Community standard value for water for injection. Polymyxin B, a cationic antibiotic that displaces Ca2+ from anionic phospholipids, was obtained from Calbiochem, Bad Soden, Germany. CS C from shark cartilage for clinical application was kindly provided by Sankyo Pharma (Munich, Germany) and was subjected to enzymatic digestion with 10 U of Chondroitinase ABC from Proteus vulgaris (Sigma, Deisenhofen, Germany) overnight. HS-Na Braun 10,000 IU (Braun, Melsungen, Germany) for clinical application, was digested with 2 U heparanase III from Flavobacterium heparinum (Sigma) at 37°C. Glyoxal and glyceraldehyde were purchased from Sigma. The polyclonal rabbit anti-mouse receptor for Flavobacterium heparinum

Preparation of human DCs

Buffy coats from healthy blood donors were obtained from the Department of Transfusion Medicine, Freiburg University Medical Center (Freiburg, Germany). White blood cells were separated on a endotoxin-free Ficoll-Paque® gradient (Pharmacia, Freiburg, Germany) and incubated with a nonactivating, nonblocking anti-human CD14 mAb (clone 26C1, American Type Culture Collection (ATCC), Manassas, VA) for 45 min on ice. The cells were washed several times in PBS before incubation with the appropriate secondary anti-mouse MACS-Beads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14+ cells were purified by magnetic cell sorting using a V8 column MACS system (Miltenyi Biotec). Purity of separated monocytes was >90% as determined by flow cytometry. Monocytes (5 × 10^6/well) were seeded into six-well flat-bottom plates (Costar, Bodenheim, Germany). CD14+ cells were purified by magnetic cell sorting using a V8 column MACS system (Miltenyi Biotec). Purity of separated monocytes was >90% as determined by flow cytometry. Monocytes (5 × 10^6/well) were seeded into six-well flat-bottom plates (Costar, Bodenheim, Germany) and cultured for 4 days in RPMI 1640 (Life Technologies, Eggenstein, Germany) supplemented with 5% foetal calf serum, 100 U/ml penicillin, 100 ng/ml streptomycin, 1% l-glutamine (all Seromed, Berlin, Germany) and 1% human serum albumin (Bayer Diagnostics, Munich, Germany), GCP/GMP quality 1000 U/ml GM-CSF (Leucomax, Novartis, Nürnberg, Germany) and 100 U/ml IL-4 (kindly provided by Schering-Plough, Madison, NJ) at 37°C, 5% CO2. The CD14+ mAb was removed completely from the cell surface of monocytes at day 3 of culture, as determined by flow cytometry.

Preparation of the HA fragments

Hyaluronic acid (HEALON) for clinical application (endotoxin content, <0.1 ng/ml) was kindly provided by Pharmacia (Erlangen, Germany). Two types of HA fragments from HEALON were generated: 1) INT-HA was prepared for 2 min on ice using a Branson sonifier with the output set at the microtip limit, as described previously (18, 24). Samples were then separated by 0.5% agarose gel electrophoresis and visualized with the cationic dye Stains All (3,3′-dimethyl-9-methyl-4,5,4,5′-dibenzothiacarbocyanine, Bio-Rad, Munich, Germany), as described (24, 25). 2) sHA were generated by enzymatic digestion of INT-HA with bovine testis hyaluronidase (Sigma) for 12 h in 1 M sodium acetate buffer, pH 5.0, 37°C. The fragments were separated on a Bio-Gel P-10 (Bio-Rad) 3.5 × 115-cm column overnight. Samples were collected from the column with a Pharmacia LKB Frac. 100 fraction collector for 12 h at 20 min each. The HA concentration of each sample was analyzed by determination of uronic acid, as described (26). A 100-μl sample was added to 600 μl 0.0025 M bisodiam tetraborate in concentrated H2SO4 and stirred for 10 min at 90°C. The product was cooled to 4°C, 20 μl 0.1% carbazole in ethanol were added, and the sample was stirred again for 10 min at 90°C. After development of staining, the concentration of uronic acid was measured photometrically at 520 nm against distilled water. The detection of HA fragment size in each sample was determined by 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) labeling technique (27). In brief, 100 μl of each sample were dried in a Speed Vac vacuum drier (Life Sciences International, Frankfurt, Germany), and 5 μl 0.15 M ANTS and 5 μl 1 M NaCNBH4 dissolved in DMSO were added (all from Sigma). After 16 h at 37°C, the samples were dried, resuspended in 50 μl 25% glycine solution, and analyzed by 30% acrylamide gel electrophoresis.

LPS stimulation

Because LPS is known to induce activation and maturation of DCs (21), we stimulated human DC with 10 μg/ml LPS (Sigma) from Escherichia coli

Immunostaining and flow cytometry

DC were incubated with primary mAb for 30 min at 4°C, washed, and stained with the appropriate FITC-labeled secondary mAb. To determine cell viability and to exclude dead cells, 1 × 10^6 cells/ml propidium iodide (Sigma) was added. With the use of a FACSscan, 5 × 10^6 cells were collected and analyzed with Cell Quest research software (both Becton Dickinson).

T cell isolation/T cell proliferation assay

Resting T cells were obtained from the CD14-depleted cell fraction of PBMCs. The remaining APCs were removed by positive selection with mAbs against HLA-DR (clone HB 145) and CD11b (OKT-6) (both ATCC) for 45 min at 4°C and washed in PBS, followed by incubation with a T cell isolation/T cell proliferation assay

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** Characterization of HA preparations. HMW-HA was sonicated as described in Materials and Methods. A. 0.5% agarose gel electrophoresis revealed that the HMW-HA preparation has a m.w. of 1,000,000–200,000, whereas INT-HA fragments were m.w. 200,000–80,000. B. Fractions containing sHA oligosaccharides produced by enzymatic digestion and gel column separation were separated by 30% PAGE. Fractions collected at earlier time points contain larger oligosaccharides, e.g., fraction 12 fragments of 4- to 14-oligosaccharide size, whereas samples collected late contain only small fragments, e.g., fraction 22 fragments of 4- to 6-oligosaccharide size.
secondary goat anti-mouse Dynal bead-labeled mAb under the same conditions (Dynal, Hamburg, Germany). Flow cytometry showed purity of CD3ε cells >85%, HLA-DR cells were <1%, and the cells did not respond to PHA stimulation (10 ng/ml). The unlabeled CD3ε cells were collected after magnetic sorting, washed, and plated at 1 × 10⁶/well with 5 × 10⁴/well mature allogeneic DCs in round-bottom 96-well plates (Costar, Cambridge, MA) in cRPMI 1640 and incubated at 37°C, 5% CO₂. On day 4, 1 μCi/well [³H]thymidine (Amersham, Freiburg, Germany) was added for the final 18 h of culture. Finally, the plates were harvested onto 96-well glass fiber filter plates, and the radioactivity was determined by liquid scintillation spectroscopy using a TopCount beta counter (all Canberra Packard, Dreieich, Germany).

ELISA assays

Supernatants from unstimulated, LPS-stimulated, or HA-stimulated DCs were collected at the indicated time points to determine the content of human IL-1β, IL-12, TNF-α, and IFN-γ. The ELISA assays were developed according to the manufacturer’s instructions (R&D Systems, Wiesbaden, Germany) and measured at an extinction of 630 nm in a MR 5000 ELISA reader and analyzed using the Bio-Linx Software (both Dynatech, Chantilly, VA).

TNF-α-blocking experiments

For TNF-α-blocking studies, day 4 DC were seeded into cRPMI 1640 containing 500 pg/ml soluble TNF-α receptor 1 (sTNF-α R1) (R&D Systems) after subsequent stimulation with LPS or HA fragments.

RT-PCR

Total RNA was isolated from dendritic cells and the RHAMM-positive breast cancer cell line T47D (21) using the Quick-Prep kit (Pharmacia, Erlangen, Germany) according to the manufacturer’s instructions. cDNA was synthesized from 5 μg total RNA using Superscript II reverse transcriptase (Life Technologies, Eggenstein, Germany). The product was subjected to 30 cycles at 94°C for 1 min, 72°C for 2 min, and 55°C for 3 min using Taq DNA polymerase (Life Technologies). PCR products (25-μl aliquots) were analyzed by 1% agarose gel electrophoresis. The primer were generated following the sequence published by Assmann et al. (21): lower primer, position 951: 5’-CAG GAA TAG AGA ACA CAA CG-3’; upper primer, position1719: 5’-TCT TCC TTC TTC ATC TTC CAG C-3’.

Results

Generation of HA fragments

HA preparations of three different sizes were used: HWM-HA (endotoxin-free HEALON); sonified HEALON yielding INT-HA; sHA generated by hyaluronidase digestion of INT-HA. The 0.5% agarose gel electrophoresis of HMW-HA or INT-HA demonstrated a m.w. of 1,000,000–200,000 or 200,000–80,000, respectively (Fig. 1A). The sHA preparation was further separated on a Bio-Gel P-10 (Bio-Rad) gel column, and fractions eluted at 20 min intervals were collected. A 30% acrylamide gel electrophoresis revealed that fractions collected at earlier time points contained larger oligosaccharides, e.g., fraction 12 fragments of 4- to 14-oligosaccharide size (Fig. 1B), whereas samples collected late contained only small fragments, e.g., fraction 22 fragments of 4- to 6-oligosaccharide size (Fig. 1B). For experimental use, three fractions covering a range of different sizes from 4- to 14-oligosaccharide length were adjusted to an HA content of 1 mg/ml. Late fractions eluted from
for their TNF-α, IL-1β, and IL-12. ELISA were performed with cell-free supernatants for their content of IL-1β, TNF-α, IL-12, and IFN-γ. Results are shown in picograms/ml ± SD of triplicate wells. *p > 0.001 compared with untreated DC (–).

the column, which contained no detectable amounts of HA, served as negative control.

s-HA, but not INT-HA or HMW-HA stimulate immunophenotypic DC maturation and cytokine secretion

The effects of the different HA preparations on the immunophenotype of human blood-derived DC was determined by flow cytometry. Day 4 immature DCs displayed a high surface expression of CD1a, CD44, and ICAM-1 and intermediate levels of B7-1, B7-2, HLA-DR, and CD115, whereas CD83 expression was low (Fig. 2). Stimulation for 48 h with three different sHA fractions led to dose-dependent phenotypic changes in DC including a marked up-regulation of HLA-DR, B7-1, B7-2, ICAM-1, and CD83 as well as a down-regulation of CD1a and CD115 (Fig. 2). sHA concentrations as low as 10 μg/ml matched exactly the phenotypic maturation induced in the same DC by a 48-h treatment with 10 μg/ml LPS (Fig. 2). Interestingly, sHA fractions containing 4–16, 4–10, or 4–6 oligosaccharides all had similar effects (Fig. 2 and data not shown). Dose titration experiments showed 10 μg/ml of each sHA fraction to induce complete phenotypic DC maturation (data not shown). This suggests that sHA fragments of 4–6 oligosaccharides, which were present in all fractions (Fig. 1B), are predominantly responsible for the DC maturation. However, early fractions contain relatively less 4–6 oligosaccharides. We can therefore not exclude that sHA fragments of 8–16 oligosaccharides might also be effective.

By contrast, HMW-HA or INT-HA obtained from the same stock of endotoxin-free HEALON at concentrations as high as 100 μg/ml had no effect on DC. Additionally, DC were not affected by the column control, which did not contain detectable amounts of HA, excluding the possibility that components of the Bio-Gel P-10 gel induced DC maturation (Fig. 2). The maturation stimulated by sHA fragments was long-lasting and irreversible, because sHA-treated DC could be cultivated until day 14 without further addition of IL-4 and GM-CSF and kept their mature phenotype (data not shown).

Because phenotypic DC maturation is accompanied by the enhanced production of proinflammatory cytokines (15, 17), ELISA were performed to determine the cytokine content in supernatants of sHA-stimulated DCs. The same three sHA-fractions, but not HMW-HA, INT-HA, or the column control, up-regulated the IL-1β and TNF-α production in a dose-dependent manner similar to the effect induced by LPS (Fig. 3 and Table I). We found that 10 μg/ml sHA effectively induced a significant TNF-α release, which was saturated at concentrations of >50 μg/ml (Table I). By contrast, sHA treatment resulted only in a modest IL-12 secretion compared with LPS (Fig. 3). IFN-γ or IL-4 production by DC was not affected by either sHA or LPS (data not shown).

sHA enhances the capacity of DC to stimulate T cell responses

The maturation of DC is also accompanied by their enhanced ability to stimulate T cell mediated immune responses in vitro and in vivo (15, 16). To test the effects of sHA on these functional properties, we first assessed the capacity of human DC to stimulate the proliferation of resting allogeneic T-cells in a standard MLR. sHA enhanced the allostimulatory potential of DC (Fig. 4). Again, this stimulatory activity was conferred by sHA fragments of 4–16 oligosaccharides (Fig. 4).

sHA effects on DC are LPS independent

Because LPS at concentrations as low as 50 pg/ml has been described to activate cells of the myelomonocytic lineage (29), we wished to exclude that the sHA preparations used in this study contained trace amounts of LPS. All materials used during sHA generation as well as during DC cultures were endotoxin free as confirmed by Limulus amebocyte lysate assay (data not shown). Furthermore, addition of 10 μg/ml of the LPS-inhibitor polymyxin B had no effect on the sHA induced up-regulation of MHC class II (Fig. 5A) as well as B7-1, B7-2, or CD1a (data not shown), but in

Table I. sHA-induced DC maturation is specific for HA oligosaccharides

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>5 μg/ml</th>
<th>10 μg/ml</th>
<th>20 μg/ml</th>
<th>50 μg/ml</th>
<th>100 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMW-HA</td>
<td>5.3 ± 0.5</td>
<td>3.6 ± 0.9</td>
<td>ND</td>
<td>6.9 ± 1.5</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>sHA</td>
<td>5.7 ± 1.6</td>
<td>563.6 ± 45.8</td>
<td>1875.3 ± 201.5</td>
<td>2174.8 ± 185.2</td>
<td>2076.2 ± 104.8</td>
</tr>
<tr>
<td>Untreated</td>
<td>1.7 ± 0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS (10 μg/ml)</td>
<td>2234 ± 145</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS</td>
<td>ND</td>
<td>3.4 ± 0.6</td>
<td></td>
<td>cyt</td>
<td>cyt</td>
</tr>
<tr>
<td>sHS</td>
<td>ND</td>
<td>2.6 ± 1.5</td>
<td></td>
<td>cyt</td>
<td>cyt</td>
</tr>
<tr>
<td>CS-C</td>
<td>4.7 ± 1.6</td>
<td>6.3 ± 2.6</td>
<td>ND</td>
<td>1.8 ± 0.6</td>
<td>3.8 ± 2.6</td>
</tr>
<tr>
<td>sCS-C</td>
<td>8.4 ± 3.2</td>
<td>2.4 ± 0.4</td>
<td>3.1 ± 1.7</td>
<td>4.8 ± 1.1</td>
<td>8.3 ± 3.7</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>6.6 ± 0.7</td>
<td>6.2 ± 1.0</td>
<td>4.4 ± 1.0</td>
<td>2.6 ± 0.5</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>5.5 ± 0.8</td>
<td>5.7 ± 1.3</td>
<td>4.9 ± 0.6</td>
<td>cyt</td>
<td>cyt</td>
</tr>
</tbody>
</table>

*Human DC were incubated for 24 h with different concentrations of the indicated substances, 10 μg/ml LPS, or left untreated, and the cell-free supernatants were assayed for their TNF-α content. All ELISA were performed with triplicate wells; a representative of three independent experiments is shown. Results are shown in picograms per milliliter TNF-α ± SD. cyt, Cytotoxic.
the same experiment inhibited all LPS effects (Fig. 5B). In aggregate, these experiments rule out the possibility that the sHA effects on DC were due to LPS contamination.

**sHA-induced DC maturation is independent of CD44 or RHAMM**

CD44 has been shown to be the major cellular HA receptor (30, 31). This raised the question whether CD44, which is expressed at high levels on DC (Fig. 2), mediates sHA-induced DC maturation. Anti-CD44 mAbs, either complete or as Fabs, shown to block HA binding to human DC (CD44) (30), were revealed to be an inappropriate tool to address this issue, because they induced DC-clustering and partial activation (data not shown and Ref. 30). As an alternative approach, we studied DC from CD44-deficient mice (23). First, we confirmed that sHA also induced maturation of murine DC prepared from the bone marrow of wild-type C57BL/6 mice. This included up-regulated expression of Iab, B7-1, and B7-2 as well as the down-regulation of CD115 (Fig. 8 and data not shown). Because we found TNF-α to be a main mediator of sHA-induced DC maturation, we used the TNF-α release by DC to further examine the specificity of the sHA effect.

To determine whether other GAGs present in the ECM could also influence DC maturation, DC were treated with purified chondroitin sulfate C (CS-C) or heparan sulfate (HS) used either as a complete molecule or after enzymatic digestion to oligosaccharide size (sCS-C, sHS). Although CS and HS show high structural homology with HA, being built of repeating disaccharide units (32), they did not influence the TNF-α secreted by these DC, CD44 expression is not required. However, we cannot exclude the possibility, that CD44 might be involved in the sHA-mediated maturation of CD44 wild-type mice. We could further exclude that sHA exerted its effects via the second HA receptor RHAMM, because we did not detect RHAMM mRNA expression in mature or immature DC by RT-PCR (Fig. 7A) or RHAMM expression on the surface or cytoplasm of DC as determined by FACS (Fig. 7B).

**GAG-mediated DC maturation is TNF-α dependent and highly specific for small HA fragments**

Because recombinant TNF-α was shown to have an essential role for final DC maturation (17, 18), we wished to determine whether the TNF-α released by sHA-stimulated DC was involved in the maturation changes shown in Figs. 2 and 3. Blocking experiments with sTNF-R1, which was shown to effectively neutralize TNF-α, inhibited significantly sHA-mediated DC maturation, affecting all parameters induced including up-regulation of HLA-DR, B7-1, B7-2, ICAM-1, and CD83 as well as the down-regulation of CD115 (Fig. 8 and data not shown). Because we found TNF-α to be a main mediator of sHA-induced DC maturation, we used the TNF-α release by DC to further examine the specificity of the sHA effect.

**Discussion**

Here, we describe for the first time that small degradation products of the ECM component HA stimulate terminal and irreversible maturation of human and murine DC. Specifically, we demonstrate that highly purified, LPS-free HA fragments of defined size (4- to 16-oligosaccharide length) induced an immunophenotypic maturation of DC including up-regulation of surface markers CD83, B7-1, B7-2, MHC class II, ICAM-1, and CD44 and a loss of

**FIGURE 5.** sHA-induced DC maturation is not due to LPS contamination. Day 4 DC were incubated for 48 h with 20 μg/ml sHA (A) or 10 μg/ml LPS (B), stained for HLA-DR expression, and analyzed by flow cytometry. *Thin solid line*, Isotype-matched control mAb; *dashed line*, HLA-DR expression on untreated DC; *dotted line*, HLA-DR expression on sHA (A)- or LPS (B)-stimulated DC. *Bold line*, HLA-DR expression on DC pretreated with polymyxin B (10 mg/ml) 1 h before each stimulation. FL, Fluorescence. A representative of four independent experiments is shown.
CD115. Further, sHA-treated DC showed a nonadherent phenotype and an augmented capacity to stimulate alloreactive T-cells in a standard MLR and to secrete the cytokines IL-12, IL-1β, and TNF-α. By contrast, HMW-HA or larger HA fragments (INT-HA), which are abundantly expressed in normal unperturbed tissue, did not affect DC. We found this effect to be specific for small fragments of HA, because the ECM GAGs CS and HS, which are structurally highly related to HA, or CS and HS fragmented to 2–12 oligosaccharide size did not influence DC maturation. Additionally, this effect was independent of highly reactive aldehyde groups, present at the free N-acetylgalactosamine end of each HA molecule (5), because addition of the small aldehydes glyoxal or glycuronaldehyde failed to induce DC maturation.

It has been shown that complete DC maturation can be induced by coincubation of immature DC with MCM or LPS (17, 28). This DC maturation is dependent on high levels of TNF-α secreted by MCM- or LPS-activated DC. Here, we show that the sHA-induced DC maturation is also TNF-α dependent, because high levels of TNF-α are produced after coincubation with sHA, and neutralization of TNF-α by the addition of a sTNF-α-R1 completely prevented DC maturation. It is known, however, that the presence of TNF-α alone is not sufficient to induce DC maturation (18). This suggests that MCM, LPS, or sHA, in addition to TNF-α, stimulate other cofactors, which are essential for complete, irreversible and long-lasting DC maturation. Putative candidates for these cofactors include PGE2 or chemokines like platelet-activating factor or monocyte chemoattractant protein-1 (33, 34).

We have shown that only small HA of 4- to 16-oligosaccharide size, but not INT-HA or HMW-HA, induce DC maturation. This is in agreement with studies related to inflammation and wound healing (35–40). SHA fragments of 3- to 10-oligosaccharide size accelerated neoangiogenesis during wound repair within 48–72 h after injury (35). In agreement with our results, HMW-HA did not affect angiogenesis, even acting antiangiogenic at higher concentrations (35). Additionally, the ability of sHA, but not HMW-HA, to stimulate directly the growth and tube formation of endothelial cells was demonstrated in a number of models including chick chorioallantoic membranes, rat skin, and cryoinjured murine skin grafts (35–37). Furthermore, sHA fragments of 10- to 16-oligosaccharide size had no effect on endothelial cell proliferation (35), thus paralleling our findings that only sHA fragments of 4–16, but not of 10–16, oligosaccharides activate DC.

On the other hand, both INT-HA fragments with a peak molecular size of 200,000 as well as sHA of 6-oligosaccharide length have been described to activate murine alveolar macrophages via a NFκB/IkB-dependent pathway (38). Moreover, they were shown to up-regulate mRNA synthesis and protein secretion of the chemokines macrophage-inflammatory protein-1α, macrophage-inflammatory protein-1β, and monocyte chemoattractant protein-1 (39) and to trigger NO synthase activity (40). This is in partial contrast to our finding that INT-HA (m.w. 80,000–200,000) had no effect on DC. These discrepant results could be due to the different cell types studied, i.e., murine alveolar macrophages (38–40) vs human or murine dendritic cells (our study). On the other hand, they may be due to the method of INT-HA generation used by McKee et al. (38–40), which does not exclude the possibility...
that their preparations contain substantial amounts of sHA fragments.

Further, we wished to determine whether sHA-induced DC maturation involves the known HA receptors CD44 or RHAMM (21, 30, 31, 41). Importantly, the sHA response of DC generated from CD44-deficient mice was identical with that of DC from CD44-expressing mice, demonstrating the sHA effect to be independent of CD44. Similar conclusions were drawn when the sHA-stimulated proliferation of vascular endothelial cells was studied (35–37). Specifically, perinuclear CD44 staining of sHA-treated endothelial cells was not different from that of untreated cells (36), indicating that the sHA uptake and specific NF-kB-dependent signaling was not mediated by CD44. The notion that sHA fragments interact with cells independent of CD44 is supported by recent findings from Culty et al. (31) and Tammi et al. (42). On the basis of HMW-HA competition studies, these investigators concluded that only sHA fragments of at least 6- to 10-oligosaccharide length, but not smaller fragments, bind to CD44 on endothelial cells or keratinocytes, respectively. Further, we found no evidence that RHAMM is involved in sHA-mediated DC maturation, because RHAMM mRNA or protein could not be detected in human DCs. However, we cannot exclude the possibility that novel cell type-specific HA-receptors might exist on DC, which mediate sHA-induced DC maturation. For example, a new HA-binding receptor with specificity for lymphatic endothelium was recently described (43).

In this paper, we have shown that sHA fragments induce a complete and irreversible DC maturation which also results in their augmented capacity to stimulate primary T cell responses in vitro. However, it remains to be determined whether sHA-matured DC are also better at stimulating T cell responses to protein Ags in vivo. In such a case, sHA-matured DC could be of use in DC-based vaccination models against tumor or viral Ags (15, 17). Interestingly, the in vivo application of sHA oligosaccharides of 4- to 10-saccharide length was already shown to inhibit tumor growth (29). In a recent report, it was shown that the sHA response of DC generated from INT-HA- or HMW-HA treated with LPS (10 μg/ml), sHA (50 μg/ml), or left untreated (—) in the presence or absence of 500 pg/ml sTNF-R1. After 48 h, cells were stained for their MHC class II expression and analyzed by FACS. Solid line, isotype-matched control mAb; dashed line, HLA-DR expression on untreated DC; dotted line, HLA-DR expression on sHA (A)- or LPS (B)-stimulated DC; bold line, HLA-DR expression on DC treated with 500 pg/ml sTNF-R1 during stimulation. A representative of three independent experiments is shown.

References
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