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Cutting Edge: High Molecular Weight Hyaluronan Promotes the Suppressive Effects of CD4⁺CD25⁺ Regulatory T Cells¹

Paul L. Bollyky,² James D. Lord, Susan A. Masewicz, Stephen P. Evanko, Jane H. Buckner, Thomas N. Wight, and Gerald T. Nepom

Hyaluronan is a glycosaminoglycan present in the extracellular matrix. When hyaluronan is degraded during infection and injury, low m.w. forms are generated whose interactions influence inflammation and angiogenesis. Intact high m.w. hyaluronan, conversely, conveys anti-inflammatory signals. We demonstrate that high m.w. hyaluronan enhances human CD4⁺CD25⁺ regulatory T cell functional suppression of responder cell proliferation, whereas low m.w. hyaluronan does not. High m.w. hyaluronan also up-regulates the transcription factor FOXP3 on CD4⁺CD25⁺ regulatory T cells. These effects are only seen with activated CD4⁺CD25⁺ regulatory T cells and are associated with the expression of CD44 isomers that more highly bind high m.w. hyaluronan. At higher concentrations, high m.w. hyaluronan also has direct suppressive effects on T cells. We propose that the state of HA in the matrix environment provides contextual cues to CD4⁺CD25⁺ regulatory T cells and T cells, thereby providing a link between the innate inflammatory network and the regulation of adaptive immune responses. The Journal of Immunology, 2007, 179: 744–747.

Hyaluronan (HA)³ is a widely distributed tissue extracellular matrix component characterized by a repeating disaccharide of N-acetylgalactosamine and D-glucuronic acid. It is long and ranges in molecular mass from 10⁴ to 10⁷ Da (1), is highly charged, and can bind large amounts of water. Breakdown products are bioactive, and the quantities and ratios of differently sized HA molecules form a potential conduit for intercellular communication in tissue inflammation and repair (2).

The bioactive properties of HA vary with its size. High m.w. HA (HMW-HA) (> 2,000 saccharides and >400 kDa) serves a variety of structural and regulatory functions including lubrication of joints (3), provision of scaffolding for tissue repair in injury (4), and regulation of osmosis in inflammation (5). HMW-HA is antiangiogenic (6) and anti-inflammatory (7–9) and inhibits phagocytosis by monocytes (10). HMW-HA currently has a number of clinical applications, including treatment for osteoarthritis of the knee and the prevention of postsurgical abdominal adhesions (11). In contrast, low m.w. HA fragments (LMW-HA) (<16 saccharides and <3 kDa) generated during infection and injury through the action of hyaluronidases can promote angiogenesis and proinflammatory responses (12–15). The abundance of HA within the tissue matrix and the dynamic interplay between differently sized HA molecules offers a potential signaling system reflecting the state of tissue matrix integrity during injury, inflammation, or repair.

A number of receptors bind HA. The best characterized of these is CD44, a nearly ubiquitous cell surface receptor that exists in multiple isoforms with distinct functional characteristics depending on alternative splicing and the expression of different exons (16). The CD44 expressed on resting T cells and monocytes is functionally inactive and binds HA only after TCR triggering or after activation by proinflammatory cytokines including TNF-α and IFN-γ (17). Activation with mitogenic stimuli causes the expression of CD44 isoforms CD44-v9 and CD44-v6, which are absent on resting T cells (18). In mice, TCR stimulation up-regulates an activated form of CD44 in the most actively suppressive subset of CD4⁺CD25⁺ CD127low regulatory T cells (T₅) (19).

We have asked whether the local state of HA in the extracellular matrix impacts T₅ function. We demonstrate that both HMW-HA and LMW-HA bind to T₅ and that the size of HA differentially impacts T₅ activation and T₅-mediated suppression of CD4⁺CD25⁺ proliferative responses. HMW-HA enhancement of activated regulatory T cells provides a potential sensor mechanism linking the state of tissue matrix integrity and the adaptive immune response.

Materials and Methods

Reagents
HA with a molecular mass of 1.5 × 10⁶ kDa was obtained (Genzyme). Fluorescein-labeled HA was prepared by a standard method (20). LMW-HA was

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³ Abbreviations used in this paper: HA; hyaluronan; HMW-HA, high m.w. HA; LMW-HA, low m.w. HA; T₅, CD4⁺CD25⁺ regulatory T cell.
Isolation of regulatory T cells

PBMC were prepared by centrifugation over Ficoll-Hypaque gradients. The CD4+ T cell population was first purified using the Dynal CD4 positive isolation kit (Invitrogen Life Technologies). Cells were then stained with anti-CD25 Abs (BD Pharmingen) and in most cases for CD127 (BD Pharmingen). Cell fractions were subsequently isolated using a FACSVantage flow cytometer cell sorter, resulting in the recovery of CD4+ CD25+ CD127low populations; purity was reliably >98%.

Cell marker staining and flow cytometry analysis

For detection of HA binding, cells were washed and then incubated in vitro with FITC-labeled HA for 60 min. To stain for FoxP3, cells were fixed, permeabilized, and stained using an FoxP3 Ab kit (eBioscience). Flow cytometric analyses were performed on a FACScalibur flow cytometer with CellQuest (BD Biosciences) and WinMDI (J. Trotter, University of California San Diego, La Jolla, CA) software.

Activation of regulatory T cells

CD25+ cells were activated with 5 μg/ml plate-bound anti-CD3 (OKT3) and 2.5 μg/ml soluble anti-CD28. Cells were removed from the plate-bound Ab after 24 h. Alternatively, activation was with Xcyte Dynabeads (Invitrogen Life Technologies) at a ratio of 1 bead per 10 cells. Xcyte beads are super paramagnetic beads to which anti-CD3 and anti-CD28 have been covalently linked. Media was supplemented with 200 IU/ml IL-2 (Chiron).

Characterization of CD44 isomer populations

CD4+ cells were isolated from PBMC via Dynal positive isolation and treated with the murine unlabeled anti-human Abs 11.9 (CD44-v6), 11.24 (CD44-v9), 11.10 (CD44-v4) (18), and G44-26 (promiscuous for CD44 isomers) (BD Pharmingen). Cells were stained with the goat anti-mouse IgG (whole molecule), R-PE-labeled secondary Ab P9670 (Sigma-Aldrich), sorted for CD25 expression by flow cytometry, and subsequently analyzed for FOXP3 expression using anti-FOX3 Ab (BD Pharmingen). For the characterization of CD44 isomer populations on activated cells, TCR were isolated from the same donor using anti-CD25 mAb clone Bu75, was obtained from Ancell.

Suppression of proliferation by CD25+ cells

For the suppression assay, CD4+ CD25+ cells (2,500 cells per well), CD4+ CD25- cells (2,500 cells per well), or both (2,500 cells per well each) were activated with either 5 μg/ml soluble anti-CD3 and 2.5 μg/ml soluble anti-CD28 along with T cell-depleted accessory cells (25,000 cells per well) or 300 Xcyte beads per well and no APC. HA was added to the wells at the initiation of the experiment. Alternatively, the TCR were incubated in HA overnight and washed three times before their addition to the assay (pulsed) as noted. No IL-2 was added to these assays. Proliferation was measured by adding 1 μCi of [3H]thymidine during the final day of a 5- to 6-day assay.

**FIGURE 1.** Binding of FITC-labeled HA. A. One percent agarose gel separation of 1.5 × 10^4 kDa HA (HMW-HA) and 3 kDa HA (LMW-HA). HA of 30 kDa is shown as a size marker but was not used for any of the experiments in this work. B. HA binding to CD4+ CD25- T cells (Treg). C. Blocking of CD4+ CD25- TCR expression by flow cytometry, and subsequently analyzed for FOXP3 expression using anti-FOX3 Ab (BD Pharmingen). Cells were stained with the goat anti-mouse IgG (whole molecule), R-PE-labeled secondary Ab P9670 (Sigma-Aldrich), sorted for CD25 expression by flow cytometry, and subsequently analyzed for FOXP3 expression using anti-FOX3 Ab (BD Pharmingen). For the characterization of CD44 isomer populations on activated cells, TCR were isolated from the same donor using anti-CD25 mAb clone Bu75, was obtained from Ancell.

**FIGURE 2.** CD44 isomers on TCR. A and B. Binding of Abs against CD44v6, CD44v9, and CD44v4 preactivation (A) and 72 h postactivation (B). C. The relative binding of FITC-labeled HMW-HA at 20 μg/ml to TCR with high (i) and low (ii) expression of CD44v6 as shown by fluorescence gates (i) and (ii) in B (left panel). D. The binding of a general CD44 Ab, nonrestricted to any particular CD44 isomer, in relation to FoxP3 expression on activated TCR.

**Clinical samples**

Peripheral blood samples were obtained with informed consent from healthy volunteers participating in a research protocol approved by the institutional review board of the Benaroya Research Institute at Virginia Mason Medical Center (Seattle, WA).

Prepared by digestion of HMW-HA with Streptomyces hyaluronidase, followed by filtration through Centricon microconcentrators (Amicon) to produce fragments of <3 kDa as described previously (21). HA of 30 kDa was generated for use as a size reference only (Fig. 1). Pep1, a peptide inhibitor of HA binding (22), was obtained from Sigma-Genosys. A CD44 blocking mAb, clone Bu75, was obtained from Ancell.
Results and Discussion

HMW-HA binding is enhanced on activated human T<sub>R</sub>

FITC-labeled HMW-HA or an equivalent amount of FITC-labeled LMW-HA (Fig. 1A) were added to resting (unactivated) flow cytometry-sorted T<sub>R</sub> and CD4<sup>+</sup>CD25<sup>+</sup> T cells as well as T<sub>R</sub> from the same donor 96 h following activation with anti-CD3 and anti-CD28. Binding to unactivated cells was minimal, while binding of both forms of HA was enhanced following activation (Fig. 1, B and C). This is consistent with studies which have reported FITC-HA binding to activated but not unactivated T cells (24). The specificity of HA binding to T<sub>R</sub> was demonstrated through inhibition in the setting of PE-1, a peptide inhibitor of HA binding (22), through competitive exclusion in the setting of coadministration of non-FITC labeled HA, and by binding blockade using an anti-CD44 blocking mAb (Fig. 1D).

We sought to characterize differences in CD44 that occur following TCR stimulation which might account for the differential binding of HA to activated T<sub>R</sub>. We found that CD44-v6 and CD44-v9 were substantially up-regulated upon T<sub>R</sub> activation, whereas CD44-v4 was not (Fig. 2, A and B). Fluorescein-labeled HMW-HA bound most strongly to cells most highly expressing CD44-v6 (Fig. 2C) and CD44-v9 (data not shown). Higher expression of CD44-v6 correlated with the expression of the T<sub>R</sub>-associated signaling molecule FoxP3 (Fig. 2B) as did the expression of total CD44 (Fig. 2D). These results are in keeping with the CD44 isomer expression patterns reported for activated and unactivated T cells (18) and with murine data suggesting that T<sub>R</sub> expressing an activated form of CD44 were the most potent suppressors of proliferative effects (19). CD44-v6 stimulation has been shown to provide a strong proliferative signal via MAPKs and is also associated with CD25 up-regulation (25).

FIGURE 3. FoxP3 expression is modulated by HA administration. A, T<sub>R</sub> on days 2 and 7 postisolation and activation showing intracellular staining with anti-FOX3 mAb. T cell (TC) expression of FoxP3 at 2 days postisolation also without activation is shown for reference. B, FoxP3 expression on T<sub>R</sub> 7 days postactivation following a 24-h treatment with 20 μg/ml HMW-HA or LMW-HA. Results are representative of three experiments.

Expanded regulatory T cells

In brief, flow-purified CD4<sup>+</sup> T<sub>R</sub> were generated using a previously described method (23). In brief, flow-purified CD4<sup>+</sup>CD25<sup>+</sup> T cells were stimulated with anti-CD3 and anti-CD28 coupled to Xcyte beads supplemented with 2,000 IU/ml IL-2 in complete medium. Cultures were monitored daily and maintained with IL-2 supplemented complete medium. Expanded cells were washed before their incorporation in suppression assays.

Statistical analysis

Data are expressed as mean values ± SE except where otherwise noted. A Student’s paired t test was used to determine significance.

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HMW-HA up-regulates FoxP3 and augments the suppressive capacity of human T<sub>R</sub>

FoxP3 expression falls on T<sub>R</sub> without continuous resupplementation with IL-2 (Fig. 3A). However, treatment with HMW-HA at 20 μg/ml led to the maintenance of high expression of FoxP3 (Fig. 3B). LMW-HA treatment had a more modest effect at this same concentration.

HMW-HA, but not LMW-HA, enhances the antiproliferative activity of T<sub>R</sub> (Fig. 4A). The HA concentration used (20 μg/ml) is within the physiologic range (26). For this experiment, expanded T<sub>R</sub> were pulsed in HA overnight and washed three times to remove free HA before their addition to CD4<sup>+</sup>CD25<sup>+</sup> responder T cells and APC in a T<sub>R</sub> assay. Responder cell proliferation was significantly more inhibited by T<sub>R</sub> that had been exposed to HMW-HA than by T<sub>R</sub> that had not. In a separate experiment, HMW-HA or LMW-HA (Fig. 4, B vs C) was added directly to a suppression assay using fresh T<sub>R</sub> and Xcyte Dynabeads as APC to exclude the contribution of HA-APC interactions. Over a wide variety of effector/responder cell ratios, suppression of autologous CD4<sup>+</sup>CD25<sup>+</sup> T cell proliferation was enhanced by 20 and 100 μg/ml HMW-HA, but not by LMW-HA at either concentration. In this experiment, the ability of HMW-HA to enhance T<sub>R</sub> suppression activity appears to be due to its interaction with T<sub>R</sub> and not due to an effect on APC or on the responder cells. However, HMW-HA also has direct effects on responder cell proliferation, albeit only at high concentrations (Fig. 5). Because LMW-HA, generated from identical HMW-HA, had no such effects, it is unlikely that this antiproliferative effect of HMW-HA is due to contaminants present in clinical grade HA.
Activated TR is associated with increased expression of FoxP3, activating TR helps dampening the cellular immune response following the successful resolution of tissue damage or disease.

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Disclosures

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