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# Hyaluronan Synthesis Is Required for IL-2-Mediated T Cell Proliferation[1]

**Christie L. Mahaffey and Mark E. Mummert[2]**

**Hyaluronan (HA) is a glycosaminoglycan composed of *N*-acetylglucosamine and glucuronic acid subunits. Previous studies have suggested that CD44 expressed by T cells bind exogenous HA for their proliferation. However, HA endogenously synthesized by T cells may participate in their autocrine proliferation. In this study, we examined the role of endogenous HA in T cell proliferation using the highly specific HA synthase inhibitor, 4-methylumbelliferone (4-MU). We found that 4-MU inhibited the mitogen-induced synthesis of HA by T cells. Moreover, 4-MU inhibited T cell proliferation in a dose-dependent manner when cells were cultured with different stimuli, including Con A, PMA/ionomycin, and allogeneic spleen cells. Furthermore, 4-MU inhibited mitogen-stimulated IL-2 secretion, suggesting that HA may play a role in the production of this cytokine. Addition of IL-2 to T cells treated with 4-MU and Con A reversed the block in cell proliferation, showing that impaired IL-2 production is a likely mechanism for the inhibited division of T cells. Surprisingly, an anti-CD44 Ab antagonistic for HA binding did not reduce IL-2 secretion or T cell proliferation. Importantly, 4-MU did not alter the surface expression of CD44 or the ability of CD44 to bind to HA. Thus, HA-mediated IL-2 production and T cell proliferation are CD44 independent. Our results strongly suggest that HA synthesized by T cells themselves is critical for their IL-2-mediated proliferation and have revealed a previously unrecognized role for endogenous HA in T cell biology.** ***The Journal of Immunology,*** **2007, 179: 8191–8199.**

Hyaluronan (HA)[3] is a nonsulfated glycosaminoglycan composed of alternating *N*-acetylglucosamine and glucuronic acid subunits. In the pericellular matrix, HA is noncovalently associated with HA receptors (e.g., CD44) (1) or the nascent copolymer chain remains tethered to the enzyme that synthesized the HA molecule (2). HA is synthesized by the HA synthases (HAS), which are designated as HAS1, HAS2, and HAS3. HAS1 and HAS2 synthesize high m.w. HA copolymers, whereas HAS3 synthesizes HA of an intermediate size (3).

Although originally considered an inert filling material of the extracellular matrix, HA is now known to be involved in a number of biological activities, including the following: 1) fertilization (4, 5), 2) embryonic development (6), 3) wound healing (7, 8), 4) angiogenesis (9, 10), and 5) cancer cell metastasis (11). HA may also have multiple roles in the immune response (reviewed in Ref. 12). High m.w. HA expressed in the extracellular matrix may provide an adhesive substrate for the trafficking of leukocytes to and from inflamed tissues (13). The ability of HA to support cell trafficking is a result of molecular interaction of HA with surface receptors, most notably CD44 (14). Moreover, the binding of HA to CD44 may be important for initiating biochemical cascades for cellular activation in at least some cell types (15).

Activated T cells have been shown to express functional isoforms of CD44 that can bind HA for their homing to sites of inflammation (16). Moreover, in vitro studies have shown that the molecular interaction of CD44 with high concentrations of exogenous HA may play a role in T cell proliferation (17). In contrast, T cells have been shown to express mRNAs for HAS and express HA after mitogen stimulation. In addition, a HA-binding peptide (Pep-1) and a HA-binding protein (bovine proteoglycan) significantly reduced mitogen-stimulated T cell proliferation (18). Presumably, Pep-1 or the HA binding site of proteoglycan inhibited T cell proliferation by blocking the binding of endogenously derived HA to surface receptors (e.g., CD44) on T cells. However, the functions of HA were not directly measured in those studies, and the mechanism for how HA facilitated T cell proliferation was not investigated. Lastly, the costimulatory function of CD44 was not evaluated.

To directly investigate the functions of endogenously synthesized HA in T cell biology, we have assessed the impact of an inhibitor of HA synthesis (4-methylumbelliferone (4-MU)) on the activation and proliferation of murine T cells. In this study, we show that 4-MU dramatically inhibited mitogen-induced T cell proliferation in a dose-dependent manner. In contrast, 4-MU did not globally impair T cell activation, as suggested by the high expression levels of CD69 after mitogen stimulation. Interestingly, 4-MU treatment resulted in a significant reduction in the production of IL-2. Addition of exogenous IL-2 to cultures that had been previously treated with Con A and 4-MU restored T cell mitosis, strongly suggesting that newly synthesized HA is required for IL-2-mediated T cell proliferation. Surprisingly, an anti-CD44 mAb antagonistic for HA binding had no effect on IL-2 secretion or on T cell proliferation. Importantly, 4-MU did not alter the surface expression of CD44 on T cells or the ability of CD44 on activated T cells to bind to HA. Thus, HA-mediated IL-2 production and T cell proliferation occur in a CD44-independent fashion. Our results have revealed a previously unrecognized role for endogenous HA in T cell biology.

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[3] Abbreviations used in this paper: HA, hyaluronan; HAS, HA synthase; 4-MU, 4-methylumbelliferone; RHAMM, receptor for hyaluronic acid-mediated motility.

## Materials and Methods

### *Animals and cells*

Female BALB/c mice (4–6 wk old) and C57BL/6 mice (4–6 wk old) were purchased from Harlan. $CD3^+$ T cells were isolated from the spleens of BALB/c mice using a T cell isolation column (R&D Systems). $CD3^+$ T cells from BALB/c mice were used for all mitogen stimulation assays as well as the responder cells in the mixed allogeneic lymphocyte reaction with C57BL/6 spleen cells serving as the stimulus. All cell culturing was performed in RPMI 1640 containing 10% FCS. Collections of spleens from the mice were approved by the institutional review board at the University of Texas Southwestern Medical Center.

### *Reagents and Abs*

Con A, PMA, ionomycin, 4-MU, rIL-2, and *Streptomyces* hyaluronidase were purchased from Sigma-Aldrich. Trans $^{35}S$ (composed of $^{35}S$-labeled Cys and Met) was from MP Biomedical. All Abs, isotype controls, and fluorescently labeled streptavidin conjugates were purchased from BD Pharmingen. Annexin V conjugated to Alexa Fluor 488 was from Invitrogen Life Technologies. The HA-binding peptide, Pep-1 (GAHWQFNALTVRGGGSK-biotin), and the scrambled peptide control (WRHGFALTAVNQGGGSK-biotin) were synthesized with a C-terminal amide by Invitrogen Life Technologies.

### *HA synthesis and surface expression by T cells*

To study HA synthesis, $CD3^+$ T cells were cultured in the presence of Con A (4 $\mu$g/ml) or PMA/ionomycin (20 and 500 ng/ml, respectively) for 4 days and pulsed with [$^3$H]glucosamine (25 $\mu$Ci/ml) during the last 2 days of culture. To assess HA synthesis by T cells in the allogeneic MLR, $\gamma$-irradiated spleen cells (1500 rad) from C57BL/6 mice were combined with an equal number of $CD3^+$ T cells from BALB/c mice ($10^6$/well). Samples were pulsed on day 2 of culture with [$^3$H]glucosamine (50 $\mu$Ci/ml), and culture supernatants were harvested on day 4. Supernatants were processed for HA, as described previously (19). To test the impact of 4-MU on HA biosynthesis, the T cells were cultured with their respective stimuli and this reagent at 100 $\mu$g/ml.

To examine the Con A- and PMA/ionomycin-induced surface expression of HA, T cells were incubated for 30 min on ice with biotinylated Pep-1 or the biotinylated scrambled control. Cells were incubated with FITC-conjugated streptavidin (diluted 1/100), washed three times, and analyzed by FACS. To test the HA-specific binding of peptides to T cells, some samples were treated with hyaluronidase (10 U/ml) for 30 min at 37°C before peptide incubations. We assessed the effect of 4-MU on the surface expression of HA using a concentration of 100 $\mu$g/ml.

To examine the surface expression of HA on T cells after stimulation by allogeneic splenocytes, cells were first incubated for 30 min on ice with Fc block (diluted 1/100). Cells were washed twice and incubated with FITC-conjugated anti-CD3 mAb and either biotinylated Pep-1 or the biotinylated scrambled peptide control for 30 min on ice. After washing, bound peptides were detected by incubating cells on ice with cytochrome 5-conjugated streptavidin diluted 1/100. Finally, cells were washed, stained with propidium iodide (1 $\mu$g/ml), and then subjected to FACS. To test the HA-specific binding of peptides to T cells, some samples were treated with hyaluronidase (10 U/ml) for 30 min at 37°C before peptide incubations. We assessed the effect of 4-MU on the surface expression of HA using a concentration of 100 $\mu$g/ml. For analysis of HA expression by T cells, we gated on viable (i.e., propidium iodide-negative) $CD3^+$ population.

### *CD44-mediated surface retention of HA*

To assess the role of CD44 in tethering HA to the surfaces of T cells, Con A-stimulated T cells were incubated on ice for 30 min with the anti-CD44 mAb KM114 (10 $\mu$g/ml) or the isotype control (10 $\mu$g/ml). After washing, surface-associated HA was detected using biotin-conjugated Pep-1 or the biotin-labeled scrambled control and FITC-streptavidin exactly as described above.

### *T cell proliferation assays*

T cells ($10^5$/well) were cultured with 4-MU at graded doses (0–100 $\mu$g/ml) in the presence of either Con A (4 $\mu$g/ml) or PMA/ionomycin (20 ng/ml PMA and 500 ng/ml ionomycin) for 30 h. Cultures were pulsed during the last 8 h of culture with [$^3$H]thymidine (2.5 $\mu$Ci/well), harvested onto glass fiber filter paper, and read in a beta counter (Beckman Coulter). For the allogeneic MLR, spleen cells from C57BL/6 mice were $\gamma$ irradiated (1500 rad) and combined with an equal number of $CD3^+$ T cells from BALB/c mice ($10^5$/well) in round-bottom 96-well plates. Samples were pulsed with [$^3$H]thymidine on day 3 and harvested on day 4 (20).

To test the impact of IL-2 on 4-MU-mediated T cell inhibition, T cells were cultured for 2 days in the presence of Con A (4 $\mu$g/ml) and 4-MU (100 $\mu$g/ml). Cells were washed and recultured ($10^5$/well) in medium containing IL-2 (5 U/ml) on day 3, pulsed with [$^3$H]thymidine, and harvested on day 4.

### *Cytotoxicity assays*

The cytotoxicity of 4-MU to T cells was assessed using propidium iodide staining and FACS. Briefly, T cells were cultured with Con A (4 $\mu$g/ml) in the presence of graded concentrations of 4-MU (0–100 $\mu$g/ml) for 30 h. Cells were harvested and the cells were washed three times. T cells were stained with propidium iodide (1 $\mu$g/ml) and then subjected to FACS. The percentage of viable cells was determined by gating on the propidium iodide-negative population. To test the potential of 4-MU to induce apoptosis, T cells were cultured with Con A or with Con A and 4-MU for 30 h. T cells were then stained with propidium iodide and Alexa Fluor 488-conjugated annexin V exactly as described by the manufacturer and analyzed by FACS.

### *Analysis of surface marker expression*

T cells were cultured with Con A (4 $\mu$g/ml) and 4-MU (100 $\mu$g/ml) for 24 h. Afterward, cells were stained for the surface expression of CD69, CD44, and CD25. Briefly, FITC-conjugated anti-CD69, FITC-conjugated anti-CD44 (clone IM7), and FITC-conjugated anti-CD25 as well as conjugated isotype control Abs were diluted 1/500 in PBS containing 1% FCS. T cells were stained for 30 min on ice, washed three times, and then subjected to FACS.

### *Removal of preformed HA from T cell surfaces*

To test the impact of preformed HA on T cell activation, T cells were pretreated or not with hyaluronidase (10 U/ml) for 30 min at 37°C. Cells were washed and stimulated overnight with Con A (4 $\mu$g/ml). To prevent new HA synthesis, 4-MU was included in the cultures at 100 $\mu$g/ml. The activation status of T cells was assessed by CD69 expression using FACS exactly as described above.

### *Cytokine secretion*

T cells were cultured for 24 h with Con A (4 $\mu$g/ml) and 4-MU (100 $\mu$g/ml). Culture supernatants were centrifuged and frozen at −20°C for storage. Secretion of the cytokines IL-2 and IFN-$\gamma$ was assessed by ELISA using kits purchased from R&D Systems.

### *Functional assays for CD44*

To evaluate the impact of CD44 on IL-2 secretion and T cell proliferation, T cells were cultured with Con A (4 $\mu$g/ml) and an anti-CD44 mAb (clone KM114 at 10 $\mu$g/ml) or the isotype control. To assess the impact of 4-MU on the HA-binding function of CD44, we performed T cell adhesion assays with $^{35}S$-labeled cells essentially as described (13). Briefly, 100 $\mu$l of a HA solution (0.1 mg/ml) was added to the wells of an Amine CovaLink plate (Nalge Nunc International), followed by addition of 50 $\mu$l of 0.1 N HCl and 50 $\mu$l of 0.2 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. After overnight incubation at room temperature, the wells were washed three times in PBS containing 2 M NaCl and 0.04 mM $MgSO_4$, followed by two additional washes with PBS alone. The HA-coated plates were then counter coated with 3% BSA in PBS for 3 h at 37°C. $CD3^+$ T cells were stimulated overnight with Con A (4 $\mu$g/ml) or Con A (4 $\mu$g/ml) plus 4-MU (100 $\mu$g/ml) in the presence of 30 $\mu$Ci/ml $^{35}S$-labeled methionine/cysteine. After washing, radiolabeled T cells were incubated on ice for 30 min with 10 $\mu$g/ml anti-CD44 KM114 mAb or 10 $\mu$g/ml isotype control. T cells were washed and then added to the HA-coated wells ($10^4$/well) and incubated at room temperature for 30 min. After removal of nonadherent cells, wells were washed three times with PBS, and the remaining adherent cells were solubilized in 1% SDS and counted for radioactivities. The percentages of adherent cells were calculated by dividing the recovered cpm by the total cpm added to each well.

### *Statistics*

Experimental groups were compared with two-tailed Student's *t* test. Differences between groups were considered significant for $p \leq 0.05$. Experiments were performed twice to assess their reproducibility.

## Results

### *Impact of 4-MU on HA expression*

The 4-MU is a well-known inhibitor of HA biosynthesis (21, 22). Importantly, 4-MU appears to inhibit HAS enzymes with high specificity, as assessed by the failure of this drug to impair the

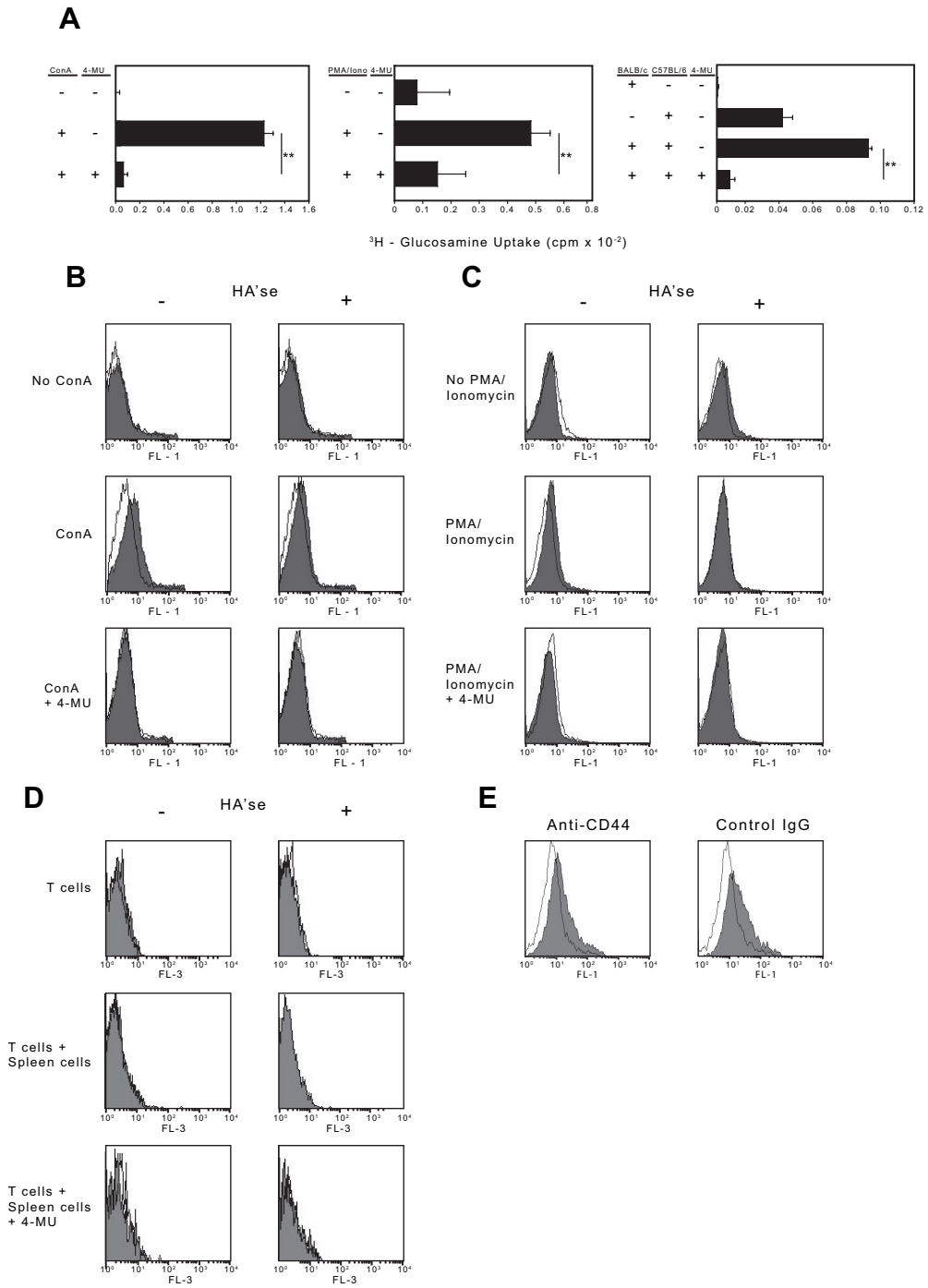


**FIGURE 1.** Impact of 4-MU on the synthesis and cell surface expression of HA. *A*, CD3$^+$ T cells isolated from BALB/c mice were stimulated with Con A (*panel 1*), PMA/ionomycin (*panel 2*), or allogeneic spleen cells from C57BL/6 mice (*panel 3*), and examined for their capacity to synthesize HA by metabolic labeling with [$^3$H]glucosamine. The data shown are the amounts of hyaluronidase-sensitive radioactivities (means ± SD) recovered from triplicate cultures. Statistically significant differences are indicated by asterisks (**, $p < 0.01$) and have been compared with cultures stimulated with mitogens or spleen cells in the absence of 4-MU. *B–D*, CD3$^+$ T cells were examined for their surface expression of HA using biotinylated Pep-1 (filled histograms) or the scrambled control (open histograms) in the presence or absence of Con A and 4-MU, PMA/ionomycin and 4-MU, or allogeneic spleen cells and 4-MU. Some samples were pretreated with hyaluronidase (HA'se) to assess the specificity of HA staining. *E*, The role of CD44 in tethering HA to T cells was investigated by examining the ability of an anti-CD44 Ab (clone KM114) or an isotype control to inhibit the binding of HA to T cell surfaces. The relative amounts of surface-associated HA were determined by staining T cells with Pep-1 (filled histograms). Staining of cells with the scrambled peptide control is shown as the open histograms.

production of other glycosaminoglycans (e.g., chondroitin sulfate) that incorporate UDP-glucuronic acid for copolymer synthesis (21, 23).

As shown in Fig. 1*A*, T cells synthesized HA after mitogen stimulation (Con A and PMA/ionomycin, *panels 1* and *2*, respectively). This finding is consistent with our previous observation that stimulated T cells express higher concentrations of HA compared with their quiescent counterparts (18). Importantly, 4-MU significantly ($p < 0.01$) inhibited HA synthesis by T cells to near background levels for both Con A- and PMA/ionomycin-stimulated T cells. Fig. 1*A*, *panel 3*, shows that CD3$^+$ T cells also actively synthesize HA following stimulation by allogeneic spleen

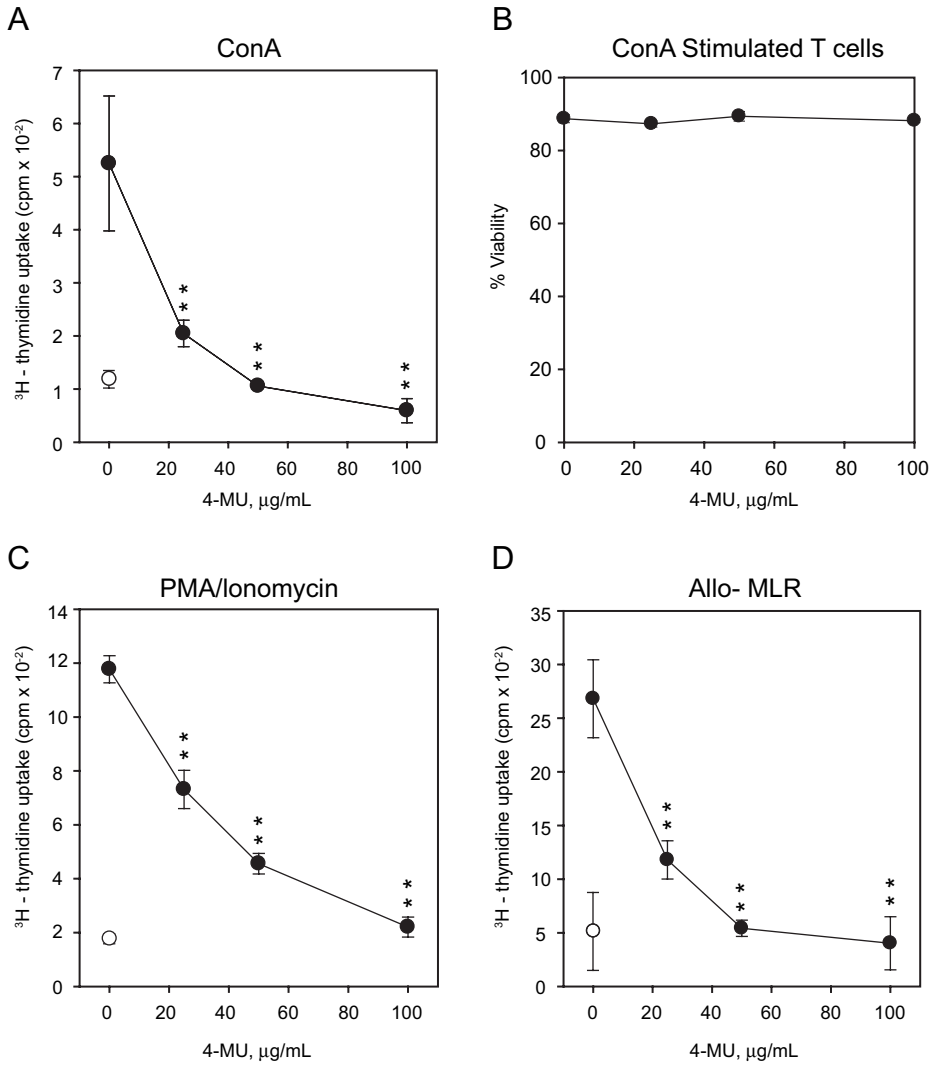


**FIGURE 2.** Impact of 4-MU on T cell proliferation and viability. *A*, $CD3^+$ T cells were treated with Con A and graded doses of 4-MU. ○, T cells cultured in medium alone (i.e., lacking Con A and 4-MU). Data shown are the means ± SD ($n = 3$) of the [$^3$H]thymidine uptake. Statistically significant differences are indicated by asterisks (**, $p < 0.01$) and have been compared with cultures stimulated with Con A alone. *B*, The viability of Con A-stimulated T cells cultured in the presence or absence of 4-MU was evaluated based on the propidium iodide staining of cells, followed by FACS. Results are presented as the means ± SD of triplicate cultures. *C*, $CD3^+$ T cells were stimulated with PMA and ionomycin with graded doses of 4-MU. Results are presented as the means ± SD ($n = 3$) of the [$^3$H]thymidine uptake. ○, T cells cultured in medium alone. Statistically significant differences are indicated by asterisks (**, $p < 0.01$) and have been compared with cultures stimulated with PMA/ionomycin without 4-MU. *D*, Splenic $CD3^+$ T cells from BALB/c mice were cocultured with γ-irradiated allogeneic spleen cells from C57BL/6 mice. Data shown are the means ± SD ($n = 3$) of the [$^3$H]thymidine uptake. ○, T cells cultured without allogeneic spleen cells. Statistically significant differences are indicated by asterisks (**, $p < 0.01$) and have been compared with cultures stimulated with spleen cells and lacking 4-MU.

cells, although the concentrations of HA were markedly less than T cells stimulated by either Con A or PMA/ionomycin. Because only ~1% of BALB/c T cells proliferate following stimulation with C57BL/6 splenocytes (24), the small concentrations of HA synthesized in the allogeneic MLR may reflect the small numbers of mitotic T cells in this assay. It is interesting to note that splenocytes alone showed synthesis of HA. Thus, unlike T cells, these results suggest that some cell types may constitutively synthesize HA. Indeed, we have previously shown that splenic dendritic cells synthesize HA regardless of their activation status (18).

Because HA polymers can associate with cell surfaces via molecular interaction with HA receptors or nascent chain interactions with HAS enzymes, we next evaluated the surface expression of HA. We failed to detect HA on the surfaces of resting T cells (Fig. 1, *B–D*). In contrast, Pep-1, but not the scrambled peptide control, showed T cell staining after stimulation with Con A (Fig. 1*B*) or PMA/ionomycin (Fig. 1*C*). Although the Pep-1-staining profiles for mitogen-induced HA expression were relatively modest, analyses of the geometric mean fluorescent intensities were significantly higher for Pep-1 binding to stimulated T cells as compared with the scrambled peptide control for both Con A (scrambled control, mean ± SD = 5.57 ± 0.44; Pep-1, mean ± SD = 14.04 ± 1.31, $p < 0.01$) and PMA/ionomycin (scrambled control, mean ± SD = 4.43 ± 0.57; Pep-1, mean ± SD = 8.37 ± 0.43, $p < 0.01$). Binding of Pep-1 to T cells was significantly reduced following treatment with hyaluronidase, showing HA specificity (Fig. 1, *B* and *C*). Finally, 4-MU markedly inhibited the cell surface expression of HA (Fig. 1, *B* and *C*). Based on these results, we conclude that 4-MU potently inhibits the mitogen-induced biosynthesis of HA in T cells. In contrast, we failed to detect surface staining for HA on T cells that were activated in the allogeneic MLR (Fig. 1*D*). Because the affinity of Pep-1 for HA is modest ($K_d = 10^{-6}$ M) (13), very low concentrations of HA cannot be detected using this reagent. Thus, our results may suggest that the surface concentrations of HA are extremely low on T cells stimulated by allogeneic spleen cells.

Finally, we assessed the potential role of CD44 to serve as an anchoring receptor for the retention of HA on T cell surfaces. As shown in Fig. 1*E*, an anti-CD44 Ab that inhibits the HA-CD44 molecular interaction failed to reduce the surface staining of HA by Pep-1. These results suggest that CD44 does not serve as a primary receptor for maintaining HA copolymers on T cell surfaces. These findings may not be totally surprising. Although CD44 can act as a receptor to retain HA on the surfaces of some cell types (1), this is not necessarily the case for all types of cells that express functional CD44 isoforms. For example, dendritic cells express both functional CD44 and surface-associated HA. However, little, if any, of the surface HA on dendritic cells is associated with CD44 (18). Previous investigations have shown that the hyaluronan synthases not only catalyze HA synthesis, but

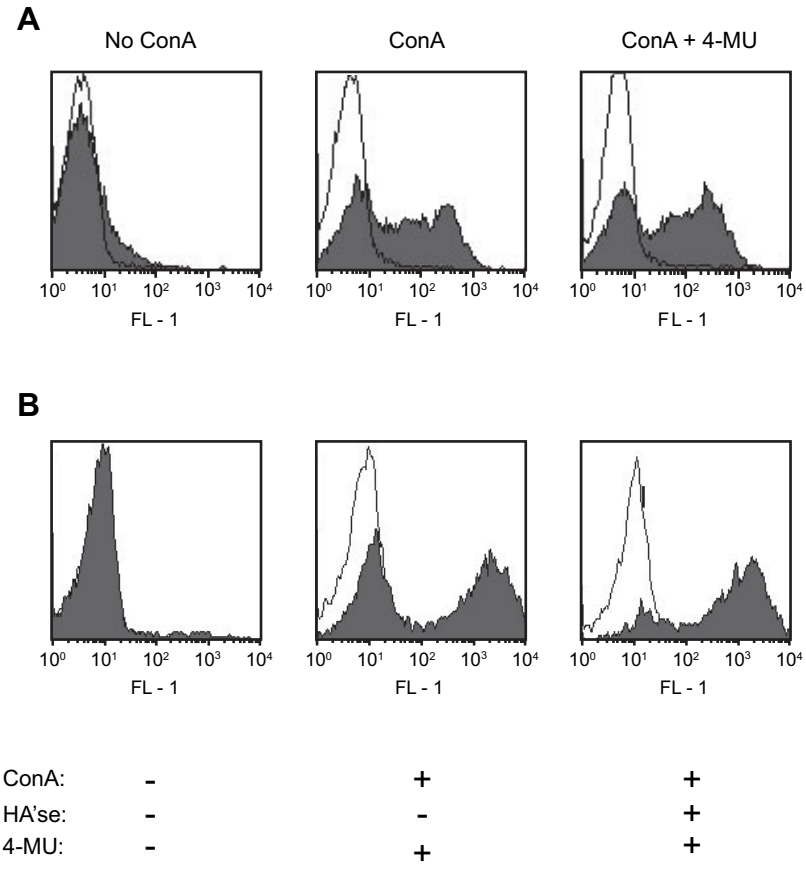


**FIGURE 3.** Effect of 4-MU on T cell activation. *A*, $CD3^+$ T cells were cultured in the presence or absence of Con A and 4-MU for 24 h. T cells were stained with anti-CD69 (shaded histogram) or an isotype control (open histogram) and evaluated by FACS. *B*, Preformed HA was removed by hyaluronidase (HA'se), and T cells subsequently were cultured in medium containing 4-MU to prevent the synthesis of new HA. T cells were treated with anti-CD69 (shaded histogram) or an isotype control (open histogram).

they can also serve to anchor HA copolymers to the cell surface (3). Thus, HA may be tethered to the surface of T cells by a similar mechanism.

*Impact of HAS inhibition on T cell proliferation and activation*

To assess the impact of HA on T cell proliferation, we cultured T cells in medium containing Con A with different concentrations of 4-MU. As shown in Fig. 2*A*, 4-MU significantly inhibited T cell proliferation in a dose-dependent fashion ($p < 0.01$). Importantly, results from the FACS analysis of propidium iodide-stained T cells showed that 4-MU was not cytotoxic at any of the concentrations relative to cultures lacking this inhibitor (Fig. 2*B*; $p > 0.05$). We also failed to detect differences between annexin V-stained T cells cultured in the presence of Con A or in the presence of Con A plus 4-MU. These results suggest that 4-MU does not induce T cell apoptosis (data not shown). To evaluate whether the 4-MU-mediated inhibition of T cell proliferation was Con A specific, we assessed the impact of this drug on the proliferation of T cells stimulated with PMA/ionomycin and allogeneic spleen cells. We found that 4-MU dose dependently inhibited T cell proliferation stimulated by PMA/ionomycin (Fig. 2*C*) and allogeneic spleen cells (Fig. 2*D*). We should note that Con A and allogeneic spleen cells most likely induce T cell proliferation via receptor-specific mechanisms, whereas PMA/ionomycin bypasses cell surface receptors (25). Because 4-MU inhibited PMA/ionomycin T cell activation, our results may suggest that newly synthesized HA plays a role in pathways downstream from the initial receptor-signaling event.

Next, we investigated the impact of 4-MU on T cell activation. As shown in Fig. 3*A*, Con A stimulation of T cells resulted in marked surface expression of the activation marker, CD69 (26). Moreover, T cells activated with Con A in the presence of 4-MU showed levels of CD69 expression that were comparable to T cells cultured with Con A alone (Fig. 3*A*). Because 4-MU inhibits new HA synthesis, we considered the possibility that preformed HA could also play a role in T cell activation. To shut down both preformed and newly synthesized HA, we pretreated T cells with hyaluronidase to remove pre-existing HA and then cultured them in medium containing Con A and 4-MU to inhibit the production of new HA. As shown in Fig. 3*B*, the surface expression of CD69 was similar between T cells treated with hyaluronidase and T cells lacking hyaluronidase pretreatment. These findings suggest that preformed HA does not play a role in T cell activation. In summary, our results show that HA does not have a global role in the activation of T cells.

*Functional roles of HA synthesis in cytokine secretion*

T cells transcribe IL-2 within hours following stimulation (27). Binding of IL-2 to the IL-2R plays a pivotal role in the proliferation of T cells (reviewed in Ref. 28). Moreover, IL-2 induces expression of the IL-2R $\alpha$-chain (CD25), and binding of IL-2 to the IL-2R constitutes an autocrine loop (29, 30). T cells also transcribe IFN-$\gamma$ at an early time point following their stimulation (27). As shown in Fig. 4*A*, 4-MU significantly reduced the secretion of IL-2 ($p < 0.01$) and IFN-$\gamma$ ($p < 0.01$) as compared with T cells stimulated with Con A alone. FACS analysis showed that T cells treated with Con A dramatically increased surface expression levels of CD25, whereas T cells cultured in the presence of Con A plus 4-MU showed a partial, but significant ($p < 0.01$) reduction in the surface expression of CD25 (Fig. 4, *B* and *C*). Our results show that impaired synthesis of HA is correlated with a reduction in IL-2 and IFN-$\gamma$ production as well as the CD25 surface expression.

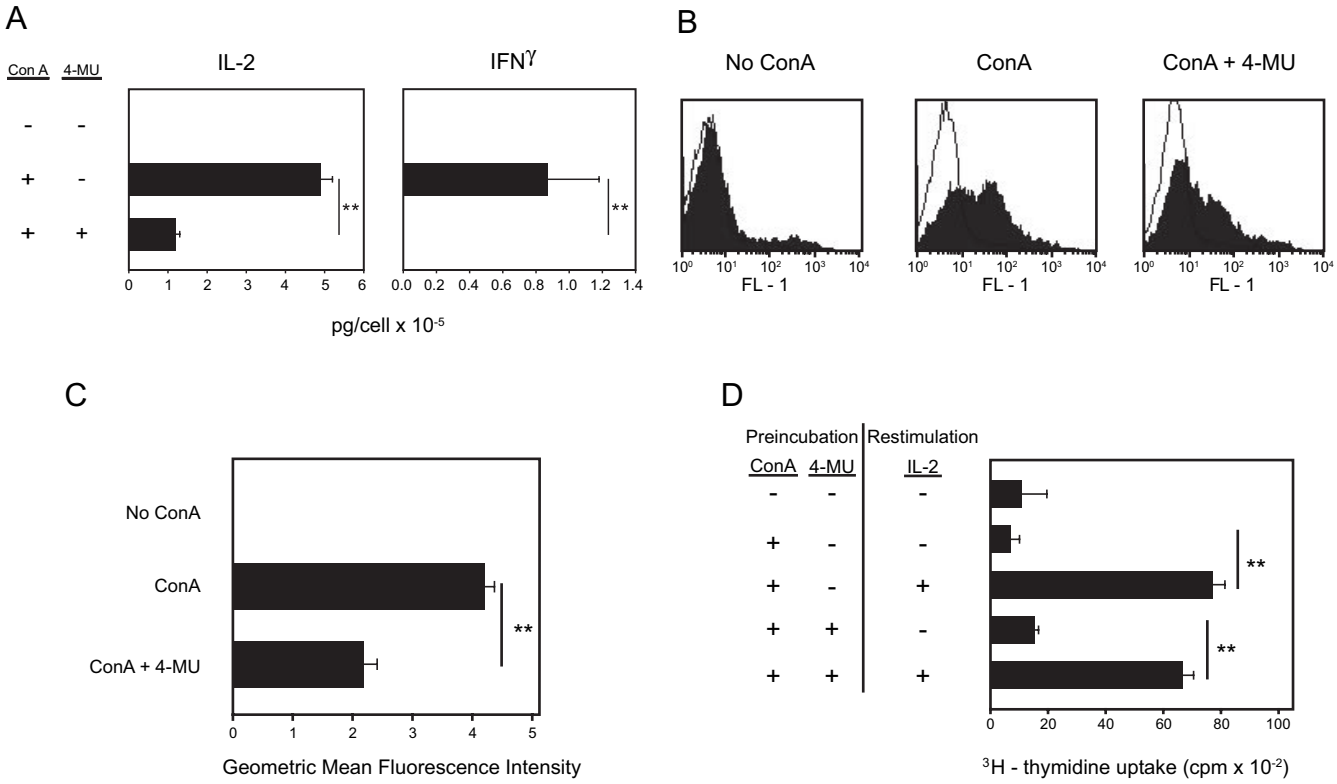


**FIGURE 4.** Impact of 4-MU on IL-2 functions. *A*, $CD3^+$ T cells were treated with Con A or 4-MU, and culture supernatants were collected 24 h later. The relative concentrations of IL-2 and IFN-γ were determined using the ELISA. The data shown are the means ± SD ($n = 3$). Significant differences are shown by asterisks (**, $p < 0.01$). *B*, The surface expression of CD25 (IL-2R α-chain) was assessed using an anti-CD25 Ab (shaded histogram) or an isotype control (open histogram). *C*, The surface expression of CD25 was evaluated quantitatively by comparing the geometric mean fluorescent intensities of T cells cultured with Con A or Con A plus 4-MU. The geometric mean fluorescence intensity of IgG isotype control-stained T cells was subtracted from the geometric mean fluorescent intensities of cells stained with an anti-CD25 mAb. The results are presented as the means ± SD of triplicate cultures. Significant differences are shown by asterisks (**, $p < 0.01$). *D*, The impact of IL-2 on T cell proliferation was ascertained by culturing $CD3^+$ T cells pretreated with Con A in the absence or presence of 4-MU with fresh medium containing exogenous IL-2. Results are presented as the means ± SD ($n = 3$) of the [$^3$H]thymidine uptake, and statistically significant differences are indicated by asterisks (**, $p < 0.01$).

We hypothesized that impaired T cell proliferation was due to the decreased production of IL-2. To test this possibility, we evaluated the impact of exogenous IL-2 on T cells that had been cultured in the presence of Con A and 4-MU. As shown in Fig. 4*D*, exogenous IL-2 significantly reversed the block in T cell proliferation ($p < 0.01$). These results strongly suggest that the 4-MU-mediated inhibition of T cell proliferation was IL-2 dependent. How can we explain reconstitution of T cell proliferation by exogenous IL-2 in light of our finding that 4-MU inhibits the expression of CD25? It is important to note that CD25 is important for modulating the IL-2R from an intermediate ($K_d = 10^{-9}$ M) to a high-affinity receptor ($K_d = 10^{-11}$ M), but most likely it does not play a role in signal transduction (31, 32). Thus, CD25 lowers the concentration of IL-2 required to elicit a T cell response. As shown in Fig. 4, *B* and *C*, 4-MU reduced, but did not abrogate, the expression of CD25. Therefore, we propose that even after pretreatment with Con A and 4-MU, the T cells still expressed high-affinity IL-2Rs, albeit at concentrations less than after stimulation with Con A alone. Because the high-affinity IL-2Rs were most likely in excess of the exogenous IL-2, T cells pretreated with Con A or Con A plus 4-MU bound similar or equivalent moles of exogenous IL-2 (i.e., the high-affinity IL-2Rs for both treatment groups had equivalent levels of IL-2 occupancy), leading to similar numbers of responsive and proliferating T cells. We should note that others have also reported that inhibition of IL-2 production does not necessarily render T cells unresponsive to the addition of exogenous IL-2. Carter et al. (33) has shown that the binding of the program death ligand-Fc fusion protein to the program death-1 receptor expressed by T cells inhibits their proliferation by suppressing IL-2 production. Importantly, exogenous IL-2 was able to restore proliferation, showing that the T cells remained capable of responding to IL-2. Based on our results, we propose that newly synthesized HA plays a role in the production of IL-2, which in turn promotes the growth and proliferation of T cells.

### *Role of CD44 in HA-mediated IL-2 secretion and cell division*

CD44 is a surface glycoprotein that is generally considered to be a major HA receptor. In fact, CD44 has been shown to play a major role for the rolling of T cells on inflamed endothelium (34) and the extravasation of T cells into inflammatory sites (35). Other reports have suggested that CD44 may also be important for regulating the activities of T cells during the inflammatory response. For example, McKallip et al. (36) has shown that $CD44^{-/-}$ T cells were resistant to TCR-mediated apoptosis, whereas CD44 in wild-type T cells induced apoptosis in activated, but not resting cells. Finally, CD44 can serve as a coreceptor for signaling in some cell types (15).

Based on the potential importance of CD44 in T cell biology, we evaluated the impact of 4-MU on CD44-mediated adhesion to HA. As shown in Fig. 5*A*, *panel 1*, Con A-stimulated T cells showed significantly better binding to wells coated with HA compared with the wells lacking the HA substrate. An anti-CD44 mAb significantly ($p < 0.05$) blocked the adhesion of T cells to HA, showing that T cell binding to HA is CD44 mediated. As shown in the

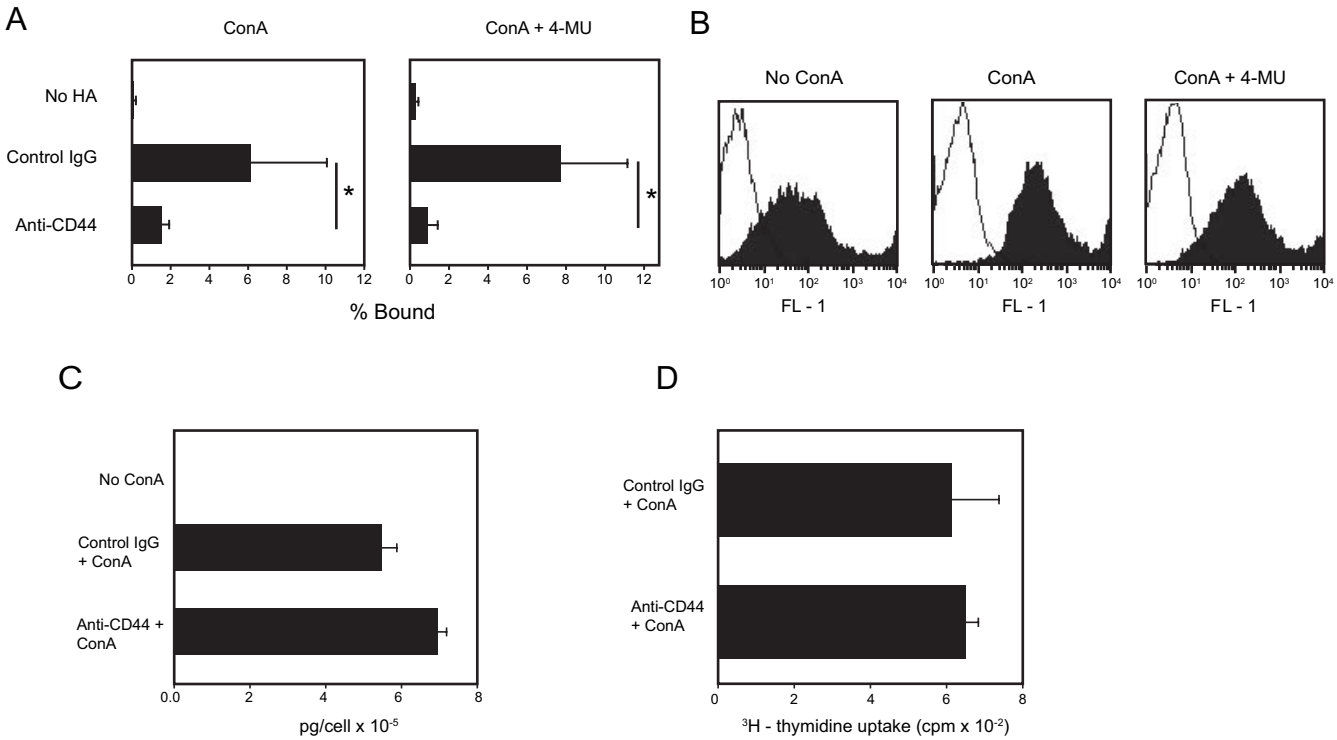


**FIGURE 5.** Assessment of CD44 to serve as a coreceptor for mitogen-induced T cell stimulation. *A*, $CD3^+$ T cells were stimulated with Con A in the absence (*panel 1*) or presence (*panel 2*) of 4-MU. The CD44-dependent adhesion of $^{35}S$-labeled T cells to bind HA-coated plates was evaluated by pretreating cells with an anti-CD44 mAb that antagonizes HA binding or an irrelevant isotype control. Results are expressed as percentage of bound cells/well and are presented as the means ± SD ($n = 4$). Statistically significant differences are indicated by an asterisk (*, $p < 0.05$). *B*, The concentration of surface-expressed CD44 on T cells after Con A stimulation in the absence or presence of 4-MU was evaluated using an anti-CD44 mAb (shaded histogram) or an isotype control (open histogram). *C*, The impact of an anti-CD44 mAb on IL-2 secretion was assessed by stimulating T cells with Con A in the presence of an anti-CD44 Ab or an isotype control. Culture supernatants were collected 24 h after stimulation, and the relative concentrations of secreted IL-2 were evaluated by the ELISA. Data are shown as the means ± SD ($n = 3$). *D*, The potential role of CD44 on T cell proliferation was evaluated by comparing the impact of an anti-CD44 mAb with an isotype control. Data shown are the means ± SD ($n = 3$) of the [$^3$H]thymidine uptake.

*second panel* of Fig. 5*A*, T cells cultured in the presence of Con A plus 4-MU showed similar levels of adhesion to HA-coated wells compared with the T cells stimulated with Con A alone (Con A alone, mean ± SD = 6.13 ± 3.94; Con A plus 4-MU, mean ± SD = 7.73 ± 3.42, $p > 0.05$). As before, T cell adhesion to HA was significantly blocked ($p < 0.01$) when the cells were preincubated with an anti-CD44 mAb. These results show that 4-MU does not impair the ability of CD44 to bind to HA.

Next, we examined the surface expression of CD44 using FACS. Our results show that Con A stimulation significantly increased the expression of CD44 on T cells. In contrast, 4-MU did not alter the Con A-induced surface expression of CD44 (Fig. 5*B*). Thus, 4-MU does not alter the ability of CD44 to bind to HA nor does it change the concentration of CD44 expressed on the surfaces of T cells.

To test the roles of CD44 in the production of IL-2 and T cell proliferation, we evaluated the impact of anti-CD44 on Con A-induced IL-2 secretion and T cell proliferation. We failed to detect a significant difference in either IL-2 production (Fig. 5*C*) or T cell proliferation (Fig. 5*D*) between anti-CD44- and isotype control-treated groups ($p > 0.05$). We interpreted these results to suggest that binding of endogenously synthesized HA to CD44 does not play a role in IL-2 production nor in T cell proliferation, the two events that were significantly impaired by inhibiting the synthesis of HA with 4-MU. Based on these findings, we propose that HA-mediated IL-2 production and consequent T cell proliferation are CD44 independent.

## Discussion

HA, a high m.w. glycosaminoglycan, plays a number of roles in various biological activities, including the immune response. CD44, a glycoprotein expressed on the surface of T cells, is generally believed to be a major receptor for HA. Previous investigations have suggested that the binding of CD44 to exogenous HA has multiple roles in T cell biology, including the following: 1) as an adhesive substrate for T cell trafficking to inflamed sites (35), and 2) as a costimulatory molecule (17). To date, studies have mostly examined the functions of exogenous HA (i.e., high concentrations of HA added to cell cultures) with relatively little or no assessment for the roles of endogenously synthesized HA. Thus, in the studies reported in this work, we have directly examined the functions of endogenously synthesized HA in T cell biology using the HAS inhibitor, 4-MU.

Our results strongly suggest that HA plays a critical role in the mitogen-induced proliferation of T cells. In contrast, 4-MU did not have an impact on the surface expression of CD69, showing that HA is most likely not globally involved in T cell activation. Both Con A and allogeneic spleen cells induce receptor-mediated T cell proliferation, whereas the combination of PMA and ionomycin bypasses receptor-mediated signaling. PMA is an analog of diacylglycerol and activates protein kinase C, whereas ionomycin causes the release of intracellular $Ca^{2+}$ (25). Our results showing that 4-MU inhibited T cell proliferation by both receptor-dependent and independent stimuli may suggest that HA plays a role in

a common cellular pathway or similar biochemical events downstream of the initial receptor-signaling event.

How does HA induce T cell proliferation? Ohno et al. (37) has shown that oligomeric HA stimulated various transcription factors in chondrocytes, including the retinoic acid receptor, retinoid X receptor, Sp1, and NF-κB. Even more recently, this same group has shown that HA oligosaccharides induce expression of matrix metalloproteinase 13 by p38 MAPK and transcriptional activation of NF-κB in chondrocytes (38). Because we found that 4-MU treatment reduces IL-2 production and addition of exogenous IL-2 to 4-MU-treated cells reversed the block in T cell proliferation, we hypothesize that newly synthesized HA may activate transcriptional factors ultimately leading to the expression of IL-2. In turn, IL-2 binds the IL-2R, facilitating T cell proliferation. Regulation of the IL-2 gene is complex and requires a number of positive and negative transcription factors (NF-AT, AP-1, NF-κB, CREB, Oct-1, and -2, Sp1, GABP, Nil-2a, STATB1, and Foxp3), chromatin remodeling, and posttranscriptional regulation (reviewed in Ref. 28). Thus, future investigations in our laboratory will seek to address how HA regulates IL-2 expression in fine detail.

Previous studies have shown that HA-coated plates (17) or HA expressed on the surfaces of APCs (i.e., dendritic cells) (18) may enhance the proliferation of T cells. Because CD44 has been shown to serve as a coreceptor in some cell types, we investigated the role of CD44 in mitogen-induced T cell proliferation. We found that 4-MU did not inhibit the ability of CD44 to bind to HA nor did it alter the expression level of CD44 on activated T cells. Finally, an antagonistic anti-CD44 mAb did not significantly inhibit IL-2 production or the proliferation of T cells. Because inhibition of HA synthesis by 4-MU dramatically reduced both IL-2 expression and T cell proliferation, we propose that endogenously synthesized HA may exert its function(s) in a CD44-independent manner.

How can we explain the discordance between previous results showing a role for CD44 in HA-mediated T cell proliferation and results in this study showing no role for CD44 in HA-mediated T cell proliferation? We propose that HA may be involved in multiple and/or overlapping cellular pathways in T cells. Thus, CD44 may bind exogenous HA and stimulate a similar or shared pathway initiated by a second HA receptor that functions by binding endogenously synthesized HA. Importantly, CD44 expressed by T cells can bind to HA only after the T cells have been activated (39). Thus, it is tempting to speculate that endogenously synthesized HA interacts with a receptor that binds HA constitutively or has the ability to bind to HA at an earlier time point than CD44. We should point out that others have also shown that HA can play a role in the activities of cells independent from molecular interaction with CD44. For example, Simon and colleagues (40, 41) have shown that HA fragments can induce the maturation of dendritic cells to highly potent APCs via molecular interaction with TLR 4. Similarly, studies in CD44-null COS-7 cells have shown that HA can induce the expression of matrix metalloproteinase 13 (38). Thus, the identification of HA receptors other than CD44 and the evaluation of their potential roles in cosignaling are required to fully appreciate the functions of HA in regulating T cell proliferation.

What receptor is responsible for the HA-mediated induction of T cell proliferation? One potential candidate is the receptor for hyaluronic acid-mediated motility (RHAMM, CD168). Like CD44, RHAMM may serve as a coreceptor for cell signaling (42). We have been able to detect the expression of RHAMM mRNA in both resting and Con A-stimulated T cells by RT-PCR (data not shown). Thus, RHAMM protein may be expressed in T cells. The development of antagonistic anti-RHAMM Abs (43) and RHAMM knockout mice (44) should allow the functions of RHAMM in T cell biology to be evaluated.

In conclusion, we have reported a unique functional role for HA in T cells. Based on data reported in this study and elsewhere, we propose that HA may use multiple receptors and/or pathways for regulating T cell proliferation. Results and experimental systems described in this study provide the conceptual and technical foundations for further investigating the roles of HA in T cell biology.

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## Disclosures

The authors have no financial conflict of interest.

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