Crosstalk between CD4 T cells and synovial fibroblasts from human arthritic joints promotes hyaluronan-dependent leukocyte adhesion and inflammatory cytokine expression in vitro

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Abstract

The content and organization of hyaluronan (HA) in the extracellular matrix (ECM) have been identified as strong indicators of inflammation in joint disease, although the source and role of HA as an effector of inflammation is not clear. In this study, we established co-cultures of activated human CD4 T cells with fibroblast-like synoviocytes (FLS) from osteoarthritis (OA) and rheumatoid arthritis (RA) subjects and examined the role of HA in promoting inflammatory events. Co-cultures of RA FLS with activated CD4 T cells generated an HA-enriched ECM that promoted enhanced monocyte adhesion compared to co-cultures of OA FLS with activated CD4 T cells. In addition, both OA FLS and RA FLS co-cultures with activated CD4 T cells elicited significant increases in the expression of IL1β, TNF, and IL6, with the increase in IL6 expression most prominent in RA co-cultures. Blocking HA synthesis and accumulation with 4-methylumbelliferone reduced expression of IL6, IL1β, and TNF in both OA FLS and RA FLS co-cultures. The increase in HA synthesis in the co-cultures was mimicked by IL6 trans-signaling of FLS in the absence of CD4 T cells. Inhibition of HA synthesis blocked the increase in IL6 by RA FLS mediated by IL6 trans-signaling, suggesting that the HA synthetic pathway may be a key mediator in IL6 expression by FLS. Overall, our study indicates that HA-enriched ECM generated by co-cultures of activated CD4 T cells with FLS from human joints creates a pathogenic microenvironment by promoting adhesion of leukocytes and expression of inflammatory cytokines including IL6.

Introduction

Rheumatoid arthritis (RA) and osteoarthritis (OA) are joint diseases characterized by chronic and persistent damage in joint tissues accompanied by pathological extracellular matrix (ECM) remodeling and inflammation [1]. Fibroblast-like synoviocytes (FLS) have been shown to play a key role in promoting persistent and chronic inflammation by producing inflammatory cytokines and ECM-degrading enzymes that lead to cartilage destruction [2–9]. The polysaccharide hyaluronan (HA) and the accumulation of its ECM protein binding partners not only alters the microenvironment to allow cells to

proliferate and migrate, but also directs immune responses by interacting with immune cells, cytokines, and cell surface receptors during immune responses and tissue inflammation [10–15]. HA is normally present in the superficial lining of healthy synovium, whereas abnormal accumulation of HA has been found in rheumatic synovium, especially in areas around blood vessels, proliferating cells, and infiltrating leukocytes to a greater degree in subjects with RA than OA [16,17]. T cells are present in areas enriched with FLS in arthritic joints, but little is known about whether the two cell types interact to drive the ECM remodeling that occurs in arthritic joints. Given that autoreactive and chronically active CD4 T cells are known to drive pathology of RA [18], we wanted to focus on whether this interaction drives events responsible for the inflammation in arthritic joints. Furthermore, whether the crosstalk between active CD4 T cells and FLS generates HA-rich ECM and whether this HA-rich ECM in the synovium of arthritis patients plays a role in regulating immune responses are not yet clear.

In this study, we established a co-culture model with activated CD4 T cells and FLS that lines the joint synovium from normal subjects and subjects with OA or RA to determine 1) the impact that activated CD4 T cells have on the expression of genes involved in the generation of an HA-rich pro-inflammatory ECM; 2) whether differences exist in the expression of these genes generated by the co-cultures derived from either OA or RA joints; 3) the cellular source of these differences in expression; and 4) the effect on the inflammatory phenotypes of these cells when HA synthesis is blocked.

Results

Activated CD4 T cells stimulate the generation of an HA-enriched ECM when co-cultured with FLS and promote pro-inflammatory cytokine expression and leukocyte adhesion

To determine whether activated CD4 T cells stimulate ECM production when cultured with FLS cells from human joints, we adapted a co-culture model with activated CD4 T cells and lung fibroblasts previously developed by our group (Fig. 1A) [19]. Normal human FLS isolated from human joints that were co-cultured with activated CD4 T cells generated an ECM that stained intensely for HA compared to the ECM deposited by FLS co-cultured with non-activated CD4 T cells. With non-activated CD4 T cells, the HA was confined primarily to the cell surface of the FLS and the cells were more elongated (Fig. 1B), whereas in co-cultures with activated CD4 T cells, there was increased staining intensity in the ECM; and the HA was organized into filamentous strands with some cable-like structures throughout the ECM (Fig. 1C). Morphologically, the FLS appeared spread out and flattened, similar to the phenotypes shown in previous studies in which double-stranded RNA-mediated TLR3 activation of fibroblasts increased production of HA [20,21]. In addition, a number of activated CD4 T cells exhibited strong HA staining of their cell surfaces and were associated with the filamentous HA-positive structures (white arrows in Fig. 1C).

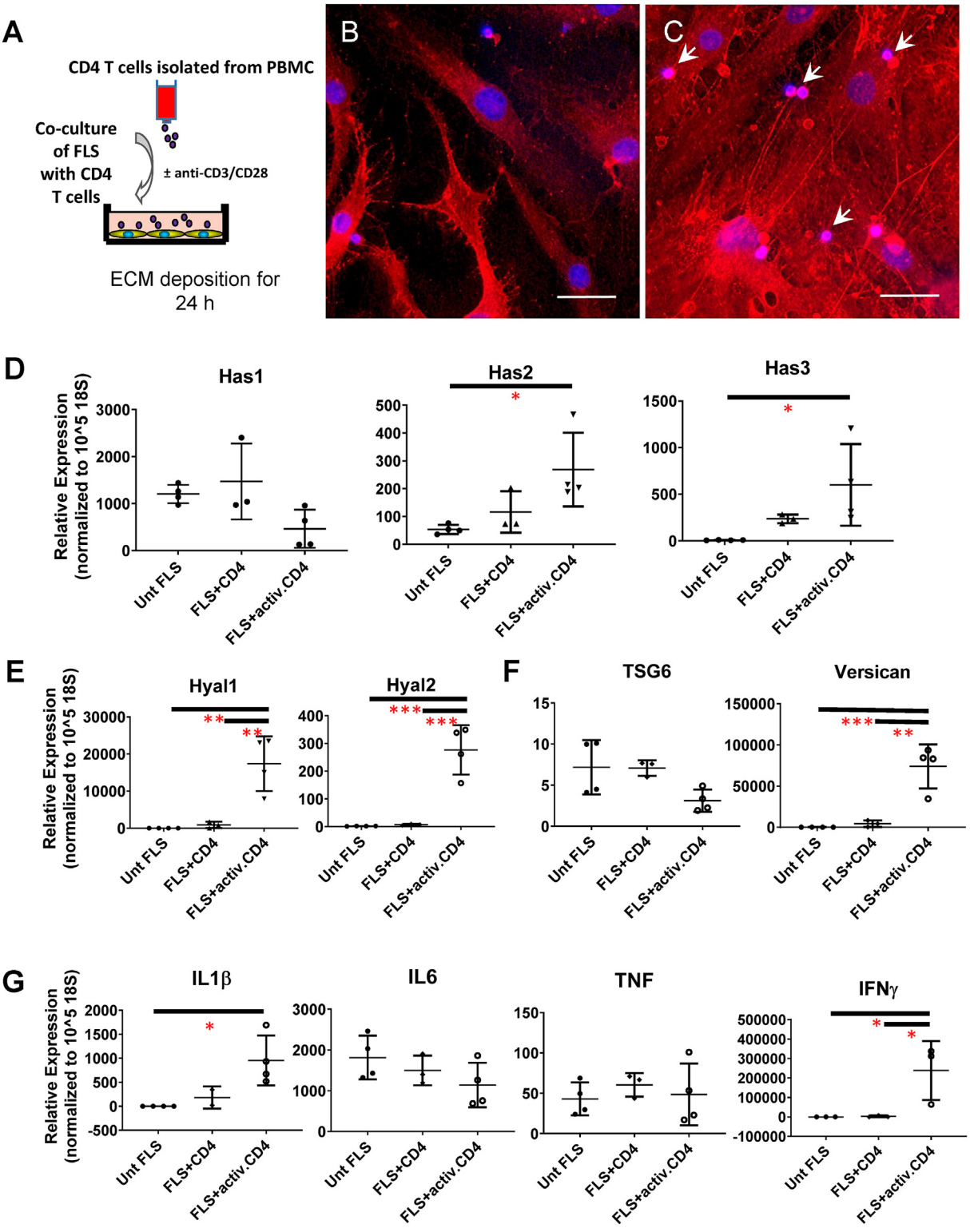
We also found that mRNA expression of HA-modifying enzymes such as hyaluronan synthase (HAS)-2 and -3 and hyaluronidase (HYAL)-1 and -2 were significantly increased in the co-cultures with activated CD4 T cells (Fig. 1D, E). In addition, the expression of HA binding proteins (also termed hyalectins), such as versican, increased while the expression of TSG-6 did not change (Fig. 1F). These findings collectively indicate that there is crosstalk between activated CD4 T cells and FLS that can regulate the expression of genes involved in the generation of an HA-enriched ECM in highly specific ways. In addition to the ECM components, we examined inflammatory cytokine expression in the co-cultures containing activated CD4 T cells and found significant increases in IL1β and IFNγ with no changes in IL6 and TNFα (Fig. 1G). These results indicate that inflammatory cytokine expression in the co-cultures is influenced by the crosstalk between these two cell types.

Another characteristic of an inflammatory ECM phenotype is in the ability to bind and retain leukocytes [22]. As shown in Fig. 2, activated CD4 T cell/FLS co-cultures bound greater numbers of monocytes compared to the co-cultures containing non-activated CD4 T cells. This binding could be reduced by *Streptomyces* hyaluronidase pretreatment of the co-cultures, indicating a primary role for HA in retaining the monocytes in these co-cultures.

Fig. 1. FLS-CD4 co-culture accumulates HA-enriched ECM and stimulates inflammatory cytokine expression. (A) Schematics of normal FLS-CD4 co-culture experiments. (B-C) Immunofluorescence images of FLS exposed to CD4 T cells in the absence (B) or presence (C) of anti-CD3/CD28 antibodies. HA staining is shown in red and nuclear staining is shown in blue. Scale bar = 50 μm. (D-F) FLS were cultured alone or co-cultured with unstimulated or anti-CD3/CD28 activated CD4 T cells and were harvested and examined for expression of (D) HA synthases, (E) HA-degrading enzymes, (F) HA binding proteins, and (G) inflammatory cytokines IL1β, IL6, TNFα, and IFNγ, by qPCR. Data are from four independent experiments using PBMCs from four healthy donors and primary FLS from one healthy donor. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in Tukey's post hoc after one-way ANOVA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Differences in the accumulation of monocyte-adhesive HA-enriched ECM generated by the co-cultures of FLS from OA and RA patients

To determine if differences existed in the capacity of co-cultures containing FLS from OA vs. RA patients to generate an HA-enriched ECM that binds inflammatory cells such as monocytes, FLS isolated from joints of individuals with OA and RA were cultured in the presence of activated CD4 T cells. Co-cultures containing OA or RA FLS



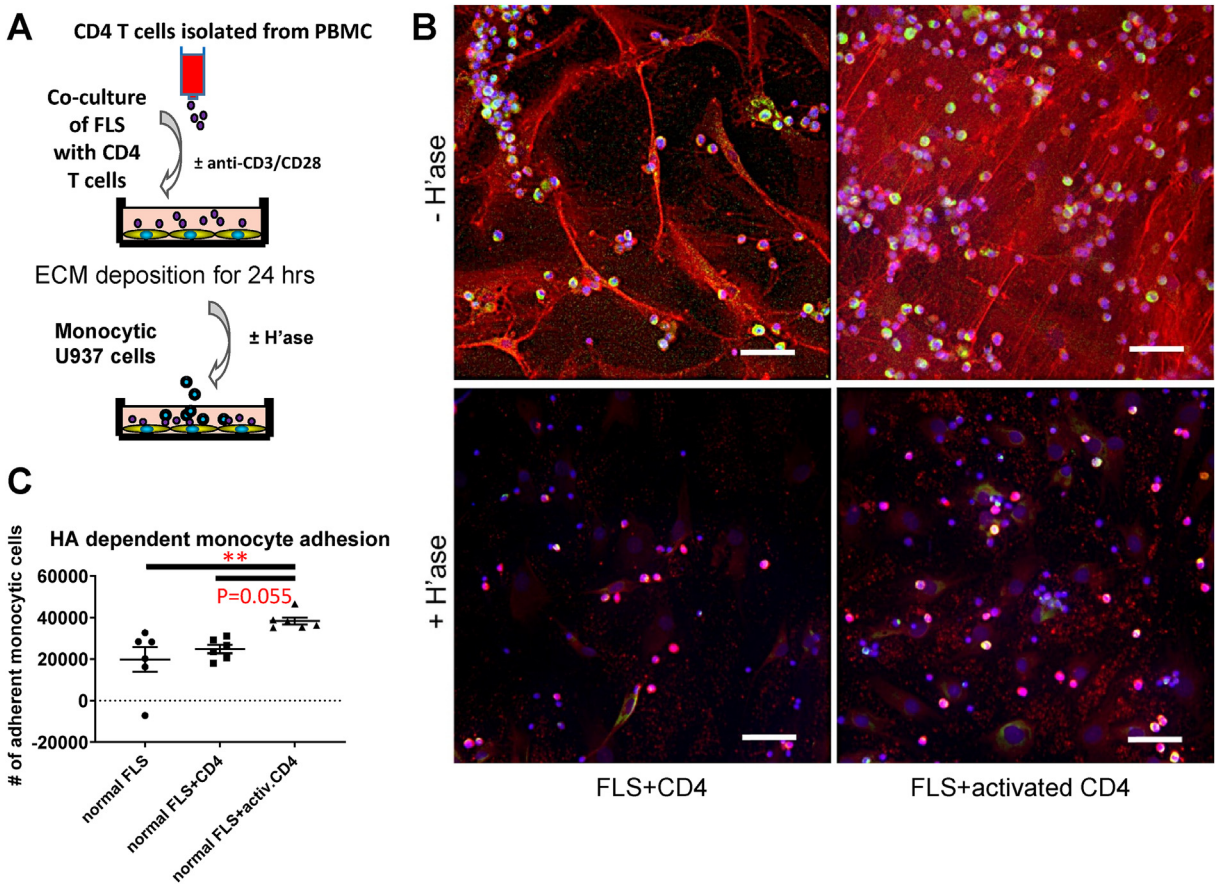


Fig. 2. Activated CD4 T cells and normal FLS generate an HA-enriched ECM that promotes monocyte adhesion. (A) Schematics of monocyte adhesion on normal FLS-CD4 co-culture. (B) Immunofluorescence images of monocytic U937 cells stained for CD68 adhering to the co-culture of activated CD4 T cells with normal FLS pretreated with or without hyaluronidase. HA staining is shown in red, monocyte marker CD68 staining is shown in green, and nuclear staining in blue. Scale bar = 50 μm. (C) Quantitation of hyaluronidase-sensitive adhesion of fluorescently labeled U937 monocytic cells examined by differences in fluorescence in the wells with and without hyaluronidase treatment. Data are from 3 OA and RA FLS cell lines from 3 subjects per group and PBMC from one healthy donor. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ in Tukey's post hoc after one-way ANOVA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

exposed to activated CD4 T cells generated an HA-rich ECM (Fig. 3A-B). No apparent differences in the amounts of HA in OA FLS and RA FLS co-cultures were found. Notably, greater levels of HA were found in media than in cell layers. When the co-cultures were treated with 4-methylumbelliferone (4MU), a pharmacological inhibitor of HA synthesis, HA accumulation was blocked in both the OA FLS and RA FLS cultures (Fig. 3). 4MU inhibits HA synthesis by covalently binding to glucuronic acid, thus preventing the enzyme UDP-glucose 6-dehydrogenase (UDPDH) from generating one of the precursors of HA, UDP-glucuronic acid (UDP-GlcUA), which is necessary for formation of disaccharide units of HA, along with UDP-N-acetyl-glucosamine (UDP-GlcNAc) [23]. As a result, 4MU blocks HA generated from UDP-GlcUA and UDP-GlcNAc by HASes [24].

The addition of human monocytes to the co-culture of activated CD4 T cells/FLS revealed interesting differences depending on whether the FLS were derived from OA or RA patients. RA FLS exposed to activated CD4 T cells showed a tendency toward an increase in the levels of monocyte adhesion compared to OA FLS (Fig. 4). Monocyte adhesion was reduced by *Streptomyces* hyaluronidase pretreatment of the co-cultures, indicating a specific involvement of HA and/or HA-associated molecules in the monocyte adhesion phenotype between OA and RA FLS co-cultures (Fig. 4).

Co-cultures of OA and RA FLS with activated CD4 T cells exhibit elevated expression of inflammatory cytokines

We found also that the expression of IFNγ, TNFα, IL1β, and IL6 were significantly increased in the

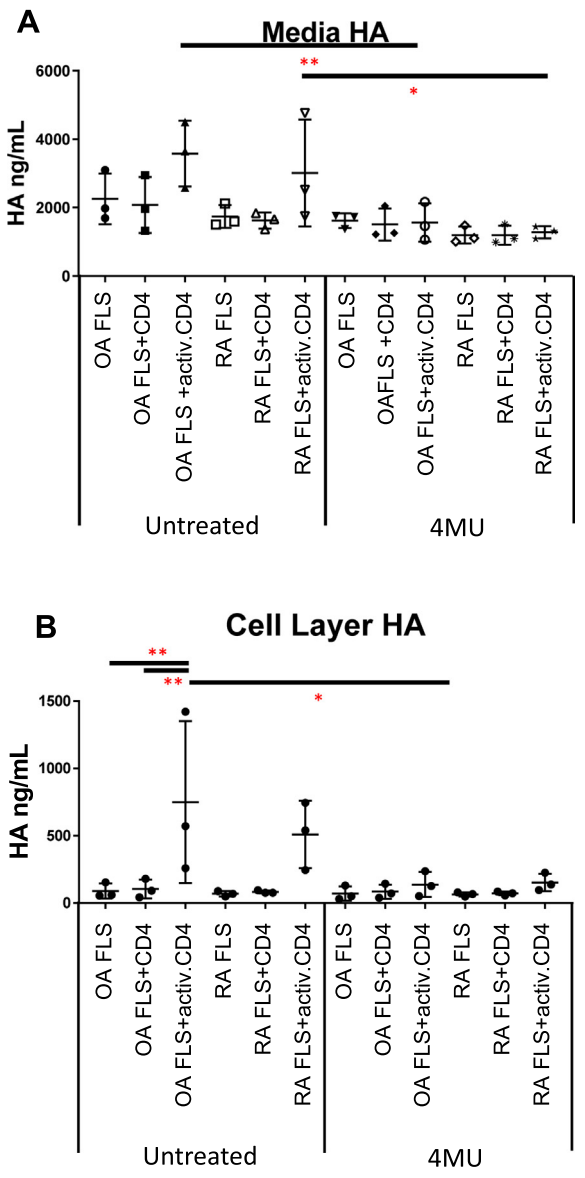


Fig. 3. OA and RA FLS co-cultured with activated CD4 T cells promotes elevated HA accumulation and secretion. Quantification of HA in (A) media and in (B) cell layers generated by OA and RA FLS alone, co-cultures of FLS with unstimulated CD4, FLS with CD4 stimulated with anti-CD3/CD28 antibodies in the absence or presence of 4-methylumbelliferone (4MU). Data are from 3 OA and RA FLS cell lines from 3 subjects per group and PBMC from one healthy donor. $^{*}P < 0.05$, $^{**}P < 0.01$ in Bonferroni's post hoc after two-way ANOVA.

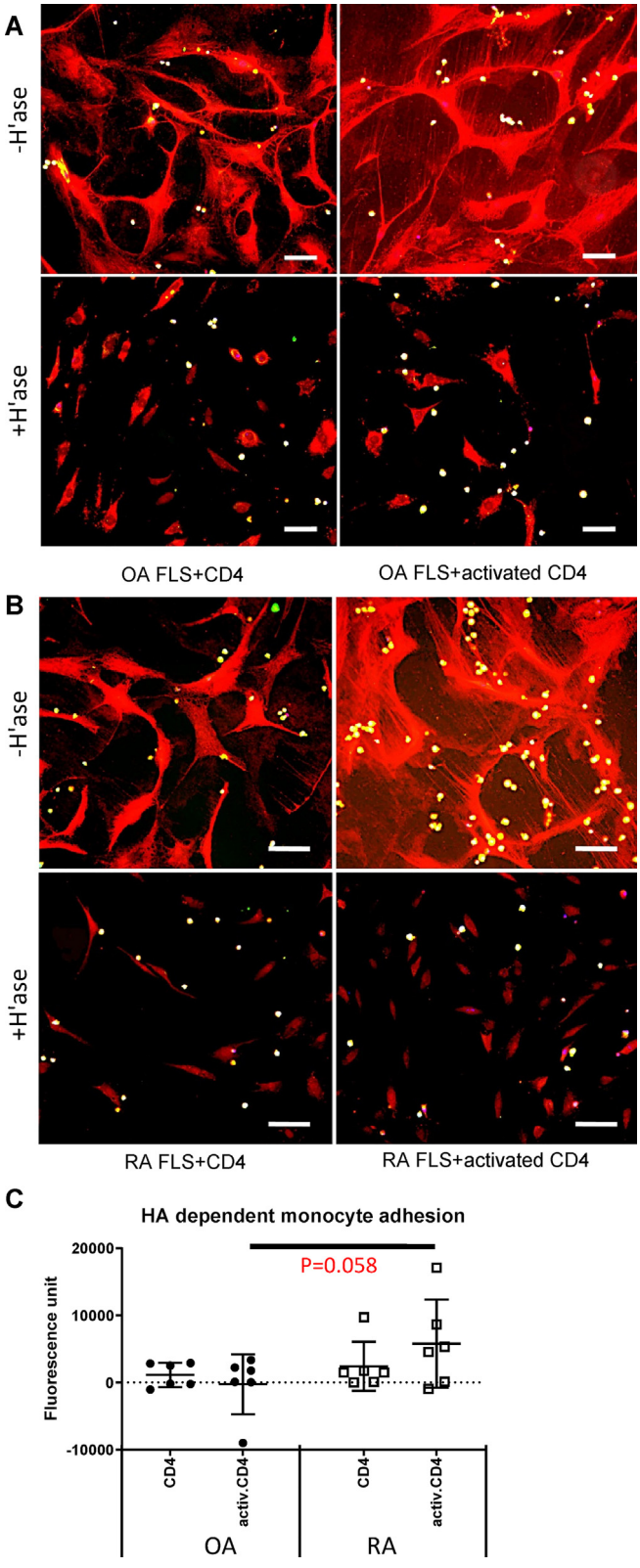
co-cultures of activated CD4 T cells with OA and RA FLS (Fig. 5). Moreover, the co-culture of activated CD4 T cells with RA FLS showed significantly elevated levels of IL6 and IL1β expression (Fig. 5A-B), whereas the co-cultures of both OA and RA FLS with activated CD4 T cells showed elevated expression levels of IFNγ and TNFα (Fig. 5C-D). Strikingly, all of these increases in cytokine expression were abolished by treatment with 4MU, in both the OA and RA FLS cultures (Fig. 5). These findings suggest that the blockade of glucuronic acid in the HA synthetic pathway may play a critical role in promoting expression of cytokines that activate type I immune responses in CD4 T cells and FLS.

Differential contributions of CD4 T cells and FLS to ECM and cytokine gene expression

Crosstalk between activated CD4 T cells and FLS can influence the inflammatory phenotype of either of the cell types in the co-culture. Given their relative size and amount of synthetic machinery, the FLS are likely responsible for the bulk of the ECM synthesized in the co-cultures. However, both FLS and CD4 T cells are capable of synthesizing and secreting components that may modify the ECM [22]. Therefore, to assess the contribution of each cell type in the production of the HA-enriched ECM and to determine whether differences existed between co-cultures of OA and RA FLS cells, we separated the CD4 T cells from the FLS and examined gene expression after 14 h of co-culture. Expression of the specific fibroblast marker PDGFRB exclusively in FLS, as well as expression of the lymphocyte marker CD3e only in CD4 T cells, confirmed the successful separation of the two cell populations (Supplemental Fig. 1).

We first examined the expression of HA-modifying genes and the effect of 4MU on these genes for each cell type in the co-cultures (Figs. 6-7). FLS and activated CD4 T cells separated from the co-culture exhibited comparable levels of HAS1, 2, and 3 expression (Fig. 6). This was unexpected since CD4 T cells, unlike stromal cells such as FLS, are not regarded as a major source of HA. 4MU treatment significantly reduced expression of selective HAS isoforms in OA and RA FLS co-cultured with activated CD4 T cells (Fig. 6). HAS2 levels in RA FLS and HAS3 levels in OA FLS showed significant decreases with 4MU treatment (Fig. 6D, F). Unexpectedly, 4MU significantly increased expression of enzymes that degrade HA, including HYAL2 and CEMIP2 (Cell Migration-Inducing Hyaluronidase 2), by both OA and RA FLS exposed to activated CD4 T cells (Fig. 7B, D). Notably, expression of the HA degradative enzyme CEMIP2 appeared more prominent in activated CD4 T cells compared to FLS as denoted by different Y-axis scales used for each cell type (Fig. 7C-D). In addition, the expression of two HA binding proteins, TSG6 and HA, as well as proteoglycan link protein 3 (HAPLN3) showed differences between RA and OA FLS. First, TSG-6 showed a tendency toward greater expression in RA compared to OA FLS (Fig. 7F). Second, HAPLN3 levels showed significant reduction with

4MU treatment in activated CD4 T cells exposed to OA or RA FLS (Fig. 7G), as well as in the RA FLS themselves (but not in the OA FLS) (Fig. 7H). Of note, expression of HAPLN3 in CD4 T cells and FLS was comparable, suggesting that activated CD4 T cells as well as FLS contribute to the HA-rich ECM by synthesizing one of the HA binding proteins. In contrast, HAPLN1 transcript levels



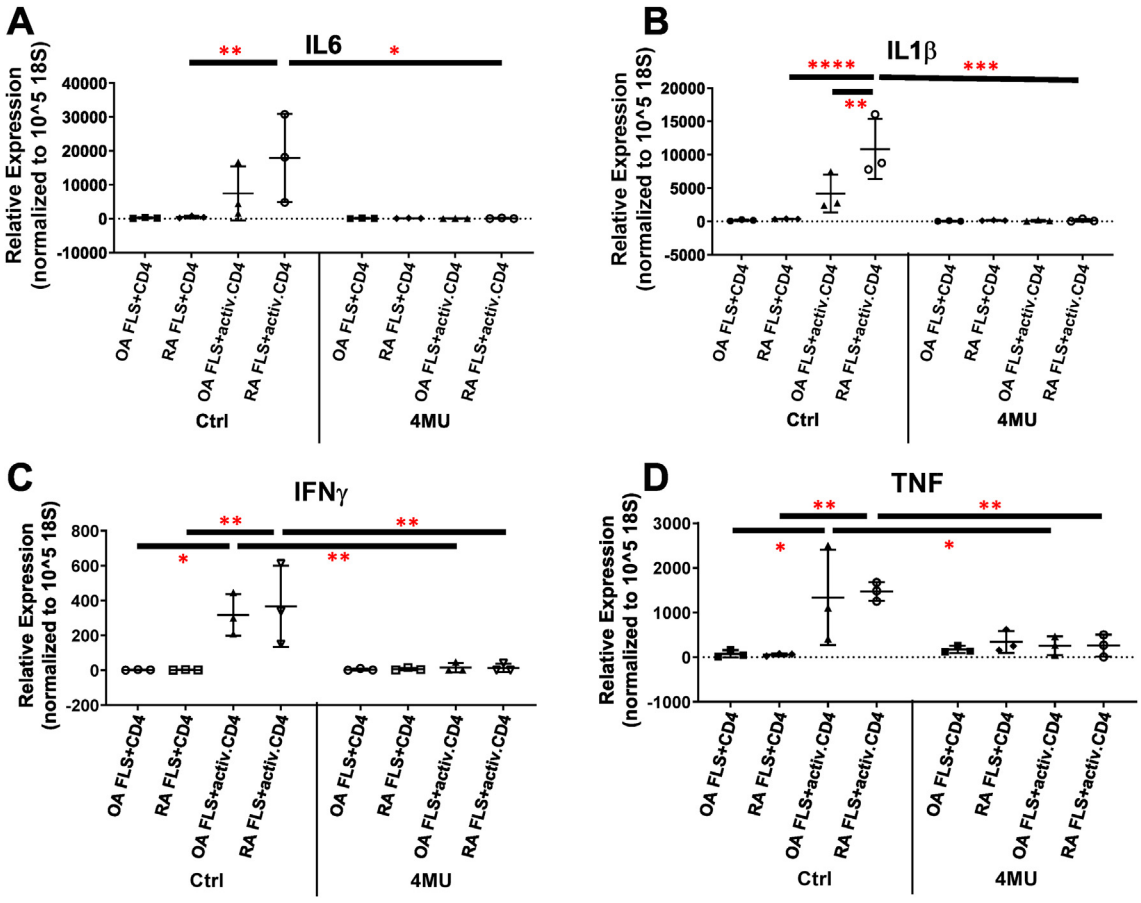


Fig. 5. Expression of inflammatory cytokines in the co-cultures of OA and RA FLS with activated CD4. (A) IL6, (B) IL1β, (C) IFNγ, and (D) TNFα mRNA levels were examined in the co-culture in the presence and absence of 4MU. $n = 3$ (OA and RA FLS cell lines from 3 subjects per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ in Bonferroni's post hoc after two-way ANOVA.

were very low or not detectable in both cell types (data not shown). These findings highlight the importance of crosstalk between activated CD4 T cells and FLS in generating a pro-inflammatory HA-enriched ECM in arthritic joints. In addition, our results demonstrate that treatment with 4MU has effects beyond inhibition of HA synthesis, including increases in the expression of HA-degrading enzymes and decreases in the expression of HA binding proteins.

To determine the cellular source of the cytokines in the co-cultures, we examined their expression separately in the activated CD4 T cells and the FLS as described above. Notably, IL6 was the only cytokine that showed higher expression levels in the FLS compared to the activated CD4 T cells, and here, RA FLS had significantly higher levels of IL6 transcript than OA FLS (Fig. 8A). In contrast, IL1β and TNFα levels were more prominent in activated CD4 T cells than in FLS but no differences were found between the OA or RA FLS co-cultures (Fig. 8B-C). IFNγ levels in FLS and activated CD4 T cells were comparable to each other and similar in the OA and RA FLS co-cultures (Fig. 8D). These results indicate that IL6, unlike IL1β, TNFα, and IFNγ, is expressed by FLS, particularly by those from RA joints.

Fig. 4. RA FLS co-cultured with activated CD4 T cells promote HA-dependent monocyte adhesion. (A-B) Immunofluorescence images of monocytes adhering to the co-culture of OA FLS with CD4 T cells (A) or to the co-culture of RA FLS with CD4 T cells (B). HA staining is shown in red and calcein AM preloaded monocytes are shown in green. Scale bar = 50 μm. (C) Quantification of hyaluronidase-sensitive monocyte adhesion measured by reading fluorescence from prelabeled monocytes. Data are from 6 OA and RA FLS cell lines from 6 subjects per group and PBMC from one healthy donor. $P = 0.058$ in Bonferroni's post hoc after two-way ANOVA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

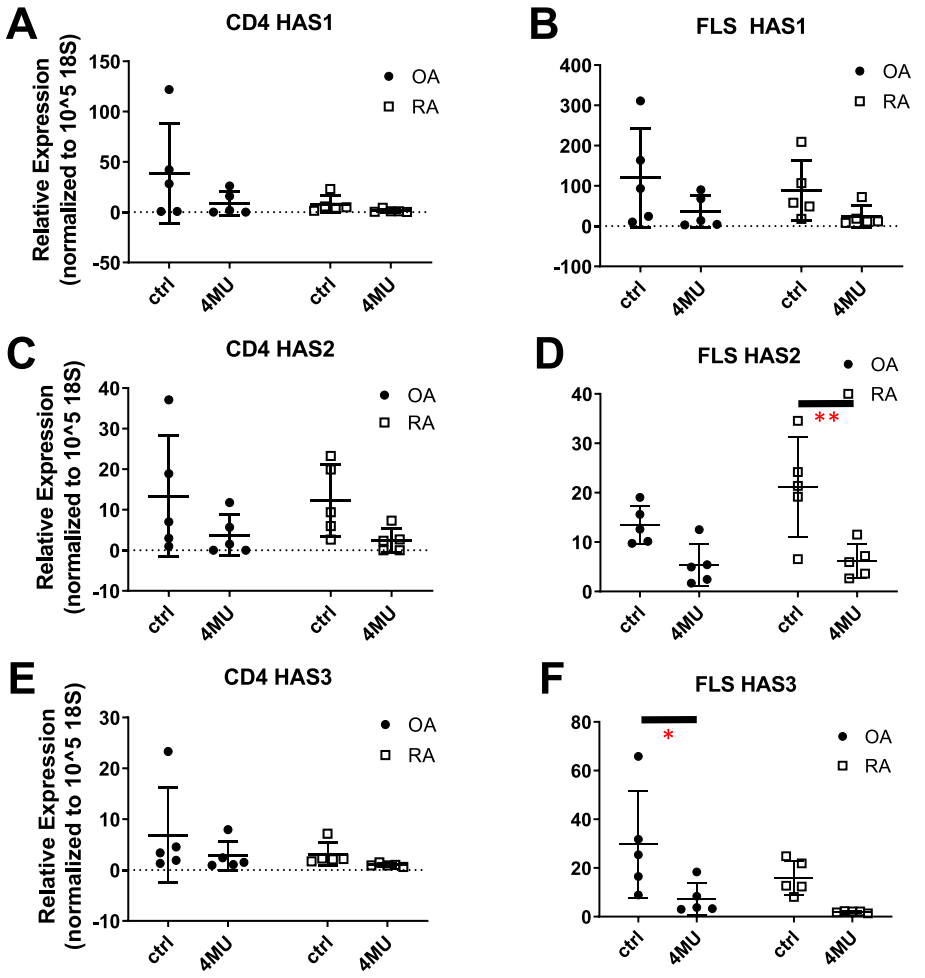


Fig. 6. Effect of 4MU on the expression of HA synthases in activated CD4 T cells and OA and RA FLS separated from the co-culture. Expression of (A-B) HAS1, (C-D) HAS2, and (E-F) HAS3, in activated CD4 (A, C, E) and OA and RA FLS (B, D, F) separated after 14 h of co-culture in the absence or presence of 4MU. Significant ($P < 0.05$) effect of 4MU was found in B, C, D, F in two-way ANOVA. $n = 5$ FLS per group (FLS cell lines from 5 RA and 5 OA subjects). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ in Bonferroni's post hoc after two-way ANOVA.

When the impact of 4MU treatment on activated CD4 T cells and FLS was determined, most differences between OA and RA FLS were also found in IL6, such that 4MU treatment significantly reduced IL6 expression, especially in RA FLS. 4MU treatment reduced IL6 levels in OA FLS and also in activated CD4 T cells (Fig. 9A-B). Both activated CD4 T cells and FLS showed significant decreases in IL1β with 4MU treatment (Fig. 9C-D). TNFα levels were also significantly reduced by 4MU treatment in FLS (Fig. 9E-F). 4MU treatment attenuated IFNγ levels in OA FLS co-cultured with activated CD4 T cells (Fig. 9G-H). These findings indicate potential linkages between pathways that affect HA metabolism and those that regulate inflammatory cytokine expression. It will be important to determine if this link is causal.

IL6 trans-signaling stimulates greater HA synthesis, secretion, and cytokine expression from RA FLS compared to OA FLS

Since our data showed that IL6 transcript levels were significantly higher in RA FLS compared to OA FLS, consistent with previous findings [25], we pursued IL6 trans-signaling, one of the key pathways unique to the crosstalk between activated leukocytes and stromal cells. IL6 trans-signaling relies on soluble IL6 receptor (IL6R), shed by activated leukocytes, forming a complex with IL6, which allows IL6 to activate stromal cells despite lacking surface expression of IL6R. In order to elicit IL6 trans-signaling in the absence of activated leukocytes, we used the covalently bound complexes of IL6R with IL6, termed hyper-IL6 (generous gift from Prof. Stefan Rose-John, Kiel University, Kiel, Germany) to stimulate arthritic FLS. We found that both

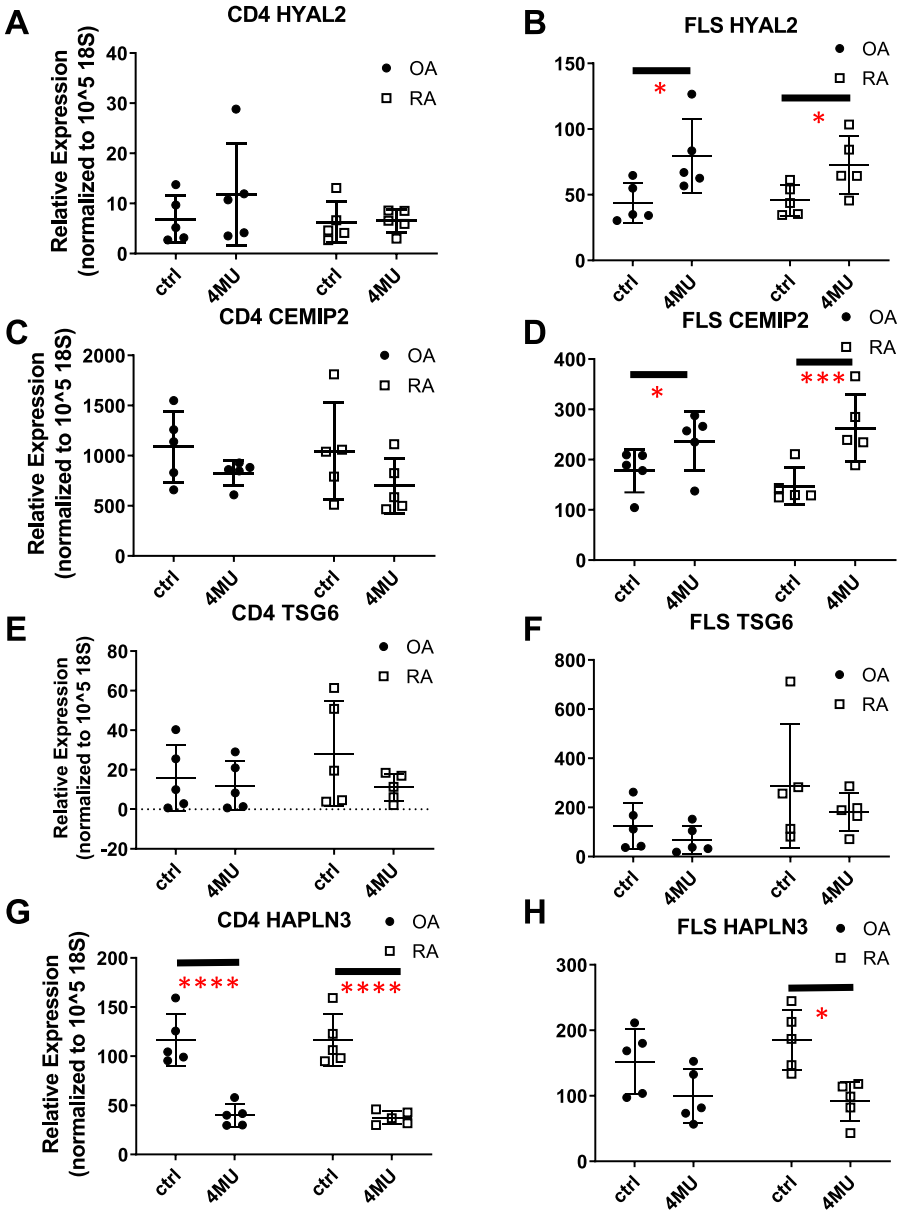


Fig. 7. Effect of 4MU on the expression of HA modifying genes in activated CD4 T cells and OA and RA FLS separated from the co-culture. (A-B) HYAL2, (C-D) CEMIP2, (E-F) TSG-6, and (G-H) HAPLN3. mRNA levels were determined in (A, C, E, G) activated CD4 T cells and (B, D, F, H) OA and RA FLS separated after 14 h of co-culture in the absence or presence of 4MU. Significant ($P < 0.05$) effect of control vs. 4MU was found in B, D, G, and H, but not in OA vs. RA in two-way ANOVA. $n = 5$ FLS per group (FLS cell lines from 5 RA and 5 OA subjects). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ in Bonferroni's post hoc after two-way ANOVA.

OA and RA FLS secreted significantly higher levels of HA when stimulated with hIL6 to trigger IL6 trans-signaling, but not with IL6 alone (Fig. 10A-B). Remarkably, RA FLS generated higher levels of HA compared to OA FLS after 24 and 48 h of hIL6 stimulation. These findings suggest that IL6 trans-signaling mimics the increase in HA induced by the crosstalk between activated CD4 T cells and RA FLS.

To determine whether blockade of HA synthesis further affects inflammatory cytokine expression induced by IL6 trans-signaling, we examined the transcript levels of IL6, IL1β, TNFα, and IFNγ in OA and RA FLS stimulated by hIL6 in the presence and absence of 4MU. We found that hIL6 significantly increases IL6 transcript levels especially in RA FLS, suggesting that IL6 trans-signaling elicits a positive feedback loop for generating more IL6 (Fig. 10C). Strikingly, when HA synthesis is inhibited by 4MU, this increase in IL6 expression in RA FLS is significantly reduced (Fig. 10C), suggesting that the HA synthetic

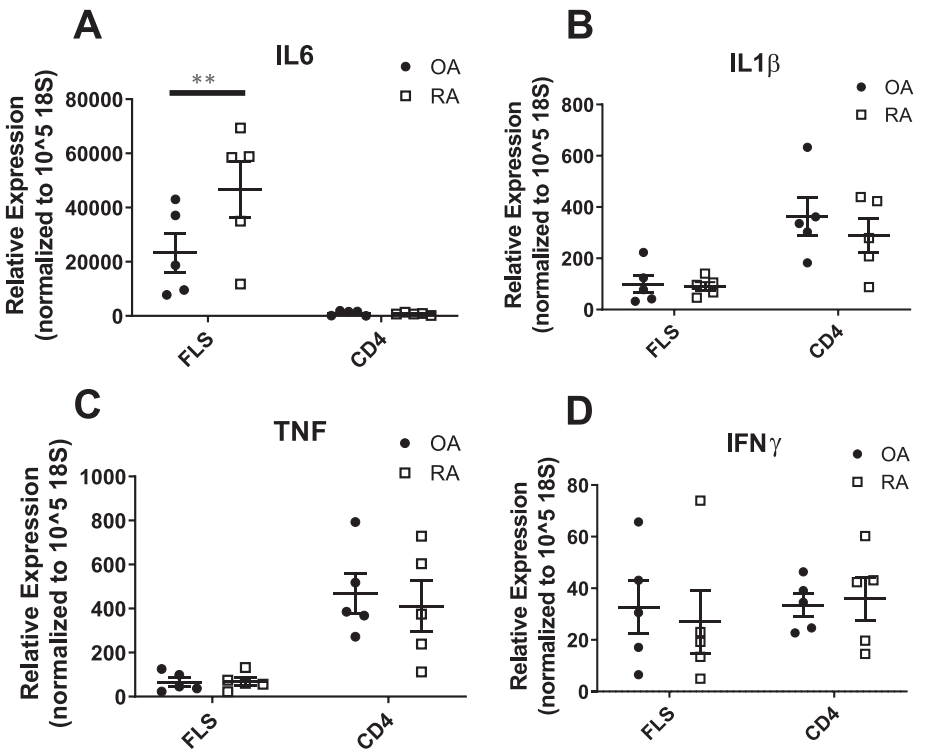


Fig. 8. Expression of inflammatory cytokines in activated CD4 T cells and OA and RA FLS separated from the co-culture. Expression of IL6 (A), IL1β, (B) TNFα (C), and IFNγ (D) in OA and RA FLS and activated CD4 T cells separated after 14 h of co-culture. $n = 5$ FLS per group (FLS cell lines from 5 RA and 5 OA subjects). $^{**}P < 0.01$, in Bonferroni's post hoc after two-way ANOVA.

pathway is a critical mediator in propagating a positive feedback loop between IL6 trans-signaling and IL6 expression, especially in RA FLS.

Discussion

In arthritic joints, HA and proteins that bind HA within the ECM undergo significant remodeling. HA is normally present in the superficial lining of healthy synovium, whereas excess accumulation of HA has been found in arthritic synovium, especially in areas around blood vessels and infiltrating leukocytes [16,17]. Synovium from OA and RA patients shows an increase in levels of enzymes involved in HA synthesis [26,27] and degradation [28–30]. Moreover, RA patients have increased levels of proteins that interact with HA, such as tenascin C, TSG-6, and IαI in their synovial fluid or serum [31–42]. Furthermore, RA patients have elevated levels of HA crosslinked with HA binding proteins in synovial fluid and serum [40,41]. FLS have been shown to play a key role in promoting persistent and chronic inflammation by producing inflammatory cytokines and ECM-degrading enzymes leading to cartilage destruction [2–9].

Immune cells are important regulators of ECM remodeling, which is a critical part of regulating the immune and inflammatory response, by producing enzymes that remodel ECM components such as matrix metalloproteinases (MMPs) [43], by generating cytokines such as IL-4 and IL-13 that induce ECM synthesis by stromal fibroblasts [44,45], and by synthesizing ECM components such as osteopontin and versican [46,47]. However, the impact of the interaction between activated leukocytes such as CD4 T cells and stromal fibroblasts on the generation of a proinflammatory ECM is not yet clear. Recently, we and others have found that stimulation of lung fibroblasts or FLS with inflammatory agonists such as polyinosinic-polycytidylic acid (poly I:C) and respiratory syncytial virus produces elevated levels of HA, which promotes adhesion and activation of CD4 T cells, monocytes, eosinophils, and mast cells [12,20,48–60]. Furthermore, we developed a co-culture model of activated CD4 T cells and FLS and found that crosstalk between these two cell types promoted the formation of an HA-rich ECM that was adhesive for leukocytes [19]. In the present study, we used this model and found that the synthesis, secretion, and formation of an HA-rich ECM are significantly increased by the interaction between activated CD4 T cells and FLS and that differences in the expression of genes responsible for this ECM depend upon whether the FLS were derived from OA or RA patients.

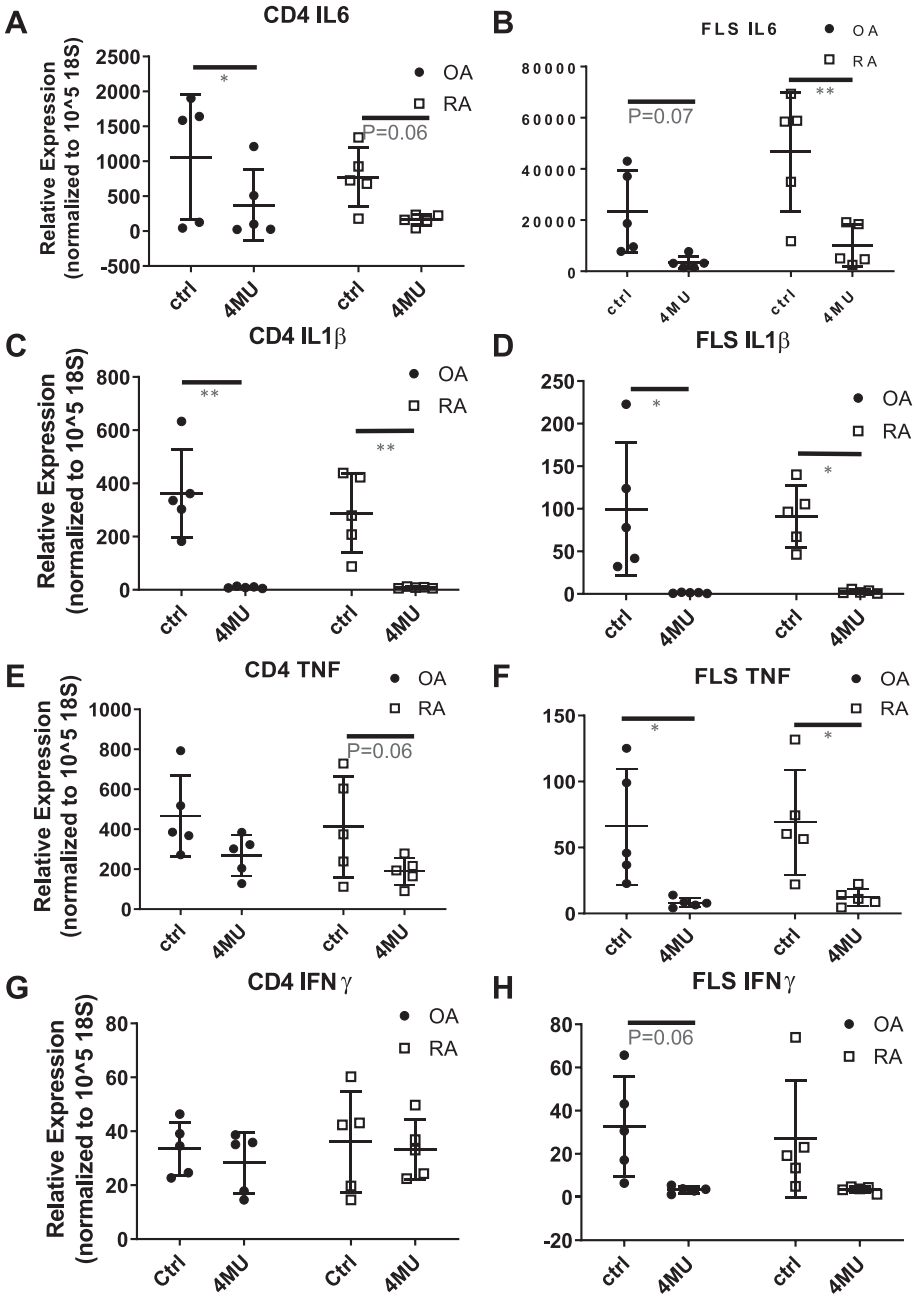


Fig. 9. Effect of 4MU on the expression of inflammatory cytokines in activated CD4 T cells and OA and RA FLS separated from the co-culture. Expression of (A-B) IL6, (C-D) IL1β, (E-F) TNFα, (G-H) IFNγ, in (A, C, E, G) activated CD4 and (B, D, F, H) OA and RA FLS separated after 14 h of co-culture in the absence or presence of 4MU was examined. Significant ($P < 0.05$) effect of control vs. 4MU was found in A, B, C, D, and F, but no significant effect of OA vs. RA was found in any groups in two-way ANOVA. $n = 5$ FLS per group (FLS cell lines from 5 RA and 5 OA subjects). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ in Bonferroni's post hoc after two-way ANOVA.

Two characteristic roles of the ECM in inflammation are: 1) the capacity to bind, activate, and retain immune cells and 2) the impact on inflammatory cytokine expression. Strikingly, we found that the HA-rich ECM generated by co-cultures of activated CD4 T cells and RA FLS, but not OA FLS, provides an adhesive substrate for monocytes. This was unexpected since the levels of HA accumulation were not different between OA and RA FLS in the co-cultures (Supplemental Fig. 1). Previously, it has been shown that the quantity of HA-rich ECM generated by the endoplasmic reticulum stress inducer tunicamycin in lung fibroblasts was much less than that generated by poly I:C treatment, whereas the degree of monocyte adhesion was similar,

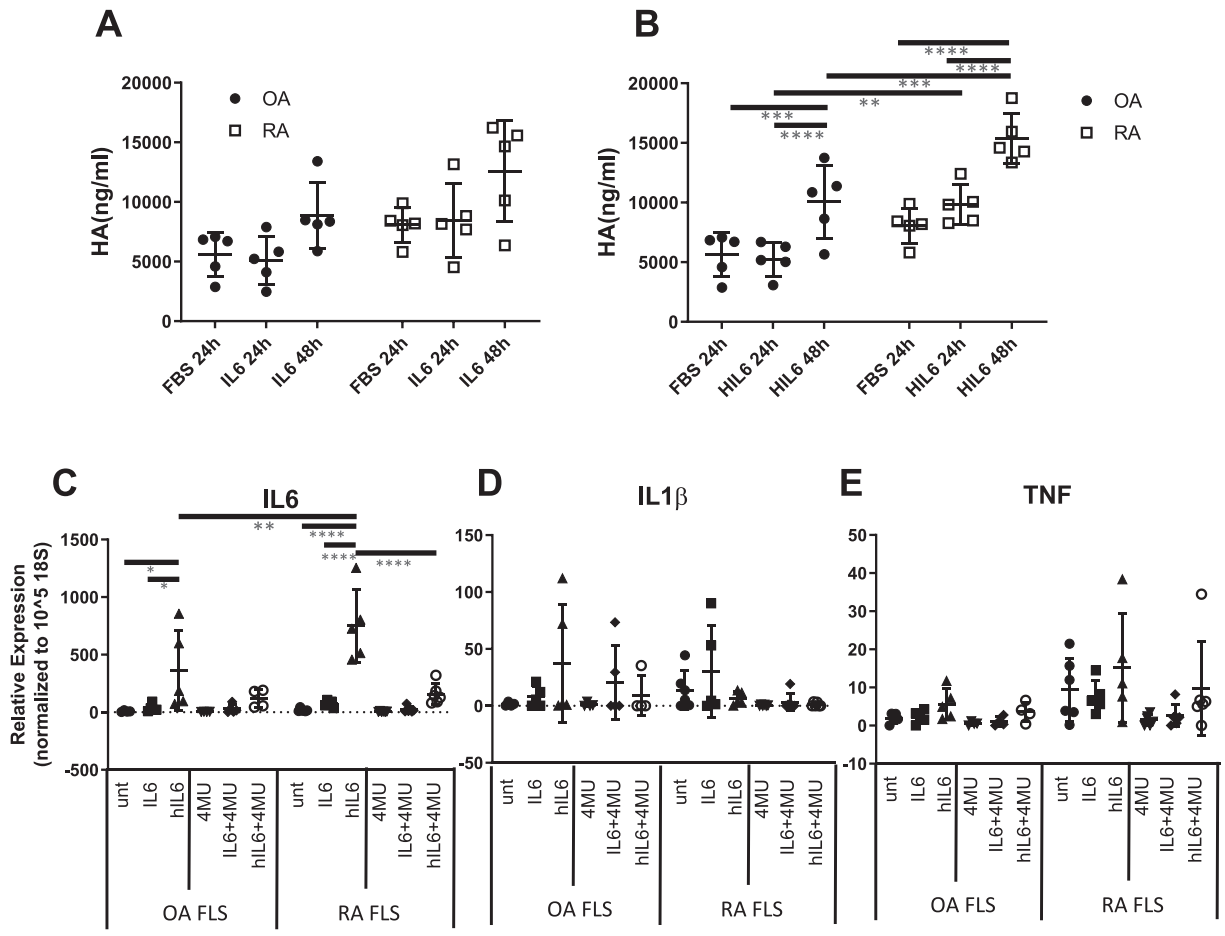


Fig. 10. HA and inflammatory cytokines generated by OA and RA FLS induced by IL6 classical- or *trans*-signaling. (A-B) HA generated by OA and RA FLS stimulated with FBS- containing media, IL6, or hyper-IL6 for 24 and 48 h. (C-E) Expression of (C) IL6, (D) IL1β, and (E) TNFα by OA and RA FLS unstimulated or stimulated by IL6 or hyper-IL6. n = 4–5 patients, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ in Bonferroni's post hoc after two-way ANOVA.

suggesting that the dominant factors for monocyte adhesion are the HA binding molecules within the HA-rich ECM and the organization of HA, rather than the amount of HA-rich ECM. One possibility explaining the difference in adhesive monocyte binding might be the increase in TSG-6 observed in RA compared to OA FLS cultures, possibly leading to enhanced HA crosslinking by TSG-6 [61,62]. In addition, we found the increase in HAPLN3 expression, which also promotes HA crosslinking, was greater in RA compared to OA FLS co-cultures. This increase may also assist in the generation of crosslinked HA-rich ECM which would promote adhesion for leukocytes (see below). HAPLN3 is a member of the HA-binding link protein family and has widespread expression in various adult tissues [63]. Accumulation of cross-linked forms of HA at sites of inflammation provides a substrate for myeloid and lymphoid cell adhesion by binding to CD44 on the surface of cells [20,48–55,64]. In other autoimmune diseases such as type 1 diabetes, HA accumulation precedes leukocyte infiltration and is prominent during early insulitis [65]. Furthermore, blocking HA synthesis and accumulation by treatment with 4MU prevented disease progression in murine models of type 1 diabetes and multiple sclerosis [66,67].

Furthermore, we were able to show that the crosstalk that occurs in the co-cultures between the activated CD4 T cells and FLS impacts both cell types. For example, unexpectedly, HAS2 and HAS3 levels expressed by activated CD4 T cells were comparable to those expressed by OA or RA FLS. This was surprising since CD4 T cells are not known to express high levels of the HAS variants and thus are not regarded as a major source of HA. One caveat is that the contaminating FLS in the CD4 T cell population after the separation may be the source of the HAS expression. However, our recent study demonstrated that CD4 T cells which infiltrated into immunogenic implants expressed significantly higher levels of HAS1 and HAS2, compared to the CD4 T cells purified from the draining lymph nodes [68]. This is consistent with the high levels of HAS2 expression by the activated CD4 T cells

in close contact with stromal cells as in our co-culture system. In a single cell RNA seq study of RA tissues, abundant expression of HAS1 and HAS2 was shown in CD55+ FLS, but not in other immune cell types including CD4+ T cells or in CD90+ FLS, a novel type of FLS identified by the study [69]. More recently, a novel type of circulating CD45- CD31- PDPN+ preinflammatory mesenchymal cells, similar to inflammatory FLS, was found in the blood from RA patients which expands 1–2 wks prior to RA flare followed by decreases during the flare [70]. HA synthases were not identified as the genes differentially expressed in these cells. However, it is possible that the CD4 T cells need to be in direct contact and within a specific time point after the activation in order to exhibit elevated levels of HAS expression, and thus a majority of CD4 T cells isolated from the inflamed synovium may not exhibit the increases in HAS of these specific subsets of CD4 T cells crosstalking to FLS as described above. In addition, and unexpectedly, 4MU significantly increased expression of HA degradative enzymes, including HYAL2 and CEMIP2, by both OA and RA FLS exposed to active CD4 T cells (see Fig. 7B, D). These enzymes have been shown to be located at cell surfaces and active in generating pro-inflammatory HA fragments in the ECM [71–74]. It is of interest that high levels of CEMIP2 were found in the CD4 T cells in the co-cultures, thus implicating these cells in turnover of HA [75,76]. In addition, our finding that HAPLN3 was generated by activated CD4 T cells suggests a novel mechanism for the remodeling of the ECM in joint disease. Such a possibility awaits experimental confirmation.

We found also that perturbation of the HA synthetic pathway in the co-cultures by 4MU affected the expression of inflammatory cytokines such as IFNɣ, IL6, and TNFα in both FLS as well as in activated CD4 T cells. The most notable difference between OA and RA FLS was that 4MU significantly reduced IL6 expression in RA FLS exposed to activated CD4 T cells, but not in OA FLS. Both OA and RA FLS showed significant reductions in TNFα when treated by 4MU. For IL1β, both OA and RA FLS as well as activated CD4 T cells showed significant reductions when treated by 4MU. There are a number of potential mechanisms whereby HA could impact inflammatory cytokine expression. HA, especially low molecular weight HA, has been shown to activate TLR2/4 and downstream signaling mediators including NFκB and to induce inflammatory cytokine production, thus acting as a danger-associated molecular pattern [12–14,77–81]. Previously, 4MU has been shown to inhibit signaling pathways downstream of HA and HA receptors (such as CD44, RHAMM, and Stabilin-2), to retard activation of ErbB2, EGFR, Akt, and NFκB, and to inhibit the expression of IL-8, CCL2, and IL1β [82–86]. When exogenous HA was added back during 4MU treatment, activation of NFκB and expression of IL8 was restored [82].

A number of studies point to an interesting parallel between 4MU and HA overloading induced by overexpressing HAS or exogenous addition of HA. For example, in corneal fibroblasts, 4MU treatment as well as the addition of high molecular weight HA significantly reduced the lipopolysaccharide-stimulated up-regulation of inflammatory cytokines including IL-1, IL-6, IL-8, TNF-α [87], suggesting that the effect of 4MU on the inflammatory cytokine was not due to depletion of extracellular HA. An alternate explanation may be the involvement of glucose metabolism including the pentose phosphate pathway and the hexosamine biosynthetic pathway, which are impacted by glucuronic acid. Indeed, inhibiting HA synthesis has been shown to enhance anaerobic glycolysis in brown adipose tissues [86]. Similarly, in a series of studies [88,89], Knudson's group has shown that the overproduction of HA driven by overexpression of HAS2 as well as inhibition of HA synthesis via 4MU had a chondroprotective effect driven by a shift in energy metabolism, with reduced glycolysis and enhanced mitochondrial respiration. It is likely that the effect of 4MU on CD4 T cells and FLS also involves a shift in energy metabolism in both cell types, especially since the increase in glucose metabolism has been identified as a crucial player of CD4 T cell activation [90]. Recent studies indicate that glucose metabolism is dysregulated in CD4 T cells as well as in FLS from RA subjects [91,92] and that targeting glucose metabolism provides therapeutic benefit to prevent synovitis [93–95]. These findings suggest that targeting the hexosamine biosynthetic pathway via 4MU may inhibit inflammatory cytokine production by altering glucose metabolism. Furthermore, it is possible that the extracellular HA interaction with HA receptors, or the dysregulated glucose metabolism affected by the hexosamine biosynthetic pathway, could potentiate inflammatory cytokine expression by activating NFκB. Further experimentation is needed to confirm this.

We found that the increase in HA could be promoted in vitro by stimulating FLS with IL6 trans-signaling. IL6 has been identified as a keystone cytokine that regulates many aspects of chronic inflammation and autoimmune diseases including RA [96]. Highly elevated levels of IL6 have been shown in serum, synovial fluid, and FLS of RA patients, the levels of which correlate with disease severity [25,97–99]. CD4 T cells from patients with type 1 diabetes show enhanced responses to IL6 and downstream IL6 target genes including cytokines involved in T cell homing as well as ECM-modifying enzymes involved in T cell trafficking [100]. In classical signaling pathways, IL6 forms a complex with membrane-bound IL6R, binds to gp130, and induces dimerization of gp130, which further recruits and activates JAK/STAT and down-

stream MAPK. However, cells lacking membrane-bound IL6R such as endothelial cells and fibroblasts can still be activated by IL6 in trans-signaling pathways. In such cases, IL6 forms a complex with soluble IL6R, often shed by activated leukocytes, that binds gp130 and further activates downstream JAK/STAT and PI3K/Akt signaling [97,101]. Anti-IL6R antibody as well as inhibitors of other signaling mediators of IL6 pathways have been a successful treatment for RA patients [102]. In the present study, our data suggest that IL6 trans-signaling stimulates RA FLS (but not OA FLS) to generate greater amounts of HA, with a concomitant significant increase in IL6 expression. In order to confirm the involvement of IL6 trans-signaling in the increases in HA generation during the crosstalk between RA FLS and CD4 T cells, blocking IL6R or gp130 during the co-culture would be required in future investigations. IL6 may enhance HAS expression by activating NFκB, which in turn would bind to NFκB binding regions in HAS promoters (50–52,74). Additionally, activation of JAK/STAT may enhance HA synthesis via an increase in glucose transporter (GLUT) expression. Previously, JAK/STAT blockade in RA FLS has been shown to block glycolysis along with reductions in expression of glycolytic enzymes as well as GLUT1 [103]. As an increase in intracellular glucose leads to enhanced glycolysis, it can also enhance the hexosamine biosynthetic pathway, leading to increased HA synthesis [104]. Thus, the increase in GLUTs induced by JAK/STAT activation is likely to mediate increases in HA. It may be that blocking HA synthesis via 4MU could break this feedback loop of IL6 expression stimulated by IL6 trans-signaling, suggesting that a positive feedback loop may exist between pathways responsible for the expression of IL6 and HA.

Overall, our study demonstrates that generation of an HA-rich ECM as a result of the crosstalk between activated CD4 T cells and RA FLS is a critical mediator of inflammatory events. These findings may lead to the identification of novel targets for therapeutic interventions for joint disease.

Materials and methods

Purification and culture of FLS. Normal FLS (Cell Applications) were cultured in Dulbecco's modified high glucose Eagle medium (DMEM; Hyclone) supplemented with 10% FBS (Atlas Biologicals), 2 mM GlutaMAX-1 (Life Technologies), 1 mM Na pyruvate (Hyclone) and penicillin–streptomycin (penicillin G sodium, 100 U/ml, and streptomycin sulfate, 0.10 mg/ml; Hyclone) complete DMEM at 37 °C in 5% CO_2 and used up to passage 10.

Primary cell lines of OA and RA FLS were either provided as generous gifts from Drs. Firestein and Boyle (UCSD, San Diego, CA) or were purified from discarded surgical specimens from subjects with OA or RA undergoing arthroplasty at Virginia Mason Medical Center (Seattle, WA). Tissue samples were collected with IRB approval by the Clinical Core of Benaroya Research Institute at Virginia Mason (BRI), following protocols from Drs. Firestein and Boyle with modifications [105]. Briefly, the surgically removed synovial specimen was cleaned to remove fat tissues, macerated into few-millimeter pieces, followed by digesting in 100 μg/ml Liberase TL (Roche), and 100 μg/ml DNA I (Roche) in RPMI 1640 (Hyclone) medium at 37 °C for 30 min. Digestion was arrested by adding 20 ml of 10% FBS in RPMI medium, the dissociated tissue was filtered through a 70 μm mesh size strainer, and centrifuged at 1500 RPM for 10 min. Isolated cells were cultured in DMEM supplemented as described above.

Isolation of CD4 T cells. CD4 T cells were isolated from cryopreserved human peripheral blood mononuclear cell (PBMC) samples obtained from healthy individuals and biobanked with IRB approval at the Clinical Core of BRI. Cells were thawed and washed twice with RPMI 1640 (Hyclone) with 10% fetal bovine serum (FBS; Atlas Biologicals), 2 mM GlutaMAX-1 (Life Technologies), 1 mM Na pyruvate (Hyclone), and penicillin–streptomycin (penicillin G sodium, 100 U/ml, and streptomycin sulfate, 0.10 mg/ml, Hyclone). CD4 T cells were isolated using the negative selection CD4 + T Cell Isolation Kit (Miltenyi Biotec) as per manufacturer's instructions.

Co-culture of FLS and CD4 T cells. Co-culture of FLS and CD4 T cells was performed as previously described [19]. Briefly, FLS were seeded at 1×10^5/well in 12-well plates in complete DMEM for 24 h at 37 °C in 5% CO_2. After 24 h, medium was replaced with fresh complete DMEM supplemented with 1 μg/ml anti-CD3 antibody clone OKT3 (eBioscience) and 0.25 μg/ml anti-CD28 antibody clone CD28.2 (eBioscience), 3×10^5 CD4 T cells in medium, or 3×10^5 CD4 T cells in medium supplemented with 1 mg/ml anti-CD3, and 0.25 mg/ml anti-CD28 antibodies. In some experiments, 100 μg/ml 4MU was added during co-culture. After 24 h of co-culture, plates were centrifuged at 300 g for 10 min, and conditioned media and cell layers were harvested. In some experiments, CD4 T cells were separated from FLS by gentle washing after 14 h of co-culture, since we found that a proportion of CD4 T cells start to transmigrate under the FLS layer after this time point, thus making separation of the two cell types difficult.

Hyper-IL6 and IL6 stimulation of FLS monoculture. FLS were seeded at 1×10^5/well in 12-well plates in complete DMEM for 24 h at 37 °C in 5% CO_2. After 24 h, medium was replaced with fresh complete DMEM supplemented with 0.1% FBS for 48 h, followed by addition of 20 ng/

ml of hIL6 (generous gift from Dr. Stefan Rose-John, Institute of Biochemistry, Kiel University, Kiel, Germany) or 10 ng/ml IL6 (equimolar concentration of IL6 contained in hIL6) for 4 h for quantifying RNA or for 24 and 48 h for quantifying HA. In some experiments, 100 μg/ml 4MU was added during hIL6 or IL6 stimulation. After 24 h of co-culture, conditioned media and cell layers were harvested separately.

HA quantification. HA secretion into the media and accumulation in the cell layer were quantified by a competitive enzyme-linked sorbent assay (ELSA), as previously described [106]. Briefly, conditioned media and cell layers from FLS-CD4 co-cultures were digested with pronase (500 μg/ml) overnight at 37 °C followed by heat-inactivation at 100 °C for 10 min. Digested media and cell layers were mixed with biotinylated HA-binding protein for 1 h at room temperature and added to microtiter plates coated with bovine serum albumin-conjugated HA. The plates were then incubated with peroxidase-labeled streptavidin for 20 min, followed by incubation with peroxidase substrate (0.03% H_2O_2, 0.5 mg/ml 2,2′-azino-bis(3-ethylbenz thiazoline)6-sulfonic acid in 0.1 M sodium citrate, pH 4.2). After 30 min, the reaction was terminated by adding 2 mM sodium azide and the absorbance at 405 nm was measured. The absorbance is inversely proportional to the level of HA in the sample, as calculated from a standard curve.

Monocyte adhesion assay. A monocyte adhesion assay was performed as previously described [51]. Monocytic U937 cells were purchased from ATCC and maintained in RPMI 1640 media supplemented with 10% FBS, 2 mM GlutaMAX-1 (Life Technologies), 1 mM Na pyruvate (Hyclone) and penicillin–streptomycin (penicillin G sodium, 100 U/ml, and streptomycin sulfate, 0.10 mg/ml; Hyclone). After 24 h of co-culture of FLS and CD4 T cells, plates were centrifuged at 300 g for 10 min and media was removed. U937 cells were labeled with 0.5 μg/ml Calcein AM (Invitrogen) for 45 min, washed in phenol red free RPMI twice, resuspended at 1×10^6/ml, and added to the co-culture at 4 °C. Some wells were pretreated with 2 U/ml *Streptomyces* hyaluronidase (Sigma-Aldrich) for 30 min prior to adding monocytes. Monocytic cells were allowed to bind for 90 min at 4 °C. Unbound monocytic cells were removed by washing with cold RPMI medium. Monocyte binding was measured by exciting the Calcein AM at 485 nm and reading absorbance at 530 nm using an EnSpire Microplate Analyzer (Perkin Elmer) [51]. HA-dependent monocyte adhesion was calculated as the fluorescence without hyaluronidase minus the fluorescence after hyaluronidase treatment. In order to calculate cell numbers from fluorescence units, a standard plate of Calcein AM-labeled cells with known numbers was examined.

Immunofluorescence staining of FLS-CD4 T cell co-culture. FLS-CD4 T cell co-cultures with or without monocytic cells were fixed with 10% formalin, 70% ethanol, and 5% acetic acid for 10 min at room temperature, washed with PBS, and incubated in blocking buffer (2% BSA, 2% normal donkey serum in PBS), prior to staining for the monocyte marker CD68 (mouse monoclonal KP-1 antibody against human CD68 (Abcam), 1:100 dilution), and biotinylated HA binding protein (2.5 μg/ml) followed by donkey anti-mouse IgG Alexa Fluor 546 and streptavidin Alexa Fluor 488 (Invitrogen) in PBS containing 1% BSA. In some experiments, monocytic cells were pre-labeled with Calcein AM instead of anti-CD68 antibodies. Nuclei were stained with DAPI. Images were acquired and merged on a Leica DM IRB inverted microscope equipped with fluorescent epi-illumination, using a Diagnostic Instruments Pursuit 4.0 megapixel chilled color CCD camera and Spot software, version 4.5.9.1.

RNA isolation and qPCR. Cells were lysed in Trizol (0.5 ml, Invitrogen) followed by addition of chloroform (0.1 ml), vigorous mixing by hand, and spinning at 14,000 RPM for 10 min at 4 °C. The aqueous phase was collected, mixed with an equal volume of 70% ethanol, and purified using EconoSpin™ columns (Epoch Life Science). cDNA was prepared from the isolated RNA with High Capacity cDNA Reverse Transcription Kit (Life Technologies) per manufacturer's instructions. Real-time PCR was performed with SYBR Select Master Mix or TaqMan® Gene Expression Master Mix (Life Technologies), as directed by the manufacturer, on an Applied Biosystems 7900HT Fast Real-Time PCR System or QuantStudio 5™ Real-Time PCR System (Thermo Fisher). For each sample, assays were run as technical duplicates. cDNA levels were then expressed as estimated copy numbers of mRNA using the master-template approach [107]. Taqman probes (Life Technologies) were HAS1 (Hs00758053_m1), HAS2 (Hs00193435_m1), HAS3 (Hs00193436_m1), HYAL1 (Hs00537920_g1), HYAL2 (Hs00186841_m1), pan VCAN (Hs00171642_m1), TSG-6 (Hs00200180_m1), HAPLN1 (Hs00157103_m1), HAPLN3 (Hs00820260_m1), and 18S rRNA (4319413E-1403063). Gene-specific SYBR primer sequences are listed in Supplemental Table 1.

Statistical analysis. All data are expressed as the average ± standard error of the mean (SEM), unless otherwise specified. Differences were identified by two-tailed Student's *t*-tests for the comparison of two groups and by one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests for the comparison of three or more groups and were regarded as significant if $P < 0.05$. In experiments in which two effects

(e.g., RA vs. OA, control vs. 4MU) were tested, two-way ANOVA for repeated measures was used followed by Bonferroni's post hoc tests and were regarded significant if $P < 0.05$.

Study approval. All individuals gave written informed consent and were enrolled in an IRB-approved protocol at the Clinical Core of Benaroya Research Institute at Virginia Mason (BRI, Seattle, WA).

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CRediT authorship contribution statement

Inkyung Kang: Conceptualization, Investigation, Writing – review & editing, Formal analysis, Writing – original draft, Visualization. **Christian Hundhausen:** Conceptualization, Writing – original draft. **Stephen P. Evanko:** Conceptualization, Writing – original draft. **Prasanthi Malapati:** Investigation. **Gail Workman:** Investigation, Writing – original draft. **Christina K. Chan:** Investigation. **Cliff Rims:** Conceptualization, Resources. **Gary S. Firestein:** Conceptualization, Resources. **David L. Boyle:** Conceptualization, Resources. **Kevin M. MacDonald:** Conceptualization, Resources. **Jane H. Buckner:** Conceptualization, Resources. **Thomas N. Wight:** Conceptualization, Writing – review & editing, Funding acquisition, Writing – original draft, Supervision.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mbplus.2022.100110.

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