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Crosstalk between T Lymphocytes and Lung Fibroblasts: Generation of a Hyaluronan-Enriched Extracellular Matrix Adhesive for Monocytes

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Abstract

In immunity and inflammation, T cells are often associated with stromal mesenchymal cells such as fibroblasts. Hyaluronan and proteins that associate with hyaluronan such as versican and tumor necrosis factor-inducible gene-6 (TSG-6) are extracellular matrix (ECM) components that promote leukocyte adhesion, accumulation, and activation. However, the factors responsible for producing this specialized ECM and its impact on inflammatory events are not well understood. In this study, we explored the role of T cells in stimulating lung fibroblasts to produce an ECM that impacts monocyte adhesion. We found that CD3/CD28-activated human CD4⁺ T cells when co-cultured with human lung fibroblasts stimulated the expression of mRNA for hyaluronan synthase 2 (*HAS2*) and decreased the expression of hyaluronidase 2 (*HYAL2*). This led to an increase in the deposition of hyaluronan that formed cable-like structures within the ECM. Co-culturing activated T cells with fibroblasts also led to increased expression and accumulation of TSG-6. Surprisingly, addition of activated CD4⁺ T cells to the fibroblasts reduced the expression of mRNA for versican, and increased the expression of enzymes that degrade versican, such as ADAMTS-4 and ADAMTS-9 (a disintegrin and metalloproteinase with a thrombospondin type-1 motif) leading to a decrease in versican in the ECM of the co-cultures. Furthermore, addition of human monocytes to these co-cultures resulted in elevated monocyte adhesion to the cable-like structures in the ECM when compared to controls. These results illustrate the importance of crosstalk between T cells and fibroblasts in promoting the generation of a matrix that is adhesive for monocytes.

Keywords

ADAMTS; Co-culture; Extracellular matrix; Fibroblasts; Hyaluronan; Inflammation; Leukocytes; Lymphocytes; Monocytes; TSG-6; Versican

Inflammatory responses require the emigration of leukocytes from the vasculature into the underlying damaged tissue promoting extracellular matrix (ECM) remodeling which can lead to chronic inflammatory disease [Gill et al., 2010; Parish, 2006; Sorokin, 2010; Vaday

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Conflicts of Interest

The authors declare no competing interests.

et al., 2001; Wight et al., 2014]. T cells are often found in close proximity to stromal mesenchymal cells during the ECM remodeling phases of inflammation, but it is not clear whether T cells “talk” to fibroblasts in such a way as to regulate specific changes in the ECM that impact the inflammatory process [Parsonage et al., 2005; Rezzonico et al., 1998]. A number of studies have shown that co-culturing T cells with fibroblasts leads to an upregulation of a number of different pro-inflammatory cytokines by fibroblasts, either by direct contact between the two cells [Bombara et al., 1993; Chizzolini et al., 2006; Rezzonico et al., 1998] or through release of soluble factors in the conditioned media by the T cells [Loubaki et al., 2013; Mikko et al., 2008]. Furthermore, a few studies have shown that co-culture of leukocytes with fibroblasts leads to changes in the expression of genes for ECM components such as collagen and the enzymes that degrade these components such as collagenase [Casini et al., 1985; Mikko et al., 2008]. However, there have been no studies that have evaluated whether other ECM components are involved and if these ECM changes further alter the pattern of leukocyte adhesion to the ECM.

Hyaluronan and versican are two ECM components implicated in leukocyte adhesion [de la Motte et al., 1999; Petrey and de la Motte, 2014; Potter-Perigo et al., 2010; Wight et al., 2014]. Hyaluronan is an ECM glycosaminoglycan that serves as a ligand for CD44 and accumulates in a variety of tissues during inflammation [Toole, 2004]. Hyaluronan synthases (HAS) are enzymes responsible for the synthesis of ECM hyaluronan [Weigel et al., 1997], whereas hyaluronidases (HYAL) [Stern and Jedrzejewski, 2006] are enzymes responsible for hyaluronan degradation. Versican is a chondroitin sulfate proteoglycan that interacts with hyaluronan [Wight, 2002; Zimmermann and Ruoslahti, 1989], and can be degraded by a number of proteases including members of the a disintegrin and metalloproteinase with a thrombospondin type-1 motif (ADAMTS) family [Apte, 2009; Kenagy et al., 2006]. Versican modulates cellular adhesion, migration, and proliferation [Wight, 2002] and increases during inflammation [Wight et al., 2014].

In addition, other proteins that interact with hyaluronan (termed “hyaladherins”) [Toole, 1990] are often found associated with hyaluronan in inflammation such as tumor necrosis factor-inducible gene 6 (TSG-6) [Day and de la Motte, 2005; Lauer et al., 2013; Milner and Day, 2003]. TSG-6 is a 35 kDa protein that is synthesized and secreted by many types of cells after treatment with TNF α and type I interferon [Wisniewski et al., 2005], and is known to participate in cross-linking hyaluronan into higher order structures [Day and de la Motte, 2005; Lauer et al., 2013; Milner and Day, 2003]. Previous studies by our group and others have shown that hyaluronan, versican, and TSG-6 are ECM components that promote leukocyte adhesion, accumulation, and activation during inflammation in a variety of tissues such as in the lung [Jiang et al., 2011; Lauer et al., 2013; Lauer et al., 2008; Potter-Perigo et al., 2010; Reeves et al., 2016; Savani et al., 2000; Teder et al., 2002], colon [de la Motte et al., 1999], kidney [Selbi et al., 2004; Wang and Hascall, 2004], skin [Milinkovic et al., 2004; Mummert et al., 2000], and cells from the synovium [Evanko et al., 2012]. However, the factors responsible for the production of this specialized ECM and the involvement of leukocytes in its generation are not well understood. In this study, we have explored the role of human CD4 $^{+}$ T cells in the production of hyaluronan, versican, and TSG-6 by human lung fibroblasts (HLFs) and whether the ECM produced in this co-culture system further impacts leukocyte adhesion and accumulation.

Materials and Methods

Isolation of CD4⁺ T cells

CD4⁺ T cells were isolated from human peripheral blood mononuclear cell (PBMC) samples, collected with informed consent from healthy volunteers taking part in a research protocol approved by the Institutional Review Board of the Benaroya Research Institute at Virginia Mason (BRI, Seattle, WA). Human PBMCs were obtained by centrifugation of whole blood over Ficoll gradients, and then frozen for future use. Samples were used fresh or thawed just before use and washed twice with RPMI 1640 (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO), 50 μ M β -mercaptoethanol, 2 mM GlutMAX-1 (Life Technologies, Carlsbad, CA), 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 EasySep Human CD4⁺ T Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada) or the negative selection CD4⁺ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as per manufacturers' instructions.

Co-culture of fibroblasts and CD4⁺ T cells

HLFs were derived from explants of the lung, after removing the pleura and parenchyma, a generous gift from Professor Ganesh Raghu, Division of Pulmonary and Critical Care Medicine, University of Washington (Seattle, WA). HLFs were isolated in accordance with approval from the human subjects review committee of the University of Washington, as described [Raghu et al., 1988]. For later experiments, normal HLFs were purchased from Lonza (Basel, Switzerland). HLFs were maintained in Dulbecco's modified high glucose Eagle medium (DMEM; Hyclone) supplemented with 10% FBS (Atlas Biologicals), 50 μ M β -mercaptoethanol, 2 mM GlutMAX-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) at 37°C in 5% CO₂. Cells were passaged with trypsin (0.25%, Hyclone) and were used for experiments between passages 4 and 21 after initial isolation. HLFs were seeded at between 1 and 3 $\times 10^5$ /well in 24-well plates in 10% FBS DMEM for 24 h at 37°C in 5% CO₂ before adding the T cells.

After 24 h, medium was removed from the 24-well plate containing HLFs and replaced by either fresh medium, fresh medium supplemented with 1 μ g/ml anti-CD3 antibody clone OKT3 (eBioscience, San Diego, CA) and 0.25 μ g/ml anti-CD28 antibody clone CD28.2 (eBioscience), 1 $\times 10^6$ CD4⁺ T cells in medium or 1 $\times 10^6$ CD4⁺ T cells in medium supplemented with 1 μ g/ml anti-CD3 and 0.25 μ g/ml anti-CD28 antibodies. Anti-CD3 and anti-CD28 antibodies are commonly used to activate CD4⁺ T cells. 1 $\times 10^6$ CD4⁺ T cells with and without anti-CD3 and anti-CD28 antibodies at 1 μ g/ml and 0.25 μ g/ml, respectively, were added to empty wells as controls in early experiments. Cells were cultured for 24 h at 37°C in 5% CO₂ for most experiments, 48 h for hyaluronan enzyme-linked sorbent assay (ELSAs, see below) and versican Western blots, and 24 h, 48 h and 72 h for time course experiments.

For imaging and immunostaining, fibroblasts were seeded on 2- or 4-well chamber slides (Nunc Lab-Tek Chamber Slide system, Thermo Scientific, Waltham, MA) at 6×10^4 /chamber and 4×10^4 /chamber, respectively, in 10% FBS DMEM. After 24 h, medium was removed and fresh medium or CD4+ T cells were added, with and without anti-CD3 and anti-CD28 antibodies at 1 µg/ml and 0.25 µg/ml, respectively. Cells were cultured for 48 h at 37°C in 5% CO₂, then medium was removed and slides were washed once with PBS.

Quantitative PCR and RT-PCR

When collecting cells for RNA, the supernatant was collected first and centrifuged to isolate non-adherent cells. Adherent and non-adherent cells were homogenized together in TRIzol (Ambion Life Technologies, Carlsbad, CA), and total RNA was extracted on an Econospin column (Epoch Life Science Inc., Missouri City, TX) and using Direct-zol™ RNA Kit (Zymo Research, Irvine, CA), according to the manufacturer's directions. cDNA was prepared by reverse transcription of equal amounts of RNA, using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (ABI; Foster City, CA). QPCR was performed with an ABI Prism 7900HT Sequence Detection System. Taqman gene expression Master Mix from ABI was used with the following ABI gene expression assays: 18S Hs99999901_s1; *HAS1* Hs00758053_m1; *HAS2* Hs00193435_m1; *HAS3* Hs00193436_m1; *HYAL1* Hs00537920_g1; *HYAL2* Hs00186841_m1; TSG-6 Hs00200180_m1; total versican Hs00171642_m1; ADAMTS-1 Hs00199608_m1; and ADAMTS-4 Hs00192708_m1. Sybr Select Master Mix from ABI was used with the following gene expression assay: ADAMTS-9 (forward primer AGACCGGAGTCGGAACGCGA, reverse primer CGCCGCAGGTCTTGGTGCAT), TNFα (forward primer GACAAGCCTGTAGCCCATGT, reverse primer TTATCTCTCAGCTCCACGCC), IL-6 (forward primer TACATCCTCGACGGCATCTC, reverse primer TTTTCTGCCAGTGCCTCTTT), IFN-γ (forward primer ACCAGAGCATCCAAAAGAGTGT, reverse primer GCGTTGGACATTCAAGTCAGT). The change in gene expression was calculated using the comparative Ct method (Applied Biosystems).

Hyaluronan Enzyme-Linked Sorbent Assay

Hyaluronan ELSA was performed as described [Wilkinson et al., 2004]. Hyaluronan was released from the media and cell layers of each sample by digestion with pronase at 0.5 mg/ml overnight at 37°C. The pronase was then inactivated by heating to 100°C for 20 min, and samples were mixed with an equal volume of biotinylated hyaluronan binding protein (b-HABP) at 3 µg/ml in 10% calf serum in PBS for 1 h at room temperature. Each well of a 96-well plate was previously coated with hyaluronan conjugated to bovine serum albumin (BSA) for 1 h at room temperature, then washed three times with PBS, blocked for 1 h at room temperature with 10% in PBS, and rinsed again once with PBS. Samples and b-HABP mixes were added to the 96-well plate for 1 h at room temperature, then the wells were washed four times with water. Peroxidase-labeled streptavidin (2 µg/ml) was added for 20 min. Wells were washed as before, then 0.03% H₂O₂ and 0.5 mg/mL 2,2-azino-bis(3-ethyl-benzthiozoline sulfonic acid) in 0.1 M sodium citrate pH 4.2 were added. The resulting optical densities (OD₄₀₅), being inversely proportional to the amount of hyaluronan, were read using an OPTImax microplate reader (Molecular Devices, Sunnyvale, CA).

Immunohistochemistry and hyaluronan staining

Cells were fixed in acid-formalin-ethanol (3.7% formaldehyde-PBS, 70% ethanol and 5% glacial acetic acid, all v/v) for 10 min at room temperature, as described previously [Lin et al., 1997] to allow maximum retention of the hyaluronan and its associated proteins. After fixation, slides were washed once with PBS and blocked at room temperature with 1% BSA and 2% goat or donkey serum in PBS for at least 1 h. Primary antibody solutions diluted in 1% BSA and PBS were then added for at least 1 h at room temperature. b-HABP was used at 4 µg/ml to detect hyaluronan. Versican was detected using either the mouse monoclonal antibody 2B1 (Associates of Cape Cod, East Falmouth, MA) at 1:250 or the rabbit monoclonal antibody ab177480 (Abcam, Cambridge, UK) at 1:200. For TSG-6, the rabbit polyclonal antibody RAH-1 was used at 1:500, a kind gift from Dr. Anthony Day, University of Manchester [Fujimoto et al., 2002; Kuznetsova et al., 2005]. After washing the slides twice with PBS, the following secondary antibody solutions were added for 1 h at room temperature while protected from light: Alexa Fluor 488 streptavidin (3 µg/ml), Alexa Fluor 594 goat anti-mouse antibody (3 µg/ml) or Alexa Fluor 555 donkey anti-rabbit antibody (all at 3 µg/ml and all from Molecular Probes, now ThermoFisher). After two washes in PBS, slides were mounted using anti-fade mounting media Fluorogel with Tris buffer (Electron Microscopy Sciences, Hatfield, PA) containing 4',6'-diamidino-2-phenylindole (DAPI; 1 µg/ml; Molecular Probes, now ThermoFisher). Cells were examined using a Leica DMIRB inverted microscope or Leica DMR microscope (Wetzlar, Germany), and images were acquired using a Spot Pursuit or Spot Insight CCD camera and imaging program (Diagnostic Instruments Inc., Sterling Heights, MI). Quantitation of hyaluronan and TSG-6 was performed by measuring the area of staining per 200× field from 10 different pictures per condition using NIH ImageJ.

Western analysis

The Western analysis was performed as described previously [Potter-Perigo et al., 2010]. Briefly, versican present in the media and on cell layers was digested using chondroitin ABC lyase (Associates of Cape Cod), then applied to SDS-PAGE and transferred to 0.2 µm nitrocellulose membranes (GE Healthcare, Piscataway, NJ) using a BioRad Transblot SD Semi-Dry Transfer Cell (BioRad, Hercules, CA). Versican was detected using the 2B1 primary antibody described above, and enhanced chemiluminescence (Western-Light Chemiluminescent System with CSPD proprietary luminescent substrate from Applied Biosystems). Ponceau staining was performed to ensure equal loading between lanes.

Cytokine assay

Supernatant was collected from each well and kept at -80°C until use in the BD Bioscience Cytometric Bead Array Human Th1/Th2/Th17 cytokine kit (Franklin Lakes, NJ), following the manufacturer's protocol. Samples were acquired on a dual-laser BD FACSCalibur cytometer and analyzed with FCAP Array software (BD).

Monocyte adhesion assay

The monocyte adhesion assay was run as previously described [Potter-Perigo et al., 2010]. Briefly, HLFs were plated at 3×10^4 cells per well in 10% FBS DMEM in 96-well plates

and incubated for 24 h at 37°C in 5% CO₂. Medium was then removed from each well and replaced by either fresh medium, fresh medium supplemented with 1 µg/ml anti-CD3 and 0.25 µg/ml anti-CD28 antibodies, 1×10^5 CD4⁺ T cells in medium with or without anti-CD3/CD28 antibodies, as above. Cells were incubated for 24 h. Before the assay, the human monocyte cell line U937 was incubated with calcein-AM (Invitrogen, Carlsbad, CA) at 0.5 µg/ml for 45 min, washed twice with serum-free RPMI medium, and resuspended to 3×10^6 /ml. Half of the HLFs were treated with *Streptomyces* hyaluronidase (Sigma-Aldrich, St. Louis, MO) at 0.66 U/ml for 30–45 min before the adhesion assay. Then, medium was removed, and wells were gently washed once with serum-free RPMI. 100 µl of the U937 cell suspension was added to each well, and allowed to adhere for 1 h at 4°C. Finally, plates were washed three times with cold serum-free RPMI and the fluorescence of each well was measured using a Fusion Series Universal Microplate Analyzer (Packard Bioscience Co., Meriden, CT) with excitation wavelength of 485 nm and detection wavelength of 535 nm. The amount of U937 adherent cells per well was determined by running fresh standards for each separate experiment [de la Motte et al., 1999; Potter-Perigo et al., 2010].

Statistical analyses

Statistical significance was determined by multiple unpaired *t*-tests, using logarithmic values when considering fold changes, or 2-way ANOVA with Sidak's multiple comparison test.

Results

Co-culture of HLFs and activated CD4⁺ T cells caused increased accumulation of hyaluronan and TSG-6 on the cell layer

We first assessed hyaluronan metabolism by looking at the expression of the *HAS* and *HYAL* families respectively. *HAS2* and *HYAL2* mRNA levels were similar for cultures of HLFs only and co-cultures of HLFs and non-activated CD4⁺ T cells over time, and were almost not detectable in cultures of CD4⁺ T cells only. On the contrary, *HAS2* was significantly upregulated and *HYAL2* was downregulated in co-cultures of HLFs and activated CD4⁺ T cells at 24, 48 and 72 h (Fig. 1A). No *HAS1* and very little *HAS3* and *HYAL1* were detected (data not shown). The most notable difference was the more than 10-fold upregulation of TSG-6 (Fig. 1A). As these changes in gene expression occur as soon as 24 h, we kept this time point and looked at the amount of activated CD4⁺ T cells necessary for gene activation. A ratio of HLFs to activated CD4⁺ T cells of 1:1 did not significantly modify the expression of *HAS2*, *HYAL2* and TSG-6 compared to HLFs alone. However, a ratio of 1:3 was sufficient to significantly upregulate *HAS2* and TSG-6 expression, and significantly downregulate *HYAL2* expression (Fig. 1B). Downregulation of *HYAL2* and upregulation of TSG-6 was further enhanced at a ratio of 1:10, whereas *HAS2* expression peaked at a ratio of 1:3, so for the following experiments we kept the ratio of HLFs to activated CD4⁺ T cells between 1:3 and 1:10.

We next verified our findings by applying the same protocol (24-h co-culture, with the ratio of HLFs to CD4⁺ T cells between 1:3 and 1:10) using CD4⁺ T cells from 7 different individuals. As seen before, only co-cultures of HLFs and activated CD4⁺ T cells showed a significant upregulation of *HAS2* expression, downregulation of *HYAL2* expression, and

upregulation of TSG-6 expression (Fig. 2). These results suggest an increase in the synthesis of both hyaluronan and TSG-6, as well as a decrease in hyaluronan degradation, resulting in an accumulation of hyaluronan in the ECM. To confirm this, we performed hyaluronan staining, which increased when activated CD4⁺ T cells and HLFs were co-cultured (Fig. 3, A–D, quantification in Fig. 7F). The most striking difference in structure was the presence of hyaluronan-rich cables in the HLFs and activated CD4⁺ T cell co-cultures, whereas the hyaluronan in the other cultures was not in the form of cables (Fig. 3, D, arrows). When using a higher magnification, we could observe activated CD4⁺ T cells localized on these hyaluronan-rich cables (Fig. 3E, arrows heads). We also performed a hyaluronan ELSA to quantify the amount of hyaluronan present in the media and on the cell layer. The amount of hyaluronan found in the media was similar for each condition. However hyaluronan accumulated several fold in the cell layer in co-cultures of HLFs and activated CD4⁺ T cells, compared to the other conditions (Fig. 3, G), consistent with our RNA and hyaluronan staining data.

Similarly, there was a significant increase in TSG-6 staining intensity when activated CD4⁺ T cells were co-cultured with HLFs (Fig. 4, A–D, quantification Fig. 4E). Hyaluronan and TSG-6 staining were codistributed in the cell layer and in the larger cable-like structures, where hyaluronan was predominant (Fig. 4F, arrows).

Co-culture of HLFs and activated CD4⁺ T cells alters expression and accumulation of versican

We also examined gene expression of versican and the versican-degradative members of the ADAMTS family. As expected, the expression of versican and the ADAMTSs was similar for cultures of HLFs only, co-cultures of HLFs with resting CD4⁺ T cells, and very little expression was detected in cultures of CD4⁺ T cells only. On the contrary, 24-h co-cultures of HLFs and activated CD4⁺ T cells showed a significant downregulation of versican expression, downregulation of ADAMTS-1, and upregulation of ADAMTS-4 and –9 (Fig. 5A). This effect was increased at 48 and 72 h except for ADAMTS-9 expression, but since the changes were already significant at 24 h, we kept this time point to examine the influence of the amount of activated CD4⁺ T cells added. As with hyaluronan accumulation, downregulation of versican and ADAMTS-1, and upregulation of ADAMTS-4 and –9 were not significant at a ratio of 1:1 for HLFs to activated CD4⁺ T cells, but became significant at a ratio of 1:3 and 1:10 (Fig. 5B). Thus, for the following experiments, we kept the cell ratio between 1:3 and 1:10.

Next we confirmed our results by using CD4⁺ T cells from 7 different individuals. As observed before, co-cultures of HLFs and activated CD4⁺ T cells significantly decreased the expression of versican and ADAMTS-1, and increased the expression of ADAMTS-4 and –9 (Fig. 6, A–D). These results are consistent with a decrease in versican in the co-cultured ECM observed in the present study. Western blotting confirmed that versican levels were lower in the media of co-cultures of HLFs and activated CD4⁺ T cells (Fig. 6E). Versican was not detectable by Western blot in the cell layer; this was not surprising as most of the versican produced by the cells is secreted in the media.

Co-culture of HLFs and activated CD4+ T cells promotes the secretion of pro-inflammatory cytokines, as well as monocyte adhesion

We next examined the cytokine expression profile of our co-cultures. qPCR analysis revealed increased gene expression of pro-inflammatory cytokines such as IL-6 and IFN- γ (Fig. 7, A–B) in co-cultures of HLFs and activated CD4+ T cells, and TNF α was increased in the presence of activated CD4+ T cells (Fig. 7C). qPCR data was confirmed by cytometric bead array, showing a significant increase in the secretion of the pro-inflammatory cytokines IFN- γ , IL-6, and TNF α in the supernatant of 24-h co-cultures of HLFs and activated CD4+ T cells (Fig. 7, D–F).

Finally, we wanted to examine whether this new ECM was adherent for monocytes. After 48 h of co-culture, we added fluorescently-labeled U937 monocytes and incubated the plate for 1 h at 4°C. After three washes, there were twice as many U937 adherent cells in the co-cultures of HLFs and activated CD4+ T cells than in cultures of HLFs alone, or in co-cultures of HLFs with non-activated CD4+ T cells (Fig. 8A, black bars). Before adding the U937 cells, we also treated half the wells with hyaluronidase for 45 min – 1 h, to see if the higher monocyte adhesion was due to the increased presence of hyaluronan and hyaluronan-rich cables. Indeed, the addition of hyaluronidase significantly decreased the number of U937 adherent cells on the HLFs and activated CD4+ T cell co-cultures (Fig. 8A, gray bars). Hyaluronidase-resistant adhesion was still significantly higher than the number of adherent cells in the other conditions however, suggesting that hyaluronan might not be the only factor promoting monocyte adhesion. Increased monocyte adhesion could be visualized by DAPI staining and phase contrast images (Fig. 8B), where a clear increase in hyaluronan staining, CD4+ T cells and U937 cells were observed. Addition of hyaluronidase abolished hyaluronan staining and reduced adhesion of CD4+ T cells and U937 cells.

Discussion

The present study examines the effects of human CD4+ T cells on ECM remodeling, comparing their resting state to a simulated disease state by activating T cells with anti-CD3 and anti-CD28 antibodies. Co-cultures of HLFs and activated CD4+ T cells exhibited an upregulation of *HAS2* mRNA expression, and a decrease in *HYAL2* expression, leading to an accumulation of hyaluronan on the cell layer. The increase in *HAS2* expression in the co-cultures is significant given previous findings that *HAS2* overexpression resulted in an invasive myofibroblast phenotype that promoted lung fibrosis [Li et al., 2011] and more recent work from the same group showing that *HAS2* depletion promoted myofibroblast senescence [Li et al., 2016], which is required for resolution of fibrosis. However, myofibroblasts were not induced in our experiments. *HAS2* transcription can be finely regulated. Recent studies found *HAS2* expression to be dependent on expression of *HAS2* antisense (*HAS2*-AS1) and epigenetic modifications such as *O*-GlcNAcylation [Vigetti et al., 2014]. Further studies will be required to determine the mechanism of *HAS2* regulation in our co-cultures.

Since other hyaluronan binding proteins such as TSG-6 and versican have been shown to increase during inflammation [Day and de la Motte, 2005; Wight et al., 2014], the expression of these ECM components was also examined. Co-culture of activated T cells

with fibroblasts led to increased TSG-6 expression and accumulation, which, as shown by staining, was codistributed with hyaluronan to cable-like structures within the ECM. TSG-6 has already been identified as a protein that promotes the crosslinking of hyaluronan into larger cable-like structures [Day and de la Motte, 2005; Jessen and Odum, 2003; Mukhopadhyay et al., 2004]. This protein increases in inflammatory states and has been observed to increase in bronchoalveolar lavage fluid from asthmatic patients [Forteza et al., 2007]. Involvement of TSG-6 with the formation of hyaluronan cables that bind leukocytes has been described previously [Lauer et al., 2013].

Surprisingly, we found a reduction of versican in the ECM of the co-cultures of activated CD4⁺ T cells and fibroblasts. This reduction appears to result from decreased synthesis due to downregulation of versican mRNA. Furthermore, the expression of two enzymes capable of degrading versican, ADAMTS-4 and -9, was upregulated suggesting an increase in turnover of versican in the co-cultures. Since both T cells and fibroblasts were present when RNA was extracted from the co-cultures, it is possible that the trends we observed by qPCR were influenced by a dilution effect due to varying amounts of T cells. However, this is unlikely as no dilution effect was observed when the same number of non-activated T cells was added, such as in Figures 2 and 6. Moreover, we consistently measured a much higher RNA concentration (3- to 5-fold) when extracting mRNA from wells with fibroblasts alone compared to wells with activated T cells alone, suggesting that fibroblasts in general make more RNA than T cells, and that the mRNA collected from the co-cultures mostly comes from the fibroblasts. Nonetheless it was important to confirm our findings by staining, western blot, and hyaluronan ELSA. Collectively, these results indicate that the ECM produced and deposited in these co-cultures was uniquely enriched in hyaluronan and TSG-6, but deficient in versican. Finally, we have found that the activated CD4⁺ T cells and HLFs co-culture environment was rich in pro-inflammatory cytokines, and filled with cable-like structures that can trap monocytes.

Our finding that versican was decreased in the ECM of the co-cultures of HLFs and activated CD4⁺ T cells while at the same time this ECM still supported monocyte adhesion is somewhat surprising since our earlier work showed that versican produced by lung fibroblasts stimulated with polyinosine-polycytidylic acid (poly I:C) together with hyaluronan was necessary for monocyte adhesion to the ECM [Potter-Perigo et al., 2010; Wight et al., 2014]. The difference, of course, when comparing these two studies is that different agonists were used to generate the adhesive ECM. Such differences, although permissive for monocyte adhesion to the ECM have different effects on the phenotype of the cells. For example, poly I:C treatment leads to the formation of myofibroblasts [Evanko et al., 2012; Sugiura et al., 2009] which were not observed in our co-culture system. Indeed, our qPCR data show a dramatic increase in IL-1 β expression in co-cultures of HLFs and activated CD4⁺ T cells, but no change was observed in the expression of TGF β or in α smooth muscle actin expression, a marker for myofibroblasts (data not shown). In this regard, this ECM was more similar to the IL-1 β -induced ECM described by Meran and colleagues in which this ECM accumulated hyaluronan and promoted monocyte binding, suggesting that fibroblasts may respond differently to immune activation than to agonists that promote tissue fibrosis [Meran et al., 2013]. Furthermore, we did find that unlike monocytes [Potter-Perigo et al., 2010], T-cell binding was sensitive to versican since

versican interfered with T-cell binding to hyaluronan and blocked the ability of hyaluronan to support T-cell migration and invasion into collagen gels [Evanko et al., 2012]. Thus, in this context, ECMs enriched in versican may affect different types of leukocytes differently. A “stromal cell address code” may exist to regulate the types of leukocytes that accumulate during the inflammatory response [Parsonage et al., 2005].

A major caveat of our study is that we do not know precisely the cellular origins of the factors that are responsible for the generation of the ECM microenvironment that binds monocytes in these co-cultures. CD4⁺ T cells alone produce very little hyaluronan and versican compared to HLFs, so it is likely that hyaluronan and versican are derived from the fibroblasts. On the other hand, the cellular source of the cytokines and chemokines in the co-culture conditions is not clear. While activated T cells produce a host of inflammatory cytokines and chemokines [Brouty-Boye et al., 2000; Kim et al., 2007; Zhang and Phan, 1996], fibroblasts are also capable of upregulating cytokine and chemokine production in the presence of myeloid and lymphoid cells [Kim et al., 2007]. Despite this uncertainty, our findings show that culturing activated T cells and fibroblasts together produces a specialized ECM, enriched in hyaluronan and TSG-6, which is adhesive for monocytes.

In many inflammatory diseases, activated T cells can be found in the tissue in close contact with stromal cells in most inflammatory diseases, such as chronic obstructive pulmonary disease [Grumelli et al., 2004; Saetta et al., 1998; Turato et al., 2002], asthma [Del Prete et al., 1993], rheumatoid arthritis [Panayi et al., 1992; Tak et al., 1997] systemic sclerosis [Fleischmajer et al., 1977; Prescott et al., 1992; Roumm et al., 1984], diabetes during insulinitis [Bogdani et al., 2014]. These regions are often enriched in hyaluronan and associated proteins. The present study indicates that crosstalk between activated T cells and stromal cells can generate a microenvironment that further promotes inflammatory cell recruitment and invasion. Targeting this pro-inflammatory ECM may be a therapeutic strategy to block chronic inflammation in the future.

In this study, we have addressed using *in vitro* conditions in a well-choreographed series of interactions involving T cells, fibroblasts, and monocytes that creates an ECM microenvironment that regulates, in part, the phenotypes of these cells. The inflammatory state is characterized by a number of modifications in the tissue microenvironment which translate into whether inflammation will resolve or become chronic. These characteristics include persistent cytokine and chemokine production, persistent infiltration and accumulation of immune cells, and changes in the production and accumulation of the ECM, which, in large part, dictates the inflammatory outcome.

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Abbreviations

ADAMTS	a <u>d</u> isintegrin and <u>m</u> etalloproteinase with a <u>t</u> hrombos <u>p</u> ondin type-1 motif
b-HABP	biotinylated hyaluronan binding protein
BSA	bovine serum albumin
DAPI	4',6'-diamidino-2-phenylindole
ECM	extracellular matrix
HA	hyaluronan
HAS	hyaluronan synthase
HLF	human lung fibroblast
HYAL	hyaluronidase
PBMC	peripheral blood mononuclear cell
TSG-6	tumor necrosis factor-inducible gene 6

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Figure 1. Co-cultures of HLFs and activated CD4+ T cells cause upregulation of *HAS2* and *TSG-6* and downregulation of *HYAL2* over time and with increasing amounts of T cells

(A) 1.5×10^5 HLFs were plated on a 24-well plate for 24 h in 10% FBS DMEM, then media was removed and replaced with either fresh media (black line), fresh media supplemented with 1 $\mu\text{g}/\text{ml}$ anti-CD3 and 0.25 $\mu\text{g}/\text{ml}$ anti-CD28 antibodies (blue line), 1×10^6 CD4+ T cells in fresh media (pink line), or 1×10^6 CD4+ T cells in fresh media supplemented with 1 $\mu\text{g}/\text{ml}$ anti-CD3 and 0.25 $\mu\text{g}/\text{ml}$ anti-CD28 antibodies (green line). Control wells were also added with 1×10^6 CD4+ T cells only, with and without anti-CD3/28 antibodies (purple and orange lines respectively). mRNA was extracted at 24 h, 48 h and 72 h, normalized to 18S RNA for each condition, and $2^{-\text{Ct}}$ (relative quantification compared to HLF alone; no stim) was plotted. Data are presented as mean $\pm$ SEM of three independent experiments with duplicate wells. (B) 1.5×10^5 HLFs were plated on a 24-well plate for 24 h in 10% FBS DMEM, then media was removed and replaced with either fresh media (1:0 ratio of HLF to CD4+ T cells), 1.5×10^5 CD4+ T cells (1:1 ratio), 4.5×10^5 CD4+ T cells (1:3 ratio), or 1.5×10^6 CD4+ T cells (1:10 ratio). Each well was activated with 1 $\mu\text{g}/\text{ml}$ anti-CD3 and 0.25 $\mu\text{g}/\text{ml}$ anti-CD28 antibodies. mRNA was extracted at 24 h, normalized to 18S RNA and $2^{-\text{Ct}}$ (relative quantification compared to HLF alone) was plotted. Data are presented as mean $\pm$ SEM of three independent experiments with duplicate wells (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Figure 2. Upregulation of *HAS2* and *TSG-6* and downregulation of *HYAL2* are still observed when HLFs are co-cultured with CD4+ T cells from 7 different individuals

1–3 × 10⁵ HLFs were plated on a 24-well plate for at least 24 h in 10% FBS DMEM, then media was removed and replaced with either fresh media or 1 × 10⁶ CD4+ T cells, with and without anti-CD3/28 antibodies. For some experiments, control wells were also set with 1 × 10⁶ CD4+ T cells alone, activated or non-activated. mRNA was extracted after 24 h and normalized to 18S RNA for each condition and 10⁵ × 2^{-Ct} was plotted. Data are presented as mean ± SEM of 6 (*HYAL2*) or 7 independent experiments using CD4+ T cells from a different individual each time (ns, *P* > 0.05; *, *P* < 0.05; ****, *P* < 0.0001; n.d., not defined).

Figure 3. Hyaluronan accumulates on the cell layer of co-cultures of HLFs and activated CD4+ T cells, forming cable-like structures

(A–E) Following culture of HLFs with and without activated and non-activated CD4+ T cells on chamber slides, cells were fixed with acid/alcohol/formalin and stained for hyaluronan (HA, green) using HABP. Nuclei were counterstained with DAPI. Original magnification: 200×. Arrows in (D) indicate hyaluronan-rich cables. (E) Higher magnification (600×) photo of activated CD4+ T cells binding to hyaluronan-rich cables (arrows heads). All images are representative of 5 independent experiments. (F) Quantitation of hyaluronan by measuring the area of staining per 200× field from 10 different pictures per condition. (G) After 48 h of co-culture, media and cell layers were harvested for hyaluronan ELSA after digestion with pronase. One well for each condition was set in parallel for cell count. Data are presented as mean ± SEM of three independent experiments with duplicate or triplicate wells, each condition reported as a fold compared to HLF alone (ns, $P > 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

Figure 4. Staining and quantification show accumulation of TSG-6 in co-cultures of HLFs and activated CD4+ T cells

(A–D) Following culture of HLFs with and without activated and non-activated CD4+ T cells on chamber slides, cells were fixed with acid/alcohol/formalin and stained for TSG-6 using the polyclonal antibody RAH-1. Original magnification: 200×. Bar equals 100 μm. (E) Quantitation of TSG-6 by measuring the area of staining per 200× field from 10 different pictures per condition. (ns, $P > 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$). (F) Higher magnification (630× original) image of activated CD4+ T cells binding to the hyaluronan and TSG-6 rich cables (arrows, yellow color indicates codistribution). Green, hyaluronan;

Red, TSG-6; Blue, DAPI. Bar equals 50 μ m. All images are representative of 3 independent experiments.

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Figure 5. Co-cultures of HLFs and activated CD4⁺ T cells cause downregulation of versican and alter expression of versican-related ADAMTSs over time and with increasing amounts of T cells (A) 1.5×10^5 HLFs were plated on a 24-well plate for 24 h in 10% FBS DMEM, then media was removed and replaced with either fresh media (black line), fresh media supplemented with 1 $\mu\text{g/ml}$ anti-CD3 and 0.25 $\mu\text{g/ml}$ anti-CD28 antibodies (blue line), 1×10^6 CD4⁺ T cells in fresh media (pink line), or 1×10^6 CD4⁺ T cells in fresh media supplemented with 1 $\mu\text{g/ml}$ anti-CD3 and 0.25 $\mu\text{g/ml}$ anti-CD28 antibodies (green line). Control wells were also added with 1×10^6 CD4⁺ T cells only, with and without anti-CD3/28 antibodies (purple and orange lines respectively). mRNA was extracted at 24 h, 48 h and 72 h, normalized to 18S RNA and $2^{-\text{Ct}}$ (relative quantification compared to HLF alone; no stim) was plotted. Data are presented as mean $\pm$ SEM of three independent experiments with duplicate wells. (B) 1.5

$\times 10^5$ HLFs were plated on a 24-well plate for 24 h in 10% FBS DMEM, then media was removed and replaced with either fresh media (1:0 ratio of HLF to CD4⁺ T cells), 1.5×10^5 CD4⁺ T cells (1:1 ratio), 4.5×10^5 CD4⁺ T cells (1:3 ratio), or 1.5×10^6 CD4⁺ T cells (1:10 ratio). Each well was activated with 1 μ g/ml anti-CD3 and 0.25 μ g/ml anti-CD28 antibodies. mRNA was extracted at 24 h, normalized to 18S RNA and 2^{-C_t} (relative quantification compared to HLF alone) was plotted. Data are presented as mean $\pm$ SEM of three independent experiments with duplicate wells (ns, $P > 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Figure 6. Downregulation of versican and ADAMTS-1 and upregulation of ADAMTS-4 and ADAMTS-9 are still observed when HLFs are co-cultured with CD4⁺ T cells from 7 different individuals, leading to lower levels of versican in the media

(A–D) $1\text{--}3 \times 10^5$ HLFs were plated on a 24-well plate for at least 24 h in 10% FBS DMEM, then media was removed and replaced with either fresh media or 1×10^6 CD4⁺ T cells, with and without anti-CD3/28 antibodies. For some experiments, control wells were also set with 1×10^6 CD4⁺ T cells alone, activated or non-activated. mRNA was collected after 24 h and normalized to 18S RNA for each condition and $10^5 \times 2^{-C_t}$ was plotted. Data are presented as mean $\pm$ SEM of 7 independent experiments using CD4⁺ T cells from a different individual each time (ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$; n.d., not defined). (E) $3\text{--}4 \times 10^5$ HLFs were plated on 60-mm dishes for 24 h in 10% FBS DMEM, then media was removed and replaced with either fresh media or 1×10^6 CD4⁺ T cells, with and without anti-CD3/28 antibodies. After 48 h, cell layers and conditioned media were harvested and analyzed by Western blot after digestion with chondroitin ABC lyase. Ponceau staining ensures that material from an equal number of cells was used in each lane, which represents a pool of two 60-mm dishes. No versican was detected on the cell layer, but a decreasing level of versican was detected in the media. *Lanes 1 and 5*, HLF alone; *lanes 2 and 6*, HLF + aCD3/28; *lanes 3 and 7*, HLF + CD4⁺ T cells; *lanes 4 and 8*, HLF + CD4⁺ T cells + aCD3/28, *lanes 9–10*, versican-negative and -positive controls. Results are representative of two independent experiments.

Figure 7. Co-cultures of HLFs and activated CD4+ T cells have increased pro-inflammatory cytokine expression and secretion

1–3 × 10⁵ HLFs were plated on a 24-well plate for 24 h in 10% FBS DMEM, then media was removed and replaced with either fresh media or 1 × 10⁶ CD4+ T cells, with and without anti-CD3/28 antibodies. Control wells were also set up with 1 × 10⁶ activated or non-activated CD4+ T cells alone. (A–C) mRNA was collected after 24 h and normalized to 18S RNA for each condition and 10⁵ × 2^{-Ct} was plotted. Data are presented as mean ± SEM of 3 or 4 (IL-6) independent experiments. (D–F) After 24 h of co-culture, supernatants were collected and cytometric bead array was performed to examine cytokine secretion. Data are presented as mean ± SD of one experiment with duplicate wells (n.d., not defined; ns, *P* > 0.05; *, *P* < 0.05; ***, *P* < 0.001; ****, *P* < 0.0001).

Figure 8. Monocyte adhesion is increased in co-cultures of HLFs and activated CD4+ T cells
 (A) 3×10^4 HLFs were plated on a 96-well plate for at least 24 h, then media was removed and replaced by fresh media or 1×10^5 CD4+ T cells in fresh media, with or without anti-CD3 and anti-CD28 antibodies as in previous experiments. After 24 h, media was removed again and replaced by 3×10^5 U937 monocytes fluorescently-labeled with calcein-AM. Plates were incubated for 1 h at 4°C, washed three times then read (black bars). Half the wells were treated with hyaluronidase (Hyal) for 30–45 minutes before addition of monocytes (gray bars). Data are presented as mean $\pm$ SD of one experiment with at least 6 replicate wells per condition, representative of 3 independent experiments (ns, $P > 0.05$; *, $P < 0.05$; ****, $P < 0.0001$). (B) The same experiment was performed once on two-well chamber slides, then cells were stained for hyaluronan (HA) using HABP (green) and nuclei were counterstained with DAPI (blue). Lower panels show separate phase contrast images. Bars equal 100 μ m.