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HYALURONAN ON AIRWAY EPITHELIAL CELLS

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DEDICATION

I dedicate my dissertation work to my family especially my mother, EVH TSOLAKIDOU, whose loving words of encouragement and push for tenacity are still ringing in my ears. My mother, sister and best friend whose words I will never forget and memories I will always cherish.

May her soul rest in peace.

"ΟΣΟ ΖΩ ΕΛΠΙΖΩ"

To my father Dr. Mouaz Abbadi for the continuous support and inspiration. My sister Rania who was there for me all the way, with her encouraging words. She was the source of hope, joy and motivation. My brothers, Rami and Tareq Abbadi for believing in me. Joman Jbour Abbadi and baby Evelina Abbadi who brought happiness to our lives when we most needed it to keep us going.

I love you all very much.

ACKNOWLEDGEMENT

I would like to express my deepest and most sincere appreciation to my mentor, Dr. Vincent Hascall, whose deep knowledge and expertise inspired me and set the foundation of this achievement. Whose invaluable advice and constructive criticism have contributed to my growth as a scientist. Whose kindness and humanity touched my heart and have shaped my personality. Without his guidance, and persistent help, this dissertation work wouldn't have been possible and I wouldn't be where I am today. Thank you for being patient and for this rewarding experience and opportunity in your research lab. I thank you and Vanja for being there for me in my most difficult times, for treating me like a daughter and being my family in the US.

I wish to thank the members of my dissertation committee for their valuable time and precious suggestions to help improve my research. I thank Dr. Yan Xu, Dr. Aimin Zhou, Dr. David Anderson, and Dr. Xiaoxia Li. I especially would like to thank the efforts of my academic adviser Dr. Yan Xu for the continuous follow-up on my work and valuable advice. Chairperson Dr. David Anderson, and the department secretaries Richelle Emery, and Michele Jones for all their administrative help to make this achievement possible.

I wish to greatly acknowledge Dr. Shadi Swaidani whose knowledge and expertise contributed crucially to my knowledge. He taught me the skills necessary to confidently work on my project. Dr. Mark Lauer, Program of Excellence in Glycosciences (PEG) Research Core Director, who closely helped me by providing advice, ideas, and technical support that tremendously helped in my project. Dr. Mark Aronica, Dr. Georgiana Cheng, Dr. Alana Majors and Lisa Ruple for providing us with the TSG6-/-

mice and for their support. Special thanks to Dr. Cheng for providing us with the primers sequences used for this work. Thank you for your kindness to me, I will never forget that.

I thank all the PEG trainees (alphabetically), Dr. Aaron Petrey, Dr. Jarrod Barnes, Dr. Johanne Dubail, Sajina Shakya, Dr. Simon Foulcer, Dr. Sumeda Nandadasa, Dr. Yaoyao Du, Dr. Yan Wang, and Yeojung Kim for making this incredible journey full of lovely memories. I thank all the PEG PIs especially to Dr. Carol de la Motte for her kindness and encouraging words and Dr. Suneel Apte for believing in me and for his valuable advice. I would also like to extend my thanks to all the individuals of the Biomedical Engineering Department at the Cleveland Clinic, who have helped me in my day- to-day activities and provided me with great support to be able to accomplish my work especially Lisa MacDowell, Izzy Delgado, Laura Kelly, Katie Root, Jackie Loftis, Ellie Lamkin, and Valbona Cali.

I would like to thank all my colleagues from Cleveland State University, especially those who have helped me along the way, Dr. Hanan Mohammad, Dr. Saleem Bani Hani, and Ghaith Tawalbeh.

I would also like to give special praise to all my previous mentors who have inspired me to pursue my dream especially Dr. Shteiwi Abbadi and Dr. Youssef Bilto from the University of Jordan.

Finally, I would also like to express my infinite gratitude to my dear friends Elona Gusho, and Andrea Qudah who have been like sisters to me. They have helped me overcome one of the most painful and difficult times of my life by being very compassionate, loving, and supporting. Thank you for giving me the courage to go on.

Special thanks to my close friends (alphabetically) Alicia Mathewson, Dr. Joe El-Khoury, Mahassen Alamine, Monica Plunkett, Maryam and Sami Al-Beiroti, Sahithya Wintrich, and Yagoda Benic for the lovely times. Thank you all.

HYALURONAN RAFTS ON AIRWAY EPITHELIAL CELLS

AMINA M. ABBADI

ABSTRACT

Many cells, including murine airway epithelial cells, respond to a variety of inflammatory stimuli by synthesizing leukocyte-adhesive hyaluronan cables that remain attached to their cell surfaces. This study shows that air-liquid interface cultures of murine airway epithelial cells (AECs) also actively synthesize and release a majority of their HA onto their ciliated apical surfaces to form a heavy chain-hyaluronan (HC-HA) matrix in the absence of inflammatory stimuli. These matrices do not resemble the rope-like HA cables, but occur in distinct sheets, or rafts, that can capture and embed leukocytes from cell suspensions. The HC-HA modification involves the transfer of heavy chains from the inter-α-inhibitor (IαI) proteoglycan, which has 2 heavy chains (HC1 and HC2) on its chondroitin sulfate (CS) chain. The tranesterification transfer of HCs from CS to HA is mediated by tumor-necrosis-factor-induced-gene 6 (TSG-6), which is upregulated in inflammatory reactions. Because the AEC cultures do not have TSG-6 nor serum, which is the source of IαI, assays for HCs and TSG-6 were done and showed that AECs synthesize TSG-6 and their own heavy chain donor (pre-IαI) with a single heavy chain 3 (HC3), which is the substrate for transfer to HA to form the H3-HA rafts. This HC3 pre-IαI is also constitutively expressed by human renal proximal tubular epithelial cells. These leukocyte adhesive HC3-HA structures were also found in the bronchoalveolar lavage (BAL) of naïve mice, and were observed on their apical ciliated surfaces. Thus, these

leukocyte-adhesive HA rafts are now identified as HC3-HA complexes that could be part of a host defense mechanism filling some important gaps in our current understanding of murine airway epithelial biology and secretions.

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ABBREVIATIONS

Extracellular matrix (ECM), GlycosAminoGlycan (GAG) Inter-alpha-inhibitor (IαI), Heavy Chain (HC), hyaluronan (HA), Tumor-Necrosis-Factor-stimulated-gene-6 (TSG-6), Tumor necrosis factor α induced protein-6 (Tnfaip6), Fluorophore-Assisted-Carbohydrate-Electrophoresis (FACE), Airway Epithelial Cells (AECs), and Proximal Tubular Epithelial Cells (PTECs), pre-Inter-alpha-Inhibitor (pre-IaI), Heavy Chain 3-bikunin (HC3-bikunin), Chondroitin-4-Sulfate (C4S), hyaluronidase (hyase), Madin-Darby canine kidney (MDCK) cells, Fetal Bovine Serum (FBS), Bovine Serum Albumin (BSA), High Molecular Weight (HMW), Low molecular weight (LMW), N-acetyl-glucosamine (glcNAc), Amniotic Membrane (AM), Airway Smooth Muscle Cell (ASMC), Airway Epithelial Cell (AEC)

CHAPTER I

BACKGROUND

I.1. Hyaluronan

Hyaluronic acid or hyaluronan (HA) is an abundant glycosaminoglycan (GAG) in the extracellular matrices (ECMs). GAGs are long, linear and heterogenous polysaccharides, which consist of repeating units of disaccharides, with sequences that vary in composition of the disaccharide unit, type of linkage and degree of acetylation and sulphation. While most GAGs are attached to proteins and are highly sulfated (i.e. heparin, heparan sulphate, dermatan sulphate, keratan sulphate and chondroitin sulphate) [1], HA is uniquely a much larger, non-sulfated GAG, lacking a core protein. It is a linear polymer of repeating disaccharides composed of D-glucuronic acid and D-N-acetylglucosamine linked by β (1,4) and β (1,3) glycosidic bonds [2]. HA is not simply an inert structural molecule. Rather, it is implicated in many physiological and pathological tissue responses such as ovulation and fertilization, embryonic development,

tissue fluid homeostasis, inflammation, tissue repair and remodeling, tumor progression and metastasis [3]. HA is synthesized by membrane-bound synthases on the inner surface of the plasma membrane with the chains being extruded through pore-like structures into the ECM. There are three mammalian hyaluronan synthases (HAS1-3). They incorporate UDP-sugars (UDP-N-acetyl-D-glucosamine and UDP-D-glucuronate substrates) onto the non-reducing end of the growing chain [4,5].

I.2. TSG-6 Mediated Hyaluronan Modification

Tumor necrosis factor α (TNFα) induced protein-6 (Tnfaip6), also known as TNFa stimulated gene-6 (TSG-6), is a member of the hyaladherin family (HA binding proteins). TSG-6 is a 35 kDa secreted protein that is not constitutively expressed in most normal adult tissues but is rapidly induced in many different cell types such as monocytes, fibroblasts, vascular smooth muscle cells, synoviocytes, chondrocytes, and proximal tubular epithelial cells by inflammatory mediators such as interleukin-1 (IL-1) and TNFα, lipopolysaccharides and prostaglandin E2 [6,7]. It consists of two domains: the N-terminal link (link module) through which it binds to HA and the C-terminal (CUB) domain of uncertain function [8]. Besides binding to HA, it can also bind to several components of the ECM including chondroitin-4-sulfate (C4S) and inter-α-inhibitor (IαI). IαI is a proteoglycan produced by liver hepatocytes and released in the serum. It is composed of the trypsin inhibitor bikunin with heavy chain 1 (HC1) and heavy chain 2 (HC2) that are covalently bound by ester linkages to bikunin via its single chondroitin sulfate chain [9]. During inflammation, IαI can ingress into extracellular tissue spaces as a consequence of increased vascular permeability where it serves as a

heavy chain donor. TSG-6 catalyzes the transfer of HC1 and HC2 from IαI to HA, forming the HC-HA complex [**Fig. 1**] [10,11].

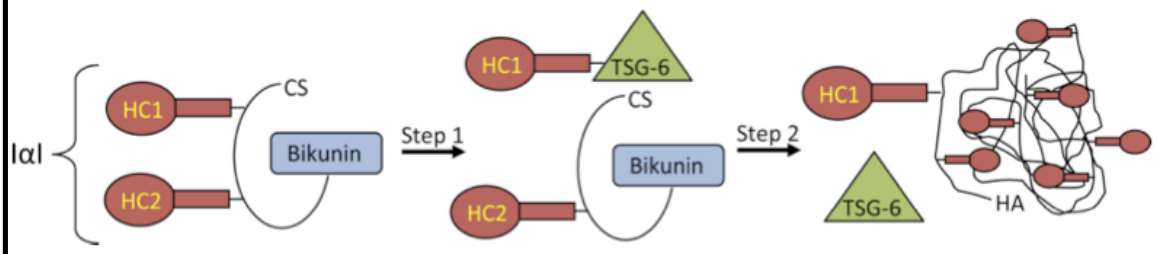

Fig. 1. **A Modified Schematic Illustration of the Heavy Chains Transfer to Hyaluronan via TSG-6.** Inter-α-Inhibitor (IαI) has two HCs (HC1 and HC2) attached by ester bonds to N-acetyl-galactosamine residues in the chondroitin sulfate (CS) chain on bikunin. TSG-6 removes a HC from IαI to form a HC-TSG-6 intermediate and transfers it to form an ester bond to a N-acetyl-glucosamine residue in HA. [15]

These HC-HA complexes have been identified in synovial fluids from patients with rheumatoid arthritis, osteoarthritis [12], and in bronchial secretions of asthmatics [13]. Furthermore, the expression of TSG-6 is upregulated in inflamed synovial tissue of arthritis [14] and in lungs of asthmatics [15]. The expression of TSG-6 is among the genes upregulated > 4 fold in allergen-challenged patients [16] emphasizing the important role TSG-6 has during inflammation. HC-HA matrices promote leukocyte adhesion to HA matrices as demonstrated in pathological conditions, including, rheumatoid arthritis [14], idiopathic arterial pulmonary hypertension [17,18] and asthma [15].

I.3. Pathological Hyaluronan Matrices

Adhesive HA structures on their cell surface with coalesced HA strands are referred to as HA cables [19]. These modified HA cables have structural information recognized by mononuclear leukocytes [20]. Leukocytes can bind to HA cables at 4° C [**Fig. 2**] and rapidly phagocytose the matrix at a physiological temperature of 37° C [19, 20].

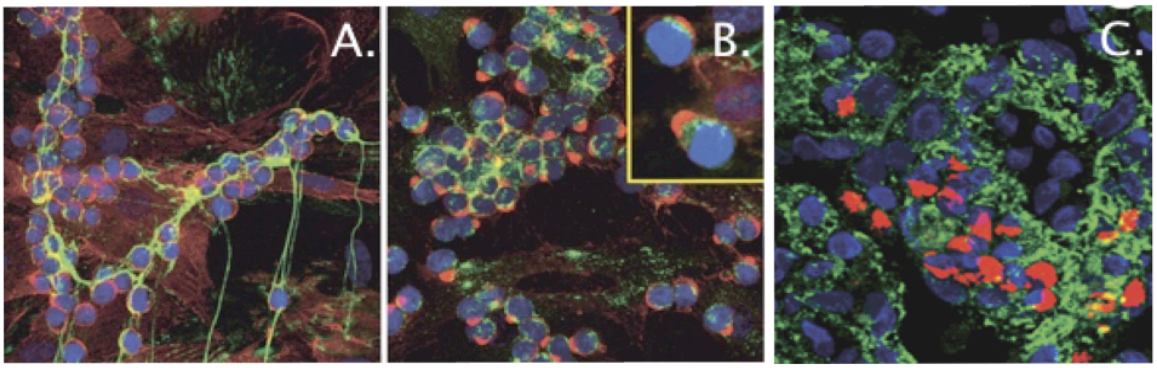

Fig. 2. Confocal Image of Leukocytes Bound to HA "Cable" Structures showing CD44 "Capping." Smooth muscle cells (colon) were treated with poly (I:C) to induce the synthesis of HA "cable" structures (green) (panel A). U937 monocytic leukocytes (CD44, red) bind these structures like "beads on a string" while not adhering to HA "coats" found on the cell surface. At permissive temperatures (panel B), the monocytic cells relocated, or "capped" CD44 to one pole and internalized HA fragments as shown in the enlarged inset. CD44 capped leukocytes were also found embedded in an HA matrix from an asthmatic lung biopsy (panel C). Nuclei are shown in blue. (Mag. 100X) [19,20].

These leukocyte adhesive HA matrices are seen in lung biopsies from asthmatic patients [15] or in response to ER stress at physiological normal levels of glucose [21], and in wound healing [22], idiopathic pulmonary hypertension [17,18], airway smooth muscle cells in vitro [23, 24] and airway interstitial cells in mouse asthma models [15], adipocytes in adipose tissue in a diabetic mice model [25,26] and renal tubular endothelial stress [27,28]. The binding of leukocytes to HA cables is essential for its removal, which occurs in a CD44-dependent manner [29]. This was demonstrated by showing that CD44-/- mice subjected to bleomycin inhalation to induce inflammation synthesized and accumulated HA matrix that couldn't be removed by the influx of monocytes and macrophages. Furthermore, the irradiated CD44-/- mice repopulated with normal monocytes and macrophages, were able to remove this matrix after the bleomycin treatment [30].

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CHAPTER II

MURINE AIRWAY EPITHELIAL CELLS RELEASE THE VAST MAJORITY OF THEIR HYALURONAN APICALLY

II.1. ABSTRACT

Murine airway epithelial cells behave differently than any other cell type reported so far. Besides making leukocyte adhesive HA cables that remain attached to the cell surface when stressed, they can also uniquely release HA rafts from their apical surface into their extracellular space. This unique release of HA occurs even when the cells are unstimulated. This implies that synthesis of HA rafts may be a normal process of airway epithelial biology, though it may be modified during periods of stress and inflammatory stimuli. We have developed a physiologically relevant *in vitro* air-liquid interface (ALI) culture model of murine primary airway epithelial cells that closely resembles the *in vivo* airway epithelium. We used this model to characterize these cell-free HA rafts and the

mechanism by which this chapter, we show that AECs constitutively release significant amounts of HA from their apical surface over the course of their *in vitro* differentiation in comparison to their basal compartment. HA released apically into the extracellular space was of a wide range in size with a median of 495 kDa.

II.2. INTRODUCTION:

II.2.1. Airway Epithelium and its Extracellular Matrix

The primary function of airway epithelium is to function as a physical barrier between the external and internal environment. There are three barrier functions: (i) mucociliary clearance by trapping and removing inhaled foreign particles from the airways [1], (ii) intercellular apical junctional complexes that regulate epithelial pericellular permeability [2], and (iii) secretion of antimicrobial substances such as enzymes, protease inhibitors, oxidants, and antimicrobial peptides that kill inhaled pathogens [3,4].

The airway epithelium sits upon a specialized matrix structure called a basement membrane. It is composed of type IV collagen, proteoglycans, laminins and [5]. The basement membrane has several important roles in maintaining epithelial integrity: (i) it acts as an anchor facilitating adhesion of epithelial cells; (ii) it establishes and maintains correct cellular polarity, (iii) it acts as a barrier between the surface epithelium and the underlying mesenchymal compartment, and (iv) it provides essential survival signals to the epithelium [6].

II.2.2. Air-Liquid Interface (ALI) Culture Model of Murine Airway Epithelial Cells

We have developed a physiologically relevant *in vitro* air-liquid interface (ALI) culture model of murine primary airway epithelial cells that closely resembles the *in vivo* airway epithelium. Primary AECs are isolated from murine tracheas by limited proteolytic digestion. After a series of purification steps, they are cultured on a native basement membrane. This basement membrane on the transwell insert is pre-deposited by Madine-Darby Canine Kidney (MDCK) epithelial cells. These steps are summarized as shown in [**Fig. 3**].

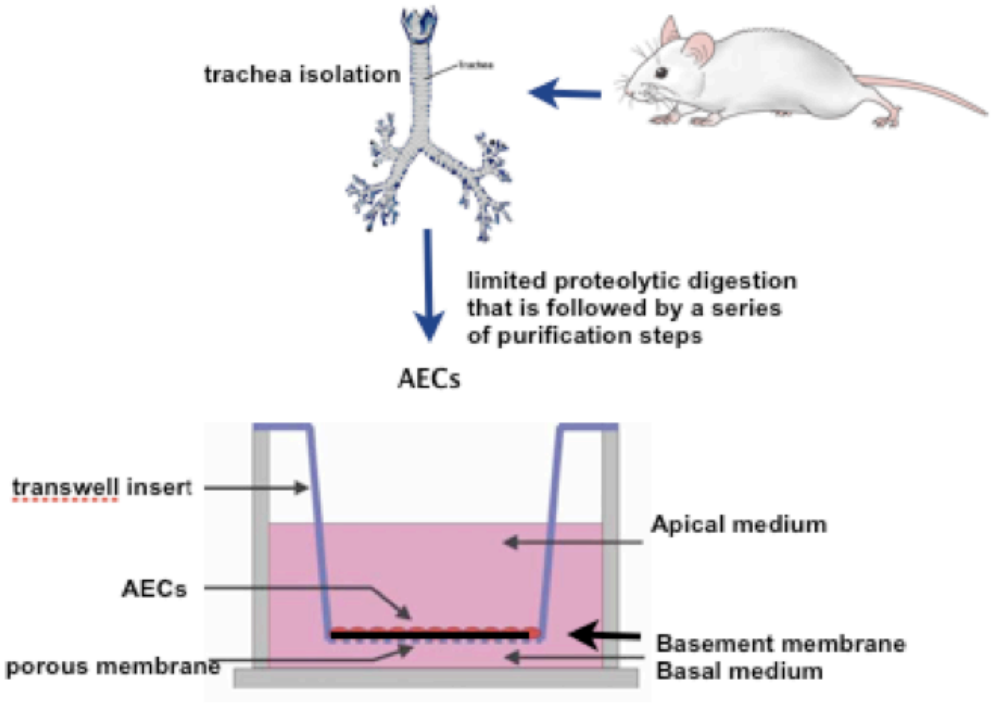

Fig. 3. A Schematic Illustration of the Main Steps in Murine Airway Epithelial Cell Culture.

After the AECs reach confluence, the culturing medium is removed (i.e. lifted) from the apical chamber, creating an "air-liquid interface" which promotes the differentiation of these cells into a mature, pseudostratified, and ciliated airway epithelium (10 – 14) days following lift [**Fig. 4**] [7]. It is also very important at this point to note that these cells are grown in serum free medium that lacks the source of IαI, the HC donor, and TSG-6.

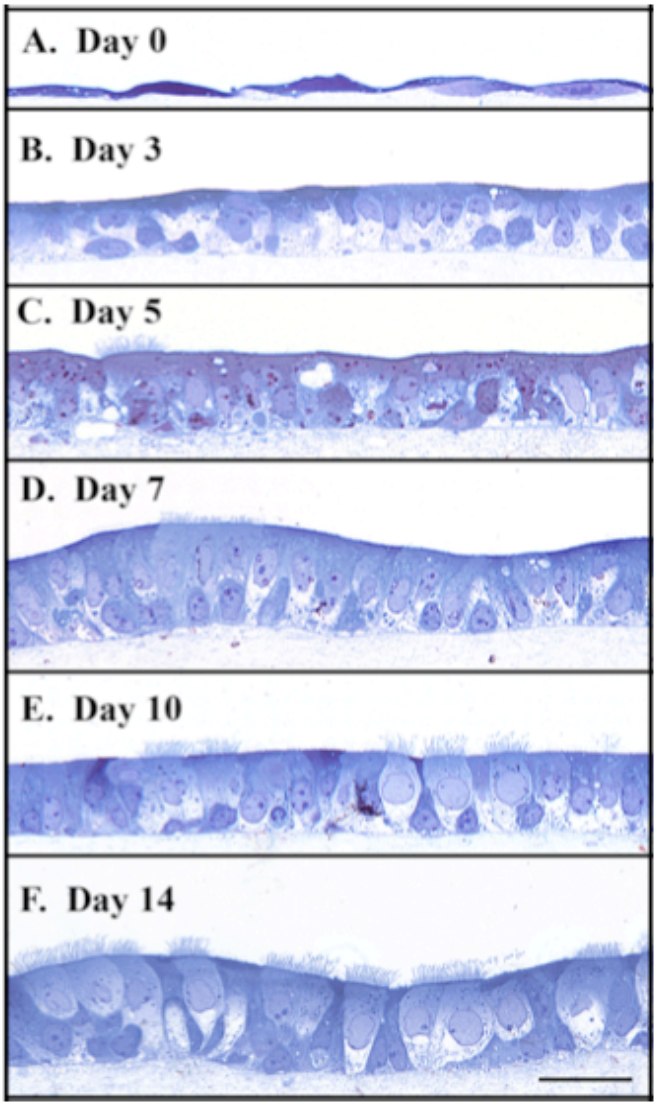

Fig. 4. Murine Airway Epithelial Cell Differentiation Time Course on Native Basement Membrane. Toluidine blue staining of 1 μm epoxy embedded AECs were harvested 0, 3, 5, 7, 10, and 14 days after exposure to an air-liquid interface (*A-F*, respectively). Differentiation progressed from squamous (day 0) to cuboidal (day 3) to pseudostratified (days 5-14). Cilia formation was apparent by day 5 and reached a plateau between 10 and 14 days. (Mag. 40 X, Magnification bar of 100 μm). [7]

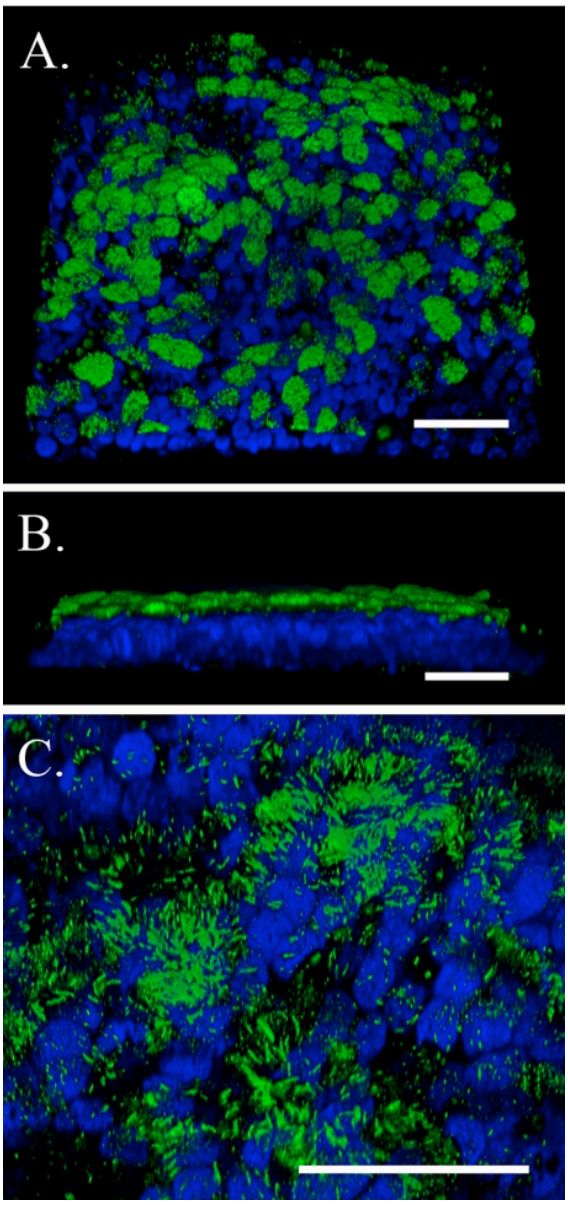

Fig. 5. Three-dimensional Confocal Images of Differentiated Murine Airway Epithelial Cells Cultured on a Native Basement Membrane. Cilia are highlighted by β-tubulin IV staining (*green*), and DAPI-stained nuclei are blue. (A) the apical surface of the epithelial cells (Mag. 63X). (B) a lateral cross-section of the epithelial cells (magnification is ×63). (C) the apical surface zoomed from 63X to ~160X, where the gain for the green channel was lowered to highlight individual cilia. All magnification bars are 50 μm. [7]

II.3. MATERIALS AND EXPERIMENTAL PRPCEDURES:

II.3.1. Materials:

100 mM ammonium acetate, pH 7.0, 400 mM Tris-acetate (T-6791, 500 g, MW 121.1; Sigma Aldrich, TRIZMA BASE,), AMAC (2-aminoacridone) Solution (A-6289, 25 mg, MW 246.7; Molecular Probes,), sodium cyanoborohydride (15615-9; Sigma Aldrich), glacial acetic acid, 10x Proteinase K (Storeroom #155490, 100 mg; Invitrogen,), Hyaluronidase, SD (100741-1A; Seikagaku) of a final concentration: 2.5 mU/µl, Chondroitinase ABC (100330-1A; Seikagaku) of final concentration: 25 mU/µl, *Streptomyces* hyaluronidase (100740-1; Seikagaku, East Falmouth, MA) of a final concentration: 0.5 TRU/mL, 1X TBE (electorphoresis buffer), ammonium persulfate (161-0700, 10 g; BioRad), TEMED (N,N,N'N'-Tetra-methylethylenediamine) (161-0801; BioRad), Select HA™ Hi ladder (HL0503; Hyalose, Oklahoma), Select HA™ Lo ladder (LL0401; Hyalose, Oklahoma), Mega- HA™ ladder (200505; Hyalose, Oklahoma, ML), Stains all (1-Ethyl-2-[3-(1-ethylnaphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-d]thiazolium bromide, 3,3′-Diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine) (E9379; Sigma Aldrich).

II.3.2. Mice:

All mice were maintained in the Biological Resource Unit of the Cleveland Clinic Lerner Research Institute in a temperature-controlled facility with an automatic 12 h light-dark cycle and were given free access to food and water. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic. TSG-6-/-

mice [8] were a generous gift from Dr. Tibor T. Glant (Rush University Medical Center, Chicago, IL), and (4-6)-week-old female TSG-6 -/- mice were used. Wild-type controls on a BALB/c background were purchased from Jackson laboratories and used for preparing airway epithelial cell cultures.

II.3.3. MDCK basement membrane:

Basement membranes on the transwells were generated by applying Madin-Darby canine kidney (MDCK) cells to the surface of the porous membranes of 12-well tissue culture inserts (353180; BD Falcon). After reaching confluence, the MDCK cells were removed by detergent lysis using PBS with 0.5% IGEPAL® CA-630 (I8896; Sigma) leaving behind a native basement membrane on the transwell surfaces. The basement membranes on the inserts were washed three times to ensure complete removal of the cellular debris. This method is similar to our previous method for basement membrane formation, with the exception that the basement membrane is not synthesized on a collagen matrix [7]. We found that the absence of the performed collagen layer did not hinder the AEC adhesion and that the AECs grew more uniformly in the absence of collagen layer.

II.3.4. Primary Cell Culture:

After the mice were sacrificed using either pentobarbital or isofluorane, the tracheas were excised from the mice and placed in Ham's F-12 nutrient medium with 50 units/ml penicillin, 50 μg/ml streptomycin, and maintained at 4° C on ice. Under a dissecting microscope, the esophagus and surrounding connective tissue were removed. The tracheas were cut longitudinally with a scalpel to expose the lumen and transferred to 5

ml of 0.15% pronase (10165921001; Roche Applied Science) in Ham's F-12 nutrient medium with 50 units/ml penicillin and 50 μg/ml streptomycin for an overnight incubation at 4° C in a 15-ml centrifuge tube. The next day, fetal bovine serum (FBS) was added to the tracheas (final concentration 10%) to inhibit further protease degradation. The medium, containing the released epithelial cells, was transferred to a 50-ml centrifuge tube. Washing medium (5 ml), containing Ham's F-12 nutrient medium with 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FBS, was added to the tracheas. The 15-ml centrifuge tube, containing the tracheas, was inverted several times to dislodge remaining epithelial cells. The supernatant was pooled with the original isolate, and the washing step was repeated twice, pooling all supernatants. The pooled epithelial supernatants were centrifuged at 300 × g for 10 min. The pellet was resuspended in 2 ml of Ham's F-12 nutrient medium with 50 units/ml penicillin, 50 μg/ml streptomycin, 10 mg/ml bovine serum albumin (BSA) (BP-1605-100; Fisher), and pancreatic DNase I (0.5 mg/ml) (DN-25; Sigma), and was incubated for 5 min on ice before it was centrifuged at 300 × g for 5 min. The pellet was resuspended in 12 ml of DMEM/F-12 with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 20 mM L-glutamine, and 10% FBS and transferred to a 100-cm^2 Petri dish and incubated at 37° C, 5% CO_2, and 100% humidity for 3 h to allow for the attachment of any contaminant smooth muscle cells or fibroblasts onto the dish. Afterwards, the culturing medium, containing the non-adherent epithelial cells, was transferred to a 10-ml tube. The adherent cells were washed once with 5 ml of medium to remove remaining non-adherent epithelial cells and pooled with the first isolate. The purified epithelial cells were centrifuged at 300 × g for 10 min. The pellet was re-

suspended at 100,000 cells/ml in DMEM/F-12 medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 20 mM L-glutamine, 5 mM glucose (physiological levels), 5% FBS, 10 μg/ml insulin (I-6634; Sigma), 5 μg/ml transferrin (T-1147; Sigma), 0.1 μg/ml cholera toxin (C-8052; Sigma), 25 ng/ml epidermal growth factor (354001; BD Biosciences), 30 μg/ml bovine pituitary extract (P-1476; Sigma), and 80 nM retinoic acid (R-2625; Sigma). The cells were seeded onto the basement membrane-covered tissue culture inserts at 50,000 cells/well of a 12-well plate. AECs were cultured with medium replacement every other day. After the AECs reached confluence, which is normally within 6-7 days from the day they were seeded on transwell inserts, the culturing medium is removed (i.e. lifted) from the apical chamber. This lift promotes the differentiation of these cells into a mature, pseudostratified and ciliated airway epithelium by day 10 after lift.

II.3.5. Immunohistochemistry

Paraffin embedded sections of lung tissues were previously fixed overnight in 10% formalin. The sections were rehydrated in PBS for 30 min and then blocked with 1% BSA in PBS. HA was labeled with a HA biotinylated binding protein (HABP) (5 μg/mL; 385911; EMD Chemicals, Gibbstown, NJ) in blocking solution, and then with streptavidin conjugated to Alexa Fluor® 488 (1:500, product S11223; Invitrogen, Carlsbad, CA). These slides were simultaneously incubated with anti-KDEL (PA1-013; Affinity Bioreagents, Golden, CO) conjugates with Alexa Fluor® 594 secondary antibody (1:500, product A21203, Invitrogen, Carlsbad, CA). Vectashield mounting medium with DAPI (H-1200; Vector Laboratories, Burlingame, CA) was applied to the apical surface of the samples followed by coverslip application before visualizing using

fluorescent microscopy.

II.3.6. Apical Washes Collection:

After the culturing medium is removed apically on day 10 of lift, the AECs are being cultured on an air-liquid interface, and the apical surfaces are gently washed every other day with 200 μL of phosphate buffered saline (PBS) per insert of a 12-well plate. Apical washes were pooled and frozen at -80° C until the time of use.

II.3.7. Fluorophore-Assisted - Carbohydrate Electrophoresis (FACE)

HA contents in the apical washes and basal media were measured by FACE as previously described [9]. This technique was developed in our laboratory and has many advantages over other methods of HA analysis since it can be used to quantify picomolar amounts of HA from multiple samples using common vertical gel electrophoresis equipment.

Briefly, 10X Proteinase K was added to the apical washes or basal conditioned media and mixed to give a final concentration of 1X PK then incubated at 37° C overnight. 4 volumes of pre-chilled (–20° C) 200 proof ethanol was added to each sample, which were then incubated at – 20° C overnight. Next day, samples were centrifuged at 14,000 g for 10 minutes at RT. Supernatants were discarded, and the pellets, which contained the glycosaminoglycans, were washed by adding 4 volumes of pre-chilled (–20° C) 75% EtOH followed by a short vortex/spin. Samples were then centrifuged at 14,000 g for 10 minutes as before. Washes were discarded as before, with the residual ethanol removed with a pipet. The pellets were dried at RT for 20 minutes. 35 μl 100 mM ammonium acetate was added to each sample followed by a vortex/spin, and then incubated at RT for

20 minutes followed by a vortex/spin. The PK was heat inactivated by incubating the samples at 100° C for 5 min, which were then chilled on ice for 5 min. 1 µl of chondroitinase ABC and hyaluronidase SD was added to each 35 µl sample to digest the hyaluronan and chondroitin sulfate followed by a vortex/spin, and then incubated at 37° C overnight (at least 18 hrs). 160 µl of pre-chilled (–20° C) 200 proof ethanol was added to each sample followed by a vortex/spin, and then incubated overnight at –20° C. Samples were centrifuged at 14,000 g for 10 minutes at RT. Supernatants were saved in a separate 1.5 ml tube *(Note: hyaluronan and chondroitin sulfate disaccharides are now in the supernatant; heparan sulfate, nucleic acids and other carbohydrates are in the pellet)*. The pellets were then washed with 160 µl of pre- chilled (–20° C) 75% ethanol and centrifuged as before. Washes were pooled with the previous supernatants for each sample. Pooled supernatants of each sample were lyophilized (using a "Speed-Vac" or equivalent), and 2.5 µl of the AMAC solution was added to each sample and incubated at 37° C for 18 hrs in the dark. The samples were run on a gel prepared by mixing 5 mL of (500 mL 40% acrylamide (37.5:1) (161-0148; BioRad), 100 ml Tris-Acetate (400 mM, pH 7.0), 370 ml distilled water and 25 ml of glycerol) with 25 µl ammonium persulfate (10%) and 5 µl TEMED. The run of the gel was at 500 V constant voltage for 55 minutes using the BioRad Mini- PROTEAN® Tetra Cell.

A detailed protocol is found on our Program of Excellence in Glycosciences at Cleveland Clinic webpage:

http://pegnac.sdsc.edu/cleveland-clinic/files/PEGNAC_FACE_Prot_GAGsv1_1.pdf

Images of the gels were taken using myECL Imager (Thermo Scientific), and the band intensities were quantified using ImageJ software that can be downloaded using the link: http://imagej.nih.gov/ij/

II.3.8. HA sizing

10X Proteinase K was added to pooled apical washes, from 12 transwell inserts of AECs every other day during the time course of their differentiation, and mixed to give a final concentration of 1X PK, and then incubated at 37° overnight. 4 volumes of ice cold 200 proof ethanol were added to the samples followed by a Vortex/Spin and then incubated overnight at -20° C. The samples were centrifuged at 13,200 g for 10 min, and the supernatants were decanted. The pellets were washed with 1 ml ice cold 75% ethanol and centrifuged as before, and then air dried for 20 minutes before re-suspending them in 0.1 ml of 100 mM ammonium acetate (pH 7.0). The proteinase K in the samples was heat inactivated on a boiling water bath for 10 minutes, and then spun down. 3 μl of DNAse (2224, 2 U/ul; Ambion) and 3 μl of RNAse A (109169, 1.28 mg/ml; Roche) were added to each sample and incubated overnight at 37° C. The enzymes were heat inactivated on a boiling water bath for 5 min and then spun. 400 μl cold 200 proof ethanol was added to each sample and incubated overnight at -20° C. Samples were centrifuged at 13,200 g for 10 min, and then washed with 1 ml cold 75% ethanol and centrifuged as before. A p200 tip was used to remove any residual ethanol, and the pellet air-dried. Pellets were re-suspended in 20 μl 100 mM ammonium acetate (pH 7.0) and transferred to a 200 μl PCR tube. 1 μl of each sample was transferred to a separate tube and saved for subsequent FACE analysis. 1 μl sialidase (N8271-0.2UN; Sigma) was added, and each sample was

divided in half by transferring 9.5 μl to another tube to which 1 μl *Streptomyces* hyaluronidase (100740-1, 0.2 TRU/μl stock; Seikagaku) was added and incubated overnight at 37°C. The other half was incubated without the enzyme. The enzymes were heat inactivated on a boiling water bath for 5 minutes and then spun down. The samples were lyophilized by "Speed-Vac" and re-suspended in 10 μl of 10 M formamide (F-476, 10 M; Sigma-Aldrich,), and then incubated overnight at 4° C. Samples are run on a 1% agarose gel (SeaKem HGT Agarose, 50041; Cambrex) in 1x TAE that is pre-run for 6 hours at 80 V (constant voltage) to remove impurities that stain strongly with Stains-All. 2 μl tracking dye (0.2% bromophenol blue in 10 M formamide) was added to the samples. The entire sample (now at 12 μl) was loaded to a well. The gels were run at 100 V (constant voltage) until the tracking dye has migrated about 75% the length of the gel (1-1.5 hr). The gels are then transferred into a dish with 30% ethanol to equilibrate the gel into ethanol, which prevents the formation of Stains-All precipitates, and then rocked for 1 hour. The ethanol was decanted and replaced with Stains-All solution (E-9379; Sigma) for overnight incubation. The gel was totally covered to prevent light from degrading the dye. The stain was removed and replaced with water followed by shaking for 1 hour. The gel was briefly (less than 1 hour) exposed to light to decrease background as needed. The gel was scanned on a standard color scanner.

II.3.9. PCR Analyses

RNA was isolated using the RNAeasy kit (74104; Qiagen), and cDNA was prepared using Superscript @ First-Strand Synthesis System (11904-018; Invitrogen). Primers of HAS 1-3 used are listed in [Table 1] as previously reported in Cheng et al [10]. The PCR

conditions were 1 cycle at 94° C for 3 min, 40 cycles of 95° C for 30 sec, 55° C for 45 sec, 72° C for 1 min, followed by 1 cycle of 72° C for 1 min. These conditions were used for all primers. PCR products were run on 3% ethidium bromide gels (E1510; Sigma) and 1% agarose gels (SeaKem® HGT Agarose, 50041; Lonza).

Table 1. The Sequences of the Polymerase Chain Reaction (PCR) Amplification Primers

Name of Primers	Primer Sequence	Expected PCR product
β-actin	5'-GGTCATCACTATTGGCAACG-3' 5'-ACGGATGTCAACGTCACACT-3'	133 bp
HAS1	5'-GGTGGTTACCGTGTCCAAGTAT-'3 5'-GAAGGTAAACTGAGTCCCCAGA-3'	120 bp
HAS2	5'-GGTCCAAGTGCCTTACTGAAAC-3' 5'-TGTACAGCCACTCTCGGAAGTA-3'	96 bp
HAS3	5'- AGACCGAGCTAGCCTTCCTAGT-3' 5'-TAATGGCCAGATACAGCATGAG- 3'	84 bp

II.3.10. *Statistical Analysis*

Data are presented as the mean ± SEM; n is indicated in figure legends in representative experiments.

II.4. RESULTS AND DISCUSSION

II.4.1. *HA rafts on apical surfaces of murine airway epithelial cells (AECs) in vivo and in vitro.*

HA is located on the apical surfaces (white arrows) of murine AECs as distinct clumps, or HA rafts (green), that can be seen both *in vivo* on a normal WT epithelium [Fig. 6A] and on an air/liquid day 10 AEC culture *in vitro* [Fig. 6B]. Furthermore, the air/liquid AEC culture model closely resembles its *in vivo* counterpart, as previously shown [7] in terms of its pseudo-stratification, cellular morphology, presence of cilia, and KDEL (a Endoplasmic Reticulum marker) distribution. Thus, it represents a compatible model to study the HA rafts that murine AECs make.

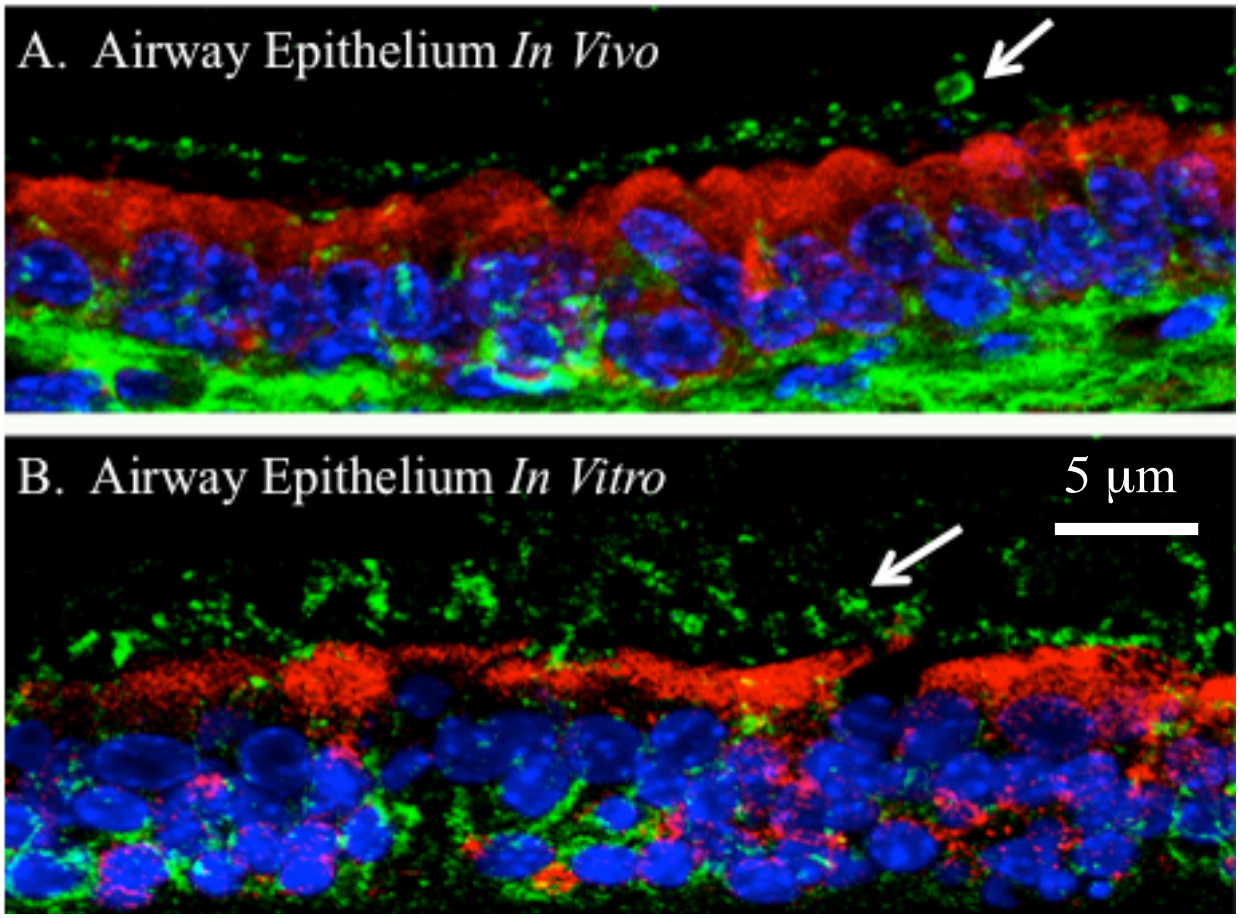

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Fig. 6. HA Rafts are Loosely attached to the Apical Surface of Murine Airway Epithelia *in vivo* and on AEC Lift Cultures *in vitro*. Paraffin embedded sections of a murine airway epithelium (A) *in vivo*, and a 10-day differentiated air/liquid AEC culture (B) *in vitro*, were stained for HA with a fluorescent conjugate of a biotinylated HA binding protein (green). Nuclei were stained with DAPI (blue) and for endoplasmic reticulum (ER) (red) with an antibody against KDEL. The arrows indicate the apical HA rafts. HA is also apparent in the basal areas with most of it in the underlying connective tissue *in vivo*. Images are from a zoomed in micrograph (Mag. 63X , magnification bar of 5 μm).

II.4.2. *Murine AECs release the majority of HA from their apical surface.*

The apical surfaces of air/liquid AEC cultures were gently washed during their course of differentiation over 10 days and analyzed for HA [Fig. 7]. HA in the basal media was also measured for comparison. The results indicate that the HA shown to be on the surface [Fig. 6B] is loosely attached, and that only small amounts are present in the basal medium. This implies that the synthesis of this loosely attached HA on the apical surface of AECs, which also appears to be present *in vivo* [Fig. 6A], may be a normal process of airway epithelial biology, though it may be modified during periods of stress and inflammatory stimuli.

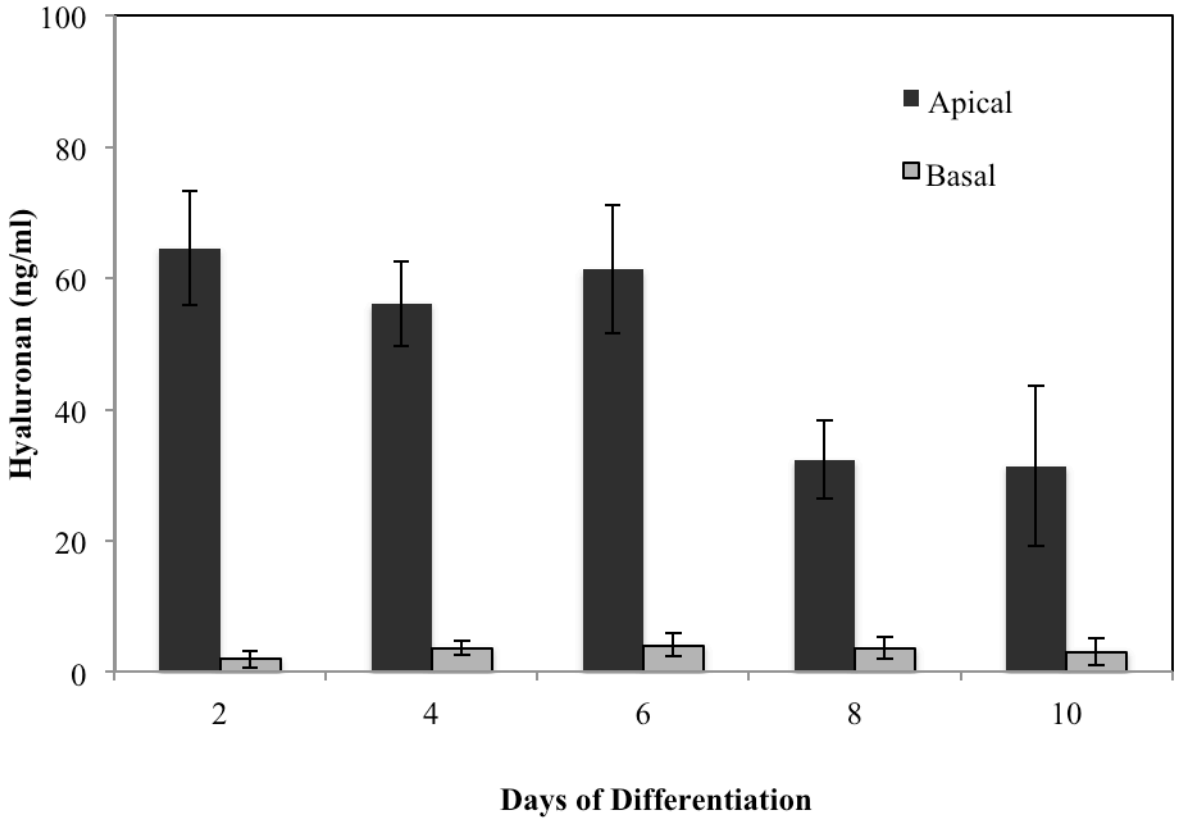

Fig.7. Murine AECs release Most of their HA from their Apical Surface. The bar graph shows the amounts of HA determined by FACE in the apical washes (black bars), and basal media (grey bars) collected during the differentiation phase of AEC cultures (N=6). The values in the apical washes are statistically greater $p<0.01$ than those in the basal media from 2 through 10 days of differentiation with apical washes taken every 2 days.

II.4.3. *Murine AECs release a wide range of HA sizes from their apical surface.*

The size of HA varies between cell/tissue type and can have a profound effect on the function of HA [11]. HA fragments generated by multiple mechanisms throughout the course of inflammatory pathologies, elicit cellular responses distinct from intact HA [12]. Typically, the full-length polymer has a very high molecular mass, ranging from 105 to 107 Da. The high molecular mass HA serves as a space filler, osmotic buffer, and viscoelastic structure [13]. Under certain physiological and pathological conditions, HA oligosac- charides exhibit quite different biological activities from the polymers, including induction of angiogenesis [14] and cell proliferation [15]. AECs uniquely release the vast majority of their HA from the apical surface in comparison to what is released basally during their differentiation time course. To determine the size of HA in the HA raft, we analyzed the HA in the apical washes of murine WT AECs collected every other day during their differentiation time. The size of HA of the HA raft is variable and ranges between very high molecular weight HA to as low as 27 kDa with a median of 495 kDa as shown in [**Fig. 8**]. This implies that AECs dynamically metabolize the HA, synthesizing very high molecular weight (HMW) HA ranging between (1520-6100 kDa) and breaking it down by hyaluronidases into smaller fragments.

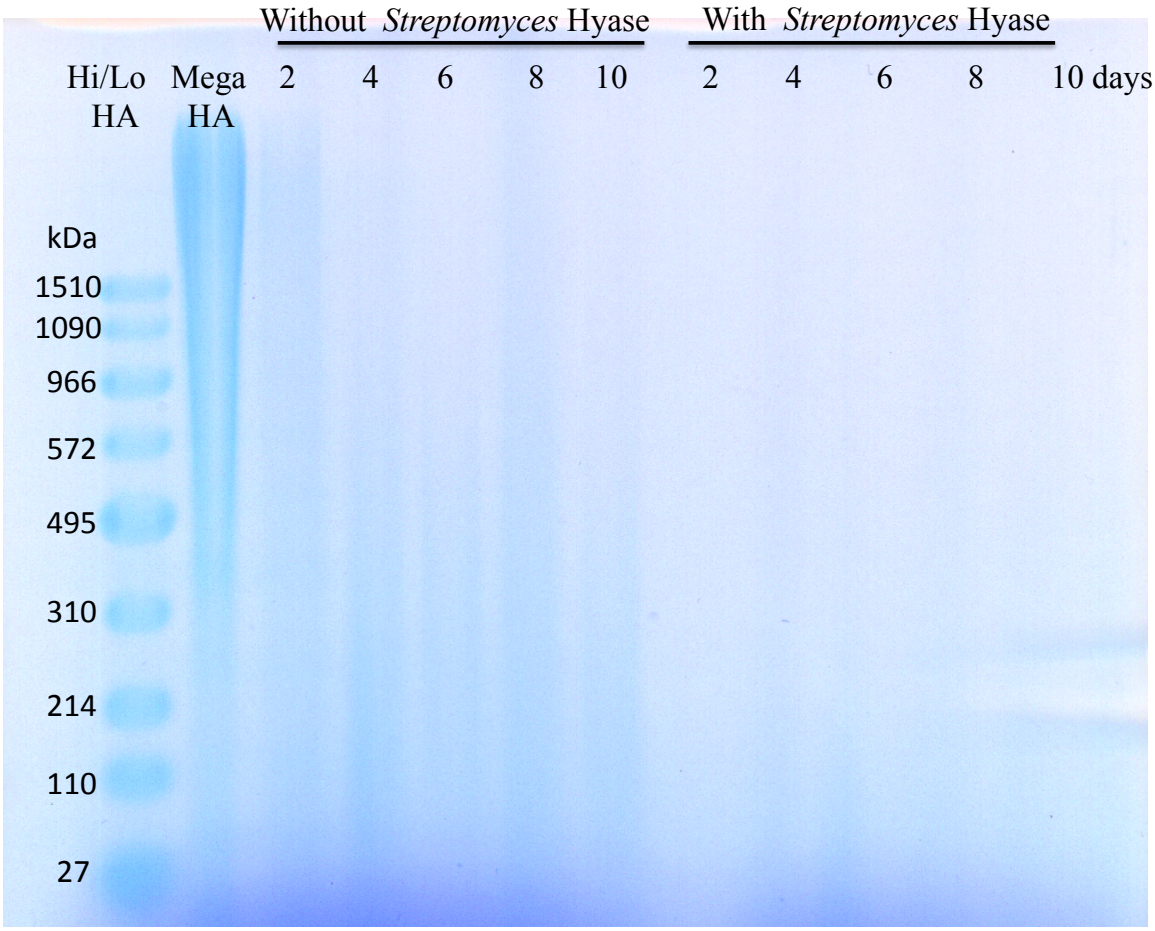

Fig. 8. Hyaluronan Size Analysis by Agarose Profiling. The size of hyaluronan of the HA raft is analyzed on a 1% agarose gel stained with Stains all. The agarose profiling shows both high and low molecular weights (Hi and Lo HA) of hyaluronan standards. Mega HA standards range between (1520 – 6100) kDa. The left lane shows a ladder of HA MW standards. The *Streptomyces* hyaluronidase (hyase) specifically breaks down the hyalronan into disaccharide units and was used in this experiment to demonstrate that the detected smears on the gel are of hyaluronan composition.

II.4.4. ***Murine AECs express HAS1-3 that are involved in the synthesis of HA rafts.***

HA is synthesized by membrane-bound synthases on the inner surface of the plasma membrane with the chains being extruded through pore-like structures into the ECM. There are three mammalian hyaluronan synthases (HAS1-3) [16,17]. HAS2 is the most crucial enzyme in HA synthesis since mice lacking the ability to express HAS2 die at mid-gestation[18] while HAS1 or HAS3 knock-out mice show no effect on fetal development [19].

AECs can express all three HAS isoforms especially HAS2 and HAS3 that are involved in the HA synthesis as shown is **[Fig. 9]**. This HA could be part of the ECM of the AECs but is mostly released into the extracellular space as HA rafts as shown in **[Fig. 7].**

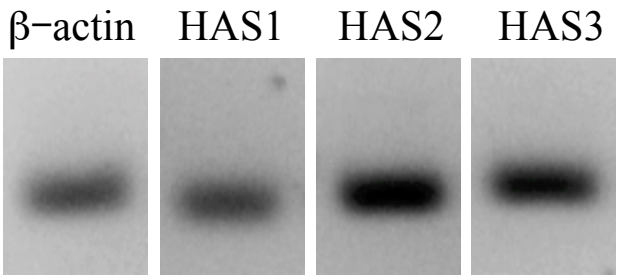

Fig. 9. Relative Gene Expression of Hyaluronan Synthases by AECs. Hyaluronan Synthases (HAS) 1-3 expression of a 10 day murine AECs is shown by the ethidium bromide stained PCR product of HAS1-3 separated on a 1% agarose gel. (N=3).

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CHAPTER III

MURINE AIRWAY EPITHELIAL CELLS MAKE LEUKOCYTE-ADHESIVE HYALURONAN RAFTS LOCATED ON THEIR APICAL SURFACE

III.1. ABSTRACT

For the past decade, a variety of cell types were shown to synthesize leukocyte- adhesive HA structures on their cell surface, which resemble braided rope made of individual HA strands known as HA cables, in response to a variety of inflammatory stimuli. In all cell types studied to date, the leukocyte-adhesive HA cables synthesized by these cells remain attached to the cell surface. However, while AECs also form HA cables on their cell surface in response to a variety of inflammatory stimuli, they also actively release a majority of their leukocyte-adhesive HA structures into the extracellular space from their apical surface even when they are not stimulated. In this chapter, we demonstrate that AECs can make leukocyte adhesive HA rafts that are loosely attached to the cell surface

because of the heavy chain (HC) transfer onto the HA raft that is mediated by TSG-6. These structures are not only found in our *in vitro* culture model but also *in vivo*. Leukocyte-,adhesive HA rafts were found in the bronchoalveolar lavage (BAL) of naïve wild type mice, and were observed on their apical ciliated surfaces. HA rafts were also found in the BAL of a TSG-6 null mouse, but the HC transfer onto the HA was absent.

III.2. INTRODUCTION

HA "cables" were first identified as HA rope-like structures that were attached to the surfaces of colon smooth muscle cells treated with poly (I:C), a viral mimic, and that promote leukocyte adhesion [1]. HA "cables" formed at the cell surface of a variety of cells in response to a variety of inflammatory stimuli and function as a ligand for inflammatory cells. We have previously found that transfer of HCs during the formation of these HA "cable" structures on airway smooth muscle cells (ASMCs), upon treatment with poly (I:C), stabilizes them, resulting in a more pronounced cable formation which promotes greater leukocyte adhesion **[Fig. 10]** [2]. The addition of TSG-6 catalyzed the transfer of HCs from IaI present in the serum in the medium onto the HA that is being induced by poly (I:C) treatment. Leukocytes adhesion to these cables is in a HC-independent manner as shown in **[Fig. 10B and 11],** but substitution of these cable structures with HCs significantly promoted leukocyte adhesion as shown in **[Fig. 10C].**

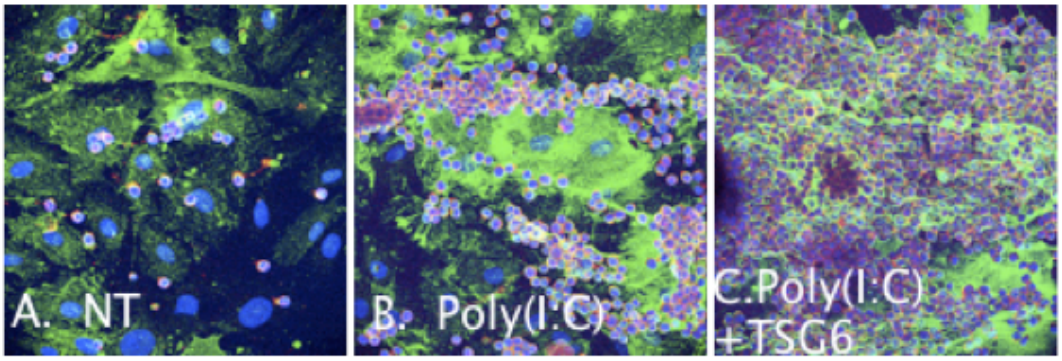


Fig. 10. The HC-HA Complex promotes Leukocyte Adhesion to HA Cables. Confocal images showing mononuclear U937 leukocytes (red with CD44 staining) bound to HA cables in ASMCs (green, with hyaluronan binding protein staining), induced by poly (I:C), or a combination of poly (I:C) and TSG-6 compared to untreated controls (NT). Nuclei are blue (DAPI staining). The addition of TSG-6 significantly enhanced leukocyte adhesion to HA cable structures via the formation of the HC-HA complex [2].

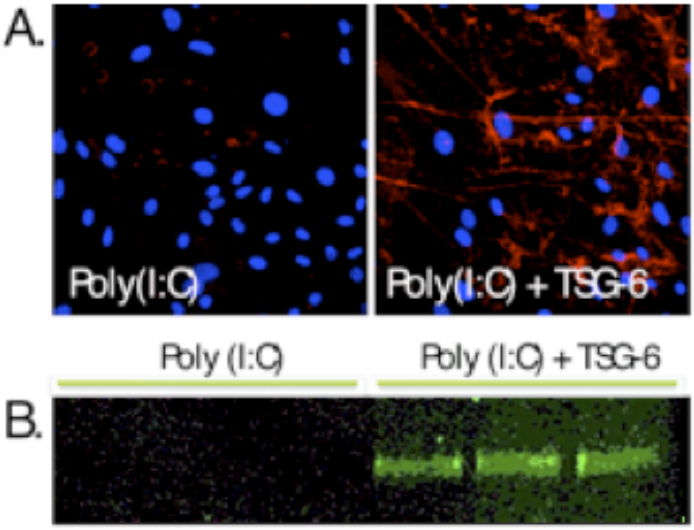


Fig. 11. Addition of HCs to HA Cables via Recombinant TSG-6. HA "cables" produced by ASMCs in response to poly (I:C) lack the HC modification. The addition of recombinant TSG-6 was used to artificially add HCs to HA "cables" to form the HC-HA complex. (A) Confocal images of ASMCs probed with an antibody against HCs (red) with nuclei in blue (Mag. 63x). (B) Western blot of *Strepotmyces* hyaluronidase extracts from these cables, thereby releasing the HCs (green) into the extract (N = 3) [2].

AECs produce leukocyte adhesive HA "cables" like other cell types in response to inflammatory stimuli such as tunicamycin, which results in an endoplasmic reticulum (ER)-stress **[Fig. 12]** [3]. Interestingly, we report for the first time the release of leukocyte- adhesive HA rafts from the apical surface of AECs that occurs in the absence of inflammatory stimuli. This implies that the synthesis of HA rafts is a part of normal respiratory secretions by AECs.

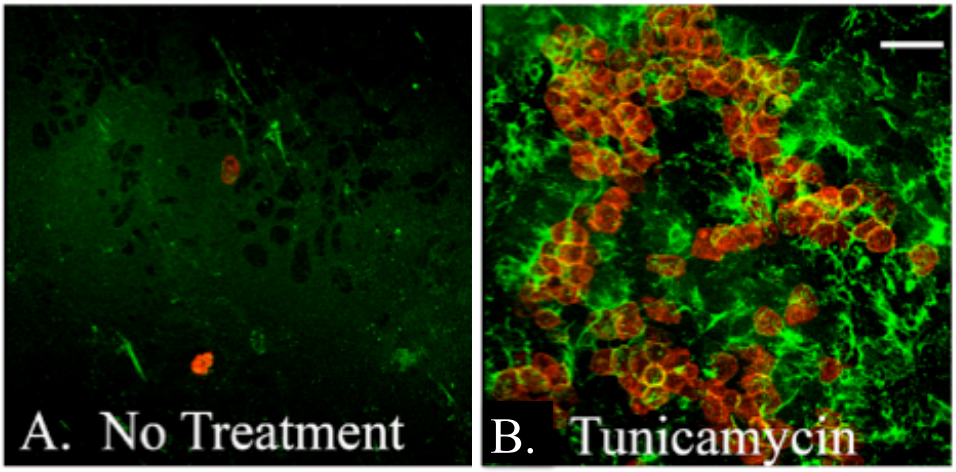


Fig. 12. U937 Monocytic Cells bind to Hyaluronan on the Apical Surface of Differentiated Murine Airway Epithelial Cells treated with Tunicamycin. CM-DiI-labeled U937 cells (red) were applied to the apical surface of epithelial cells treated without (A) or with tunicamycin (B). Hyaluronan (green) was labeled with fluorescence-tagged streptavidin on a biotinylated hyaluronan binding protein. (Mag. 40X, magnification bar of 100 μm) [3].

III.3. MATERIALS AND EXPERIMENTAL PROCEDURES

III.3.1. Mice

As described in section II.3.2. [4].

III.3.2. Apical Washes Collection

Apical washes were collected by gently washing the apical surface of differentiated AEC cultures of day 10 after lift, removal of apical media, with 500 µL of phosphate buffered saline (PBS).

III.3.3. Leukocyte Binding to Apical Washes Collected from AEC cultures

U937 cells, a human monocytic leukocyte cell line, suspended in RPMI medium with 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B and 20 mM L-glutamine were used for this binding assay. Around 50,000 U937 cells were incubated with 500 µL apical wash, which was collected from a day 10 AEC culture, for an hour at 4° C then transferred to a glass slide via cytospin and left to air dry completely before the processing for immunohistochemistry **[Fig. 13].**

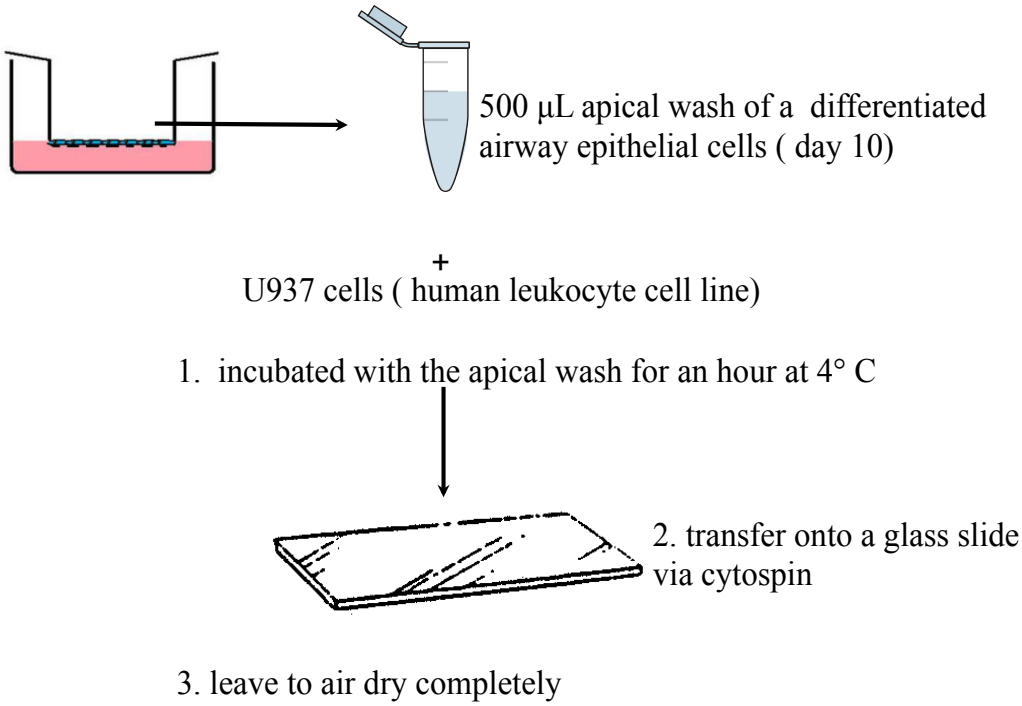


Fig. 13. A Schematic Illustration of the Main Steps in U937 Cells Binding Assay to the HA rafts *in vitro.*

III.3.4. Bronchoalveolar Lavage (BAL) Collection

Mice were euthanized by using isofluorane or pentobarbitol. BALs were obtained by cannulating the trachea with a 24 gauge-feeding needle and lavaging the lungs with 600 μL of PBS. The typical BAL fluid returned was 400-500 μL. A 100 μL aliquot of the collected BAL was transferred to a glass slide via cytospin and left to air dry completely before processing for immunohistochemistry.

III.3.5. Immunohistochemistry

Aliquots of BAL or apical washes from day 10 AEC cultures were transferred to a glass slide via cytopsins and were air dried completely. Samples were then fixed with 100% methanol at -20° C for 10 min and air dried for 1 h. Methanol fixed cytospins of BAL and apical washes of AECs were rehydrated in PBS for 30 min and then blocked with 1% BSA in PBS. HA was labeled with a HA biotinylated binding protein (HABP) (5 μg/mL; 385911; EMD Chemicals, Gibbstown, NJ) in blocking solution, and then with streptavidin conjugated to Alexa Fluor® 488 (1:500, product S11223; Invitrogen, Carlsbad, CA). These slides were simultaneously incubated with a cocktail of antibodies to HC1, HC2 and HC3, using Alexa Fluor® 594 donkey anti-mouse secondary (1:200; product A11058; Invitrogen) or anti-CD44 (1:250; product C7923, Sigma-Aldrich, St. Louis, MO) or anti-KDEL (PA1-013; Affinity Bioreagents, Golden, CO). Vectashield mounting medium with DAPI (H-1200; Vector Laboratories, Burlingame, CA) was applied to the apical surface of the samples followed by coverslip application before

visualizing using fluorescent microscopy.

III.3.6. Western Blot Analysis

Samples were electrophoresed on 10% SDS–PAGE and transferred onto Immobilon-P membranes (Millipore). All blots were blocked for 1 h with 5% milk (Blotting Grade Blocker, 170-6404; BioRad) and probed overnight at 4° C with antibodies against HC1 (SC-33944; Santa Cruz Biotechnology), HC2 (SC-21978; Santa Cruz Biotechnology), HC3 (SC- 21979; Santa Cruz Biotechnology), and IαI (A0301; Dako Cytomation, Denmark). Blots were washed with 1X Tris Buffered Saline (TBS) with TWEEN®20 (0777-2 L; AMRESCO) 3 times for 7 minutes each time. Blots were then incubated with donkey anti-goat IgG-HRP (SC-2020; Santa Cruz Biotechnology) as secondary antibody for 1 h at room temperature then washed again for three times for 7 minutes each time. TSG-6 was assessed using a biotinylated anti-mouse TSG-6 antibody (BAF2326; R&D systems, Inc.) followed by the treatment with streptavidin-horse radish peroxidase (HRP) (P0397; Dako Cytomation) for 1 h at room temperature. Recombinant mouse TSG-6 (2326-TS; R&D systems, Inc.) was used as a positive control for the TSG-6 antibody. All blots were developed using the ECL Prime Western Blotting Detection Reagent (RPN2232, Amersham) and detected protein band intensities were measured using ImageJ software (http://imagej.nih.gov/ij/). Protein standards (Fermentas Page Ruler Prestained Protein Ladder, SM0671; Thermo Pierce) were used with each gel that was run.

III.3.7. *Hyaluronidase Extraction of HCs from the HC-HA Complex*

HCs were determined in bronchoalveolar lavage (BAL) samples and in apical washes of 10 day air/liquid interface cultures of primary differentiated AECs by treatment with or without *Streptomyces* hyaluronidase (0.5 TRU/mL; 100740-1; Seikagaku, East Falmouth, MA) overnight at 37° C. The supernatants were then analyzed by Western blotting.

III.3.8 *Statistical Analysis*

Data are presented as the mean ± SEM; n is indicated in figure legends in representative experiments.

III.4. RESULTS AND DISCUSSION

III.4.1 *Murine AEC cultures release leukocyte adhesive HA rafts from their apical surface.*

HA cables are formed at the cell surface of a variety of cells including AECs [5] during inflammatory processes. These structures are known to function as a ligand for inflammatory cells [1]. However, the majority of the apical HA produced by AECs can be released into the extracellular space as HA rafts. To investigate whether these rafts are also leukocyte adhesive, we incubated the apical washes of 10 day AEC cultures with a human cell line of monocytic U937 cells for an hour at 4° C. Subsequently, an aliquot was transferred to a slide via cytospin, and stained for CD44, a HA receptor. Multiple clusters of U937 cells were embedded in the HA rafts as shown in **[Figure 14A-C].**

Murine AECs behave differently than any other cell type reported so far. Besides making leukocyte adhesive HA cables that remain attached to the cell surface in response to a variety of inflammatory stimuli, they also actively release a majority of their HA matrices into the extracellular space from their apical surface. These matrices do not resemble the rope-like HA cables, but occur in distinct sheets or rafts of HA that are leukocyte-adhesive.

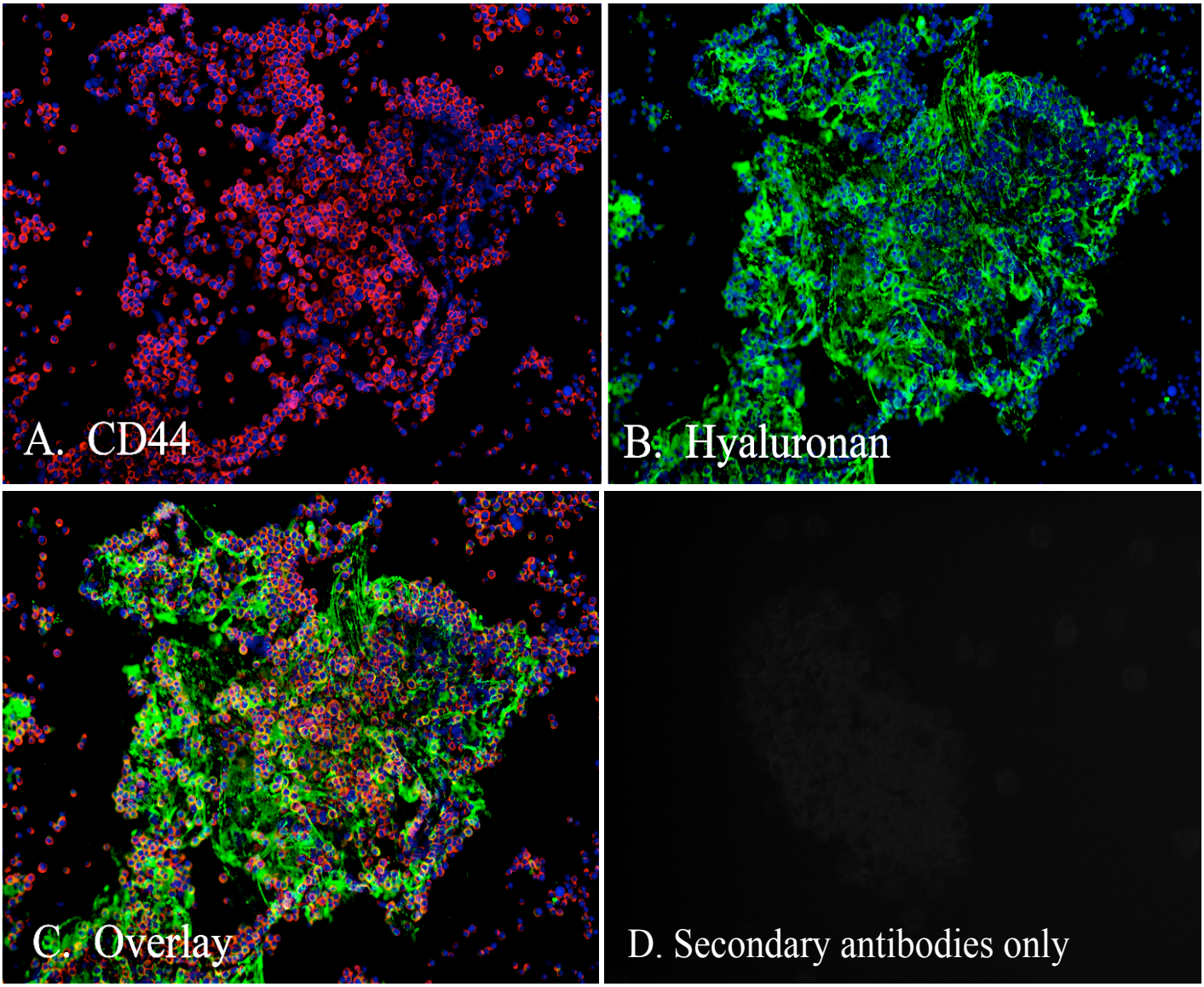


Fig. 14. Leukocyte-Adhesive HA Rafts *in vitro*. (A-C) Monocytic U937 cells were suspended in the apical wash from a 10 day AEC culture for 1 h at 4° C and subsequently cytospinned onto a slide. HA was stained with a fluorescent conjugate of biotinylated HA binding protein (green). U937 cells were probed with antibodies against CD44 (red). This fluorescent micrograph shows multiple clusters of U937 cells embedded in HA rafts (Mag. 20X). (N=3).

Furthermore, similar adhesion of U937 cells was observed when they were added to the apical surface of an AEC culture **[Fig. 15]**. This indicates that the HA rafts released by the AECs are also leukocyte-adhesive similar to the HA cables that are known to form in other pathologies. However, in this case, they are not being formed in response to a pathological stimulus.

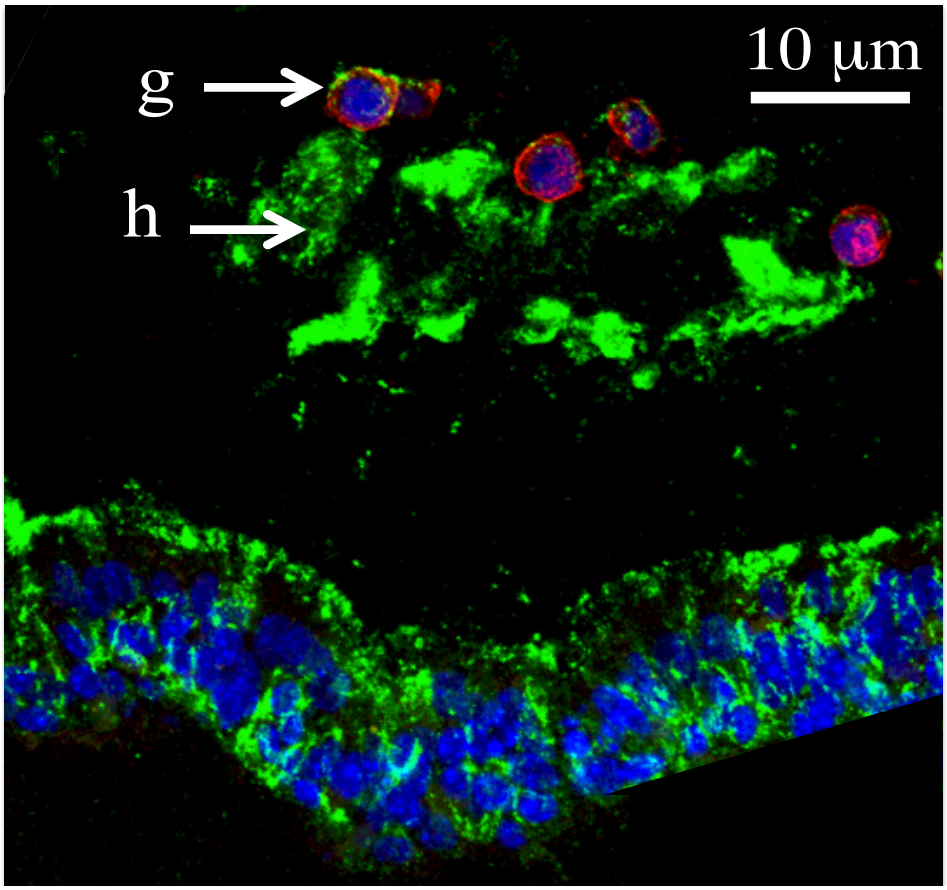


Fig. 15. Murine AECs release Leukocyte-Adhesive HA Rafts from their Apical Surface. The micrograph of a section of non-stimulated 10 day AEC culture (Mag. 63X, magnification bar of 10 µm) shows an example of U937 monocytes (g) binding to the loosely attached HA raft (h).

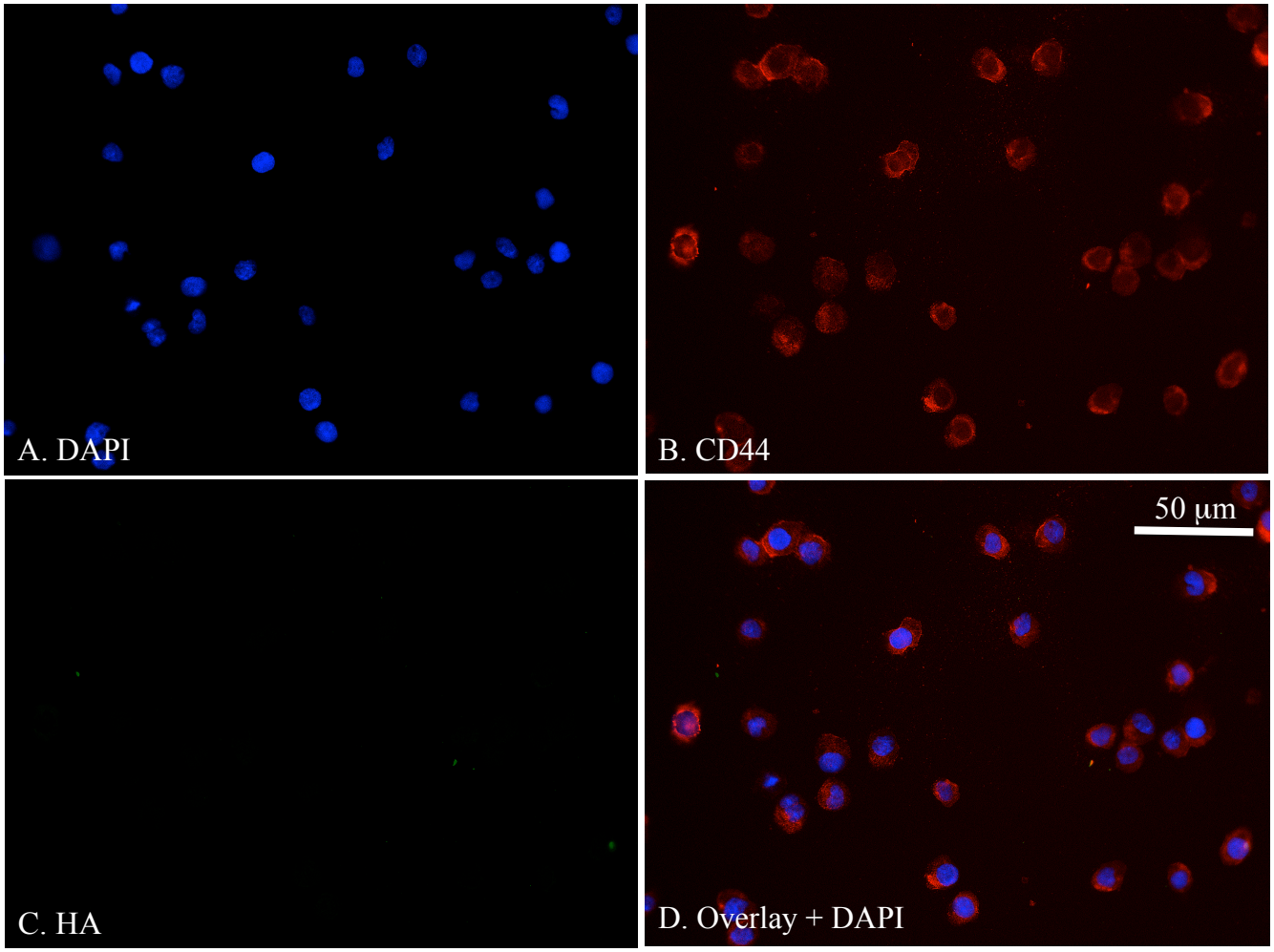


Fig. 16. U937 Cells don't cluster in the Absence of HA Rafts. (A-D) U937 cells in RPMI medium were transferred to a glass slide after one hour incubation at 4° C without the addition of day 10 AEC culture's apical wash that contains the HA rafts. Absence of HA was shown by lack of staining with a fluorescent conjugate of biotinylated HA binding protein (green). U937 cells were probed with antibodies against CD44 (red). This fluorescent micrograph shows that U937 cells don't cluster, as in **[Fig. 14]**, in the absence of HA rafts due to the centrifugal force resulting when the cells are transferred to the glass slide via cytospin. (Mag. 20X, magnification bar 50 µm). (N=3).

III.4.2. *HA rafts contain HC-HA complexes*.

One possible explanation for the HA adhesiveness to leukocytes is due to a HA modification that often occurs during inflammations, namely the transfer of HCs from IαI to form HC-HA complexes, which is mediated by TSG-6 [6,7]. Apical washes of 10 day AEC cultures were collected at the indicated times (2-48 h) after replacing the medium on day 10. They were then treated with *Streptomyces* hyaluronidase (specific for HA digestion into disaccharides) and analyzed for HCs by Western blots **[Fig. 17A]**. The accumulation of HCs at ~85 kDa over the time course is apparent. This band was not present in the absence of the hyaluronidase digestion as shown in the analysis of a 12 day apical wash **[Fig. 17C]**. This provides evidence that monocyte binding is due to a large extent by the presence of HCs [8,9], and that the HA rafts contain HC-HA complexes. Furthermore, TSG-6 was also present in the apical washes and absent in apical washes from TSG-6 null AECs **[Fig. 17B]**. The absence of HC-HA in the TSG-6 null cultures is also apparent **[Fig. 17A]**.

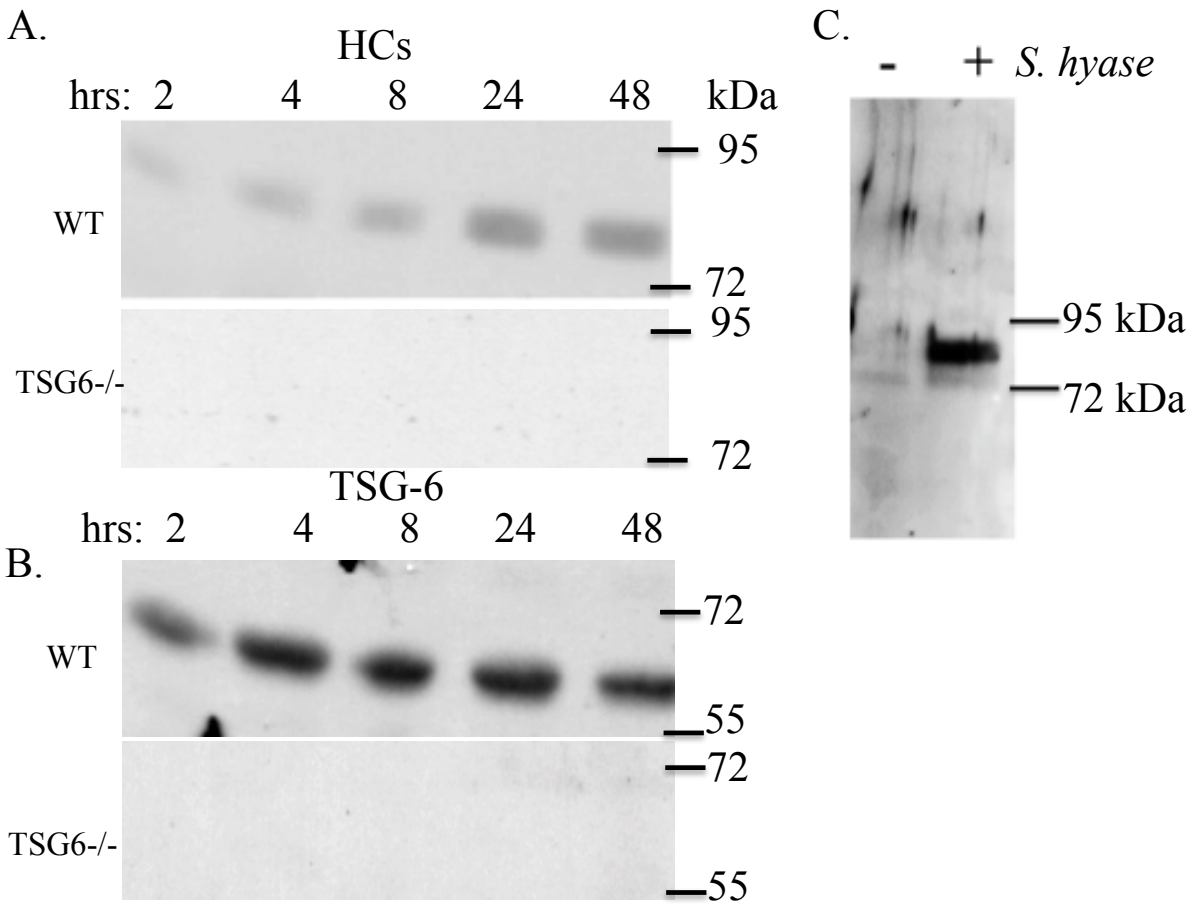


Fig. 17. Murine AECs make HA rafts that contain HC-HA complexes. Western blots of apical washes of unstimulated AECs were collected at 2, 4, 8, 24 and 48 h after day 10 of their differentiation and treated with *Streptomyces* hyaluronidase *(S.* hyase*)* and then (A) probed with a cocktail of HC1, 2 and 3 antibodies, and (B) re-probed with TSG-6 antibody. Absence of HC transfer and TSG-6 from a parallel experiment with AECs from TSG-6 null mice is apparent in (A) and (B) consecutively. (C) Western blot of an apical wash collected on day 10 of differentiation, 48 h after the previous apical wash, treated without or with *S.* hyase. This blot was probed with an antibody against IαI.

III.4.3. ***HA rafts in murine bronchoalveolar lavage (BAL) require TSG-6 for HC transfer onto HA.*** To determine whether HA rafts are HC-HA complexes released by the airway epithelium *in vivo,* we analyzed BAL samples from a naïve WT mouse and found clusters of HA that are monocyte adhesive similar to the HA rafts that AECs make *in vitro* as shown in **[Fig. 18A-C]**. Furthermore, these HA rafts lacked HCs in the absence of TSG-6 as shown in the BAL collected from a TSG-6 null mouse **[Fig. 18D-F],** which is consistent with the fact that HC transfer onto HA requires TSG-6. Moreover, the size of the HA raft in the TSG-6 null BAL fluid was much smaller, suggesting that the lack of TSG-6 may have an effect on the synthesis or aggregation of HA. This is consistent with the significant decrease of pulmonary HA concentration measured in the BAL and lung tissues of TSG-6 null mice subjected to the ova asthma model [10]. Furthermore, digesting the BAL collected from naïve WT mouse with *S.* hyase resulted in the release of significantly more HCs into the gel that were detected on Western blots **[Fig. 19A,B]** while HC bands were nearly absent (<10% of control) in BAL from a TSG-6 null mouse. This demonstrates the role of TSG-6 in mediating HC transfer onto HA in rafts located on the apical surface. Moreover, we have previously shown that TSG-6 can transfer all of the HCs from serum-derived HC-bikunin to HA1000K *in vitro* at early time points while TSG-6 released free HCs from HA1000K at later time points to a lesser extent [11]. This may explain the observation of free HCs in the presence of TSG-6 when WT BAL was not treated with *S.* hyase.

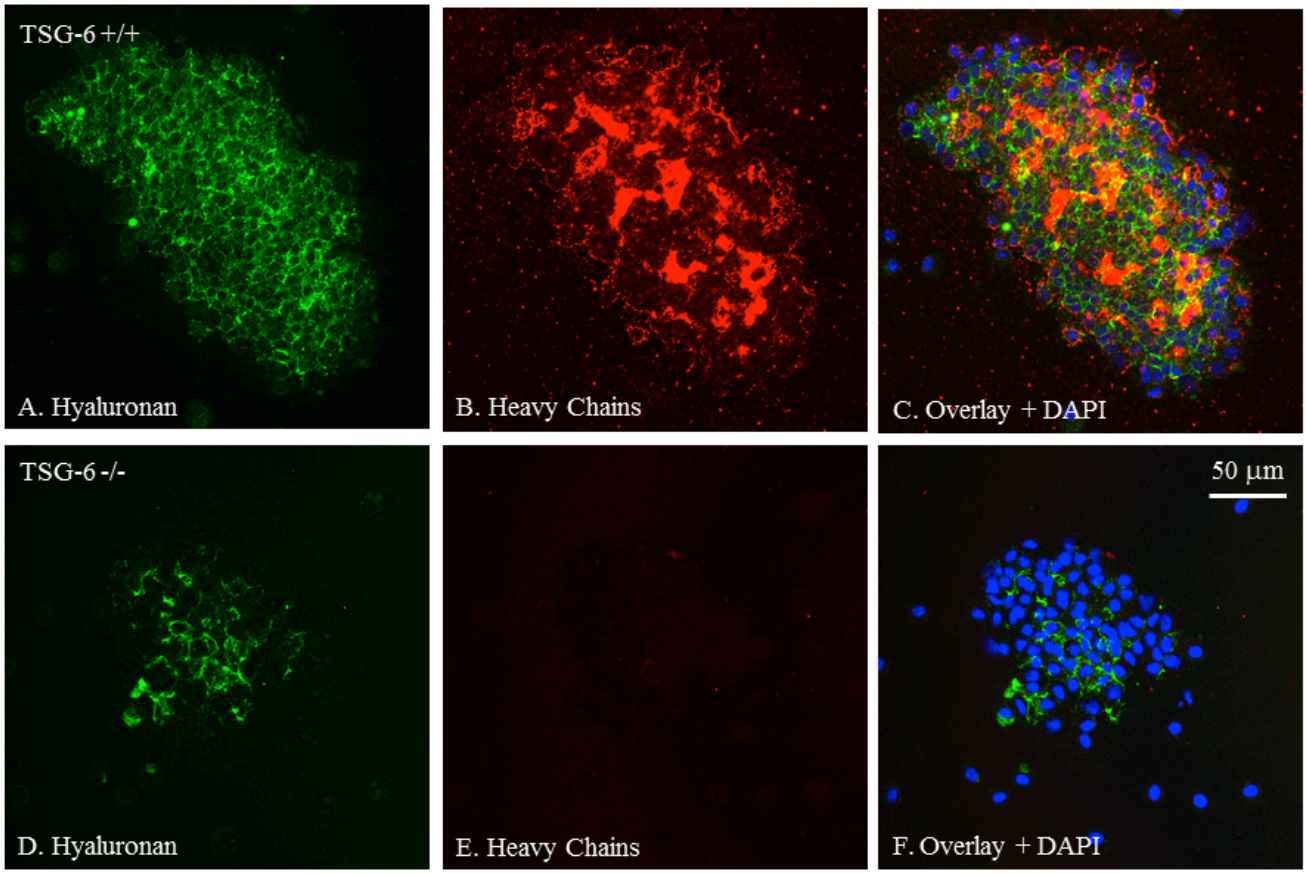
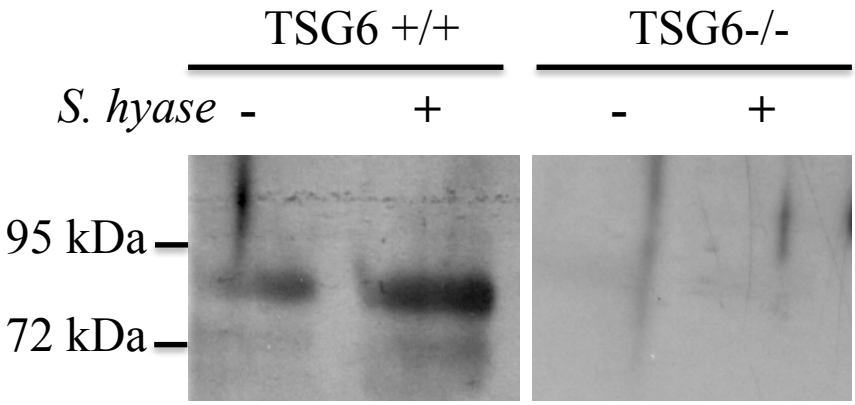


Fig. 18. HA Rafts in Murine BAL require TSG-6 for HC Transfer onto HA. HA rafts are present in BAL from naïve wild type (WT) (A-C) and from TSG-6 null mice (D-F). HA was stained with a fluorescent conjugate of biotinylated HA binding protein (green), and the presence (B) and absence (E) of the HC-HA complex was shown by staining with a cocktail of HC1, 2 and 3 antibodies (red). Leukocytes were stained with DAPI (blue). (Mag. 20X, magnification bar of 50 μm).

A.



B.

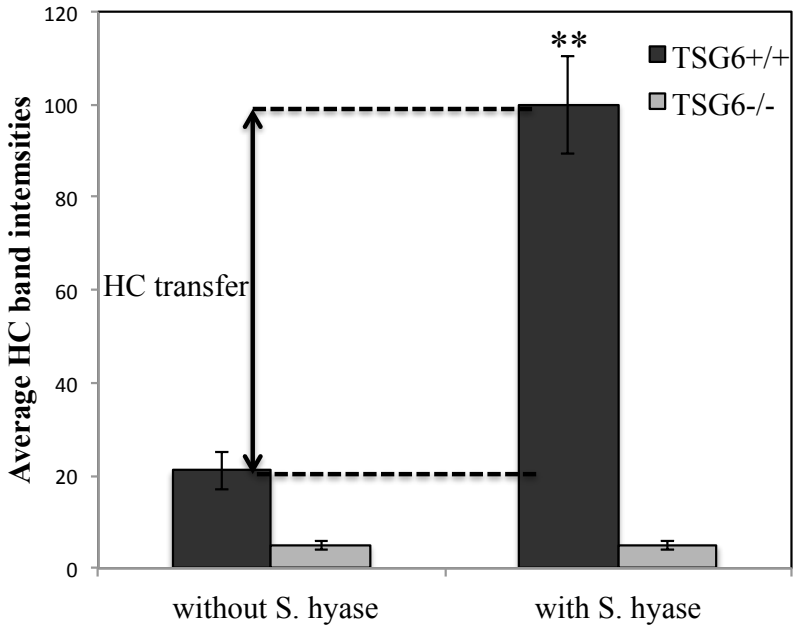


Fig. 19. HC-HA Complex is found in BAL of Naïve WT and not in that of TSG-6 Null Mice. (A) One representative out of 3 Western blots of BAL from naïve WT and TSG-6 null mice that were incubated without or with *S.* hyase. (B) Bar graph representation of the HCs released by *S.* hyase indicates the amount of HC-HA in BAL from naïve WT mice, which is absent in the BAL of TSG-6 null mice (N=3).

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CHAPTER IV

MURINE AIRWAY EPITHELIAL CELLS CONSTITUIVELY SYNTHESIZE THEIR OWN HEAVY CHAIN DONOR AND TSG-6

IV.1. ABSTRACT

The HC-HA modification involves the transfer of heavy chains from the inter-α-inhibitor (IαI) proteoglycan, which has 2 heavy chains (HC1 and HC2) on its chondroitin sulfate (CS) chain. The transesterification transfer of HCs from CS to HA is mediated by tumor-necrosis-factor-induced-gene 6 (TSG-6), which is upregulated in inflammatory reactions. IαI, the heavy chain donor, is normally produced by liver hepatocytes and released into the plasma. Very few other cell types have been reported to constitutively express their own heavy chain donor. In this study, we show that murine AEC cultures synthesize TSG-6 and their own heavy chain donor (pre-IaI) with a single heavy chain 3 (HC3)

linked to a bikunin as HC3-bikunin, which are also constitutively expressed by human renal proximal tubular epithelial cells. Therefore, AECs synthesize HC3-bikunin to act as a HC donor that is involved in the HC transfer mediated by TSG-6 onto the HA forming HC3-HA rafts. These HC3-HA rafts that are located on the apical surface of AECs are leukocyte adhesive. Thus, these leukocyte-adhesive HA rafts are now identified as HC3-HA complexes that could be part of a host defense mechanism filling some important gaps in our current understanding of murine airway epithelial biology and secretions.

IV.2. INTRODUCTION

IV.2.1. Inter-trypsin-α-Inhibitor (IαI) Family

In mammals, the bloodstream is a major carrier for many glycoproteins that act as protease inhibitors. Inter-α-inhibitor (IαI) and related molecules, collectively referred to as the IαI family, are a fascinating group of such plasma protease inhibitors. The discovery of an increasing number of such molecules has raised numerous questions about their pathophysiological functions [1]. Until recently, the knowledge of the IαI family was mostly structure oriented. Indeed, IαI was for many years described as a single entity, and a major breakthrough in the late 1980s was the emergence of a set of related molecules. At least four heavy chains (HCs) designated HC1, HC2, HC3 and HC4 [2-4], are involved in the synthesis of IαI family members **[Fig. 20]** [5,6].

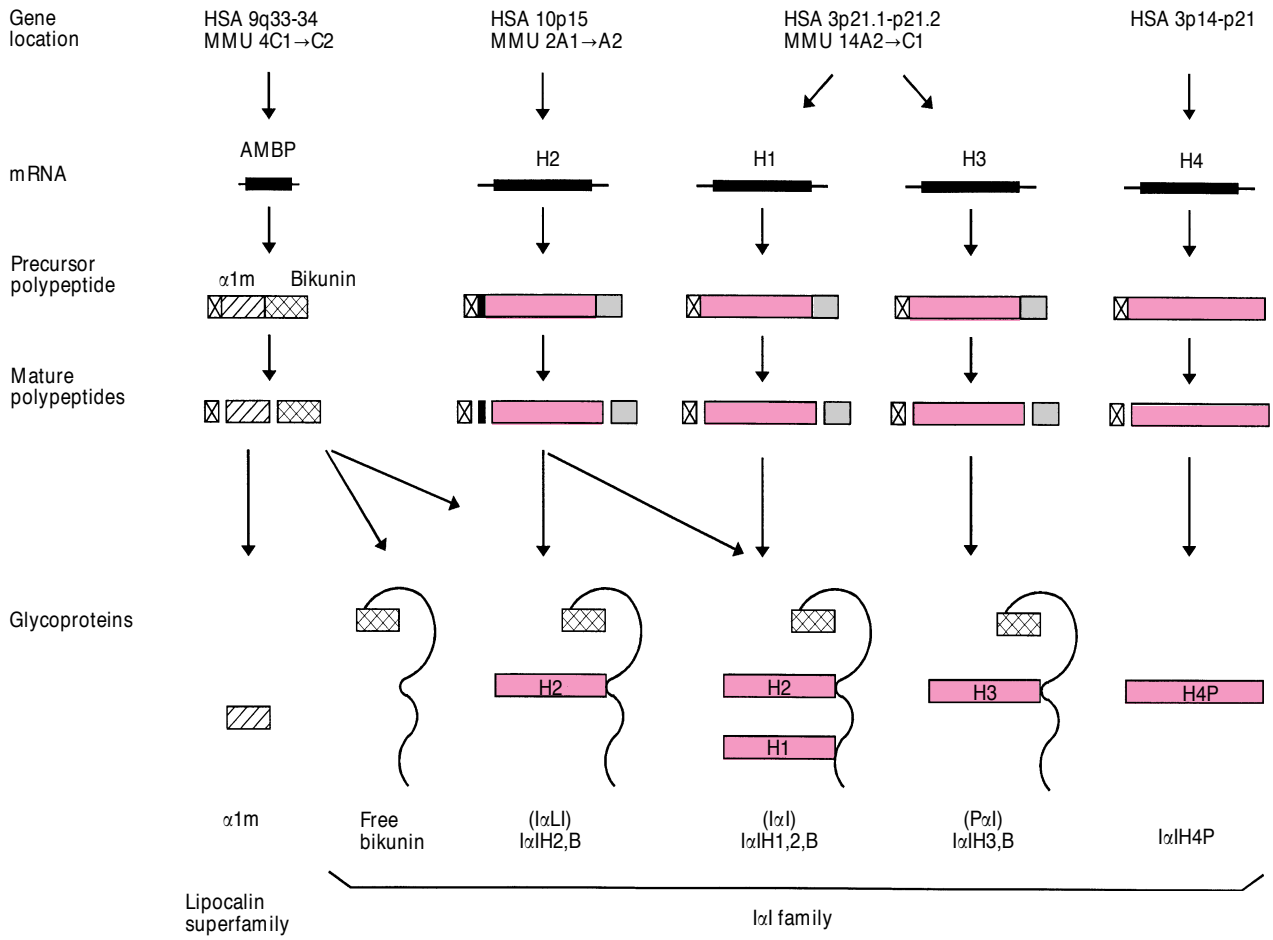

Fig. 20. A Current View of the IαI Family, from Genes to Glycoproteins. [5]

IαI is an abundant proteoglycan produced by liver hepatocytes and released in the serum. It is reported to circulate in serum at concentrations between 150 and 500 μg/mL comprising ~5% of total protease inhibitory activity of plasma, and elevated levels are reported in inflammatory pathologies [7,8]. It is normally composed of the trypsin inhibitor bikunin with heavy chain 1 (HC1) and heavy chain 2 (HC2) that are covalently bound by ester linkages to bikunin via its single chondroitin4-sulfate (C4S). The chondroitin sulfate chain is linked through an ester bond between C-terminal aspartate and the 6-hydroxyl of an N-acetylgalactosamine. The transesterification transfer of HCs from C4S of the IαI to a 6-hydroxyl of an N-acetylglucosamine of HA is mediated by TSG-6, a protein secreted by various cells upon stimulation by inflammatory cytokines [9-16].

IV.2.2. TNFα Stimulated Gene-6 (TSG-6)

Tumor Necrosis Factor α (TNFα)-induced protein-6 (Tnfαip6) also known as TNFα-stimulated gene-6 (TSG-6) is a member of HA binding proteins [17]. It is a 35 kDa secreted protein that is involved in both physiological and pathophysiological tissue responses. Besides interacting with HA [18-20], this protein also interacts with a broad spectrum of GAGs and protein ligands, including C4S [21], heparin [22], IαI [23-24], aggregan [25] and pentraxin-3 (PTX3) [26]. TSG-6 is produced by many different cell and tissue types in response to a wide variety of stimuli [27]. The expression of TSG-6 is induced by a number of factors other than TNF-α including IL-1β, TGF-β, and LPS in a highly cell type-dependent manner, and in many cases is coordinated with the synthesis of HA. TSG-6 expression has been observed in physiological and pathological contexts

that are associated with inflammation and tissue remodeling, such as in the sera and joints of arthritis patients [28]. Recently, the activity of TSG-6 in osteoarthritis synovial fluid has been demonstrated as a biomarker for OA progression and the need for knee replacement [29]. TSG-6 has a diversity of functions. It has been described to inhibit neutrophil migration [30], has anti-inflammatory effects as it is considered chondroprotective in murine model of arthritis [31], and has a major role in stabilization of the HA matrix of the cumulus cell-oocyte complexes that is crucial for fertility [32]. Further, TSG-6 expression is among the genes upregulated > 4 fold in allergen-challenged patients as shown in **[Table 2]** emphasizing the important role that TSG-6 has also during inflammation [33]. It has been shown that TSG-6 catalyzes the transfer of HCs from the C4S chain to the N-acetyl-glucosamine (glcNAc) residues on the HA to form HC-HA complexes that have been implicated in many pathological responses. HC-HA complexes have been shown to accumulate within inflamed tissues and correlate with disease severity [34,35]. During many pathological processes, these HC-HA matrices contain structural information recognized by mononuclear leukocytes potentially explaining the increased leukocyte binding [36,37].

Table 2. Bronchial Epithelial Cell mRNAs with Fourfold or Greater Upregulated Expression after Allergen Challenge

Gene Class	Common Name(s)	GenBank Identifier	Expression Ratio Allergen/Control ($n = 5$) Geometric Mean (*95% CI*)
Cell growth, proliferation, and differentiation	G0S2	M69199	13 (3.9–44)
	S100A8 (calgranulin A)	AI126134	5.3 (1.8–16)
	TM4SF1	M90657	4.8 (2.7–8.6)
		AI445461	4.5 (2.0–10)
	EMP1	Y07909	4.7 (2.3–9.9)
	S100A9 (calgranulin B)	W72424	4.7 (1.4–16)
	RAI3	AF095448	4.6 (2.5–8.3)
	CYR61	Y11307	4.0 (1.3–13)
Cytoskeleton	KRT6A	L42611	6.9 (1.5–33)
Extracellular protein	TFF1	AA314825	5.3 (1.7–16)
Immune function	IL-1RN	X52015	16 (1.9–132)
	IL-1β	X04500	13 (3.8–42)
		M15330	7.9 (2.2–29)
	IL-8	M28130	10 (3.1–35)
		M17017	5.2 (1.6–16)
	DAF (CD55, Cromer blood group system)	M31516	7.3 (2.5–21)
	TNFAIP6, TSG-6	M31165	5.5 (2.2–14)
Metabolism	ALDH1A3	U07919	4.6 (2.0–10)
Protein degradation	SCCA2, leupin	U19557	10 (2.4–45)
	PAI-2	Y00630	9.0 (2.0–41)
	MMP-10 (stromelysin 2)	X07820	7.1 (2.7–19)
Signal transduction	PDE4B	L20971	7.7 (1.9–31)
	LCP2	U20158	4.3 (1.5–13)
Transcription	MAFF, v-maf	AL021977	6.2 (2.4–16)

IV.2.3. Constitutive Expression of Heavy Chain Donors and TSG-6

At the present time, the only well characterized source of IαI is in the serum being produced by the liver hepatocytes. Amniotic membrane (AM) is another tissue in addition to the liver that constitutively produces IαI. The HC1-HA complex made by this tissue is different from that found at inflammatory sites (e.g. in asthma and arthritis) that potentially could explain for the anti-inflammatory, anti-scarring, and anti-angiogenic actions of the AM [38].

In this study we show that AECs can synthesize their own heavy chain donor as HC3-bikunin that is used for the HC transfer onto the HA without the requirement of serum exudate that could occur during inflammation. This donor is the same as, the pre-IαI made and secreted by renal epithelium, which is also composed of a bikunin with a HC3 covalently linked by an ester bond to the chondroitin-4-sulfate chain [39]. AECs can also constitutively express TSG-6 without being stimulated with TNFα as shown is the results section VI.4.

IV.3. MATERIALS AND EXPERIMENTAL PROCEDURES

IV.3.1. Mice

As described in section II.3.2.

IV.3.2. Primary Cell Culture

As described in section II.3.4.

IV.3.3. Cell Lysate Preparation

200 μL of cold cell lysis buffer (1% Triton X-100, 40 mM HEPES, of pH 7.4, 300 mM NaCl, 3 mM $MgCl_2$, 4 mM EGTA), with protease (Complete Mini, 11826153001; Roche Applied Science) and phosphatase (PhosSTOP, 04906845001, Roche Applied Sciences) inhibitors, was added to the AECs cultured on transwell inserts at day 10 of their differentiation. The AECs were lysed by pipetting the added volume of lysis buffer up and down, and avoiding the formation of air bubbles as much as possible. The volume of lysis buffer added was left on the transwell insert for 40 minutes on ice before it was collected and spun down by centrifugation for 10 minutes at 14,000 g and at 4° C. The supernatant was transferred to a new 1.5 mL eppendorf tube and then stored at -80° C till the time of use. The samples were then processed for Western Blotting as described in section IV.3.5.

IV.3.4. Basement Membrane Protein Extraction

900 μL of 4x Laemmli Sample Buffer (161-0747; BioRad) was mixed with 100 μl β-mercaptoethanol (BME) (161-0710; BioRad). 200 μl of this mix was added to one transwell insert, which was has a pre-deposited basement membrane by MDCK cells as described in section II.3.3. Extraction of the proteins of the basement membrane was done by pipetting up and down the added volume of the sample buffer 3 times, before transferring the same volume of sample buffer to the next transwell insert. This was repeated to extract the proteins from a total number of 12 transwell inserts in a total volume of 200 μL sample buffer with a final protein concentration of nearly 25 μg. This was run on a gel as described in section IV.3.5, and stained for HC-3 and TSG-6 protein detection as described in section IV.3.6 for protein detection on gel by Coomassie blue staining.

IV.3.5. Western Blot Analysis

Samples were electrophoresed on 4-5% Mini-PROTEAN TGX gels (456-8084; BioRad) and blotted using PVDF membranes (170-4156 – PVDF mini; BioRad) and the Trans-Blot Turbo System. All blots were blocked for 1 h with 5% milk (Blotting Grade Blocker,170-6404; BioRad) and probed overnight at 4° C with antibodies against HC1 (SC-33944; Santa Cruz Biotechnology), HC2 (SC-21978; Santa Cruz Biotechnology), HC3 (SC- 21979; Santa Cruz Biotechnology), and bikunin (a kind gift from Anthony Day, University of Manchester, UK). Blots were washed with 1X Tris Buffered Saline (TBS) with TWEEN®20 (0777-2 L; AMRESCO) 3 times for 7 minutes each time. Blots

were incubated with secondary antibody, and Biotin-SP-conjugated donkey anti-goat IgG was used as secondary antibody (705-065-147; Jackson ImmunoResearch), against primary antibodies against HC1-3 for 1 h at room temperature. Blots were washed again with 1X TBS with TWEEN®20 3 times for 7 minutes each time before the incubation with streptavidin-horse radish peroxidase (HRP) (P0397; Dako Cytomation) for 1 h at RT Blots were washed again with 1X TBS with TWEEN®20 3 times for 7 minutes each time.

TSG-6 was assessed using a biotinylated anti-mouse TSG-6 antibody (BAF2326; R&D systems, Inc.). Recombinant mouse TSG-6 (2326-TS; R&D systems, Inc.) was used as a positive control for the TSG-6 antibody. All blots were developed using the ECL Prime Western Blotting Detection Reagent (RPN2232, Amersham).

IV.3.6. Coomassie Blue Staining

The protein extracts of the basement membrane, deposited by the MDCK cells, were electrophoresed on 4-5% Mini-PROTEAN TGX gels (456-8084; BioRad). The gel was washed with distilled water for 10 minutes and then stained with Bio-Safe Coomassie Stain (161-0786; BioRad) overnight. The gel was washed with distilled water every 10 minutes for 30 minutes before it was visualized using myECL imager from Thermo Scientific.

IV.3.7. PCR Analyses

RNA was isolated using the RNAeasy kit (74104; Qiagen), and cDNA was prepared using Superscript @ First-Strand Synthesis System (11904-018; Invitrogen). Primers used are listed in **(Table 3)** below. The PCR conditions were 1 cycle at 94° C for 3 min, 40 cycles of 95° C for 30 sec, 55° C for 45 sec, 72° C for 1 min, followed by 1 cycle of 72° C for 1 min. These conditions were used for all primers. PCR products were run on 3% ethidium bromide gels (E1510; Sigma) and 1% agarose gels (SeaKem® HGT Agarose, product 50041; Lonza).

Table 3. The Sequences of the Polymerase Chain Reaction (PCR) Amplification Primers

Name of Primers	Primer Sequence	Expected PCR product
β-actin	5'-GGTCATCACTATTGGCAACG-3' 5'-ACGGATGTCAACGTCACACT-3'	133 bp
Heavy chain 1	5'TTCTCAGCCCTTAGAGATGGTG'3 5'-GAGTGGCAACTTTGAGTCTATGG-3'	100 bp
Heavy Chain 2	5'-GGTGATAGAGAATGATGCTGGA-3' 5'-CAACCTTGGTGCCATAATACAG-3'	101 bp
Heavy Chain 3	5'- CCCAGAAAGATTACAGGAAGGA-3' 5'-TCGGTATGGACACCATCAATTA- 3'	100 bp
TSG-6	5'- CTCCATATGGCTTGAACAAGCAGC-3' 5'- ACCACCTTCAAATTCACATACG- 3'	112 bp

IV.4. RESULTS AND DISCUSSION

IV.4.1. *Murine AECs make their own heavy chain3 (HC3) donor as HC3-bikunin*

IαI is a proteoglycan that normally contains two HCs (HC1 and HC2). It is produced by the liver hepatocytes where it is released into the serum at relatively high concentrations. It serves as a HC donor for TSG-6 mediated transfer of HCs to HA during many inflammations. The AEC culture medium lacks serum, the source of IαI, which is the source of HCs. Therefore, the presence of HC-HA in the HA rafts from unstimulated AEC cultures suggests that AECs can make their own HC donor. From the three different HC subtypes (HC1-3) analyzed by RT-PCR, murine AECs express only HC3, as shown in **[Fig. 21A]**. **[Fig. 21B]** shows that the donor, which has a MW of ~132 kDa, contains HC3 and bikunin, which is equivalent to the pre-IαI complex. This donor is the same as the pre-IαI made and secreted by renal epithelium, which is composed of a bikunin with a HC3 covalently linked by an ester bond to the chondroitin-4-sulfate chain [9-16]. Therefore, AECs synthesize their own HC3-bikunin, in a manner similar to renal epithelial cells, and are able to generate HC3-HA rafts without the requirement of a serum exudate produced during inflammations.

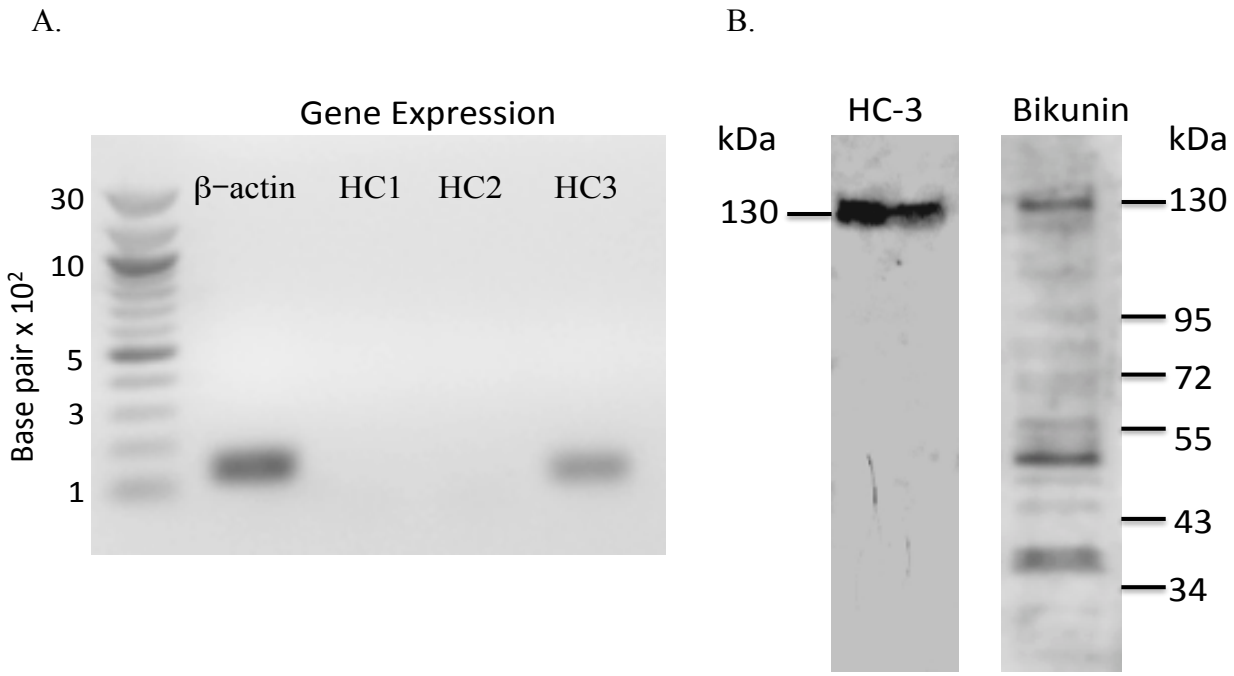

Fig. 21. Murine AECs make their Own Heavy Chain 3 (HC3) Donor as HC3-Bikunin, a Member of the PαI/IαI family. (A) PCR products produced using primers for the 3 heavy chain subtypes (HC1-3 as listed in Table 3), were prepared from mRNA isolated from day 10 unstimulated murine AECs, and then separated on a 1% agarose gel and stained with 3% eithidium bromide. β-actin mRNA production was used as the housekeeping gene. One representative gel of 3 is shown. (B) Western blots of AEC lysates demonstrate the presence of HC3 protein using a HC3 antibody (blot on the left). Western blots with antisera to HC1 and HC2 were negative (data not shown). A biotinylated secondary antibody (1:5000 dilution) was used after the primary antibody application followed by a streptavidin-HRP (1:2000 dilution). The detected band has a molecular weight of 132 kDa, equivalent to that of PαI (with one HC) of the IαI (with two HCs) family. The blot in the right also labeled the 132 MW band when it was probed

with an antibody to bikunin, the core protein of IαI, providing evidence for the pre-IαI member of the family that is HC3-bikunin.

IV.4.2. ***TSG-6 is expressed by unstimulated murine AECs and released apically and not basally***.

The HC3-HA data in figures 5 and 6 provide evidence that AECs synthesize TSG-6 as well as their own HC3-bikunin. Therefore, we isolated total cellular RNA isolated from day 10 differentiated AECs and examined TSG-6 mRNA by RT-PCR. We used cellular RNA isolated from mouse dermal fibroblasts, known to express TSG-6 only when treated with TNFα [38], as our positive control **[Fig. 22A]**. Furthermore, we analyzed Western blots of apical washes and basal media of AEC cultures for TSG-6 protein levels as probed with a biotinylated mouse TSG-6 antibody. This antibody detected the recombinant mouse TSG-6 at the expected molecular weight ~35 kDa **[Fig. 22B]**. However, a larger band (~65 kDa) was detected in the apical washes of AECs **[Fig. 22B]**. No band was detected in the basal medium, which indicates that neither the defined culture medium nor basally secreted products from the AECs contain reactive bands. The ~60 kDa band was present in the apical washes from WT AECs but absent in the apical washes of TSG-6 null AECs **[Fig. 17B]**, indicating that the antibody is likely detecting TSG-6 in a dimerized form. Therefore, AECs can express TSG-6 on both transcriptional **[Fig. 22A]** and translational level **[Fig. 22B, Fig. 17B]**.

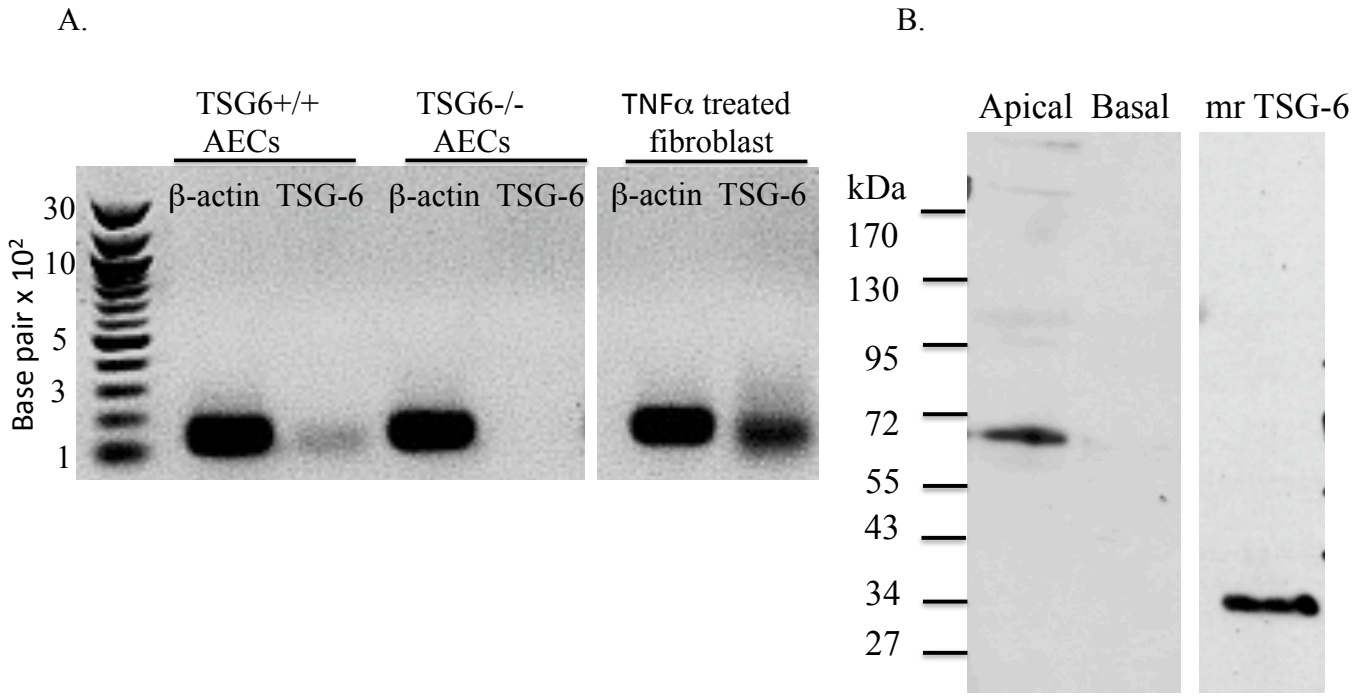

Fig. 22. AECs produce TSG-6 Apically that is required for the HC3-HA Complex Formation. (A) TSG-6 expression of a 10 day murine AECs is shown by the ethidium bromide stained PCR product of TSG-6 separated on a 1% agarose gel and by comparison to murine extracts of dermal fibroblast cultures treated with TNFα, using β-actin as the housekeeping gene. (B) Western blot of basal medium and apical washes of a 10 day AEC culture were probed with an antibody against TSG-6 that can also detect the murine recombinant TSG-6 (mrTSG-6) used as a positive control.

IV.4.3. *Basement membrane deposited by MDCK cells contains neither TSG-6 nor HC3*

We also analyzed the basement membrane deposited by MDCK cells that the AECs are cultured on during their differentiation, by Western blotting for HC3 and TSG-6 protein levels since MDCK cells are also kidney epithelial cells. Protein levels of HC3 and TSG-6 were not detected as shown in **[Fig. 23]** confirming that the source of these two proteins is from the AECs and not the basement membrane, and that the proteins found in the basement membrane are not interfering with the method of detection of the antibodies used in this study.

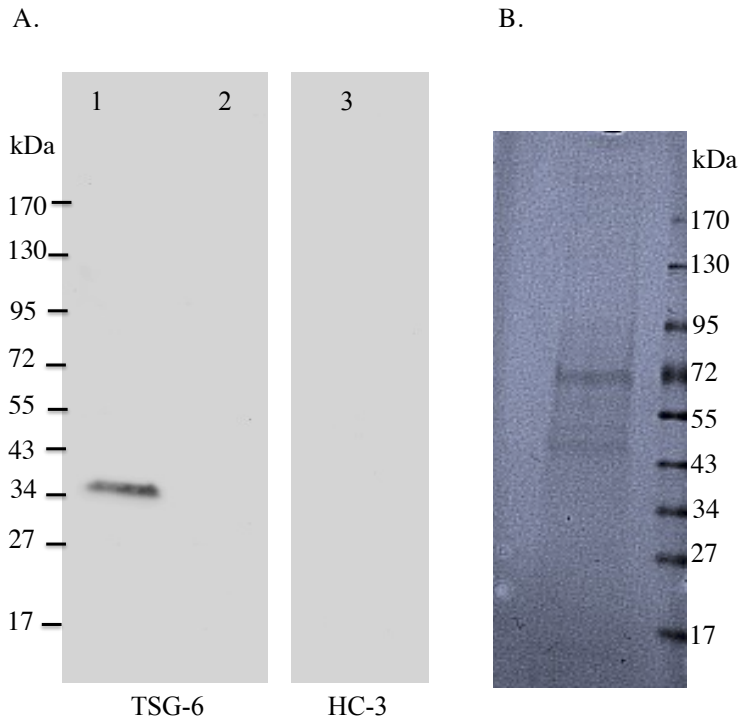

Fig. 23. Basement Membrane Deposited by MDCK Cells does not contain Detectable Levels of HC3 or TSG-6. (A) Western blot of protein extracts of the basement membrane synthesized by the MDCK cells on the transwells probed with antibodies against TSG-6 (lane 2) and HC-3 (lane 3) with recombinant TSG-6 (lane 1) as a positive control for the TSG-6 antibody used. (B) Gel of protein extracts of the deposited basement membrane by the MDCK cells stained with Coomassie Blue stain for protein detection.

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CHAPTER V

CONCLUDING REMARKS

HA can be uniquely modified during inflammation by the transfer of HCs from IαI to form HC-HA complexes mediated by TSG-6. HC-HA matrices are leukocyte adhesive as demonstrated in many pathological conditions. Further, TSG-6 has an essential role in normal biology because the lack of the TSG-6 mediated HC-HA matrix in normal cumulus cell-oocyte complexes causes female infertility in TSG-6-/- female mice [1]. This demonstrates that HC-HA matrices can be part of a normal extracellular matrix as well as an inflammatory response to stimuli.

Unlike any other cell type reported so far, AECs uniquely release leukocyte-adhesive HC3-HA complexes from their apical surface into their extracellular space even in the

absence of inflammatory stimuli. These HC3-HA rafts were not only observed in the air/liquid AEC culture model but also in the bronchoalveolar lavage of naïve mice implying that these rafts are part of murine normal secretions and can have a role as a host defense mechanism.

Leukocyte adhesion to HA rafts was also observed to a lesser extent in the absence of a TSG-6 mediated HC transfer suggesting that HC attachment to the HA raft is not the sole explanation for leukocyte binding. This adhesion could be due to the involvement of other HA binding proteins that could be further investigated or, similar to HA cables formed without HC addition, HA rafts could bind leukocytes even in the absence of binding proteins such as HCs [2].

In this study, we show that murine AECs can synthesize their own heavy chain donor as pre-IαI complex of HC3 and bikunin, which was previously reported to be constitutively expressed by human proximal tubular epithelial cells (PTECs) [3]. Furthermore, we also show that murine AECs constitutively express TSG-6 as do PTECs. However, in this study the production of HC3-bikunin and TSG-6 by human PTECs was investigated in the context of their contribution to the pathogenesis of renal fibrosis. At present it is unclear whether PTECs behave in a similar manner as AECs by forming HA rafts. Because both cell types have a similar cell lineage, this would be worth investigating in the future.

The study by Forteza et al. [4] also provided evidence that normal human epithelial cell cultures have low levels of mRNA for TSG-6, bikunin and HC3, which were increased moderately by treatment with TNF-α. This study also showed that epithelia in sections of trachea from smokers showed increased staining for IαI and bikunin compared with normal tracheal sections. This provides evidence that human airway epithelia may also normally make HC3-HA rafts, which are amplified during inflammatory processes such as smoking and asthma.

Furthermore, we have developed a culture model where AECs are cultured on a basement membrane deposited by MDCK cells. This basement membrane closely resembles the one *in vivo* and results in an airway epithelium that resembles its counterpart *in vivo.* The presence of a basement membrane allows primary cells to polarize and differentiate, in this case, into pseudotratified ciliated epithelial cells. This unique culture model has the potential to become a widely used tool not only for culturing AECs but also for other primary cell types such as Human Umbilical Vein Epithelial Cells (HUVECs).

Airway Epithelium is one of our first lines of defense that provides both a chemical and a physical barrier. A lot of studies have shown the responsiveness of the AECs to external pathogenic attack by the release of different cytokines. We have demonstrated that these cells are also equipped with HA rafts because they can make their HC donor and TSG-6, and it would be interesting to investigate their contribution during inflammation and their effect on subjacent cells. We have developed a co-culture system that uses our model of culturing AECs to investigate the cross-talk between AECs and ASMCs as shown in

[Fig. 24]. We can also extend our investigation to include AECs from different knock-out mice for proper dissection of the different pathways involved.

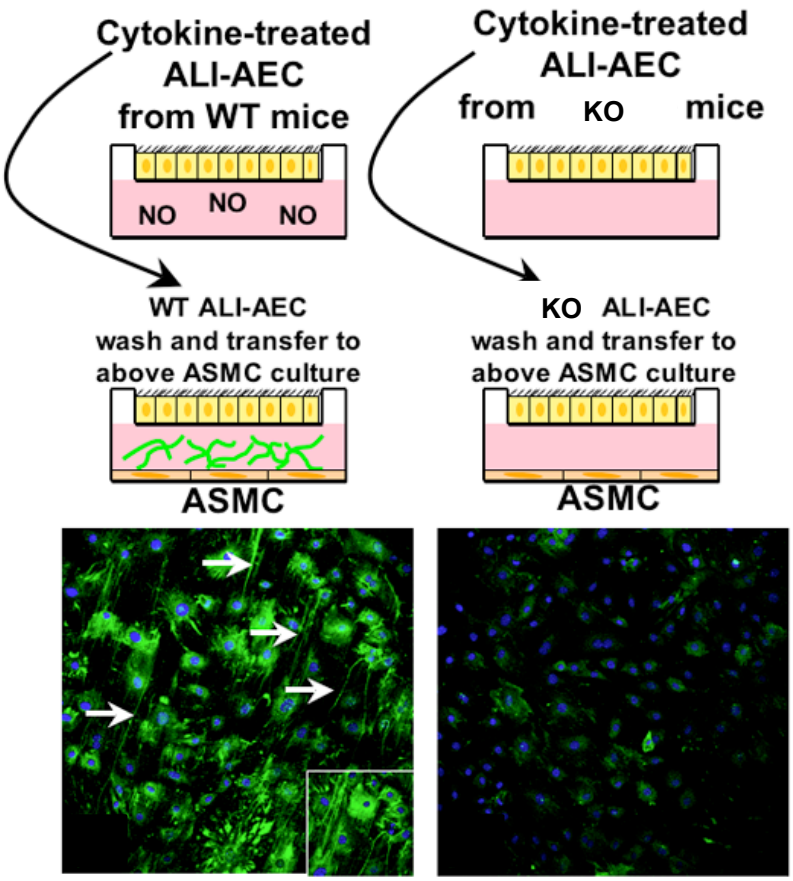

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Fig. 24. A Schematic Illustration of the Use of Co-culture Model in Investigating the Cross-Talk between AECs and ASMCs.

In summary, this study provides the first description of leukocyte-adhesive HA rafts located in the extracellular space on murine AEC apical surfaces. This unique release mechanism of HA rafts defines the HC-HA complex as a previously unidentified component of respiratory secretions. Murine HA rafts are identified as HC3-HA complexes that AECs can make in the absence of inflammatory stimuli. Thus, HA rafts perhaps serve as a broad anchor for leukocytes at the airway surface ready to be utilized upon a pathogenic attack or could be an anchor to other proteins and enzymes that might be involved in a host defense mechanism [6]. Therefore, this study fills important gaps in our current understanding of murine airway epithelial biology and secretions that relate to human health and disease.

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APPENDIX A

KDEL

KDEL The abbreviation KDEL is formed by the corresponding letters to each amino acid, **K** for Lysine, **D** for Aspartic acid, **E** Glutamic acid, **L** for Leucine.

KDEL is a sequence in the amino acid structure of a protein which keeps it from being secreted from the endoplasmic reticulum (ER). Proteins can only leave the ER after this sequence has been cleaved off. It is accumulation in the ER is indicative of an ER stress.

APPENDIX B

CD44 " Capping"

The presence of HA cables at sites of tissue inflammation can still function as an anti-inflammatory polymer. Monocytes adhere tightly to the HA cables regardless of their activation state, and following adhesion the dis- tribution of CD44 on the monocyte cell surface polarizes to form a "cap" while a portion of the HA cable is internalized.

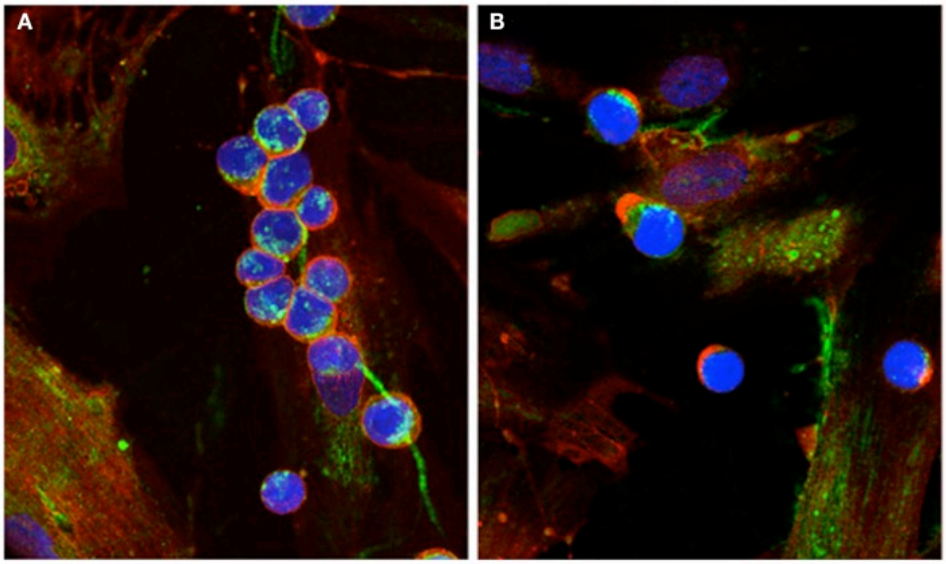

Fig 25. Capping of CD44 on leukocytes. (A) Monocytes bind to HA cables (green) produced by SMC in response to poly I:C. CD44 (red) is dispersed on the monocyte surface. (B) After 15 min incubation at 37°C, CD44 (red) is capped to one pole on the leukocytes, HA cables appear ragged (center panel) and HA is internalized. (Nuclei are blue).

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