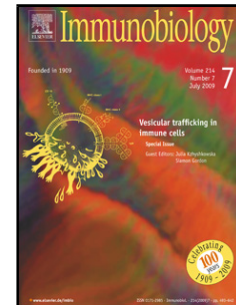


Accepted Manuscript

Title: Hyaluronan stimulates *ex vivo* B lymphocyte chemotaxis and cytokine production in a murine model of fungal allergic asthma

Author: Sumit Ghosh Scott A. Hoselton Steve B. Wanjara
Jennifer Carlson James B. McCarthy Glenn P. Dorsam Jane
M. Schuh



PII: S0171-2985(15)00019-4
DOI: <http://dx.doi.org/doi:10.1016/j.imbio.2015.01.011>
Reference: IMBIO 51296

To appear in:

Received date: 17-12-2014
Accepted date: 24-1-2015

Please cite this article as: Ghosh, S., Hoselton, S.A., Wanjara, S.B., Carlson, J., McCarthy, J.B., Dorsam, G.P., Schuh, J.M., Hyaluronan stimulates *ex vivo* B lymphocyte chemotaxis and cytokine production in a murine model of fungal allergic asthma, *Immunobiology* (2015), <http://dx.doi.org/10.1016/j.imbio.2015.01.011>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Title: Hyaluronan stimulates *ex vivo* B lymphocyte chemotaxis and cytokine production in a murine model of fungal allergic asthma

Running title: Hyaluronan stimulates B lymphocyte responses

Authors: Sumit Ghosh^{1,*}, Scott A. Hoselton¹, Steve B. Wanjara¹, Jennifer Carlson², James B. McCarthy², Glenn P. Dorsam¹, and Jane M. Schuh¹

¹Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND 58108, USA

²Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455, USA

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of North Dakota State University or the granting agencies.

Funding source: These studies were funded by grants from the NIH (1R15HL117254-01 to JMS and 1R15AI101968-01A1 to GPD).

***Corresponding author:**

Sumit Ghosh, Ph.D.

Department of Veterinary and Microbiological Sciences, North Dakota State University

P.O. Box 6050, Dept 7690

Fargo, ND 58108-6050

FAX: 701-231-9692

Office: 701-231-7696

Email: Sumit.Ghosh@ndsu.edu

ABSTRACT

Allergic asthma is a chronic inflammatory disease of the airways characterized by excessive eosinophilic and lymphocytic inflammation with associated changes in the extracellular matrix (ECM) resulting in airway wall remodeling. Hyaluronan (HA) is a nonsulfated glycosaminoglycan ECM component that functions as a structural cushion in its high molecular mass (HMM) but has been implicated in metastasis and other disease processes when it is degraded to smaller fragments. However, relatively little is known about the role HA in mediating inflammatory responses in allergy and asthma. In the present study, we used a murine *Aspergillus fumigatus* inhalational model to mimic human disease. After observing *in vivo* that a robust B cell recruitment followed a massive eosinophilic egress to the lumen of the allergic lung and corresponded with the detection of low molecular mass HA (LMM HA), we examined the effect of HA on B cell chemotaxis and cytokine production in the *ex vivo* studies. We found that LMM HA functioned through a CD44-mediated mechanism to elicit chemotaxis of B lymphocytes, while high molecular mass HA (HMM HA) had little effect. LMM HA, but not HMM HA, also elicited the production of IL-10 and TGF- β 1 in these cells. Taken together, these findings demonstrate a critical role for ECM components in mediating leukocyte migration and function which are critical to the maintenance of allergic inflammatory responses.

Keywords - Hyaluronan, *Aspergillus fumigatus*, B lymphocytes

INTRODUCTION

Asthma is a debilitating disease of the airways that affects over 300 million people globally [1]. Asthmatic airways sensitized to a specific allergen respond aggressively to subsequent exposures, resulting in episodic asthma attacks that can be fatal. In the context of allergic asthma, sensitization to fungi often presents a severe clinical scenario that is difficult to treat and accounts for a disproportionately large number of emergency center visits and hospitalizations [2-5].

The hallmarks of chronic airway inflammation in patients with severe or persistent fungal asthma include the accumulation of activated eosinophils [6-10], neutrophils [11], lymphocytes [11-13], and extracellular matrix components (ECM) components [14]. As a consequence of repeated and persistent inflammation, lung morphology eventually becomes altered even in mild forms of the disease, causing chronic dysfunction [15]. Many of the acute and chronic features of clinical fungal asthma are recapitulated in the experimental model that we have developed in our laboratory in which sensitization with fungal extracts is followed by allergy challenge via inhaled *Aspergillus* [16-18]. We observe IgE production, eosinophilia, leukocyte recruitment, and pronounced peribronchial ECM changes that are exaggerated upon subsequent exposure to inhaled conidia [7, 8, 12, 16, 19, 20].

ECM components are involved in the attachment of cells [14, 21, 22], tissue growth and repair [14], proliferation and differentiation [14, 21], cell migration and activation [14, 21, 23-25], cell survival/delay of apoptosis [14, 26], and chemotaxis [14, 23] indicating the important role that ECM components play in the development and persistence of inflammation. Hyaluronan (HA), a non-sulfated glycosaminoglycan polymer and a major component of the

ECM [27], undergoes dynamic regulation during inflammation [14, 28-33] and is synthesized by a wide variety of cell types, including stromal cells [34], fibroblasts [35-37], epithelial cells [38], and smooth muscle cells [39]. HA exists as a high molecular mass (HMM) linear polymer in excess of 10^7 Da in the native form found in the lung [23]. Under conditions of inflammation, HMM HA is broken down into low molecular mass HA (LMM HA) (35-400 kDa), which exhibits pro-inflammatory effects on cells and tissues [14, 40-45]. As HMM HA is reconstituted, it blocks the pro-inflammatory effects of LMM HA and supports tissue integrity [14, 29, 40, 46-48].

HA binding proteins are expressed on structural and immune cells, they include: CD44 [49, 50], the receptor for hyaluronan-mediated motility (RHAMM) [51, 52], tumor necrosis factor- α -stimulated glycoprotein-6 TSG-6 [53-55], the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) [56], and the toll-like receptors (TLRs) [14, 29, 57]. CD44 is the best characterized HA receptor. It is a structurally variable and multifunctional glycoprotein whose expression may be induced on most cell types [14, 29, 58, 59]. There is a growing body of evidence that HA/CD44 binding regulates both lymphoid and myeloid cells [14, 26, 28, 60-62]. In B lymphocytes, CD44 facilitates adhesion to HA and proliferation in the spleen [33, 60, 63]. However, the contribution of HA/CD44 to B lymphocyte migration and activation in the context of pulmonary responses to fungus has not been investigated.

In the present study, we observed a temporal association between LMM HA in the airway lumen after eosinophil egress and B lymphocyte recruitment to the allergen-challenged lung. We hypothesized that eosinophil migration through the HMM HA surrounding the airways generates pro-inflammatory LMM HA that impacts the movement and function of B cells in the context of the allergic lung. To test our hypothesis we used *ex vivo* cultures of B cells from allergic mice to

determine the extent to which HA/CD44 binding facilitates B lymphocyte migration and the effect of HA on the production of pro-allergy cytokines by B lymphocytes. Our data show that after fungal challenge B lymphocytes are recruited to the lung after eosinophil egress to the lumen at a time point when LMM HA levels are readily detected in the lung. We show that LMM HA, acting mainly through the CD44 receptor, has a pronounced effect on *ex vivo* B lymphocyte migration and production of the cytokines transforming growth factor-1 (TGF- β 1) and IL-10. The results presented in this study reveal previously unrecognized roles of B lymphocytes and LMM HA during the inflammatory process of fungus-induced allergic processes. These findings expand our understanding of the contribution of residential matrix components in asthma pathogenesis and have the potential to inform therapeutic advances for patients with asthma.

MATERIALS AND METHODS

Ethics Statement

All experiments were performed in accordance with the Office of Laboratory Animal Welfare guidelines and were approved by the North Dakota State University Institutional Animal Care and Use Committee, Fargo, ND, USA.

Experimental animals

C57BL/6 male and female mice (6-9 weeks of age) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed on Alpha-driTM paper bedding (Shepherd Speciality Papers, Watertown, TN, USA) in micro filter-topped cages (Ancare, Bellmore, NY, USA) in a specific pathogen-free facility with ad libitum access to food and water.

Allergen sensitization and challenge by a nose only inhalation model

Animals were sensitized as per published protocol [16]. Briefly, mice were sensitized with 10 µg of *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC, USA) in 0.1 ml sterile phosphate- buffered saline (PBS) mixed with 0.1 ml of Imject Alum (Pierce, Rockford, IL, USA), which was injected subcutaneously (0.1 ml) and intraperitoneally (0.1 ml). After two weeks, mice were given a series of three, weekly 20-µg doses of *A. fumigatus* antigen in 20 µl of PBS by an intranasal route. Animals were challenged as previously described with a 10-min, nose-only inhalation exposure to live *A. fumigatus* conidia (strain NIH 5233) [16]. Each anesthetized mouse was placed supine with its nose in an inoculation port and allowed to inhale live conidia for 10 min. This challenge was repeated 2 weeks after the first. Mice were then separated into groups of five for analysis at days 5 and 28 after the second aerosol challenge.

These time points were chosen based on previously published results showing that B lymphocyte recruitment peaks 5 days after the second conidia challenge [12] and that changes to the lung architecture continue to accrue through at least day 28 after the second inhalation of fungal conidia [64]. Naïve controls were age-matched mice that were neither sensitized nor challenged. The experimental protocol is depicted in Figure 1.

Serum and BAL sample collection

Approximately 500 µl of blood was collected from each mouse via ocular bleed and centrifuged at $13,000 \times g$ for 10 min to yield serum. Bronchoalveolar lavage (BAL) was performed on five mice per group with 1.0 ml PBS. The BAL contents from each animal were centrifuged at $2000 \times g$ for 5 min to separate cells from fluid. Cells were resuspended in 200 µl of PBS, cytopun (Shandon Scientific, Runcorn, UK) onto glass microscope slides, and counted after staining with Quik-Dip differential stain (Mercedes Medical; Sarasota, FL, USA). The mean number of each cell type per high-powered field (hpf, 1000×) was determined by morphometric analysis. Serum and BAL fluid were stored at -20°C until use.

Quantification of HA in serum and BAL

Total HA levels were quantified via specific competitive ELISA in undiluted serum and BAL fluid samples according to the manufacturer's guidelines (Echelon Biosciences, Salt Lake City, Utah, USA). The HA detection range of the kit was 12.5-3200 ng/ml. The kit detects a minimum HA size of 10 disaccharide units (4,500 Da).

Tissue harvest and staining

Paraffin-embedded lungs were sectioned at 5 µm and stained with hematoxylin and eosin (Thermo Scientific, Rockford, IL, USA) and Gomori's trichrome (Richard-Allan Scientific,

Kalamazoo, MI, USA) stains to determine inflammation and subepithelial collagen deposition, respectively. Immunostaining for HA was performed as previously described [64, 65].

HA size determination

HA molecular mass was determined in lung samples as described previously [62, 66-68]. Briefly, right lungs from mice at each time point were homogenized in 1.0 ml PBS. The samples at each time point were pooled and concentrated with a centrifugal filter (10,000 Da cut-off; Millipore, Billerica, MA, USA). The lung samples were then digested with Pronase (100 U/ml, Pronase from *Streptomyces griseus*; Sigma-Aldrich, St. Louis, MO, USA) at 55°C for 2 h, followed by inactivation of protease activity by boiling the samples for 10 min. Following pronase digestion, the samples were digested with 6 µl of 2 U/µL DNase (Ambion, Austin, TX, USA) and 6 µl of 1.28 µg/µl RNase (Roche, Indianapolis, IN, USA) as described [62]. After nuclease digestion, we divided 80 µl of our sample from various time points in half by transferring 40 µl to another tube. Added 4 µl of *Streptomyces* hyaluronidase to ½ of your sample and incubated overnight at 37 °C. Heat inactivated enzymes on a boiling water bath for 5 minutes. Concentrated samples were then analyzed by electrophoresis on a 0.8 % agarose gel. HA molecular weight standards (Select-HATM LoLadder, and HiLadder; Hyalose, Oklahoma City, OK, USA) were run on the same gel for comparison. Electrophoresis was carried out at room temperature at a constant voltage of 80 V for 3 h. Immediately after the run, the gel was stained with 0.005% Stain-All (Sigma-Aldrich) in 50% ethanol. The gel was stained overnight under light-protective cover at room temperature. For destaining, the gel was transferred to 10% ethanol solution. Final destaining of any residual background was accomplished by placing the gel on a light box for a few minutes. Agarose gels were photographed on an Alpha Innotech Imaging System (ProteinSimple, Santa Clara, CA, USA).

Characterization and purification of B lymphocytes

Mice were anesthetized using sodium pentobarbital (Butler, Columbus OH; 100 mg/kg of mouse body weight), and lungs and spleens were removed from five animals to prepare the single cell suspensions. For lung preparations, minced lungs were subjected to collagenase IV (Sigma-Aldrich) digestion in DMEM at 37°C for 1 h with gentle agitation. The cells were then dispersed through a 10-ml syringe and passed through a 40-µm cell strainer (BD Biosciences, San Jose, CA, USA). The cells were washed with sterile PBS twice before they were treated with ammonium chloride cell lysis buffer (ACLB) to remove the red blood cells. To prepare a single cell suspension of splenocytes, spleens were perfused with DMEM. Spleen cells were washed with sterile PBS and treated with ACLB to lyse red blood cells. Lung and spleen cell preparations were counted and re-suspended in PBS with 1% BSA (Sigma Aldrich) to a final concentration of 1×10^7 cells/ml. Fc receptors were blocked with 1 µg of anti-mouse CD16/CD32 per 10^6 cells (eBioscience, San Diego, CA, USA) for 10 min on ice.

The following Abs were used for phenotypic characterization of B lymphocytes using flow cytometry: FITC-anti-CD19, PE-anti-CD44, PE-anti-CD23, PE-anti-CD49d (eBioscience) and PE-CyTM 7 anti-CD11a (BD Biosciences). The samples were stained with labeled Abs (1 µg/million cells) for 30 min in the dark at 4°C and then washed with PBS 1% BSA twice before analyzing using a BD FACSCalibur (BD Biosciences) or an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA). The data was analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

For the purification of B lymphocytes, spleen and pooled lung cells were incubated with 0.0625 µg of FITC-anti-CD19 antibody per million cells for 30 min in the dark at 4°C (eBioscience). The cells were then washed with sterile PBS and incubated with 10 µl of anti-FITC Microbeads per 10^7 cells (Miltenyi Biotec, Auburn, CA, USA), and CD19-positive B lymphocytes were positively selected using the quadro MACS system with LS columns (Miltenyi Biotec) according to the manufacturer's guidelines. After sorting, B lymphocytes were re-suspended in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 50 µM 2-mercaptoethanol to a final concentration of 2×10^7 cells/ml. The purity of the samples was 90-95% via BD FACSCalibur (BD Biosciences) flow cytometry, and data was analyzed using FlowJo software. These cells were used for chemotaxis and cell culture experiments.

Chemotactic activity of low and high molecular mass HA and ex vivo culture of B lymphocytes

To assess the chemotactic activity of LMM HA (40 kDa - 60 kDa) and HMM HA (351 kDa - 600 kDa) (Lifecore Biomedical LLC, Chaska, MN, USA), isolated spleen B lymphocytes were subjected to an *in vitro* chemotaxis assay in a 96-well modified Boyden chamber (ECM512, Millipore Corporation, Billerica, MA, CA, USA). The LMM HA and HMM HA used for the chemotaxis assay were cell culture grade and certified endotoxin free. Purified B lymphocytes were re-suspended at 2×10^7 cells/ml in DMEM and each well was seeded with 5×10^5 B cells [66]. CD44 was blocked by pre-incubating B lymphocytes with 50 µg/ml of anti-CD44 neutralizing Ab (clone IM7; BD Biosciences) for 2 h [69]. B lymphocytes were also pre-incubated with 50 µg/ml isotype control Ab (Purified Rat IgG2b) for 2 h. Purified B lymphocytes (5×10^5 cells) were added to each well in the top filter plate portion of the assembly, and 150 µl

of a 400 µg/ml solution of LMM HA and HMM HA [70, 71] was added to the respective bottom wells. The chamber was incubated at 37°C with 5% CO₂ and constant humidity for 16 h. Migrated cells were detached, lysed, and labeled with a CyQuant GR dye that exhibited strong fluorescence when bound to cellular nucleic acids. Sample fluorescence was measured with a Synergy HT fluorescence microplate reader (BioTek, Winooski, VT, USA) using a 480/520nm excitation and emission filter set. For the chemotaxis assay we used CXCL13 (100 ng/ml) as a positive control [72].

For B lymphocyte *ex vivo* culture experiments, purified B lymphocytes from the spleen and lung (1 million cells /50 µl) were seeded in a 96-well cell culture plate (Corning, New York, USA). Blocking of CD44 was done as above with 50 µg/ml anti-CD44. B lymphocytes were also pre-incubated with 50 µg/ml isotype control Ab (Purified Rat IgG2b). One hundred-fifty microliters of LMM HA and HMM HA (0.5 mg/ml) [60] in complete medium was added to the wells containing B lymphocytes for a total volume of 200 µl. The plate was cultured in a humidified 37°C incubator with 5% CO₂ for 48 h.

Antibody and cytokine ELISA

IgE, IL-4, TGF-β1, and IL-10 levels were measured using ELISA kits purchased from eBioscience as per manufacturer's guidelines (eBioscience, San Diego, CA, USA).

Statistical analysis

Allergic C57BL/6 wild type animals were compared to their respective naïve controls at each time point. Results are expressed as the mean ± SEM. Data were evaluated with GraphPad Prism software (San Diego, CA) using an unpaired, student's two tailed *t* test with Welch's correction to determine statistical significance. A value of **p* < 0.05 was considered statistically significant.

RESULTS

The protocol used for the development of allergic airway disease is shown in **Fig. 1**. In allergic mice, we first ascertained that the standard hallmarks of airway disease were present. Airway hyperresponsiveness, elevated IgE in BAL fluid and serum, and elevated serum IL-4 were observed days 5 after the second conidia inhalation in allergic animals, as compared to naïve animals (data not shown). By day 28, after the second conidia challenge, the AHR, IgE, and IL-4 returned to naïve levels. This is consistent with our previously published work with this model [12, 64, 73].

Inhalational fungal challenge with *A. fumigatus* increases recruitment of lymphocytes and eosinophils to the airway

Morphometric identification of monocytes/macrophages, eosinophils, neutrophils, and lymphocytes was performed to estimate the relative makeup of the cellular inflammation and to monitor leukocyte egress into the airway lumen (Figure 2). Lymphocytes (Figure 2a) made up ~16% and eosinophils (Figure 2b) ~74% of the total inflammation at day 5 after conidia challenge, dominating the total leukocytic inflammation (Figure 2c).

The deposition of HA is increased in perivascular and peribronchial spaces, and LMM HA levels are elevated in the BAL fluid of allergen challenged mice after allergen challenge

Given the recognized role of inflammatory cells and HA in disease processes including COPD and asthma pathogenesis, we investigated the influx of inflammatory cells with HA levels in a model of fungus-induced pulmonary inflammation. Immunohistochemistry revealed that HA deposition was increased around airways (a) and blood vessels (v) after challenge in areas where inflammatory cells were present as compared to naïve lungs (Figure 3a, 3d, 3b, 3e). Basement

membrane, areas underlying the basal myocytes, and surrounding inflammatory cells showed intense HA staining at day 5 (Figure 3e). By day 28, HA was still clearly evident around blood vessels adjacent to the airways (Figure 3c) and the basement membrane (Figure 3f).

LMM HA was increased in the lung of allergic animals at day 5 after the second conidia challenge as compared to naïve mice (Figure 3g), and this HA was predominantly of LMM HA (Figure 3h), indicating HA breakdown or HA synthesis. By day 28 after challenge, HA levels were higher than naïve controls, and this HA was predominantly of HMM HA (Figure 3g). We have also shown previously that there is a parallel increase and co-localization of HA and collagen deposition in the allergic lungs after exposure to the inhaled fungus, suggesting that the regulation of these ECM components is linked [64]. This may explain the increased levels of HA at day 28 than naïve controls as peribronchial fibrosis marked with excessive collagen deposition is elevated at this time point.

CD19⁺ and CD19⁺CD44⁺ B lymphocyte numbers are increased in the allergic lung, BAL, and spleen of *A. fumigatus*-challenged mice

We next examined whether B lymphocytes were a significant component of the inflammation associated with allergic disease, when HA levels are elevated in the lung and BAL fluid of allergic animals. We had previously noted that B cell recruitment to the allergic lung peaks at day 5 after 2 inhalation challenges [12, 73]. In this study, we observed significant increases in CD19⁺ B lymphocytes in the lung (as previously reported), as well as the BAL and spleen at day 5, as compared to naïve controls (Figure 4a-d). By day 28, the number of B lymphocytes in the BAL had returned to levels comparable with naïve controls (Figure 4a, 4c), but remained elevated in the lung tissue and spleen (Figure 4b, 4d).

Our next step was to determine whether these B lymphocytes present in the allergic animals expressed the HA receptor CD44. After *A. fumigatus* conidia challenge, we analyzed the CD19⁺ B lymphocyte populations by flow cytometry for CD44 expression (Figure 4). CD19⁺CD44⁺ lung B cells were increased at day 5 after two conidia challenges (Figure 4b), as compared to controls. CD19⁺CD44⁺ B cells were also increased in the BAL and spleen of allergic animals (Figure 4c and d). However, by day 28 after the second conidia challenge, the number of CD19⁺CD44⁺ B cells returned to naïve controls in the BAL (Figure 4c). This time point coincides with the resolution of inflammation and low HA levels in BAL fluid (Figure 3).

CD19⁺CD49d⁺ and CD19⁺CD11a⁺ B lymphocyte numbers are increased in the lung, BAL, and spleen of *A. fumigatus*-challenged mice

The integrin molecules CD49d and CD11a are required for interaction with ligands on the endothelial surface to arrest and extravasate to the site of infection/tissue injury. To determine whether B lymphocytes in the lung, BAL, and spleen up-regulate or express these molecules, we analyzed B lymphocyte populations of naïve and allergic animals with flow cytometry. We found that the percentage of CD19⁺CD49d⁺ and CD19⁺CD11a⁺ B lymphocytes was increased in the allergic lung, BAL, and spleen at day 5 after the second conidia challenge (Figure 5).

CD44 is necessary for LMM HA-mediated chemotaxis of B lymphocytes

In this fungal model, we see an influx of B lymphocytes in the allergic lung at day 5 after the second conidia challenge (Figure 4). To investigate the recruitment of B lymphocytes after fungal challenge, we determined the role of HA and CD44 in HA-mediated motility of B lymphocytes. Spleen B lymphocytes were isolated and a modified Boyden chamber assay was performed using LMM HA or HMM HA as the chemoattractant. Spleen B lymphocytes were

isolated for the chemotaxis assay since the spleen compartment is dominated by B2 lymphocytes [73, 74] and all CD19⁺ spleen B cells express the CD44 receptor (Figure 4). When treated with LMM HA, B lymphocytes isolated from the spleens of allergic animals 5 days after the second conidia challenge exhibited increased chemotaxis (Figure 6a). Blocking CD44 completely abrogated this effect (Figure 6a). HMM HA had no effect on the chemotactic activity of B lymphocytes (Figure 6b).

LMM HA mediates IL-10 and TGF- β 1 production by B lymphocytes

HA and collagen deposition closely mirror each other in the context of the ECM [62, 64], and pro-allergy and pro-fibrotic cytokines have been implicated in the process of fibrotic airway wall remodeling/tissue repair process. In *in vivo* experiments, we found that BAL IL-10 and TGF- β 1 was elevated in the allergic lung as compared to naïve lungs (Figure 7a, 7b). To determine if HA acting on B cells may be contributing to the production of IL-10 and other important cytokines, we isolated B cells from allergic lung and spleen 5 days after the second conidia inhalation and cultured them for 48 h with LMM HA or HMM HA. B cells produced both IL-10 and TGF- β 1 after stimulation with LMM HA, while HMM HA had no effect (Figure 7c-f). The production of IL-10 was abrogated by blocking CD44 (Figure 7c, 7e); however, blocking CD44 did not have an effect on TGF- β 1 production (Figure 7d, 7f).

DISCUSSION

In the current work, we provide new evidence that LMM HA generated at sites of chronic inflammation may promote a cellular B cell program that supports an allergy phenotype. To our knowledge, this is the first time that LMM HA, but not HMM HA, has been shown to promote chemotaxis in B cells in allergic asthma. Further, when B cells were stimulated with LMM HA *ex vivo*, B lymphocytes produced the pro-allergy cytokines IL-10 and TGF- β 1. Our findings extend the growing body of evidence that shows the ECM's effect—and in particular LMM HA's effect—at the local site of tissue injury [14, 41].

Under disease conditions such as asthma and COPD, HA is broken down to LMM HA [14, 41], which can be detected in the BAL fluid [14, 75-77]. Previously, we have shown that *HYAL1* and *HYAL2* gene expression is significantly increased in the allergic mouse lung at day 5 after the second conidia challenge as compared to naïve controls, suggesting that these hyaluronidases are responsible for the rapid hydrolysis of HMM HA to LMM HA in this model system [64]. We have also shown that there is a parallel increase and co-localization of HA and collagen deposition in the allergic lungs after exposure to the inhaled fungus, suggesting that the regulation of these ECM components is linked [64].

CD44 is important for lymphopoiesis, lymphocyte homing, T cell activation, and metastasis [26, 50, 78]. Non-infectious lung injury in CD44-deficient mice results in sustained inflammation of the alveolar interstitium, LMM HA accumulation at 14 days after bleomycin injury, impaired clearance of neutrophils in association with decreased TGF- β 1 activation and increased mortality [29, 70], suggesting a role of CD44 in regulating inflammation, HA clearance, and cytokine production. CD44 is a prominent HA receptor and a prime target for

mediating the differential responses to low and high molecular mass HA. However, CD44 expression and engagement on B lymphocytes is not yet well defined [60, 79]. Activated B lymphocytes, particularly those stimulated with IL-5, bind HA with higher affinity than naïve B cells [50, 63, 79]. In addition, IFN- γ inhibits HA/CD44 interactions in naïve B lymphocytes [80]. Our *ex vivo* results show a role for CD44 in enabling B lymphocyte migration to LMM HA. We hypothesize that in the allergic model, elevated IL-5 and IL-4 levels may function to promote B lymphocyte interaction with HA via CD44. Similarly, elevated IL-10 levels may function to limit IFN- γ production and stimulate B lymphocyte interaction with HA via CD44.

Increased expression of the integrin molecules CD49d and CD11a allows lymphocytes to interact with ligands on endothelial cells and infiltrate into areas of allergic inflammation to mediate immune responses [81, 82]. In this model of fungus-induced airway disease, we observe an increase in the total percentage of CD19⁺CD49d⁺ and CD19⁺CD11a⁺ B lymphocytes in the allergic lung, reiterating the role of these integrin molecules, along with CD44, in B lymphocyte migration to the site of chronic inflammation/tissue injury. The mechanism through which these molecules mediate B cell migration in the allergic lung remains to be shown *in vivo*.

Our work and that of others have shown that B lymphocytes make up a significant component of inflammation associated with asthma, indicating a potential role in the deleterious effects associated with that inflammation [12, 73, 83, 84]. Although the chemokines involved in neutrophil and eosinophil recruitment in asthma are well recognized, few studies have evaluated the recruitment of B lymphocytes into the allergic lung [62, 85]. In the current study, we show *ex vivo* evidence that the ECM itself could promote B lymphocyte migration in times of HA hydrolysis.

An important outcome of this study is the increased release of IL-10 and TGF- β 1 by *ex vivo* cultured B lymphocytes in response to LMM HA. IL-10 production by B lymphocytes was dependent on CD44 indicating that CD44 may be involved in the production of this Th2 cytokine. On the contrary, TGF- β 1 production by B lymphocytes was independent of CD44, indicating that other receptors may be involved in regulating this process. It is possible that Toll-like receptors 2 and 4 (TLR-2 and TLR-4), present on B lymphocytes, provide the signals for TGF- β 1 production, as TLR-2 and TLR-4 have been shown to interact with fragmented HA [14]. The mechanisms responsible for the differential functions of low versus high molecular mass HA are still unclear, but may be related to an increased binding affinity of the smaller molecule that allows more efficient CD44 receptor crosslinking [40, 86].

Increased IL-10 production is associated with increased levels of total and specific immunoglobulins (IgE, IgG1, and IgA) and airway remodeling, reflecting a role of IL-10 in promoting a Th2 response to *A. fumigatus* antigens [15, 87]. Alternatively, IL-10 can suppress the secretion of other cytokines and control cellular activation, consistent with modulating pathology [88]. In addition, IL-10 permits the Foxp3-inducing capacity of TGF- β 1 and hence could further elevate Treg differentiation [89]. Like IL-10, TGF- β 1 is considered as an immune suppressive cytokine, and as a result local production may help modulate asthma [90]. However, its role in allergic asthma is clearly more complex. The combination of IL-4 and TGF- β 1 has been shown to induce IL-9 secreting CD4⁺ T cells, a cytokine known to exacerbate asthmatic symptoms [91]. Further, TGF- β 1 has a clear role in causing tissue remodeling changes that are associated with decreased airway responsiveness in chronic asthma.

Airway wall remodeling is a particularly unyielding symptom of chronic airway inflammation due to allergy. TGF- β 1 has long been associated with the epithelial alterations,

subepithelial fibrosis, and airway smooth muscle changes that characterize remodeling [92-94]. We hypothesize that during disruption of the ECM, whether from cells moving from the parenchyma to the lumen or microbial attack from the lumen, normally immunologically inert HMM HA is hydrolyzed and takes on a chemoattractant role, functioning to both attract and activate B cells in an area where they can most effectively enact a fibrotic repair mechanism. With subsequent rounds of allergen inhalation, this 'repair' is reinforced until intractable fibrosis results. This hypothesis will need to be tested *in vivo* and is the topic of ongoing research in our laboratory.

A variety of factors have been linked to increased HA synthesis, including prostaglandins and a multitude of cytokines many of which have roles in asthma [31, 46, 62]. Likewise, there are several possible pathways that may be involved in the degradation of HA in the asthmatic lung [95, 96]. The stimulus for LMM HA accumulation in this model is difficult, as almost every cell has the capacity to produce HA and the balance of competing HASs and HYALs is critical to overall HA deposition [14, 28, 62, 64, 85].

In conclusion, these results support a novel localized immune modulation mediated by B lymphocytes when they interact with LMM HA generated at sites of allergic inflammation after the inhalation of fungal conidia. Our data suggest that LMM HA can function as an important signaling molecule for B lymphocyte movement and cytokine production, which are critical to the maintenance of allergic inflammatory responses. Further studies examining HA's role in mediating cellular responses may help to develop targets for treatment in patients with severe asthma due to fungal sensitization.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Pawel Borowicz, co-Director of the Advanced Imaging and Microscopy Core Laboratory at NDSU, for his help with imaging using the Zeiss Z1 AxioObserver inverted microscope. We also thank Jessica Ebert for critical reading of the manuscript.

AUTHOR CONTRIBUTIONS

SG: Study conception and design, data acquisition, analysis and interpretation, and drafting and editing the manuscript; SAH and SG: FCS data acquisition and FlowJo analysis; JC and JBM: HA staining, analysis and interpretation; SBW, JBM, and GPD: HA size determination; JMS: fungal asthma model, study conception and design, and editing the manuscript. All authors read and approved the final version of the manuscript.

DISCLOSURES

The authors declare no conflicts of interest associated with this work.

Figure 1. Sensitization, challenge, and analysis schedule for the *A. fumigatus* murine model of allergic asthma. Mice were sensitized to *A. fumigatus* extract via a series of injections and intranasal inoculations, after which they were exposed to 2, nose-only inhalation doses of live conidia 2 wk apart. Groups of animals were assessed at prescribed time points after allergen challenge.

Figure 2. Effect of inhalation of *A. fumigatus* conidia on lymphocyte and eosinophil inflammation in allergic C57BL/6 mice. Airway inflammation marked by the presence of lymphocytes **(A)** and eosinophils **(B)** in naïve and allergic animals. **(C)** Represents total inflammatory cells in the BAL compartment of naïve and allergic C57BL/6 mice. Data were analyzed using an unpaired Student two-tailed *t* test with Welch correction. All values are expressed as the means $\pm$ SEM (*n* = 4-5 mice/group). **p* < 0.05. Data shown are representative of two independent experiments.

Figure 3. Effect of inhalation of *A. fumigatus* conidia on HA deposition and LMM HA levels. **(A-F)** HA deposition around the small **(A-C)** and large airways **(D-F)** is shown for lungs from naïve (unchallenged) mouse and *A. fumigatus* sensitized and challenged mice [*a* = airway, *v* = blood vessel]. ELISAs were carried out to measure HA levels in C57BL/6 mice after fungal challenge. **(G)** HA levels in the BAL fluid of naïve and allergic animals. **(H)** HA size in the lung of naïve and allergic animals. Data were analyzed using an unpaired Student two-tailed *t* test with Welch correction. All values are expressed as the means $\pm$ SEM (*n* = 4-5 mice/group). **p* < 0.05. Data shown are representative of two independent experiments.

Figure 4. Inhalation of *A. fumigatus* conidia increases CD19⁺ and CD19⁺CD44⁺ B cells in the lung, BAL, and spleen of allergic mice. Flow cytometry was used to analyze density plots (SSC

vs CD19) of cells isolated from the lungs, BAL (pooled by group), and spleens of naïve and allergic C57BL/6 mice **(A)**, and the percentage of CD19⁺ and CD19⁺CD44⁺ B cells **(B-D)** was calculated. Data were analyzed using an unpaired Student two-tailed *t* test with Welch correction. All values are expressed as the means $\pm$ SEM (*n* = 4-5 mice/group). **p* < 0.05. Data shown are representative of two independent experiments.

Figure 5. *A. fumigatus* conidia inhalation increases the number of activated CD19⁺ B cells in the allergic lung, BAL, and spleen. Lung, BAL, and spleen CD19⁺ B cells were evaluated by flow cytometry for CD49d **(A)** and CD11a **(B)** expression, and the percentage of CD19⁺CD49d⁺ **(C-E)** and CD19⁺CD11⁺ B cells **(F-H)** was calculated in the lung, BAL (pooled samples), and the spleen of naïve and allergic C57BL/6 mice. Data were analyzed using an unpaired Student two-tailed *t* test with Welch correction. All values are expressed as the means $\pm$ SEM (*n* = 4-5 mice/group). **p* < 0.05. Data shown are representative of two independent experiments.

Figure 6. LMM-HA effects B cell movement through a CD44-dependent mechanism. Using a modified Boyden chamber, chemotactic activity towards LMM HA **(A)** and HMM HA **(B)** was measured for CD19⁺ spleen B cells isolated from mice 5 days after conidia inhalation with and without CD44 blocking antibodies. Data were analyzed using an unpaired Student two-tailed *t* test with Welch correction. All values are expressed as the means $\pm$ SEM (*n* = 5 mice/group). **p* < 0.05. This result is an outcome of two independent experiments.

Figure 7. The effect of *A. fumigatus* conidia challenge/ HA fragments on IL-10 and TGF- β 1 production. IL-10 **(A)** and TGF- β 1 **(B)** levels were assessed by ELISA in the BAL fluid of naïve and allergic animals. Spleen **(C and D)** and lung **(E and F)** B cells were harvested from wild-type C57BL/6 mice at day 5 and treated with LMM HA or HMM HA with or without a neutralizing CD44 antibody. Supernatant was harvested 48 h later, and IL-10 **(C and E)** and

TGF- β 1 (**D and F**) production was assessed by ELISA. Data were analyzed using an unpaired Student two-tailed *t* test with Welch correction. All values are expressed as the means $\pm$ SEM (*n* = 5 mice/group). **p* < 0.05. No statistics are shown for cytokine production by lung B cells as the lung B cells from five mice from D5 time point were pooled and run as a single sample. Data shown are representative of two independent experiments.

Figure 8. Hypothetical model for LMM HA regulation of B lymphocyte recruitment and function in the allergic lung. At sites of chronic inflammation in the lung combined actions of HASs, HYALs, and ROS generated by eosinophils during the asthmatic response may be breaking down LMM HA to HMM HA. This LMM HA would promote the preferential recruitment of B lymphocytes in a CD44 dependent manner. These B lymphocytes in addition to CD44 express the activation markers (CD49d and CD11a) which would promote the extravasation of B lymphocytes to the site of tissue injury. In the allergic lung these B cells will interact with LMM HA and will promote the production of pro-allergy cytokines IL-10 and TGF- β 1.

506

507 **REFERENCES**

- 508 1. Manion AB. Asthma and obesity: the dose effect. *The Nursing clinics of North America*.
509 2013;48(1):151-8. doi:10.1016/j.cnur.2012.12.002.
- 510 2. Knutsen AP, Bush RK, Demain JG, Denning DW, Dixit A, Fairs A et al. Fungi and allergic
511 lower respiratory tract diseases. *The Journal of allergy and clinical immunology*.
512 2012;129(2):280-91; quiz 92-3. doi:10.1016/j.jaci.2011.12.970.
- 513 3. Hogan C, Denning DW. Allergic bronchopulmonary aspergillosis and related allergic
514 syndromes. *Seminars in respiratory and critical care medicine*. 2011;32(6):682-92.
515 doi:10.1055/s-0031-1295716.
- 516 4. Umetsu DT, DeKruyff RH. The regulation of allergy and asthma. *Immunological reviews*.
517 2006;212:238-55. doi:10.1111/j.0105-2896.2006.00413.x.
- 518 5. Hamid QA, Minshall EM. Molecular pathology of allergic disease: I: lower airway disease.
519 *The Journal of allergy and clinical immunology*. 2000;105(1 Pt 1):20-36.
- 520 6. Rosenberg HF, Dyer KD, Foster PS. Eosinophils: changing perspectives in health and disease.
521 *Nature reviews Immunology*. 2013;13(1):9-22. doi:10.1038/nri3341.
- 522 7. Ghosh S, Hoselton SA, Dorsam GP, Schuh JM. Eosinophils in fungus-associated allergic
523 pulmonary disease. *Frontiers in Pharmacology*. 2013;4. doi:10.3389/fphar.2013.00008.
- 524 8. Chaudhary N, Marr KA. Impact of *Aspergillus fumigatus* in allergic airway diseases. *Clinical*
525 *and translational allergy*. 2011;1(1):4. doi:10.1186/2045-7022-1-4.
- 526 9. Acharya KR, Ackerman SJ. Eosinophil granule proteins: form and function. *The Journal of*
527 *biological chemistry*. 2014;289(25):17406-15. doi:10.1074/jbc.R113.546218.

- 528 10. Wardlaw AJ, Brightling C, Green R, Woltmann G, Pavord I. Eosinophils in asthma and other
529 allergic diseases. *British medical bulletin*. 2000;56(4):985-1003.
- 530 11. Murdock BJ, Shreiner AB, McDonald RA, Osterholzer JJ, White ES, Toews GB et al.
531 Coevolution of TH1, TH2, and TH17 responses during repeated pulmonary exposure to
532 *Aspergillus fumigatus* conidia. *Infection and immunity*. 2011;79(1):125-35.
533 doi:10.1128/iai.00508-10.
- 534 12. Ghosh S, Hoselton SA, Schuh JM. Characterization of CD19(+)CD23(+)B2 lymphocytes in
535 the allergic airways of BALB/c mice in response to the inhalation of *Aspergillus fumigatus*
536 conidia. *The open immunology journal*. 2012;5:46-54. doi:10.2174/1874226201205010046.
- 537 13. Shreiner AB, Murdock BJ, Sadighi Akha AA, Falkowski NR, Christensen PJ, White ES et al.
538 Repeated exposure to *Aspergillus fumigatus* conidia results in CD4+ T cell-dependent and -
539 independent pulmonary arterial remodeling in a mixed Th1/Th2/Th17 microenvironment that
540 requires interleukin-4 (IL-4) and IL-10. *Infection and immunity*. 2012;80(1):388-97.
541 doi:10.1128/iai.05530-11.
- 542 14. Jiang D, Liang J, Noble PW. Hyaluronan as an immune regulator in human diseases.
543 *Physiological reviews*. 2011;91(1):221-64. doi:10.1152/physrev.00052.2009.
- 544 15. Dagenais TR, Keller NP. Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis.
545 *Clinical microbiology reviews*. 2009;22(3):447-65. doi:10.1128/cmr.00055-08.
- 546 16. Hoselton SA, Samarasinghe AE, Seydel JM, Schuh JM. An inhalation model of airway
547 allergic response to inhalation of environmental *Aspergillus fumigatus* conidia in sensitized
548 BALB/c mice. *Medical mycology*. 2010;48(8):1056-65. doi:10.3109/13693786.2010.485582.

- 549 17. Schuh JM, Hoselton SA. An inhalation model of allergic fungal asthma: *Aspergillus*
550 *fumigatus*-induced inflammation and remodeling in allergic airway disease. *Methods in*
551 *molecular biology* (Clifton, NJ). 2013;1032:173-84. doi:10.1007/978-1-62703-496-8_14.
- 552 18. Samarasinghe AE, Hoselton SA, Schuh JM. A comparison between intratracheal and
553 inhalation delivery of *Aspergillus fumigatus* conidia in the development of fungal allergic
554 asthma in C57BL/6 mice. *Fungal biology*. 2011;115(1):21-9. doi:10.1016/j.funbio.2010.09.013.
- 555 19. Ghosh S, Hoselton SA, Asbach SV, Steffan BN, Wanjara SB, Dorsam GP et al. B
556 lymphocytes regulate airway granulocytic inflammation and cytokine production in a murine
557 model of fungal allergic asthma. *Cellular & molecular immunology*. 2014.
558 doi:10.1038/cmi.2014.103.
- 559 20. Pandey S, Hoselton SA, Schuh JM. The impact of *Aspergillus fumigatus* viability and
560 sensitization to its allergens on the murine allergic asthma phenotype. *BioMed research*
561 *international*. 2013;2013:619614. doi:10.1155/2013/619614.
- 562 21. Oharazawa H, Ibaraki N, Lin LR, Reddy VN. The effects of extracellular matrix on cell
563 attachment, proliferation and migration in a human lens epithelial cell line. *Experimental eye*
564 *research*. 1999;69(6):603-10. doi:10.1006/exer.1999.0723.
- 565 22. Thannickal VJ, Lee DY, White ES, Cui Z, Larios JM, Chacon R et al. Myofibroblast
566 differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin
567 signaling via focal adhesion kinase. *The Journal of biological chemistry*. 2003;278(14):12384-9.
568 doi:10.1074/jbc.M208544200.
- 569 23. Noble PW. Hyaluronan and its catabolic products in tissue injury and repair. *Matrix Biol*.
570 2002;21(1):25-9. doi:S0945053X01001846 [pii].

- 571 24. McKee CM, Penno MB, Cowman M, Burdick MD, Strieter RM, Bao C et al. Hyaluronan
572 (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA
573 size and CD44. *J Clin Invest.* 1996;98(10):2403-13.
- 574 25. Peck D, Isacke CM. Hyaluronan-dependent cell migration can be blocked by a CD44
575 cytoplasmic domain peptide containing a phosphoserine at position 325. *Journal of cell science.*
576 1998;111 (Pt 11):1595-601.
- 577 26. Ruffell B, Johnson P. Hyaluronan induces cell death in activated T cells through CD44.
578 *Journal of immunology (Baltimore, Md : 1950).* 2008;181(10):7044-54.
- 579 27. Meyer K, Palmer JW. The polysaccharide of the Vitreous humor. *The Journal of biological*
580 *chemistry.* 1934(107):629-34.
- 581 28. Katoh S, Ishii N, Nobumoto A, Takeshita K, Dai SY, Shinonaga R et al. Galectin-9 inhibits
582 CD44-hyaluronan interaction and suppresses a murine model of allergic asthma. *American*
583 *journal of respiratory and critical care medicine.* 2007;176(1):27-35. doi:10.1164/rccm.200608-
584 1243OC.
- 585 29. Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. *Annual review of cell*
586 *and developmental biology.* 2007;23:435-61. doi:10.1146/annurev.cellbio.23.090506.123337.
- 587 30. Laurent TC, Fraser JR. Hyaluronan. *FASEB journal : official publication of the Federation of*
588 *American Societies for Experimental Biology.* 1992;6(7):2397-404.
- 589 31. Lennon FE, Singleton PA. Role of hyaluronan and hyaluronan-binding proteins in lung
590 pathobiology. *American journal of physiology Lung cellular and molecular physiology.*
591 2011;301(2):L137-47. doi:10.1152/ajplung.00071.2010.
- 592 32. Toole BP. Hyaluronan is not just a goo! *J Clin Invest.* 2000;106(3):335-6.
593 doi:10.1172/jci10706.

- 594 33. Ghosh S, Hoselton SA, Dorsam GP, Schuh JM. Hyaluronan fragments as mediators of
 595 inflammation in allergic pulmonary disease. *Immunobiology*. 2014.
 596 doi:10.1016/j.imbio.2014.12.005.
- 597 34. Yue BY, Baum JL, Silbert JE. Synthesis of glycosaminoglycans by cultures of normal human
 598 corneal endothelial and stromal cells. *Investigative ophthalmology & visual science*.
 599 1978;17(6):523-7.
- 600 35. Kitchen JR, Cysyk RL. Synthesis and release of hyaluronic acid by Swiss 3T3 fibroblasts.
 601 *The Biochemical journal*. 1995;309 (Pt 2):649-56.
- 602 36. Acharya PS, Majumdar S, Jacob M, Hayden J, Mrass P, Weninger W et al. Fibroblast
 603 migration is mediated by CD44-dependent TGF beta activation. *Journal of cell science*.
 604 2008;121(Pt 9):1393-402. doi:10.1242/jcs.021683.
- 605 37. Vistejnova L, Safrankova B, Nesporova K, Slavkovsky R, Hermannova M, Hosek P et al.
 606 Low molecular weight hyaluronan mediated CD44 dependent induction of IL-6 and chemokines
 607 in human dermal fibroblasts potentiates innate immune response. *Cytokine*. 2014.
 608 doi:10.1016/j.cyto.2014.07.006.
- 609 38. Toshida H, Tabuchi N, Koike D, Koide M, Sugiyama K, Nakayasu K et al. The effects of
 610 vitamin A compounds on hyaluronic acid released from cultured rabbit corneal epithelial cells
 611 and keratocytes. *Journal of nutritional science and vitaminology*. 2012;58(4):223-9.
- 612 39. Deudon E, Berrou E, Breton M, Picard J. Growth-related production of proteoglycans and
 613 hyaluronic acid in synchronous arterial smooth muscle cells. *The International journal of*
 614 *biochemistry*. 1992;24(3):465-70.

- 615 40. Yang C, Cao M, Liu H, He Y, Xu J, Du Y et al. The high and low molecular weight forms of
616 hyaluronan have distinct effects on CD44 clustering. *The Journal of biological chemistry*.
617 2012;287(51):43094-107. doi:10.1074/jbc.M112.349209.
- 618 41. Noble PW, Jiang D. Matrix regulation of lung injury, inflammation, and repair: the role of
619 innate immunity. *Proceedings of the American Thoracic Society*. 2006;3(5):401-4.
620 doi:10.1513/pats.200604-097AW.
- 621 42. Dentener MA, Vernooij JH, Hendriks S, Wouters EF. Enhanced levels of hyaluronan in lungs
622 of patients with COPD: relationship with lung function and local inflammation. *Thorax*.
623 2005;60(2):114-9. doi:10.1136/thx.2003.020842.
- 624 43. Papakonstantinou E, Karakioulakis G. The 'sweet' and 'bitter' involvement of
625 glycosaminoglycans in lung diseases: pharmacotherapeutic relevance. *British journal of*
626 *pharmacology*. 2009;157(7):1111-27. doi:10.1111/j.1476-5381.2009.00279.x.
- 627 44. Singleton PA, Lennon FE. Acute Lung Injury Regulation by Hyaluronan. *Journal of allergy*
628 *& therapy*. 2011;Suppl 4. doi:10.4172/2155-6121.s4-003.
- 629 45. Stern R. Devising a pathway for hyaluronan catabolism: are we there yet? *Glycobiology*.
630 2003;13(12):105R-15R. doi:10.1093/glycob/cwg112.
- 631 46. Petrey AC, de la Motte CA. Hyaluronan, a Crucial Regulator of Inflammation. *Front*
632 *Immunol*. 2014;5. doi:10.3389/fimmu.2014.00101.
- 633 47. Sokolowska M, Chen LY, Eberlein M, Martinez-Anton A, Liu Y, Alsaaty S et al. Low
634 molecular weight hyaluronan activates cytosolic phospholipase A2alpha and eicosanoid
635 production in monocytes and macrophages. *The Journal of biological chemistry*.
636 2014;289(7):4470-88. doi:10.1074/jbc.M113.515106.

48. Toole BP. Hyaluronan: from extracellular glue to pericellular cue. *Nature reviews Cancer*. 2004;4(7):528-39. doi:10.1038/nrc1391.
49. Entwistle J, Hall CL, Turley EA. HA receptors: regulators of signalling to the cytoskeleton. *Journal of cellular biochemistry*. 1996;61(4):569-77. doi:10.1002/(SICI)1097-4644(19960616)61:4<569::AID-JCB10>3.0.CO;2-B.
50. Lesley J, Hyman R, Kincade PW. CD44 and its interaction with extracellular matrix. *Advances in immunology*. 1993;54:271-335.
51. Pilarski LM, Masellis-Smith A, Belch AR, Yang B, Savani RC, Turley EA. RHAMM, a receptor for hyaluronan-mediated motility, on normal human lymphocytes, thymocytes and malignant B cells: a mediator in B cell malignancy? *Leukemia & lymphoma*. 1994;14(5-6):363-74. doi:10.3109/10428199409049691.
52. Toole BP. Hyaluronan and its binding proteins, the hyaladherins. *Current opinion in cell biology*. 1990;2(5):839-44.
53. Getting SJ, Mahoney DJ, Cao T, Rugg MS, Fries E, Milner CM et al. The link module from human TSG-6 inhibits neutrophil migration in a hyaluronan- and inter-alpha -inhibitor-independent manner. *The Journal of biological chemistry*. 2002;277(52):51068-76. doi:10.1074/jbc.M205121200.
54. Maier R, Wisniewski HG, Vilcek J, Lotz M. TSG-6 expression in human articular chondrocytes. Possible implications in joint inflammation and cartilage degradation. *Arthritis and rheumatism*. 1996;39(4):552-9.
55. Swaidani S, Cheng G, Lauer ME, Sharma M, Mikecz K, Hascall VC et al. TSG-6 protein is crucial for the development of pulmonary hyaluronan deposition, eosinophilia, and airway

- hyperresponsiveness in a murine model of asthma. The Journal of biological chemistry. 2013;288(1):412-22. doi:10.1074/jbc.M112.389874.
56. Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. The Journal of cell biology. 1999;144(4):789-801.
57. Garantziotis S, Li Z, Potts EN, Lindsey JY, Stober VP, Polosukhin VV et al. TLR4 is necessary for hyaluronan-mediated airway hyperresponsiveness after ozone inhalation. American journal of respiratory and critical care medicine. 2010;181(7):666-75. doi:10.1164/rccm.200903-0381OC.
58. Pure E, Cuff CA. A crucial role for CD44 in inflammation. Trends in molecular medicine. 2001;7(5):213-21.
59. Rothenberg ME. CD44--a sticky target for asthma. J Clin Invest. 2003;111(10):1460-2. doi:10.1172/jci18392.
60. Rafi A, Nagarkatti M, Nagarkatti PS. Hyaluronate-CD44 interactions can induce murine B-cell activation. Blood. 1997;89(8):2901-8.
61. Do Y, Nagarkatti PS, Nagarkatti M. Role of CD44 and hyaluronic acid (HA) in activation of alloreactive and antigen-specific T cells by bone marrow-derived dendritic cells. Journal of immunotherapy (Hagerstown, Md : 1997). 2004;27(1):1-12.
62. Cheng G, Swaidani S, Sharma M, Lauer ME, Hascall VC, Aronica MA. Correlation of hyaluronan deposition with infiltration of eosinophils and lymphocytes in a cockroach-induced murine model of asthma. Glycobiology. 2013;23(1):43-58. doi:10.1093/glycob/cws122.

- 681 63. Vasconcellos FC, Swiston AJ, Beppu MM, Cohen RE, Rubner MF. Bioactive polyelectrolyte
 682 multilayers: hyaluronic acid mediated B lymphocyte adhesion. *Biomacromolecules*.
 683 2010;11(9):2407-14. doi:10.1021/bm100570r.
- 684 64. Ghosh S, Samarasinghe AE, Hoselton SA, Dorsam GP, Schuh JM. Hyaluronan deposition
 685 and co-localization with inflammatory cells and collagen in a murine model of fungal allergic
 686 asthma. *Inflammation research : official journal of the European Histamine Research Society* [et
 687 al]. 2014. doi:10.1007/s00011-014-0719-3.
- 688 65. Simpson MA, Wilson CM, McCarthy JB. Inhibition of prostate tumor cell hyaluronan
 689 synthesis impairs subcutaneous growth and vascularization in immunocompromised mice. *The*
 690 *American journal of pathology*. 2002;161(3):849-57. doi:10.1016/S0002-9440(10)64245-9.
- 691 66. Bhilocha S, Amin R, Pandya M, Yuan H, Tank M, LoBello J et al. Agarose and
 692 polyacrylamide gel electrophoresis methods for molecular mass analysis of 5- to 500-kDa
 693 hyaluronan. *Analytical biochemistry*. 2011;417(1):41-9. doi:10.1016/j.ab.2011.05.026.
- 694 67. Eldridge L, Moldobaeva A, Wagner EM. Increased hyaluronan fragmentation during
 695 pulmonary ischemia. *American journal of physiology Lung cellular and molecular physiology*.
 696 2011;301(5):L782-8. doi:10.1152/ajplung.00079.2011.
- 697 68. Liang J, Jiang D, Jung Y, Xie T, Ingram J, Church T et al. Role of hyaluronan and
 698 hyaluronan-binding proteins in human asthma. *The Journal of allergy and clinical immunology*.
 699 2011;128(2):403-11 e3. doi:10.1016/j.jaci.2011.04.006.
- 700 69. Vachon E, Martin R, Plumb J, Kwok V, Vandivier RW, Glogauer M et al. CD44 is a
 701 phagocytic receptor. *Blood*. 2006;107(10):4149-58. doi:10.1182/blood-2005-09-3808.

- 702 70. Zaman A, Cui Z, Foley JP, Zhao H, Grimm PC, Delisser HM et al. Expression and role of the
703 hyaluronan receptor RHAMM in inflammation after bleomycin injury. *American journal of*
704 *respiratory cell and molecular biology*. 2005;33(5):447-54. doi:10.1165/rcmb.2004-0333OC.
- 705 71. Tamoto K, Nochi H, Tada M, Shimada S, Mori Y, Kataoka S et al. High-molecular-weight
706 hyaluronic acids inhibit chemotaxis and phagocytosis but not lysosomal enzyme release induced
707 by receptor-mediated stimulations in guinea pig phagocytes. *Microbiology and immunology*.
708 1994;38(1):73-80.
- 709 72. Rupprecht TA, Plate A, Adam M, Wick M, Kastenbauer S, Schmidt C et al. The chemokine
710 CXCL13 is a key regulator of B cell recruitment to the cerebrospinal fluid in acute Lyme
711 neuroborreliosis. *Journal of neuroinflammation*. 2009;6:42. doi:10.1186/1742-2094-6-42.
- 712 73. Ghosh S, Hoselton SA, Schuh JM. μ -chain-deficient mice possess B-1 cells and produce
713 IgG and IgE, but not IgA, following systemic sensitization and inhalational challenge in a fungal
714 asthma model. *Journal of immunology (Baltimore, Md : 1950)*. 2012;189(3):1322-9.
715 doi:10.4049/jimmunol.1200138.
- 716 74. Hsueh RC, Roach TIA, Lin KM, O'Connell TD, Han H, Yan Z. Purification and
717 Characterization of Mouse Splenic B Lymphocytes. *AfCS Research Reports*. 2002;1(1):1-11.
- 718 75. Sahu S, Lynn WS. Hyaluronic acid in the pulmonary secretions of patients with asthma. *The*
719 *Biochemical journal*. 1978;173(2):565-8.
- 720 76. Sahu SC. Hyaluronic acid. An indicator of pathological conditions of human lungs?
721 *Inflammation*. 1980;4(1):107-12.
- 722 77. Necas J, Bartosikova L, Brauner P, Kolar J. Hyaluronic acid (hyaluronan): a review.
723 *Veterinari Medicina*. 2008;8(53):397-411.

78. DeGrendele HC, Estess P, Siegelman MH. Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science*. 1997;278(5338):672-5.
79. Murakami S, Miyake K, June CH, Kincade PW, Hodes RJ. IL-5 induces a Pgp-1 (CD44) bright B cell subpopulation that is highly enriched in proliferative and Ig secretory activity and binds to hyaluronate. *Journal of immunology (Baltimore, Md : 1950)*. 1990;145(11):3618-27.
80. Marko Kryworuchko FD-MaAK. Interferon-gamma inhibits CD44-Hyaluronan interactions in Normal Human B Lymphocytes. *Exp Cell Res*. 1999(250):241-52.
81. McDermott DS, Varga SM. Quantifying antigen-specific CD4 T cells during a viral infection: CD4 T cell responses are larger than we think. *Journal of immunology (Baltimore, Md : 1950)*. 2011;187(11):5568-76. doi:10.4049/jimmunol.1102104.
82. Lang J, Kelly M, Freed BM, McCarter MD, Kedl RM, Torres RM et al. Studies of lymphocyte reconstitution in a humanized mouse model reveal a requirement of T cells for human B cell maturation. *Journal of immunology (Baltimore, Md : 1950)*. 2013;190(5):2090-101. doi:10.4049/jimmunol.1202810.
83. Hamelmann E, Vella AT, Oshiba A, Kappler JW, Marrack P, Gelfand EW. Allergic airway sensitization induces T cell activation but not airway hyperresponsiveness in B cell-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94(4):1350-5.
84. Jungsuwadee P, Benkovszky M, Dekan G, Stingl G, Epstein MM. Repeated aerosol allergen exposure suppresses inflammation in B-cell-deficient mice with established allergic asthma. *Int Arch Allergy Immunol*. 2004;133(1):40-8. doi:10.1159/000075252 [doi] 75252 [pii].

- 746 85. Cheng G, Swaidani S, Sharma M, Lauer ME, Hascall VC, Aronica MA. Hyaluronan
747 deposition and correlation with inflammation in a murine ovalbumin model of asthma. *Matrix*
748 *Biol.* 2011;30(2):126-34. doi:10.1016/j.matbio.2010.12.003.
- 749 86. Ohkawara Y, Tamura G, Iwasaki T, Tanaka A, Kikuchi T, Shirato K. Activation and
750 transforming growth factor-beta production in eosinophils by hyaluronan. *American journal of*
751 *respiratory cell and molecular biology.* 2000;23(4):444-51. doi:10.1165/ajrcmb.23.4.3875.
- 752 87. Latge JP. *Aspergillus fumigatus* and aspergillosis. *Clinical microbiology reviews.*
753 1999;12(2):310-50.
- 754 88. Donaldson DS, Apostolaki M, Bone HK, Richards CM, Williams NA. The *Escherichia coli*
755 heat-labile enterotoxin B subunit protects from allergic airway disease development by inducing
756 CD4⁺ regulatory T cells. *Mucosal immunology.* 2013;6(3):535-46. doi:10.1038/mi.2012.93.
- 757 89. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-
758 beta induces a regulatory phenotype in CD4⁺CD25⁻ T cells through Foxp3 induction and down-
759 regulation of Smad7. *Journal of immunology (Baltimore, Md : 1950).* 2004;172(9):5149-53.
- 760 90. Makinde T, Murphy RF, Agrawal DK. The regulatory role of TGF-beta in airway remodeling
761 in asthma. *Immunology and cell biology.* 2007;85(5):348-56. doi:10.1038/sj.icb.7100044.
- 762 91. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA et al. IL-4 inhibits TGF-
763 beta-induced Foxp3⁺ T cells and, together with TGF-beta, generates IL-9⁺ IL-10⁺ Foxp3(-)
764 effector T cells. *Nature immunology.* 2008;9(12):1347-55. doi:10.1038/ni.1677.
- 765 92. Halwani R, Al-Muhsen S, Al-Jahdali H, Hamid Q. Role of transforming growth factor-beta
766 in airway remodeling in asthma. *American journal of respiratory cell and molecular biology.*
767 2011;44(2):127-33. doi:10.1165/rcmb.2010-0027TR.

- 768 93. Shifren A, Witt C, Christie C, Castro M. Mechanisms of remodeling in asthmatic airways.
769 Journal of allergy. 2012;2012:316049. doi:10.1155/2012/316049.
- 770 94. Vignola AM, Gagliardo R, Siena A, Chiappara G, Bonsignore MR, Bousquet J et al. Airway
771 remodeling in the pathogenesis of asthma. Current allergy and asthma reports. 2001;1(2):108-15.
- 772 95. Agren UM, Tammi RH, Tammi MI. Reactive oxygen species contribute to epidermal
773 hyaluronan catabolism in human skin organ culture. Free radical biology & medicine.
774 1997;23(7):996-1001.
- 775 96. Atmuri V, Martin DC, Hemming R, Gutsol A, Byers S, Sahebjam S et al. Hyaluronidase 3
776 (HYAL3) knockout mice do not display evidence of hyaluronan accumulation. Matrix Biol.
777 2008;27(8):653-60. doi:10.1016/j.matbio.2008.07.006.

778

779

Figure 1

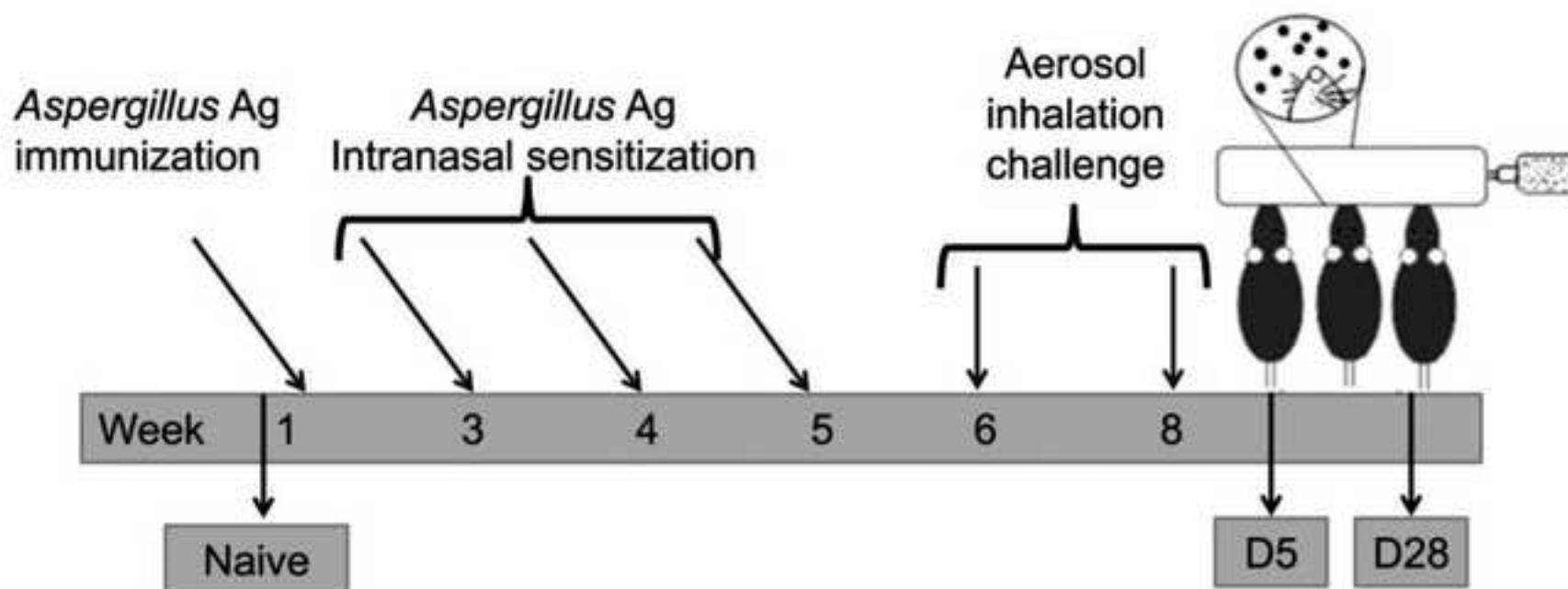


Figure 2

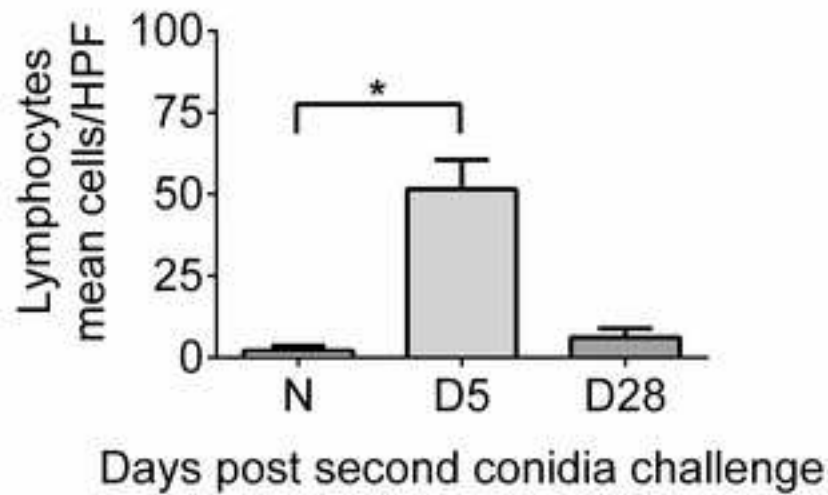
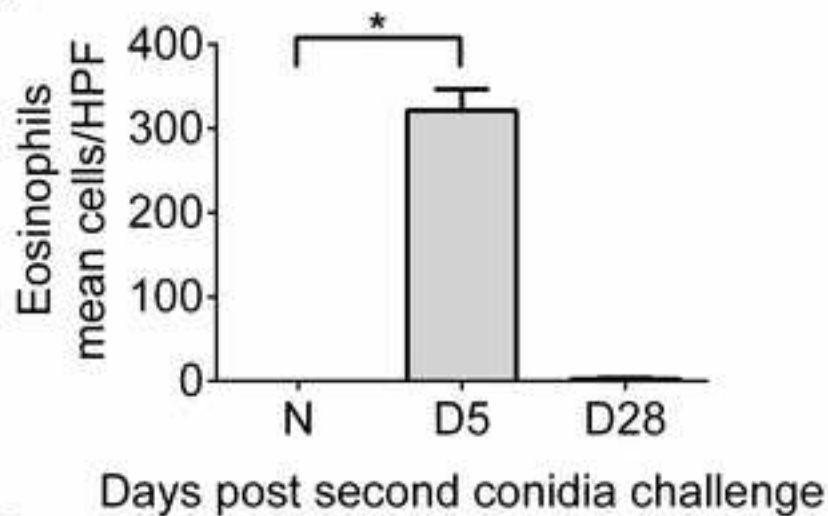
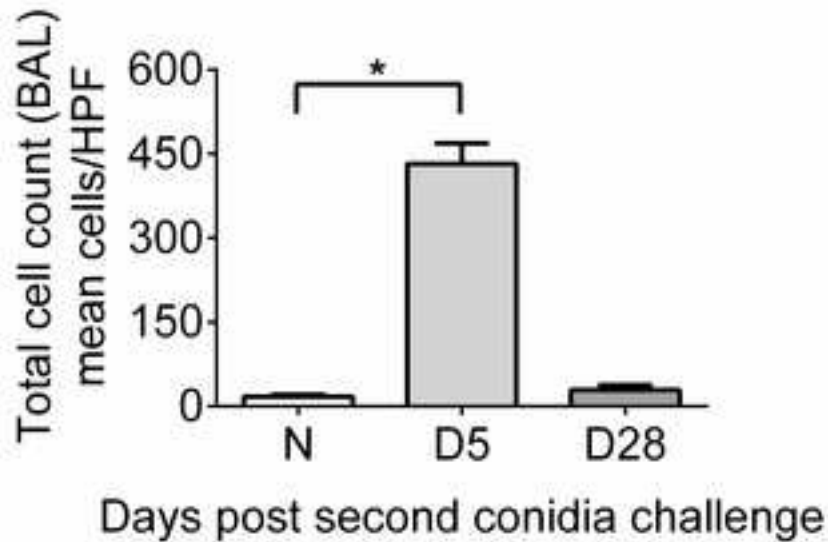
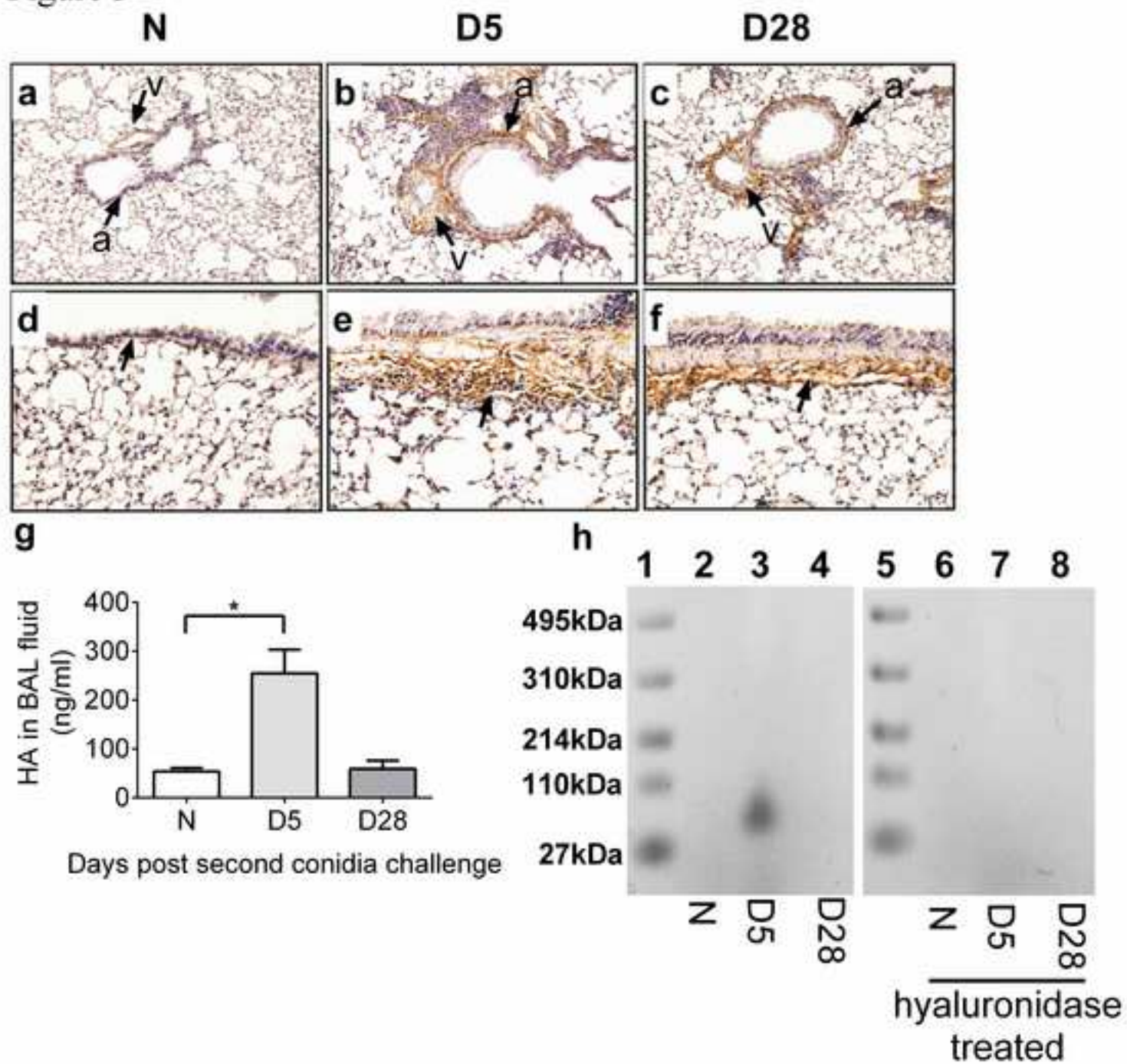
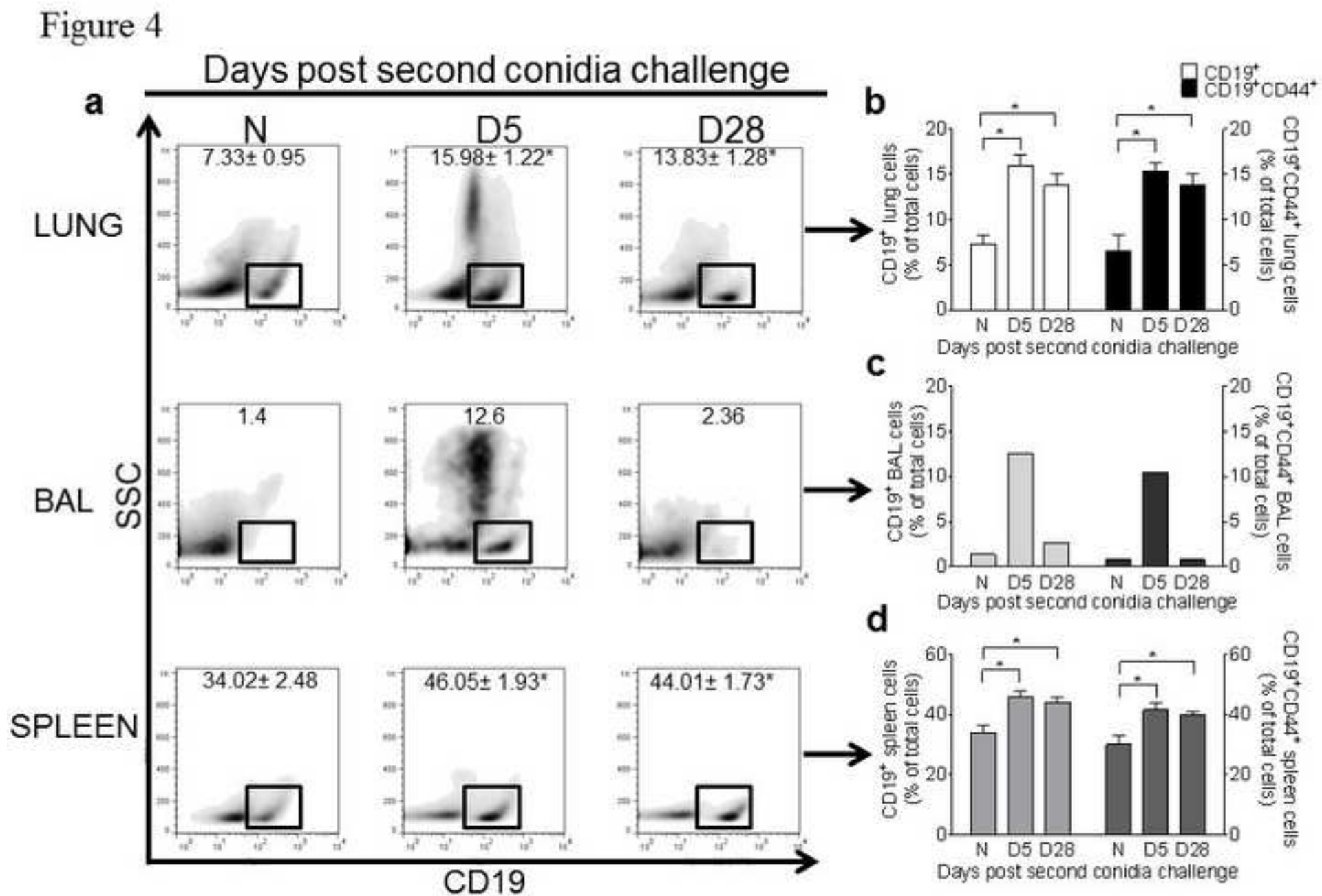
a**b****c**

Figure 3





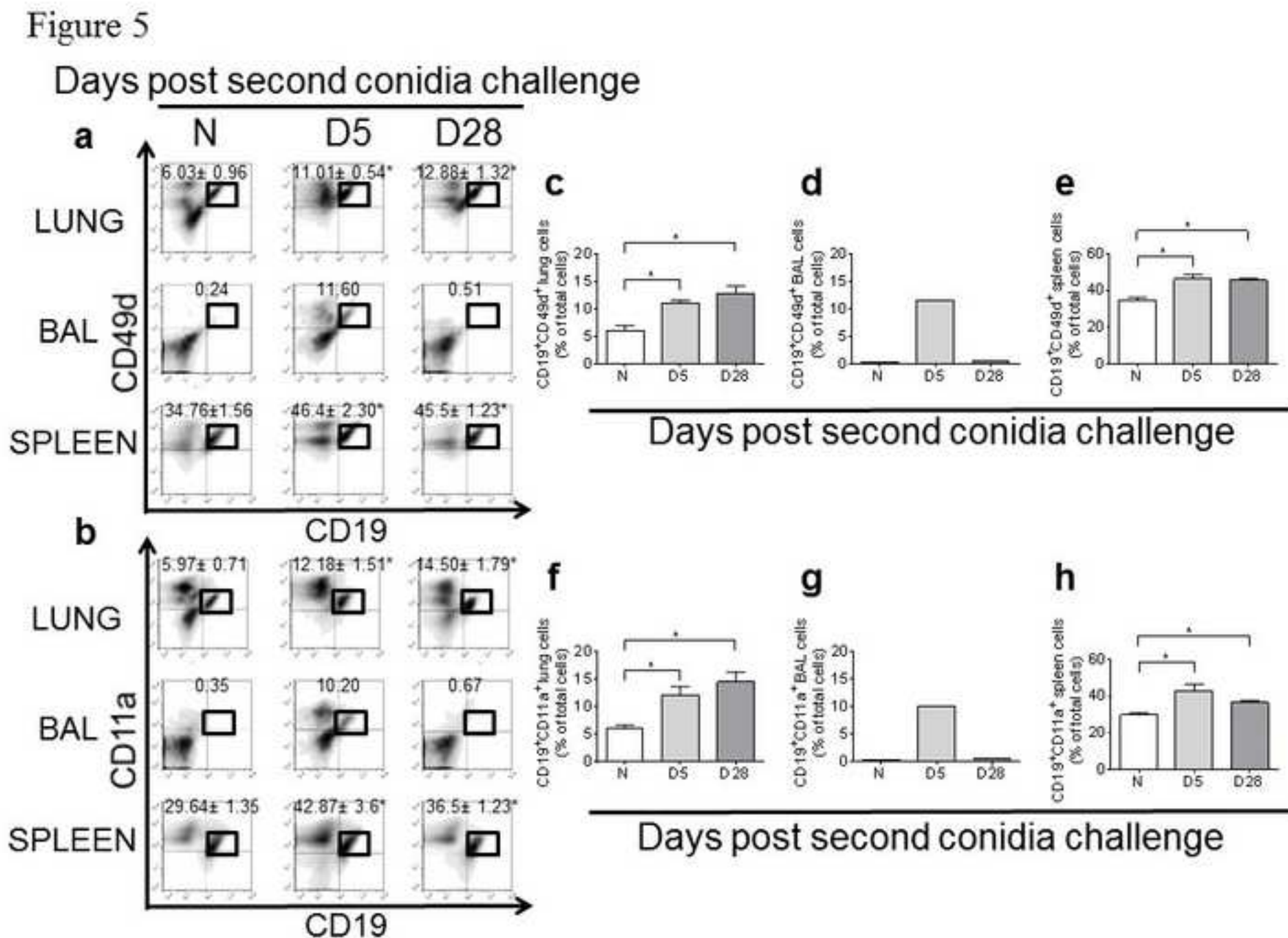
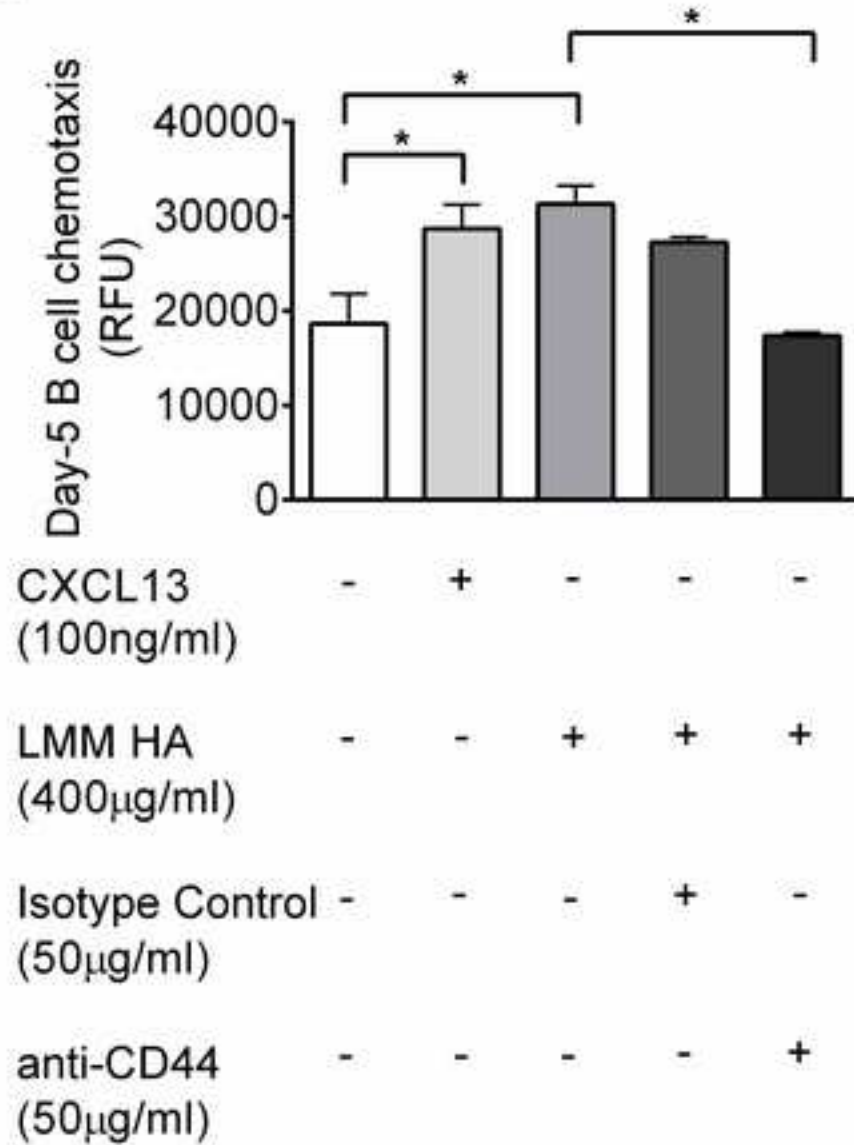


Figure 6

a



b

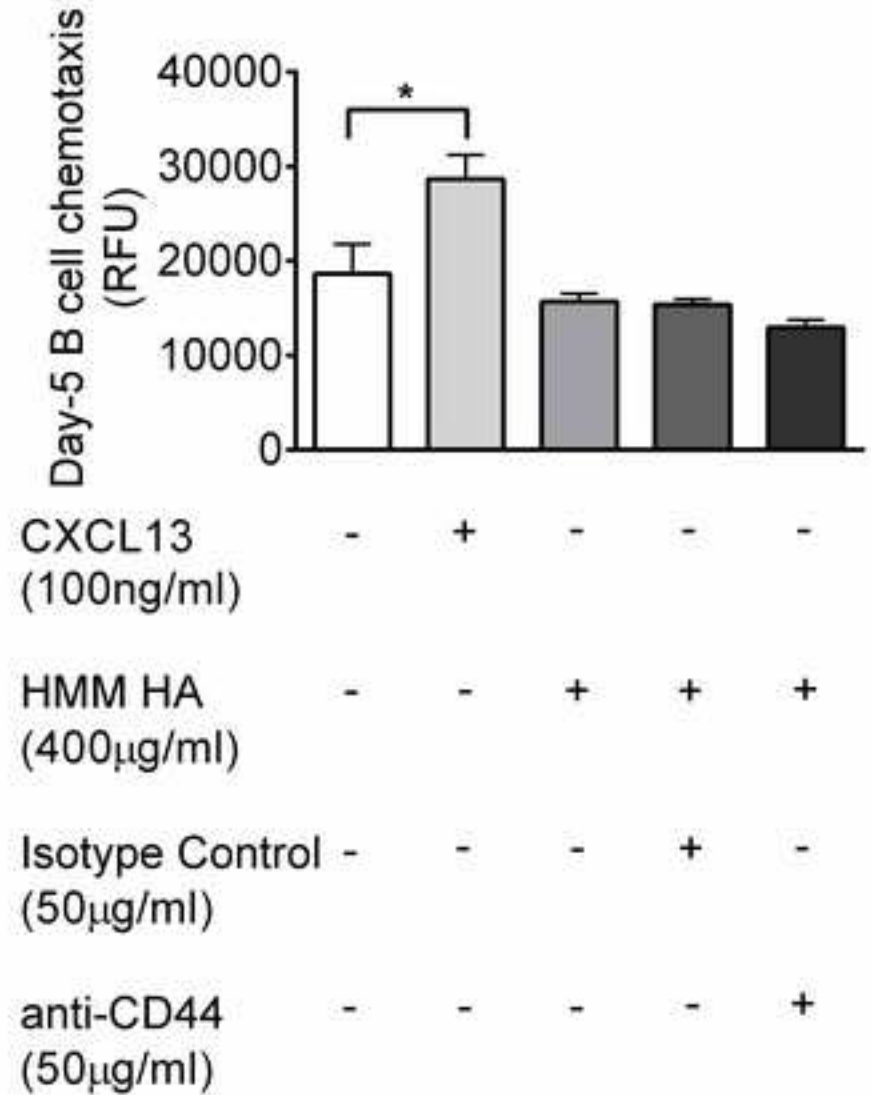


Figure 7

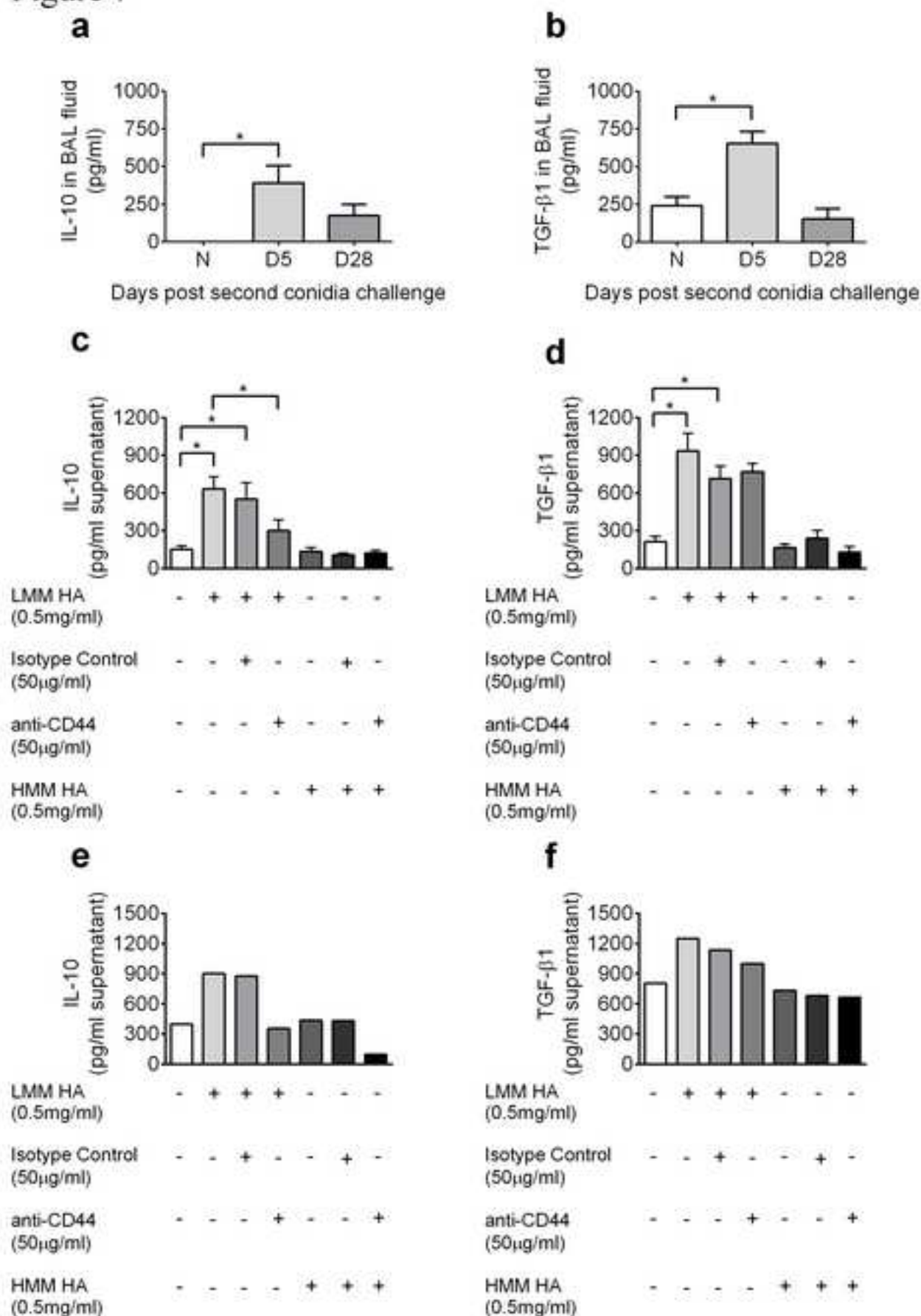
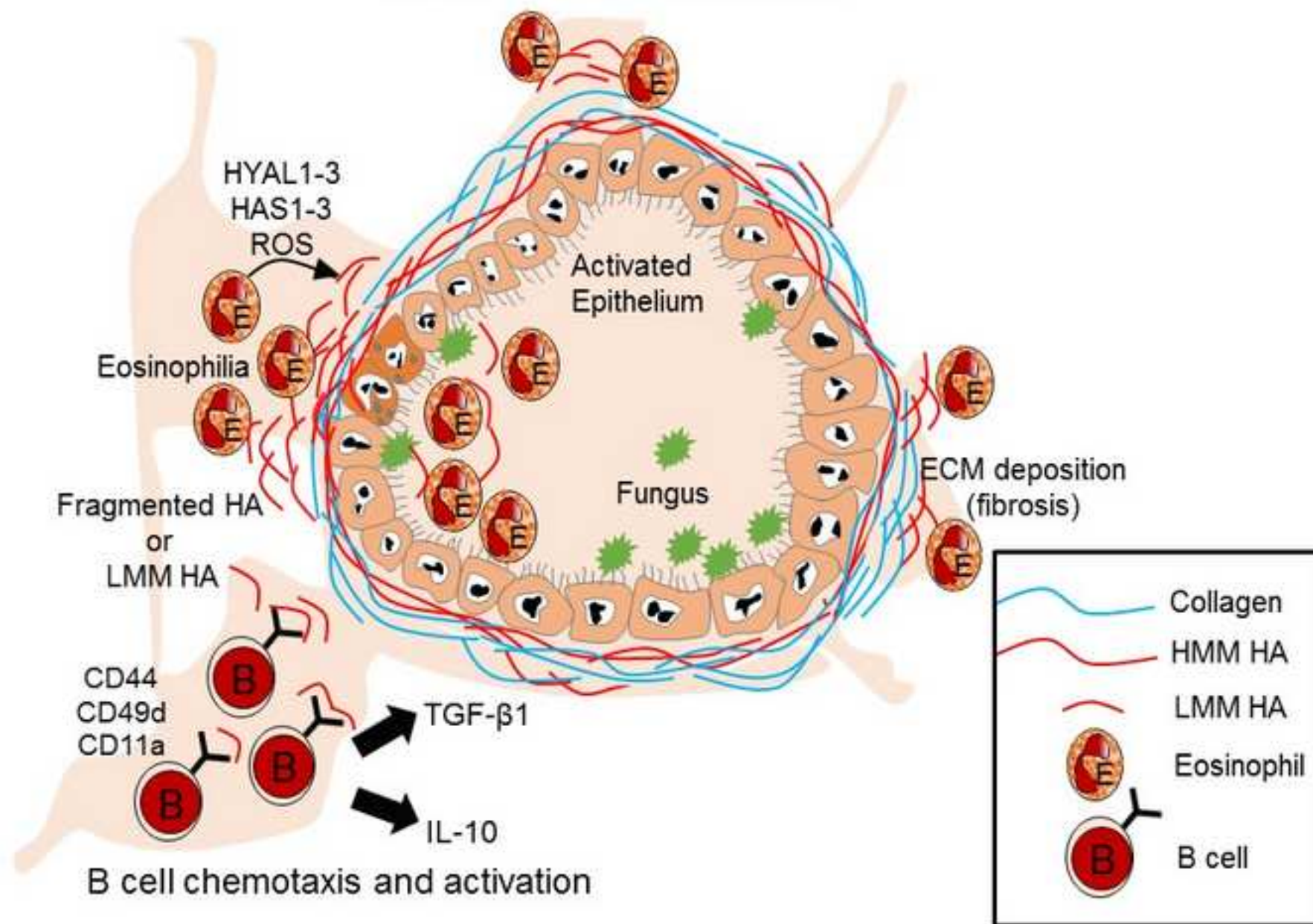


Figure 8

ASTHMATIC AIRWAY



CONFLICTS OF INTEREST

The authors report no conflicts of interest regarding the publication of this paper.

Accepted Manuscript