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Inflammation Research
Official Journal of: The International Association of Inflammation Societies + The European Histamine Research Society

ISSN 1023-3830

Inflamm. Res.
DOI 10.1007/s00011-012-0530-y

Springer

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Inflammation Research

ORIGINAL RESEARCH PAPER

Corticosteroid administration reduces the concentration of hyaluronan in bronchoalveolar lavage in a murine model of eosinophilic airway inflammation

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Received: 2 January 2012 / Revised: 30 June 2012 / Accepted: 5 July 2012

Abstract
Objective To analyze the effect of corticosteroid administration on the concentration of hyaluronan (HA) in bronchoalveolar lavage (BAL) in a murine model of eosinophilic airway inflammation and to study the mechanisms involved.
Materials and methods Untreated-mice or mice treated with 1 μg/g/day betamethasone (Bm) or 0.25 μg/g/day^{-1} budesonide (Bd) were sensitized and challenged with *Dermatophagoides pteronyssinus* (Dp) or saline (control group). The concentration of HA in BAL was determined by ELISA. In vitro migration assays were performed using a Boyden chamber and the expression of HA synthases (HAS) was analyzed by RT-PCR.
Results We found a significant increase ($P < 0.01$) in the levels of HA in BAL from Dp-treated mice that was prevented by Bm or Bd. Corticosteroids also inhibited the increase in HAS expression, and the phosphorylation of Akt and ERK in the lungs of challenged mice. Finally, we found that low molecular weight HA induces the chemotaxis of BAL cells in vitro through a mechanism mediated by CD44.
Conclusion We conclude that corticosteroids prevent the increase in HA in BAL from Dp-challenged mice. This effect is associated with reduced expression of HAS and reduced phosphorylation of Akt and ERK in the lungs of challenged mice.

Keywords Hyaluronan · Asthma · Bronchoalveolar lavage · Corticosteroids

Responsible Editor: Michael Parnham.

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Introduction

Asthma is a heterogeneous disease characterized by variable airway obstruction, bronchial hyper-responsiveness and airway inflammation. Multiple aetiologies exist for both development and symptom exacerbation [1]. Atopic asthma includes infiltration of activated eosinophils, goblet cell hyperplasia and mucus overproduction [2, 3]. Both intrinsic and extrinsic factors are involved in this phenomenon [4]. Among extrinsic factors, house dust mites [*Dermatophagoides pteronyssinus* (Dp)] are one of the agents most frequently causing allergic asthma [5].

Emphysema, pulmonary fibrosis and asthma are three chronic lung diseases that have a common imbalance between synthesis and degradation of extracellular matrix (ECM) components [6–8]. HA is an important glycosaminoglycan of the ECM, and is composed of the repeating polymeric disaccharides D-glucuronic acid and *N*-acetyl glucosamine [9, 10]. In its native state, HA exists as a high molecular weight polymer (HMW-HA) while under inflammatory conditions it is usually found as a low molecular weight form (LMW-HA) [11, 12]. Accumulation of LMW-HA occurs during tissue injury and inflammation, as a consequence of deregulated expression of HA synthesis (HAS-1, HAS-2, HAS-3) and hyaluronidase

Published online: 28 July 2012

degradation enzymes [13]. HA is present in low concentration in bronchoalveolar lavage (BAL) of healthy individuals, while increased amounts have been reported in BAL from patients with allergic asthma [14, 15]. Furthermore, airways fibroblasts from patients with asthma produced higher concentrations of LMW-HA compared with those obtained from non-asthmatic patients [16].

LMW-HA contributes to inflammation by stimulation of TLR-4, and its removal appears to be required for the resolution of inflammation [17]. CD44, the principal receptor for HA, is involved in the clearance of LMW-HA mediated by macrophages in the alveolar space [18]. However, the CD44-HA interaction promotes inflammatory responses including cell migration through a mechanism dependent on the activation of PI3K/Akt and MAP kinase pathways [19–22].

The aims of this study were to analyze the levels of HA in BAL and serum from Dp-challenged mice and to determine the effects of betamethasone (Bm) and budesonide (Bd) on the course of the allergic inflammation in a murine model. Our results showed increased HA in BAL from Dp-challenged mice associated with a reduced expression of HAS and phosphorylation of Akt and ERK in the lungs of challenged mice.

Materials and methods

Reagents

Dermatophagoides pteronyssinus (Q-Pharma, Capital Federal, Argentina). Recombinant HMW-HA (1.5–1.8 × 10^6 Da) and LMW-HA (0.25–0.45 × 10^6 Da) (Farmatrade, Buenos Aires, Argentina) were used. Anti-CD44 (KM81) antibody was kindly provided by Dr. Pauline Johnson (University of British Columbia, Vancouver).

Murine model of eosinophilic airway inflammation

Female 8-week-old BALB/c mice ($n = 5$ per group) were sensitized by intraperitoneal (i.p.) injections with 90 μg Dp allergen prepared in Al-$(OH)_3$ 3 % on day 0. Then, mice were inoculated by the intranasal route with 25 μg Dp/day on days 8–12. Finally, animals were challenged with 25 μg Dp on day 18. Control mice were injected i.p. with Al-$(OH)_3$ 3 % and inoculated by the intranasal route with saline. Both groups of animals received Bm (1 μg/g/day) administered orally through a gastric tube, or Bd (16 drops kg/day equivalent to 0.25 μg/g/day), administered by nebulizers for 18 days.

Prick tests were performed to confirm sensitization of mice. Briefly, on day 18 the backs of mice were shaved and Dp was inoculated by the intradermal (i.d.) route. Induration and redness of the skin was evaluated. Mice were sacrificed, and the skin was cut and photographed.

The experimental procedures were approved by the Review Board of the Ethics Committee of the Instituto de Estudios de la Inmunidad Humoral (IDEHU). Animal experiments were conducted according to the NIH "Guide for the care and use of Laboratory Animals" (http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf).

Bronchoalveolar lavage

Twenty-four hours after the final challenge (day 18), the animals were killed by i.p. administration of ketamine/xylazine. A tracheotomy was performed by introduction of a flexible capillary micropipette through the tracheal stoma to reach a wedge position, three aliquots of 1 ml saline were instilled, and BAL was collected. Cells were concentrated by cytocentrifugation (400 rpm, 10 min), stained with Giemsa and counted.

Histopathological analysis

Lungs were fixed in formalin and embedded in paraffin. Tissue sections (5–10 μm) were stained with hematoxylin and examined under light microscopy. To evaluate the presence of eosinophils in the tissues LUNA stain was used.

Evaluation of HA levels in BAL and serum by ELISA

HA levels in BAL and serum were measured using a competitive ELISA as described previously, with minor modifications [23]. Briefly, 96-well microtiter plates were coated with 100 μg/ml HA overnight at 4 °C and blocked with BSA 3 % for 1 h. Samples or standard HA were incubated in the presence of 1 μg/ml biotinylated HA-binding protein (bHABP) (Calbiochem, La Jolla, CA) for 18 h at 37 °C; 50 μl of this mixture was added to HA-coated wells and incubated at 37 °C for 4 h. The bHABP bound to the wells was determined using an avidin–biotin detection system (Vector Laboratories, Burlingame, CA).

Total RNA extraction and semi-quantitative reverse transcription polymerase chain reaction

Total RNA samples from the lungs (~50 mg) were isolated employing TRIZOL (Invitrogen, Carlsbad, CA). Isolated RNA was reverse transcribed and cDNA was then amplified with specific primers for HAS-1, HAS-2 and HAS-3 (Table 1) by 30 PCR cycles [30 s at 94 °C, 1 min at annealing temperature (Table 1) and 1 min at 74 °C], followed by an extension at 72 °C for 10 min. As a

Table 1 Primer sequences for mouse hyaluronan synthases (HAS) HAS-1, HAS-2, HAS-3 and β-actin

Gene	Primer sequence	T_a (°C)	Size of product (bp)
HAS-1			
Sense	5′-CATTCCTCAGCGCACACCTA-3′	56	752
Antisense	5′-TGATGCAGGACACACAGTGG-3′		
HAS-2			
Sense	5′-TGGAACACCGGAAAATGAAGAAG	57	805
Antisense	5′-GGACCGAGCCGTGTATTTAGTTGC-3′		
HAS-3			
Sense	5′-AGGTGGTCATGGTAGTGGAT-3′	58	898
Antisense	5′-CACTGTTAGCAGGAAGAGGA-3′		
β-Actin			
Sense	5′-ATGGATGACGATATCGCT-3′	52	569
Antisense	5′-ATGAGGTAGTCTGTCAGGT-3′		

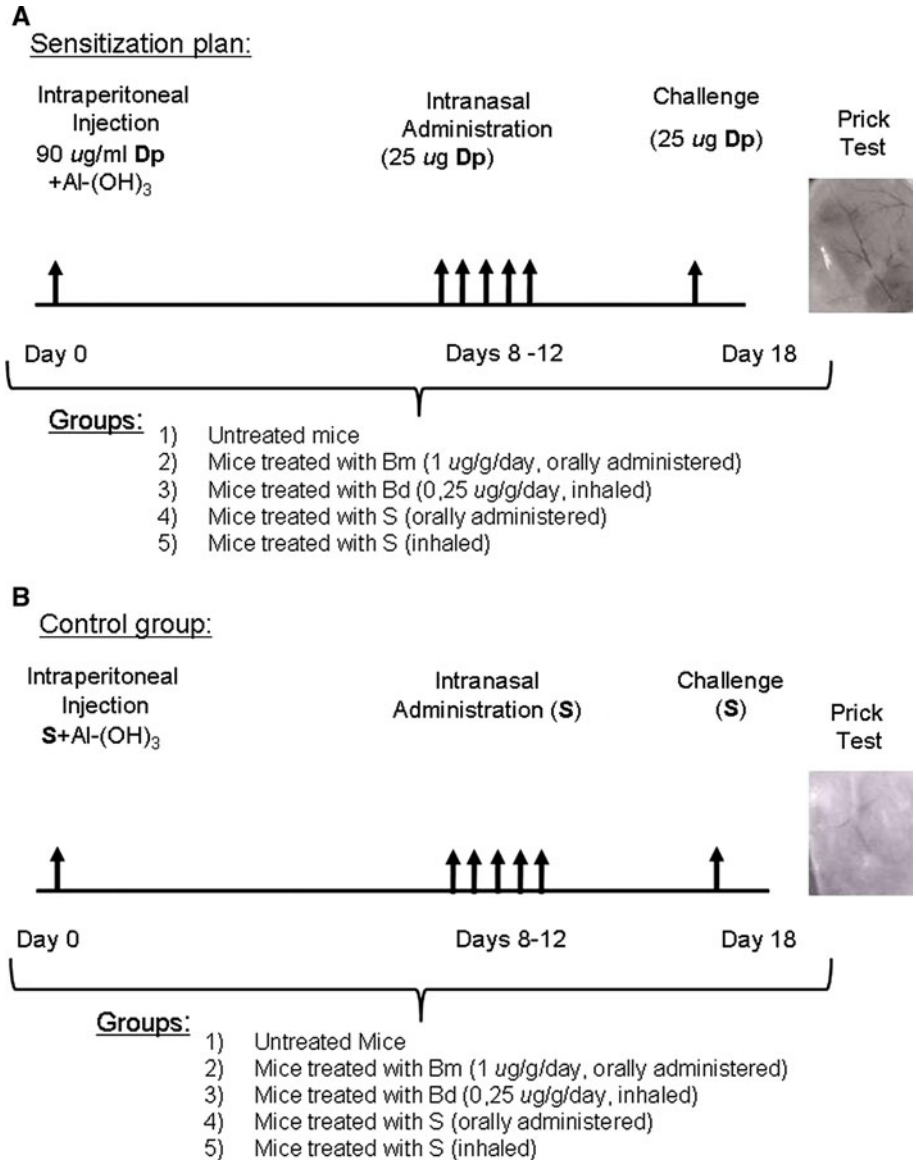

Fig. 1 Murine model of eosinophilic airway inflammation. **a** Groups of mice ($n = 5$) were sensitized with *Dermatophagoides pteronissynus* (*Dp*) according to a detailed plan, as specified in the figure. The photograph shows edema and vascularization areas in the injected skin. **b** Control groups of mice ($n = 5$) were treated similarly in parallel but with saline only (*S*). The photograph shows absence of response. To evaluate the effect of corticosteroids, mice were treated or not with betamethasone (*Bm*) administered orally, or budesonide (*Bd*) inhaled, during the period of sensitization. Placebo treatments were performed with S administered orally (oS) or inhaled (iS)

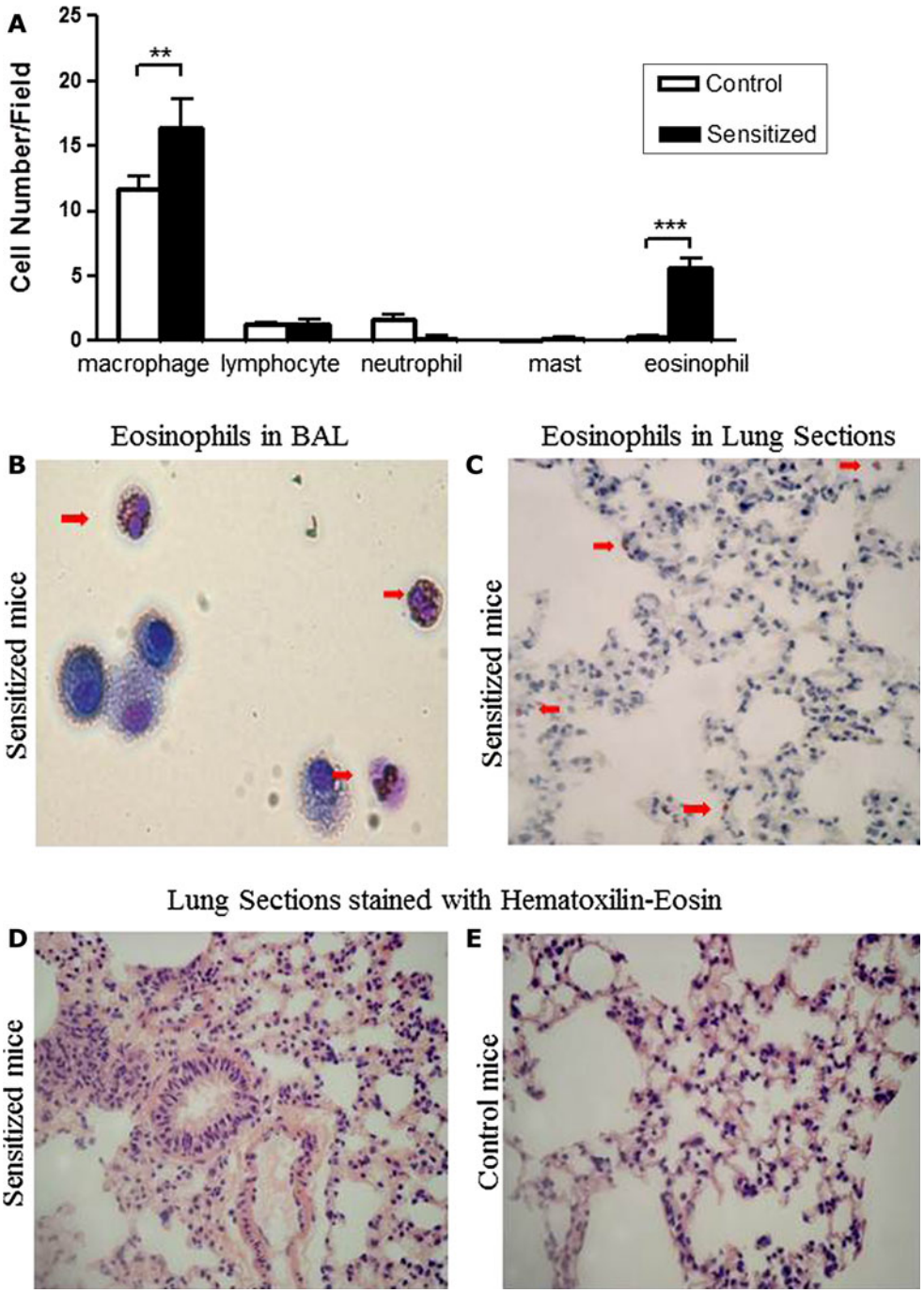

Fig. 2 Differential cell count and histological examination. **a** Number of inflammatory cells in bronchoalveolar lavage (BAL). Cells were concentrated by cytocentrifugation, stained with Giemsa and counted. Sensitization of mice with Dp induced a significant increase in eosinophils and reduced macrophages in BAL as compared to controls (***$P < 0.001$ and **$P < 0.01$, respectively). **b** Photographs of BAL cells from sensitized mice, stained with Giemsa and shown at ×400 magnification. *Red arrows* eosinophils. **c** Histological examination of lungs from mice sensitized with Dp. Sections of lung were fixed in formalin and stained with Luna. *Red arrows* eosinophils, (×400). **d**, **e** Histological examination of formalin-fixed lung sections stained with hematoxylin–eosin, (×400). Sensitized mice showed parenchyma associated to bronchus with areas of epithelial edema and moderate-to-severe lymphocyte infiltration (**d**) while preserved pulmonary parenchyma was observed in control mice (**e**) (color figure online)

reference gene, β-actin was amplified. PCR products were separated in 2 % agarose gel, stained with ethidium bromide and visualized under UV light (Cole Palmer Instrumental, Vernon Hills, IL). Densitometric analysis was performed using Image Scion Software (Scion Corporation, Frederick, MD).

Lung tissue extracts and Western blot

Lung tissue extracts were prepared 24 h after the antigen challenge (day 18) by the Perfect method [24]. Perfused lungs were placed in extraction solution (90 mM CHAPS, Research Organics, Cleveland, OH) supplemented with protease inhibitors (EDTA-free Complete, Roche Diagnostics, Mannheim, Germany) at a ratio of 2 µl/mg tissue and frozen at −70 °C. Extraction was performed for 12 h at 4 °C in a homogenizer. Samples were then centrifuged and the supernatants were collected. Equal amounts of proteins were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with specific antibodies to ERK, Akt or actin (Santa Cruz Biotechnology, Santa Cruz, CA). The reaction was revealed with

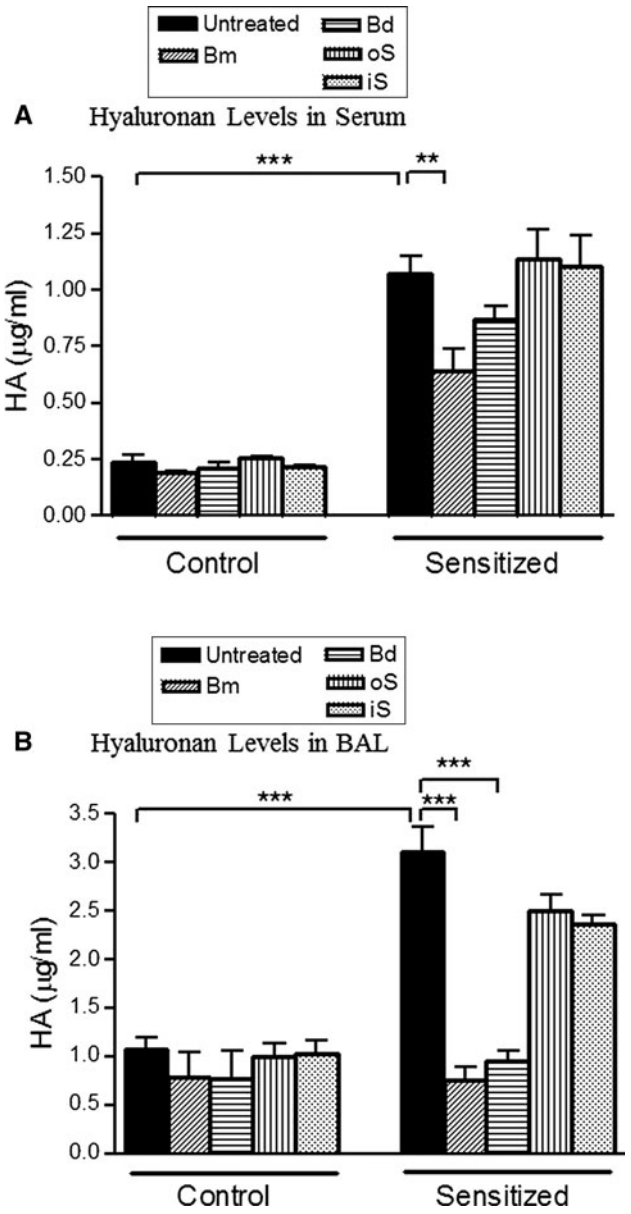

Fig. 3 Measurement of hyaluronan (HA) levels in serum (**a**) or BAL (**b**) from control mice or animals sensitized with Dp, treated with either 1 µg g/day Bm or 0.25 µg g/day Bd, and those treated with placebos. Results are expressed as µg/ml HA and are representative of three independent experiments, in duplicate. *Bars* mean ± SEM, $***P < 0.001$ and $*P < 0.05$

horseradish peroxidase-labelled secondary antibody and developed using a chemiluminescent detection system. Gel images obtained with a digital camera were subjected to densitometric analysis using Image Scion Software (Scion Corporation).

Migration assay

Briefly, a 48-well chemotaxis Boyden chamber (Neuroprobe, Gaithersburg, MD) with a polycarbonate membrane (5 µm pore size, Neuroprobe) was used. BAL cells (3×10^5 cells/ml) were placed in the upper chamber of the transwell unit while the lower wells contained 200 µg/ml HA (LMW-HA or HMW-HA) or RPMI. After incubation at 37 °C for 3 h, the membrane was removed and cells on the upper side of the membrane were scraped out with a blade. Cells attached to the lower side of the membrane were fixed in 2 % formaldehyde, stained in 10 % DAPI or GIEMSA. To evaluate the capacity of anti-mouse CD44 monoclonal antibodies to inhibit HA-mediated migration, cells were incubated with KM81 mAb (20 µg/ml) or with a rat IgG control at 4 °C for 30 min before the migration assay [25].

Statistical analysis

All the experiments with mice were performed at least three times independently ($n = 5$ animals/group). Data were expressed as mean ± SE. Differences between the groups were evaluated by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. Differences were considered significant at a level of $P < 0.05$ or less. Analysis was performed using Prism 4 software (Graph Pad, San Diego, CA).

Results

Murine model of eosinophilic airway inflammation

We used a murine model of allergic inflammation triggered by Dp to investigate the effect of corticosteroids on HA synthesis. Five groups of mice were sensitized with Dp, as indicated in Fig. 1a. Another five groups of mice were used as controls (not-sensitized with Dp) (Fig. 1b). Figure 1a shows swelling and a wheal and flare reaction in the skin of sensitized mice while Fig. 1b shows no response in the skin of not-sensitized mice.

Inflammatory cells in BAL were counted 24 h after intranasal sensitization with the allergen (day 18). BAL (300 µl) was collected from both sensitized and control mice. As expected, the number of eosinophils was significantly higher in BAL from sensitized mice compared to controls: number of eosinophils/field 5.60 ± 0.69 versus 0.25 ± 0.16; $P < 0.001$ (Fig. 2a, b). These results are in agreement with the histopathological findings. Infiltration of eosinophils in the bronchial wall was observed by selective staining (Fig. 2c). Sensitized mice showed areas of epithelial edema and moderate-to-severe leukocyte infiltration in the underlying parenchyma associated to bronchus (Fig. 2d), while the control group showed preserved pulmonary parenchyma (Fig. 2e).

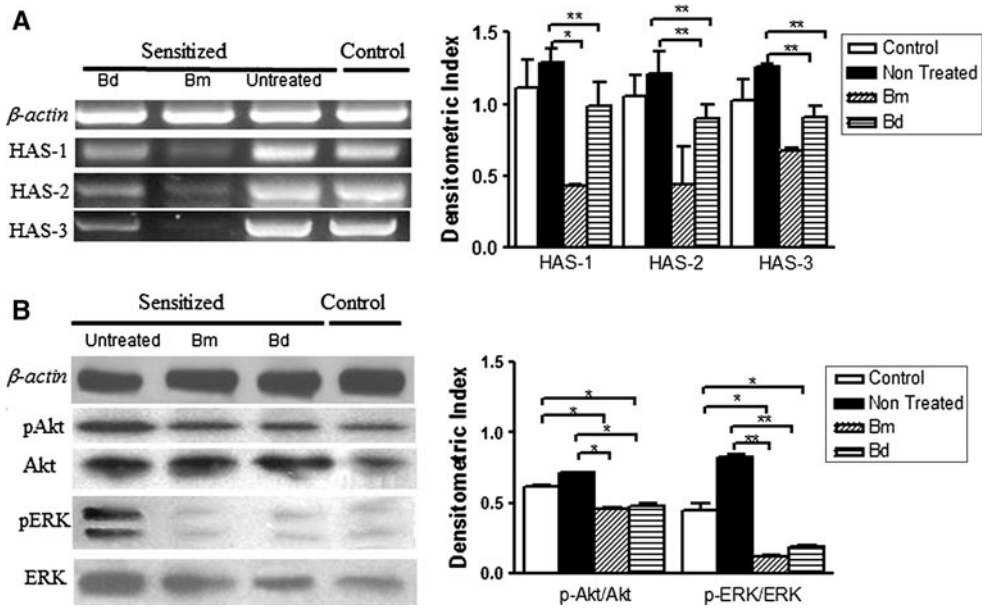

Fig. 4 **a** mRNA expression of hyaluronan synthase (HAS) HAS-1, HAS-2 and HAS-3. *Left panel* HAS (HAS-1, HAS-2 and HAS-3) mRNA expression analyzed by RT-PCR in lung tissues. One representative from three independent experiments is shown. *Right panel* densitometric analysis of the bands in the *left panel*. Results are expressed as densitometric index (HAS mRNA expression/*β*-actin mRNA expression), determined from three independent experiments and normalized to the reference gene. *Bars* mean ± SD; $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$. **b** AKT/p-Akt and ERK/p-ERK expression. *Left panel* Western blot analyzed for p-Akt and p-ERK. *Right panel* densitometric analysis of the bands in the *left panel*. Results are expressed as densitometric index (p-Akt/Akt)/*β*-actin or (p-ERK/ERK)/*β*-actin, determined from three independent experiments and normalized to the reference gene. *Bars* mean ± SD; $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$

Analysis of HA levels

As shown in Fig. 3a, serum HA levels were increased significantly in sensitized-mice compared with controls: 1.07 ± 0.07 versus 0.24 ± 0.03 μg/ml; $P < 0.001$. Bm-treatment in Dp-sensitized mice significantly prevented the increase in serum HA levels in sensitized mice: 0.64 ± 0.10 versus 1.07 ± 0.07 μg/ml; $P < 0.001$. In contrast, Bd did not exert any significant effect on the levels of serum HA in Dp-sensitized mice. A significant increase in the levels of HA was also found in BAL from Dp-sensitized mice compared with controls: 3.11 ± 0.25 versus 1.07 ± 0.12 μg/ml; $P < 0.001$. Treatment with either Bm or Bd significantly prevented the increase in BAL HA levels in sensitized mice: 0.74 ± 0.14 and 0.94 ± 0.11 versus 3.11 ± 0.25 μg/ml; $P < 0.001$ (Fig. 3b).

Analysis of HAS expression

To evaluate expression of the enzymes involved in HA synthesis (HAS-1, HAS-2 and HAS-3), RT-PCR was performed on RNA from lung samples. We found no significant differences between control and Dp-sensitized mice, while corticosteroid reduced mRNA expression of all three enzymes analyzed. Bm and Bd decreased the expression of mRNA for HAS-1 (0.58 ± 0.22 and 0.94 ± 0.11 vs. 1.36 ± 0.96; $P < 0.05$) compared to Dp-sensitized mice. Moreover, mRNA for HAS-2 was 0.49 ± 0.02 and 0.86 ± 0.05 vs. 1.31 ± 0.06; $P < 0.05$ while HAS-3 was 0.50 ± 0.09 and 0.68 ± 0.06 vs. 1.21 ± 0.13; $P < 0.05$ (Fig. 4a).

Modulation of PI3K/Akt and ERK pathways

As shown in Fig. 4b, Bm and Bd decreased Akt phosphorylation in both sensitized and control mice (0.46 ± 0.01 and 0.48 ± 0.02 vs. 0.71 ± 0.02 and 0.61 ± 0.01; $P < 0.05$). Similar results were found when the phosphorylation of ERK was analyzed (0.12 ± 0.01 and 0.18 ± 0.01 vs. 0.82 ± 0.02 and 0.45 ± 0.04; $P < 0.05$).

Migration of BAL cells towards HA

When evaluating the effect of HA, we observed that migration of BAL cells from Dp-sensitized mice towards LMW-HA was increased significantly compared to RPMI or HMW-HA (2.57 ± 0.17 vs. 1; $P < 0.001$ or 2.57 ± 0.17 vs. 1.34 ± 0.15; $P < 0.05$). Migration was inhibited by pre-treatment with anti-CD44 mAb (1.33 ± 0.11 vs. 2.57 ± 0.17; $P < 0.001$), suggesting that migration is mediated by CD44-HA interaction (Fig. 5a). HMW-HA failed to induce the migration of BAL cells (Fig. 5a) while

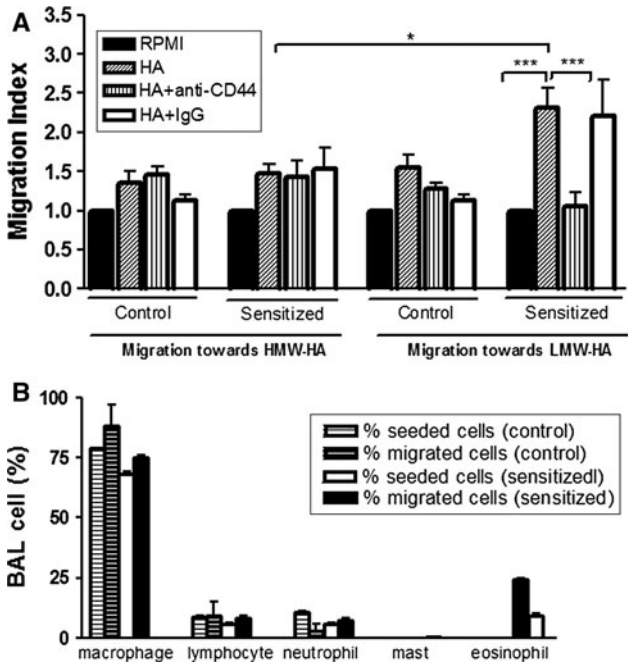

Fig. 5 Migration of BAL cells towards HA. **a** Migration assay of 3×10^5 BAL cells towards 200 µg/ml LMW-HA, HMW-HA or RPMI, with a pretreatment with either anti-CD44 KM81 antibody or rat IgG isotype (control). Results are expressed as migration index (MI) = (mean total cells/field of samples with HA)/(mean total cells/field of samples with RPMI). Basal migration was performed in the absence of HA. The results are representative of three independent experiments, in quadruplicate. *Bars* mean ± SEM; $***P < 0.001$. **b** Differential count of BAL cells seeded upper the chamber and cells migrated and attached to the lower side of the membrane

LMW-HA induced migration of macrophages, eosinophils, lymphocytes and neutrophils from BAL as shown in Fig. 5b. Corticosteroids did not modify the migration towards HA (data not shown).

Discussion

Asthma can be accompanied by prolonged inflammation and structural changes in respiratory areas [26]. This process involves an abnormal accumulation of some constituents of the ECM in the lung epithelium and submucosa, which affects the elasticity and resistance of the airways [27]. Although combined treatment with corticosteroids has proved successful in the treatment of asthma, it is not well understood whether corticosteroids participate in remodeling of the ECM [28]. Several studies have shown the role of HA in lung inflammatory processes such as lung fibrosis [18]. Moreover, in a model of murine asthma, an early increase in the levels of HA in BAL and lung tissues associated with the up-regulation of expression of HAS-1 and HAS-2 enzymes has been reported [29].

In this study, we used a previously described murine model of allergic asthma based on sensitization of mice with *D. farinae* [30]. We demonstrated that Dp induced an inflammatory response in the lung together with eosinophil infiltration. These results are in accordance with previous reports that have shown asthma induction by Df administration [31]. We also demonstrated that HA levels were increased both in serum and BAL of Dp-sensitized mice. These findings are in accordance with previous reports that have shown increased HA in BAL from mice challenged with OVA and from ozone-induced airway hyper-responsiveness [31, 32]. Our results showed a good correlation between serum and BAL HA levels in mice sensitized with Dp. It is noteworthy that HA levels were higher in BAL than in serum, probably due to local HA synthesis by type II pneumocytes and lung fibroblasts in response to the inflammatory process.

Both treatments (Bm and Bd) decreased HA levels and inhibited mRNA expression of the three synthases HAS-1, HAS-2 and HAS-3. The PI3K/Akt and ERK signaling pathways seem to be involved in these effect. Our results are in accordance with previous reports showing that dexamethasone inhibits the expression of HAS-3 in an animal model of ventilator-induce lung injury [33].

Deregulation of HAS and their activities have been found during tissue injury, which is consistent with the fact that HA accumulates during inflammatory processes [34]. In fact, expression of HAS isoforms can be regulated by growth factors and cytokines such as TNF-α and IL-1β [35]. Moreover, corticosteroids exert a wide range of anti-inflammatory effects by interfering with the ability of transcription factors such as nuclear factor-kappa B (NF-κB) to bind to the promoter regions of inflammatory genes [36]. Based on such knowledge, we suggest that in Dp murine asthma the effect of corticosteroids on HA synthesis may be a consequence of cytokine modulation.

The role of CD44-HA interaction in leukocyte extravasation under inflammatory conditions has been described recently [19, 37]. Mouse models have shown that treatment with anti-CD44 monoclonal antibodies leads to a significant decrease in the recruitment of inflammatory cells in BAL [38]. In this work, we observed that BAL cells from mice sensitized with Dp showed increased capacity to migrate towards LMW-HA but not towards HMW-HA. CD44-HA interaction is involved in this process since pretreatment of cells with anti-CD44 monoclonal antibodies inhibited cell migration. We previously reported that tumor cells exhibited a higher capacity to migrate to LMW-HA, but not to HMW-HA. Migration was mediated by CD44 since it was abrogated by anti-CD44 monoclonal antibody [39].

HA has been described as an immune regulator in human diseases. Therapeutic developments targeting HA

and its binding proteins are developing [40]. Our data support a role for HA in the inflammatory process in a murine model of asthma induced by Dp. Understanding its role in airway inflammation will contribute to the development of novel therapeutics for these inflammatory diseases.

Acknowledgments We thank Dr. Pauline Johnson of the Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada, for kindly providing the blocking anti-CD44 Mab (KM81). We also thank *Q*-Pharma, SA Argentina, for kindly providing the Dp used to sensitize the animals, and Farmatrade SA, Argentina for providing the HA used for ELISA and chemotaxis assays. We are grateful to Dr. Susana Constantino, Dr. Daniela Ureta and Victor Tomasi for their excellent technical assistance. This work was supported by grants from University of Buenos Aires (UBACYT-B021) and CONICET (PIP-0199).

Conflict of interest The authors declare that no conflict of interest existed in the development of this work.

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