

Galectin-9 in Allergic Airway Inflammation and Hyper-Responsiveness in Mice

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Key Words

Galectin-9 • Asthma • Eosinophils • Allergic inflammation • Animal model

Abstract

Background: Galectin-9 (Gal-9) is a member of the growing family of β -galactoside-binding lectins. Gal-9 is an eosinophil chemoattractant and inducer of Th1 cell apoptosis. These effects suggest its potential role in the pathogenesis of asthma. Our aim was to study the expression of Gal-9 in an ovalbumin (OVA)-induced mouse model of allergic asthma. **Methods:** To investigate the significance of Gal-9 in allergic inflammation and airway hyperresponsiveness (AHR), a group of BALB/c mice was sensitized and challenged with OVA (G_{OVA}). Another group of animals was allergized with OVA and also treated with dexamethasone (DEX) ($G_{OVA+DEX}$). The control group (G_{PBS}) received phosphate-buffered saline instead of OVA as placebo. Airway reactivity to intravenous methacholine was assessed. **Results:** The percentage of Gal-9-positive cells and their intracellular Gal-9 content and Th1/Th2 cytokine levels in the bronchoalveolar lavage (BAL) were determined by flow cytometry. Gal-9 mRNA expression and protein level were measured in the lung tissue

by real-time RT-PCR and Western blot. In G_{OVA} mice, airway inflammation and mucus hypersecretion developed. DEX treatment inhibited the main features of experimental asthma. The number of Gal-9-positive lymphocytes, eosinophil and neutrophil granulocytes and the levels of Th2 cytokines were higher in the BAL of G_{OVA} compared to G_{PBS} or $G_{OVA+DEX}$ mice. Moreover, Gal-9 protein level was elevated in the lungs of G_{OVA} mice. **Conclusions:** These results suggest that Gal-9 plays a role as a mediator contributing to the development of allergic airway inflammation. Gal-9 may serve as a recruiter of eosinophil granulocytes and promoter of Th2 dominance.

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Introduction

The incidence and prevalence of asthma have substantially increased in the developed world over the last two decades. Asthma is a complex inflammatory disease of the lung characterized by airway inflammation, airway

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hyperresponsiveness (AHR) and variable airflow obstruction [1]. The inflammatory response in the asthmatic lung is characterized by infiltration of the airway wall with lymphocytes and eosinophil granulocytes [2, 3]. Paradigms emphasize the importance of type 2 T-helper lymphocyte (Th2)-derived cytokines that promote eosinophilic inflammation [4, 5]. It is well known that the presence of eosinophil granulocytes and eosinophil-derived products in the respiratory tract correlates with the manifestations of allergic asthma [6, 7].

Galectins are a family of animal lectins that have a conserved sequence motif of β -galactoside-binding domains. Based on their biochemical structure, galectins have been classified into prototype, chimera type, and tandem-repeat type groups [8–10]. Galectins have been implicated in diverse biological processes. Their role in inflammation [11], immunomodulation [12, 13], cell adhesion and migration [14], cell growth regulation [15, 16] and apoptosis [17, 18] has been discussed previously [9, 19, 20].

Galectin-9 (Gal-9), a tandem-repeat type protein, was first cloned from the tissue of patients with Hodgkin's disease [21]. In mice, it is highly expressed in the lung, as well as in immunocompetent cells [22]. Gal-9 has multiple functions and was described as an eosinophil chemoattractant protein [23] as well as a urate transporter/channel [24], a tumor antigen [21], a regulator of thymocyte-epithelial cell interactions [25] and a mediator of apoptosis [26]. It has been demonstrated that Gal-9 induced cell death in type 1 T-helper lymphocytes (Th1), but not in Th2 cells, in a T-cell-immunoglobulin-domain and mucin-domain (Tim)-3-dependent manner in mice [27]. Gal-9 may regulate eosinophil trafficking in immune responses and allergic reactions. Yamamoto et al. [28] have recently shown that Gal-9 contributes to eosinophil accumulation in the lungs of guinea pigs.

Gal-9 has a major effect on eosinophil migration and inflammation; therefore, in the present experiment, we investigated the expression of Gal-9 in a mouse model of allergic airway inflammation and hyperresponsiveness.

Materials and Methods

Animals and Treatment Protocol

Experiments were performed on 8-week-old female BALB/c mice (Charles River Laboratories, Isaszeg, Hungary). The animals were housed in a temperature-controlled ($22 \pm 1^\circ\text{C}$) room with alternating light and dark cycles. The animals had free access to ovalbumin (OVA)-free mouse chow and water. The institutional committee on animal welfare approved all experiments. The mice

were randomized into 3 groups. In G_{OVA} ($n = 14$): mice were sensitized by intraperitoneal injections of OVA (20 μg OVA/animal) emulsified in 2.25 mg aluminium hydroxide (ALUM) in a total volume of 100 μl phosphate-buffered saline (PBS; Sigma-Aldrich Co., St. Louis, Mo., USA) on days 1 and 14. Then they were challenged on days 28, 29 and 30 for 20 min with OVA aerosol (1% OVA diluted in PBS) using an ultrasonic nebulizer (Samsung SUN-600, Seocho-Gu, Seoul, Korea) [29]. In $G_{\text{OVA+DEX}}$ ($n = 14$): the animals were sensitized and challenged with OVA using the same protocol as in G_{OVA} animals and injected subcutaneously with dexamethasone sodium phosphate (DEX, Oradexon, Organon, Oss, The Netherlands) (5 mg/kg/day/animal) diluted in a total volume of 100 μl normal saline on days 29 and 30 2 h before the OVA challenge [30]. This dosage of DEX was previously shown to inhibit all features of allergic airway disease in mice [31]. In G_{PBS} ($n = 14$): control animals were sensitized on days 1 and 14 with 2.25 mg ALUM/animal in a total volume of 100 μl PBS and challenged on days 28, 29 and 30 for 20 min with PBS.

Sample Collection

Twenty-four hours after the last OVA or PBS challenge, a group of mice ($n = 7/\text{group}$) was anesthetized with an intraperitoneal injection of 72 mg/kg pentobarbital sodium (Nembutal, Sanofi, Budapest, Hungary). The lungs were lavaged with $3 \times 600 \mu\text{l}$ PBS and then removed and immediately frozen in liquid nitrogen or fixed in formalin (4%, pH = 7.4; Sigma-Aldrich). The collected bronchoalveolar lavage (BAL) fluid samples were centrifuged (700 g, 4°C , 5 min). Supernatants were aliquoted and immediately frozen (-80°C) pending cytokine measurement. The cell pellets were washed and resuspended in PBS.

Measurement of Airway Responsiveness

Airway responsiveness to methacholine (MCh) was assessed as we previously described [29, 32]. Briefly, on day 31, 24 h after the last OVA or PBS challenge, mice ($n = 7/\text{group}$) were anesthetized with an intraperitoneal injection of pentobarbital sodium (72 mg/kg; Nembutal, Sanofi) [33]. Tracheostomy was performed to ventilate the animals mechanically at a rate of 150 breaths/min (Inspira, Harvard Apparatus, Holliston, Mass., USA). The transpulmonary pressure was measured using a whole-body plethysmograph (Buxco Electronics, Sharon, Conn., USA) and the airflow was calculated from the pressure changes of the plethysmograph by BioSystem XA software (Buxco Electronics). Lung resistance (R_L) was determined as the ratio of transpulmonary pressure and airflow during the intravenous infusion of MCh in 3-fold increasing concentrations to assess AHR. The starting dose of MCh was 43 $\mu\text{g}/\text{kg}$ [34], and the consecutive doses were administered as intravenous boluses at 2-min intervals.

Histological Analysis

The formalin-fixed (4%, pH = 7.4; Sigma-Aldrich) lung samples were embedded in paraffin. Sections were stained with hematoxylin-eosin (HE) and periodic acid-Schiff stain (PAS) according to the standard protocols. Slides were coded and then examined by a qualified pathologist in a blinded fashion.

Flow Cytometric (FACS) Analysis of Gal-9-Positive Cells

After washing the BAL cells with PBS, they were stained for Gal-9 using the following protocol. Nonspecific binding of Fc receptors was blocked with 2.5 μl anti-mouse CD16/CD32 antibody

Table 1. Nucleotide sequences of the different primers and probes applied for the detection of Gal-9 and GAPDH by real-time RT-PCR

Gene		Primer pairs and probes	Product lengths
Gal-9	Sense:	5' GCA GGA GGG ACT TCA GGT GA 3'	173 bp
	Antisense:	5' GCC CCC ACT GTC CGT TCT 3'	
GAPDH	Sense:	5' CAC CAC CAT GGA GAA GGC TG 3'	240 bp
	Antisense:	5' GTG ATG GCA TGG ACT GTG 3'	
	Probe:	5' LCRed460-CCCTGGCCAAGGTCATCCATGA-PH 3'	
	Probe:	5' TCCTGCACCACCAACTGCTTAGC-FL 3'	

(rat monoclonal IgG2, BD Pharmingen, San Diego, Calif., USA, 4°C, 10 min), then the samples were aliquoted. The cells were fixed in 4% buffered paraformaldehyde solution (pH = 7.4 at room temperature, RT, for 10 min) and then resuspended in 500 µl PBS and centrifuged (800 g, RT, 5 min). Pellets were thereafter washed with PBS and then with saponin solution (0.1% saponin, 1% FCS 0.1% NaN₃; all purchased from Sigma-Aldrich). After permeabilization, cells were centrifuged (800 g, RT, 5 min) and incubated with 0.5 µg goat antimouse Gal-9 IgG antibody (R&D Systems, Minneapolis, Minn., USA). Then the cells were washed with saponin solution, centrifuged and incubated with 0.5 µg FITC-conjugated secondary antibody (antigoat IgG FITC conjugate, rabbit polyclonal antibody, Sigma-Aldrich, RT, 20 min). Negative controls were incubated with the FITC-conjugated secondary antibody only. At the end, the cells were washed with saponin solution and then resuspended in 300 µl 2% buffered paraformaldehyde (pH = 7.4). The cytometric analysis was carried out using a FACSCalibur cytometer (Becton Dickinson, San Jose, Calif., USA). According to the forward and side scatter, we identified an intact cell gate (without debris). Inside this gate, cell populations of alveolar macrophages, lymphocytes, eosinophil and neutrophil granulocytes were determined using anti-F4/80 (AbD Serotec, Oxford, UK), anti-ECP, -CD3, -B220 (Becton Dickinson) antibodies. The results were analyzed using the CellQuest Pro Software (Becton Dickinson).

Cytokine Measurements

BAL fluid samples were assessed using Cytomix beads and assayed for IL-1α, IL-2, IL-5, IL-6, IL-10, IFNγ, TNFα, GM-CSF, IL-4, and IL-17 by Flow Cytomix Multiplex mouse Th1/Th2 10plex kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. The results were analyzed by CellQuest Pro Software (Becton Dickinson).

RNA Isolation and Real-Time RT-PCR

Total RNA was isolated from the lung samples by RNeasy RNA isolations kit (Qiagen GmbH, Hilden, Germany). One microgram RNA was reverse-transcribed using SuperScript II RNase H⁻ (Gibco/BRL, Eggenstein, Germany) to generate first-strand cDNA. Gal-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression were determined by real-time RT-PCR using Syber Green (for Gal-9) or fluorescence resonance energy transfer hybridization probes (for GAPDH) on a Light Cycler system (Roche Diagnostics, Mannheim, Germany). The PCRs

were performed in a final volume of 20 µl. The reaction mix for Gal-9 contained 4 mmol/l MgCl₂, 500 nmol/l of each PCR primers (table 1) (TibMolBiol), 2 µl of FastStart DNA Master SYBR Green enzyme mix (Roche Diagnostics) and 1 µl of cDNA sample. The conditions of the PCRs for Gal-9 were as follows: 1 cycle at 95°C for 8 min, followed by cycles at 95°C for 5 s, 55°C for 5 s and 72°C for 20 s. The reaction mix for GAPDH contained 150 nmol/l of each hybridization probe, 500 nmol/l of each PCR primer (table 1) (TibMolBiol), 50 µM dNTP, 1.6 U of the HotMaster Taq DNA Polymerase (Qiagen) and 1 µl of cDNA sample. The PCRs were carried out for GAPDH: 1 cycle at 94°C for 2 min, followed by cycles at 95°C for 20 s, 59°C for 10 s and 65°C for 20 s. Quantification was performed with the second-derivative method by monitoring the cycle number at which the fluorescent signal could be distinguished from the background (crossing point). Serially diluted cDNA samples were used as standards. Results were analyzed with Light-Cycler software, version 3.5.3 (Roche Diagnostics).

Protein Isolation and Western Blotting

Lung samples (100 mg) were solubilized in a buffer containing 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1% Triton X-100, 0.1 M Tris-HCl (pH = 8.0), 1 mM ethylene glycol-bis(2-aminoethyl-ether),N,N,N',N'-tetraacetic acid (EGTA), 5 mM NaF, 1 mM phenylmethane-sulfonyl fluoride and 10 mM Na-orthovanadate (all purchased from Sigma-Aldrich). The lysed samples were centrifuged (10,000 g for 10 min, 4°C) and then the protein concentration of the supernatants was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, Calif., USA). Forty micrograms of protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V (~40 mA) for 120 min (Penguin™ Dual-Gel Water Cooled Systems, Owl, N.H., USA). A prestained protein mixture (BenchMark™, Gibco) was used as marker of molecular mass. After SDS-PAGE, the separated proteins were transferred to nitrocellulose membrane (Hybond™ ECL™, AP Biotech, UK) in transfer buffer containing Tris, glycine and methanol at 70 V (~220 mA) for 90 min (MiniTank™ electroblotter, Owl, N.H., USA). Nonspecific binding sites of the nitrocellulose membrane were blocked for 1 h at RT in a blot solution containing 5% nonfat dry milk and PBS. Then blots were incubated for 60 min (at RT) with goat polyclonal anti-mouse Gal-9 IgG (R&D Systems) diluted to 1:200. Equal protein loading to the gel was confirmed by staining with a goat polyclonal antimouse β-actin IgG (Actin [¹¹C], Santa Cruz Biotech-

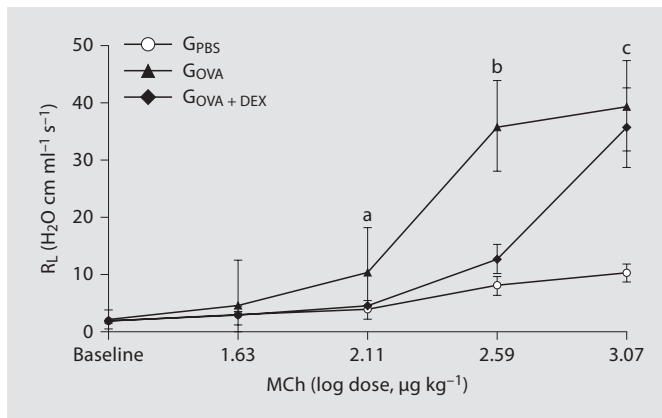


Fig. 1. Effects of OVA on airway responsiveness. Lung resistance (R_L) was registered during the infusion of MCh in 3-fold increasing concentration to assess AHR. Data are expressed as means $\pm$ SD of 7 animals in each group. ^a $p < 0.05$ G_{OVA} versus G_{PBS} and $G_{OVA+DEX}$; ^b $p < 0.01$ G_{OVA} versus G_{PBS} and $G_{OVA+DEX}$; ^c $p < 0.05$ G_{PBS} versus G_{OVA} and $G_{OVA+DEX}$.

nology Inc., Santa Cruz, Calif., USA) diluted to 1:200. Blots were then washed and incubated with peroxidase-conjugated secondary mouse anti-goat IgG (Santa Cruz Biotechnology) antibody diluted to 1:1,000 (RT, 30 min). Immunoreactive bands were visualized using the enhanced chemiluminescence Western blotting detection protocol (AP Biotech, Uppsala, Sweden). Computerized densitometry of the specific bands was analyzed by Gel-Pro Analyser 3.1 software (Media Cybernetics, Bethesda, Md., USA).

Immunofluorescent Staining

The frozen lung samples were embedded in Shandon cryomatrix (ThermoElectron Co., Waltham, Mass., USA) for immunohistochemical analysis and then cut into 5- μm slides. Slides were first incubated for 60 min at RT with anti-Gal-9 goat polyclonal IgG antibody (same antibody as in Western blot) diluted to 1:100. After repeated washing, slides were incubated with Alexa Fluor® 488 F(ab')₂ fragment of donkey anti-goat IgG (Invitrogen, Carlsbad, Calif., USA) diluted to 1:100 for 30 min at RT. DNA was stained with Hoechst 33342 (Sigma-Aldrich Company Ltd, Gillingham, UK) for 10 min at RT diluted to 1:1,000. Finally, the slides were rinsed in PBS and coverslipped with Vectashield fluorescent mounting medium (Vector Laboratories, Burlingame, Calif., USA). Appropriate controls were performed omitting the primary antibodies to assure their specificity and to avoid autofluorescence. To visualize the stained tissues on the sections, a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with an inverted Axiovert 200M microscope, 20 $\times$ Plan Apochromat (NA = 0.80) and 63 $\times$ Plan Apochromat oil immersion DIC objectives (NA = 1.4) was used.

Statistical Analysis

Data were analyzed using Statistica 7.1 software (StatSoft Inc., Tulsa, Okla., USA). After testing the normality with Shapiro-Wilk test, one-way analysis of variance (ANOVA) followed by Newmann-Keuls post-hoc test was used to determine differences

among the groups. Confidence intervals were calculated at the 95% level. Data were considered to be significantly different if $p < 0.05$.

Values for all measurements were expressed as means $\pm$ SD.

Results

Airway Responsiveness

After the three highest doses of MCh (log dose: 2.11, 2.59 and 3.07 $\mu\text{g}\cdot\text{kg}^{-1}$), significantly increased R_L values were detected in G_{OVA} compared to G_{PBS} mice (10.37 vs. 3.49 $\text{H}_2\text{O cm}\cdot\text{ml}^{-1}\cdot\text{s}^{-1}$; 35.81 vs. 7.11, and 39.46 vs. 9.00 $\text{H}_2\text{O cm}\cdot\text{ml}^{-1}\cdot\text{s}^{-1}$, respectively). The R_L values of $G_{OVA+DEX}$ animals were similar to those of G_{PBS} animals (3.11 vs. 2.69; 4.46 vs. 3.49; 10.37 vs. 7.11 $\text{H}_2\text{O cm}\cdot\text{ml}^{-1}\cdot\text{s}^{-1}$) only after the administration of the highest dose of MCh (3.07 $\mu\text{g}\cdot\text{kg}^{-1}$) were the R_L values of $G_{OVA+DEX}$ animals significantly higher than those of G_{PBS} (35.74 vs. 9.00 $\text{H}_2\text{O cm}\cdot\text{ml}^{-1}\cdot\text{s}^{-1}$) (fig. 1).

Histological Analysis

We observed inflammatory cell influx into the peribronchial and perivascular lung tissues of G_{OVA} compared to G_{PBS} mice. The elevated number of PAS-positive cells in G_{OVA} mice indicates goblet cell metaplasia and increased mucus production in the lungs compared to G_{PBS} mice (fig. 2a, b). In $G_{OVA+DEX}$ mice, the peribronchial and perivascular inflammation was diminished compared to G_{OVA} mice. PAS staining showed lower mucus production of the goblet cells in $G_{OVA+DEX}$ compared to G_{OVA} mice (fig. 2c).

FACS Analysis of BAL Cells

Cellular constitution of BAL showed that lymphocytes, alveolar macrophages, eosinophil and neutrophil granulocytes were present in G_{OVA} mice (lymphocytes: $20.3 \pm 6.7\%$; alveolar macrophages: $21.1 \pm 6.5\%$; eosinophils: $42.5 \pm 10.1\%$; neutrophils: $6 \pm 0.8\%$). In $G_{OVA+DEX}$ mice, the eosinophil and neutrophil granulocyte populations were not detectable (lymphocytes: $6.9 \pm 3.0\%$; alveolar macrophages: $90.4 \pm 3.5\%$; eosinophils: 0%; neutrophils: 0%), similarly to the BAL of G_{PBS} (lymphocytes: $5.3 \pm 2.2\%$; alveolar macrophages: $93.8 \pm 2.4\%$; eosinophils: 0%; neutrophils: 0%) (fig. 3). The percentage of the Gal-9-positive lymphocytes in BAL of G_{OVA} mice was significantly elevated compared to the G_{PBS} or $G_{OVA+DEX}$ mice ($p < 0.01$; $45.7 \pm 1.9\%$ vs. $6.1 \pm 1.4\%$ or $4.3 \pm 2.2\%$, respectively). The percentage of Gal-9-positive alveolar macrophages was significantly lower after OVA sensitization and challenge compared with

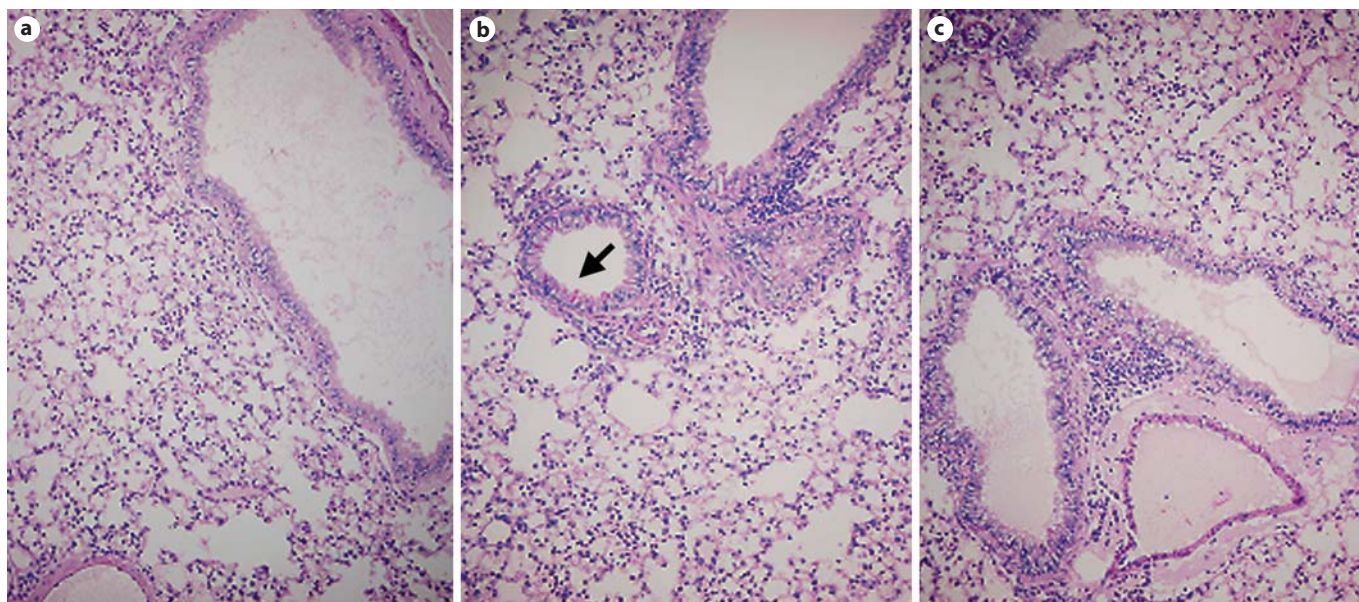


Fig. 2. Histological analysis of the lung of G_{PBS} (a), G_{OVA} (b) and $G_{\text{OVA+DEX}}$ (c) mice. The formalin-fixed (4%, pH = 7.4) lung samples were embedded in paraffin. Sections were stained with PAS according to the standard protocols. The samples were coded and examined by a qualified pathologist. The arrow indicates goblet cells. Original magnification: $\times 400$.

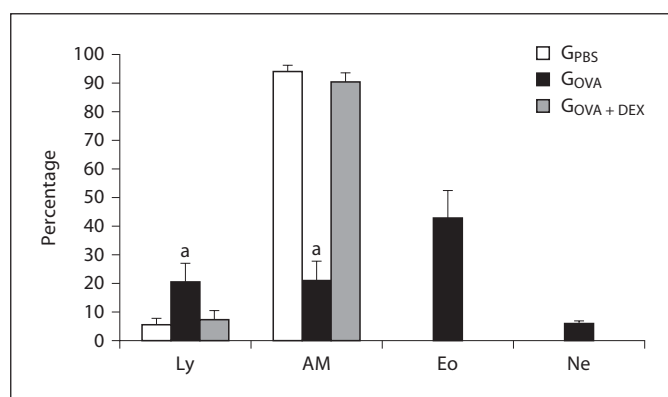


Fig. 3. Cellular content of BAL. Lymphocyte (Ly), alveolar macrophage (AM), eosinophil (Eo) and neutrophil (Ne) granulocyte populations were determined by flow-cytometric (FACS) analysis. Data are expressed as means $\pm$ SD of 7 animals in each group. ^a $p < 0.01$ G_{OVA} versus G_{PBS} and $G_{\text{OVA+DEX}}$.

the G_{PBS} or $G_{\text{OVA+DEX}}$ animals ($p < 0.01$; $2.4 \pm 1.0\%$ vs. $57.3 \pm 3.7\%$ or $48.8 \pm 6.7\%$). The percentage of Gal-9-positive eosinophil ($0.8 \pm 0.2\%$) and neutrophil granulocytes ($3.9 \pm 3.0\%$) was relatively low in the BAL of G_{OVA} , and was absent in the BAL of G_{PBS} and $G_{\text{OVA+DEX}}$

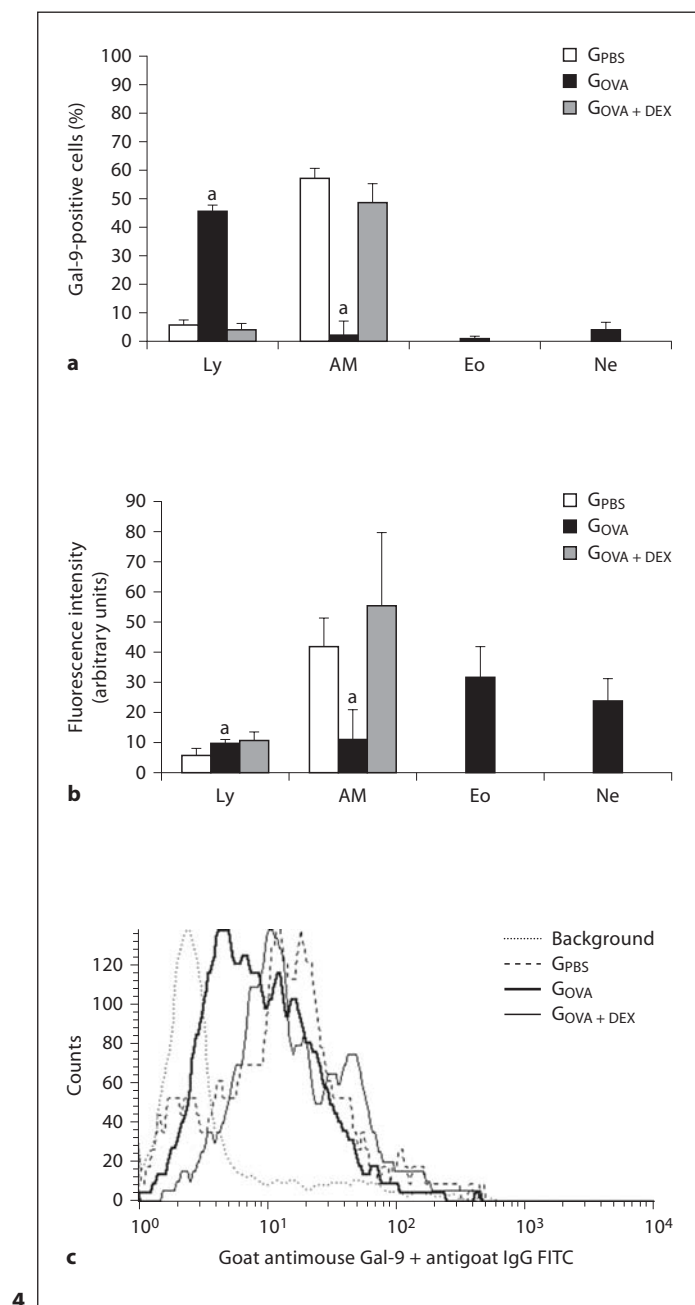
mice (fig. 4a, c). The intracellular amount of Gal-9 in the Gal-9-positive cells was determined based on the fluorescence intensity. Results are shown as arbitrary units. Eosinophil granulocytes contained 3 times more Gal-9 (30.8 ± 10.1) than lymphocytes (9.6 ± 1.1) or macrophages (11.1 ± 10.1). The amount of intracellular Gal-9 in neutrophil granulocytes was relatively high as well (24.1 ± 7.4) (fig. 4b).

Concentrations of Cytokines in the BAL Supernatant

The levels of IL-4, IL-5, and IL-6 were significantly elevated in G_{OVA} compared to G_{PBS} or $G_{\text{OVA+DEX}}$ animals (fig. 5). The levels of other cytokines did not change significantly after OVA sensitization and challenge. In $G_{\text{OVA+DEX}}$ mice, the level of IL-10 was elevated compared to G_{PBS} mice (fig. 5).

Gal-9 mRNA Expression in the Lungs

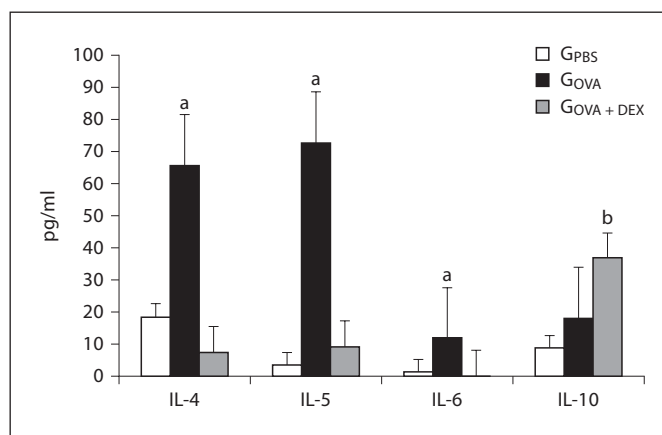
The mRNA expression of Gal-9 was lower in the lungs of G_{OVA} ($p < 0.01$) compared to G_{PBS} mice as determined by real-time RT-PCR (fig. 6a). Similarly to G_{OVA} mice, the mRNA expression of Gal-9 was lower in $G_{\text{OVA+DEX}}$ mice. There was no significant difference in mRNA expression of Gal-9 between G_{OVA} and $G_{\text{OVA+DEX}}$ animals.



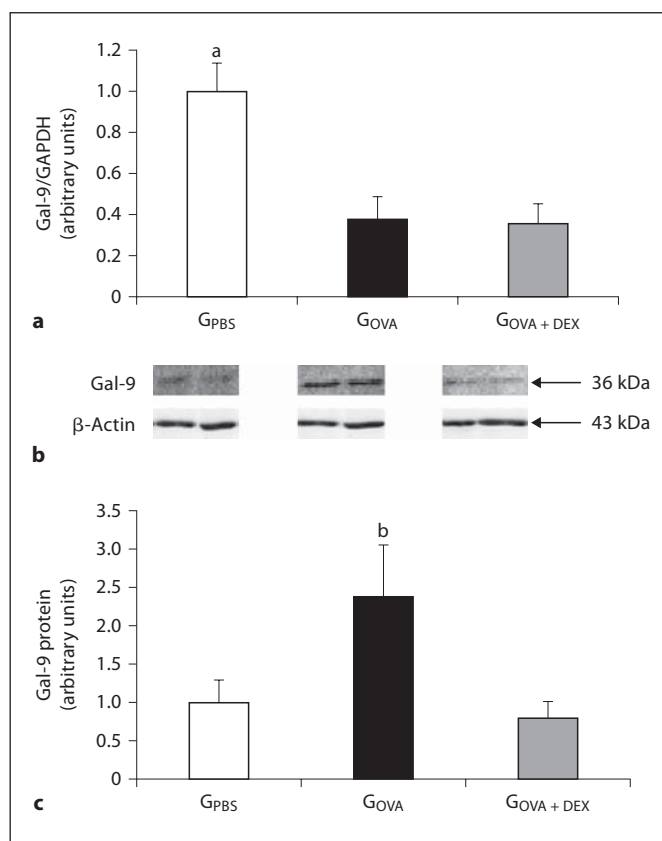
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Fig. 4. Percentage (a) and intracellular amount of Gal-9-positive cells (b), and representative histogram of Gal-9-positive lymphocytes (c) of the BAL. Gal-9-positive lymphocytes (Ly), alveolar macrophages (AM), eosinophil (Eo) and neutrophil granulocytes (Ne) and their intracellular Gal-9 amount were determined in BAL by flow-cytometric (FACS) analysis using goat antimouse Gal-9 IgG antibody as primer and rabbit anti-goat IgG-FITC as secondary antibody. Data are expressed as means $\pm$ SD of 7 animals in each group. ^a $p < 0.01$ G_{OVA} versus G_{PBS} and G_{OVA}+DEX.

Fig. 5. Concentration of cytokines in the BAL supernatant. Cytokine levels were measured with the Flow Cytomix kit (Bender MedSystems) on a flow cytometric (FACS) analyser according to the manufacturer's instructions. Data are expressed as means $\pm$



5



6

SD of 7 animals in each group. ^a $p < 0.01$ G_{OVA} versus G_{PBS} and G_{OVA}+DEX; ^b $p < 0.05$ G_{OVA}+DEX versus G_{PBS}.

Fig. 6. mRNA expression (a) and protein level of Gal-9 in the lungs (b, c). The mRNA expression of Gal-9 was determined by real-time RT-PCR in the lungs of G_{PBS}, G_{OVA} and G_{OVA}+DEX mice (a). The protein level of Gal-9 was determined by Western blot using anti-Gal-9 goat polyclonal IgG antibody as primer and donkey anti-goat IgG as secondary antibody. Analysis of the lung lysates reveals one distinct band with a molecular weight of 36 kDa (b). Data for Gal-9 protein level were obtained by computerized analysis of the Western blots (c). Data are expressed as means $\pm$ SD of 7 animals in each group. ^a $p < 0.01$ G_{PBS} versus G_{OVA} and G_{OVA}+DEX; ^b $p < 0.01$ G_{OVA} versus G_{PBS} and G_{OVA}+DEX.

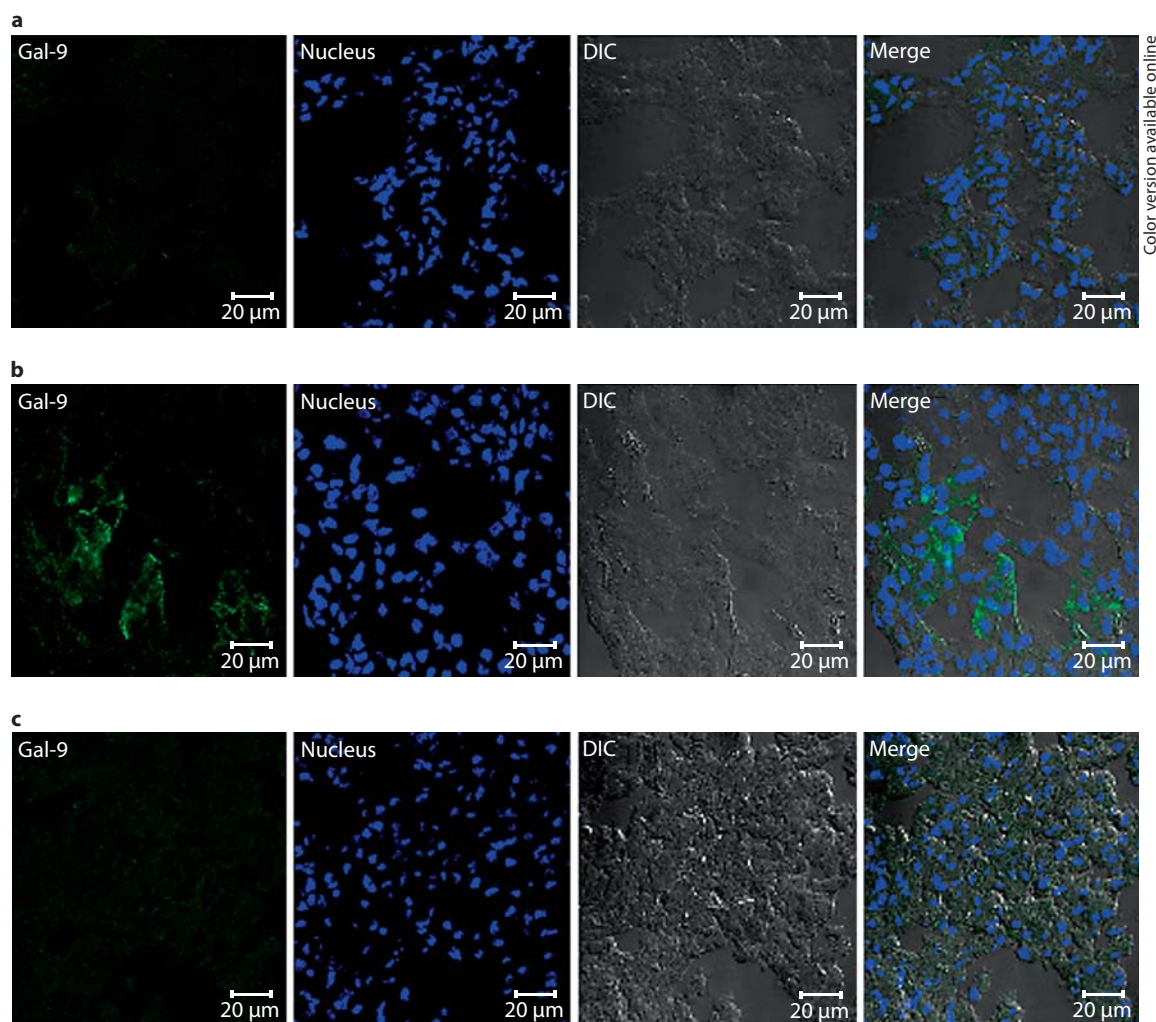


Fig. 7. Immunohistochemical localization of Gal-9 in the lung of representative samples from G_{PBS} (a), G_{OVA} (b) and $G_{\text{OVA+DEX}}$ (c) mice. Localization of Gal-9 in the lung tissue of representative samples of each group using immunofluorescence staining followed by confocal images. Slides were incubated with anti Gal-9

goat polyclonal IgG antibody as primer and Alexa Fluor® 488 F(ab')₂ fragment of donkey antigoat IgG as secondary antibody. DNA was stained with Hoechst 33342. Confocal images were taken on a Zeiss Axiovert LSM 510 confocal laser scanning microscope with the plan apochromat 63×/1.40 oil DIC.

Gal-9 Protein Levels in the Lungs

We found that the protein level of Gal-9 was significantly higher in G_{OVA} ($p < 0.01$) compared to G_{PBS} animals as measured by Western blot analysis (fig. 6b, c). In $G_{\text{OVA+DEX}}$ mice, the protein level of Gal-9 was significantly lower compared to G_{OVA} mice. There was no significant difference in the protein level of Gal-9 between $G_{\text{OVA+DEX}}$ and G_{PBS} animals.

Localization of Gal-9 in the Lung

The cellular distribution of Gal-9 proteins was determined by immunofluorescent staining. As shown in figure 7, weak Gal-9 immunoreactivity was observed in the

lungs of G_{PBS} mice (fig. 7a). In the lung tissue of G_{OVA} mice, strong Gal-9 staining intensity was found in alveolar epithelial cells compared to controls (fig. 7b). Similarly to G_{PBS} mice, weak immunoreactivity was detectable in the lung tissue of $G_{\text{OVA+DEX}}$ mice (fig. 7c).

Discussion

Allergic asthma is a complex inflammatory disorder of the airways, characterized by variable airway obstruction and AHR [35]. Several molecules take part in the development of allergic asthma; currently, sugar-binding

proteins such as galectins come into focus. Galectins have been increasingly implicated in both adaptive and innate immune responses [36–38]. However, our knowledge about the involvement of galectins in allergic asthma is incomplete.

In this study, we investigated the expression of Gal-9 in a classic OVA-induced mouse model of allergic asthma [39]. In the present model, OVA sensitization and airway challenge induced AHR, marked peribronchial and perivascular inflammatory cell infiltration, goblet cell metaplasia and increased mucus production in the lungs. Changes in the number and type of BAL cells were also representative of allergic asthma [39, 40]. The inflammatory response in the asthmatic lung is characterized by airway infiltration by lymphocytes [2, 3], especially with activated Th2 cells [41, 42]. Cytokines and chemokines derived from Th2 lymphocytes promote eosinophilic infiltration and play a pivotal role in the pathogenesis of allergic asthma [4, 5].

In our model, the percentage of Gal-9 immunopositive lymphocytes and their content of intracellular Gal-9 and the level of Th2 cytokines such as IL-4, IL-5 and IL-6 were increased in BAL of the G_{OVA} compared to G_{PBS} or $G_{OVA+DEX}$ mice. It has been demonstrated previously that Gal-9 induces cell death in Th1 lymphocytes in a Tim-3-dependent manner. Tim-3 is a Th1-specific type 1 membrane protein which is not expressed on the surfaces of naive T and Th2 cells but emerges on the cell surface of fully differentiated Th1 cells [27]. Our results suggest that an increased Gal-9 level in lymphocytes may participate in the development of Th2 dominance in allergic asthma and, as a consequence, in the increased secretion of Th2 cytokines.

In the present study, we also characterized the synthesis of Gal-9 in the lungs of allergized mice. Interestingly, while the mRNA expression of Gal-9 decreased, the protein level of Gal-9 increased in the lungs of G_{OVA} compared to G_{PBS} mice. These data suggest that OVA sensitization and airway challenge may alter both the transcription and the translation of Gal-9. Elevation in the protein level of Gal-9 in the presence of decreased mRNA expression suggests the importance of posttranscriptional regulation in the synthesis of Gal-9. Another possibility would be that the decreased mRNA expression in G_{OVA} mice results in a decreased protein level of Gal-9 at a later time point than the investigated time point. However, based on the literature, we suggest that OVA sensitization and airway challenge may lead to an increased protein level of Gal-9. Recently, Yamamoto et al. [28] also found increased pulmonary levels of Gal-9 7 h after the last air-

way challenge of guinea pigs, which was sustained for up to 24 h.

DEX treatment did not alter the mRNA expression of Gal-9 in $G_{OVA+DEX}$ compared to G_{OVA} mice. However, the protein level of Gal-9 was decreased in $G_{OVA+DEX}$ compared to G_{OVA} mice. Our findings suggest that while DEX treatment alters the translation, it does not affect the transcription of Gal-9. These results further support our above hypothesis that the synthesis of Gal-9 is tightly regulated both at the transcriptional and at the translational level.

In the lung, bronchial epithelial cells are one of the main sources of Gal-9; however, fibroblasts, endothelial cells, lymphocytes, eosinophil granulocytes, macrophages were also suggested to produce Gal-9 [28, 43]. Nonetheless, there is an apparent discrepancy in the literature, i.e., Gal-9 may participate in the pathomechanism of allergic inflammation via different ways.

In line with our results, Yamamoto et al. [28] found increased Gal-9 levels in the lungs of sensitized guinea pigs and suggested that Gal-9 may induce eosinophil granulocyte chemotaxis and T cell apoptosis. On the contrary, Katoh et al. [44] showed that different doses (10, 30, 100 μ g) of intravenously administered recombinant Gal-9 inhibit allergic inflammation by modulating CD44-dependent leukocyte recognition of the extracellular matrix. Based on the literature and our results, we suggest that the effect of Gal-9 may be dose dependent. While physiological amounts of Gal-9 may induce chemotaxis of eosinophil granulocytes and contribute to the pathomechanism of allergic asthma [28], the higher concentration of exogenously administered Gal-9 reduces the recruitment of these cells and diminishes inflammation [44].

We assume that the elevated Gal-9 level in the lungs influences the progress and severity of asthma in different manners. As mentioned above, Gal-9 may induce Th1 cell death [27], which may lead to the development of Th2 dominance and the secretion of Th2 cytokines during the allergic processes. Th2 cytokines, including IL-4, IL-5 and IL-6, play an important role in the pathophysiology of allergic asthma by promoting eosinophilic inflammation [4, 5, 45]. On the contrary, in $G_{OVA+DEX}$ mice the decreased level of Gal-9 may participate in the attenuation of the AHR and eosinophil airway inflammation.

Eosinophil granulocytes and eosinophil-derived products in the respiratory tract correlate with the exacerbation of allergic asthma [6, 7, 46]. Released eosinophilic mediators cause epithelial damage and persistence of inflammation in the lung [47–49]. They influence AHR

[50] and airway wall remodeling [5] and they induce and regulate IgE production and eosinophil airway infiltration [51]. Gal-9 has a well-known effect on eosinophil recruitment. It has been shown that Gal-9 is involved in prolonged eosinophil accumulation in the lungs of allergized guinea pigs [28]. Others have shown that Gal-9 exhibited potent chemoattractant activity on eosinophil granulocytes in vitro and in vivo but not on neutrophil granulocytes, lymphocytes, or monocytes [23].

In summary, increased levels of Gal-9 were demonstrated in the lungs and also in BAL lymphocytes of allergized mice. Moreover, the levels of Th2 cytokines were elevated in the BAL of G_{OVA} animals. These data support the role of Gal-9 in the pathogenesis of allergic asthma.

In line with current publications, we propose that Gal-9 may promote the development of Th2 dominance and might participate in the recruitment of eosinophil granulocytes. Further studies are, however, required to precisely define the mechanisms of the effects of Gal-9 in allergic asthma.

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