



Glycomacropeptide administration attenuates airway inflammation and remodeling associated to allergic asthma in rat

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Received: 29 September 2015 / Revised: 22 December 2015 / Accepted: 28 December 2015
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Abstract

Objective Glycomacropeptide (GMP) is a bioactive peptide derived from milk that has been reported to exhibit a range of anti-inflammatory and immunomodulatory properties. The aim of this study was to analyze the prophylactic effect of GMP administration on airway inflammation and remodeling in an experimental model of asthmatic rat.

Methods Animals treated orally with or without GMP (500 mg/kg/day) were ovalbumin-sensitized and -nebulized and several indicators of Th2 response, airway structural changes and inflammatory cells recruitment were evaluated.

Results Treatment with GMP prior and during asthma development resulted in reduction of allergen-specific IgE titers in serum and blood eosinophilia. Also, GMP substantially suppressed the recruitment of inflammatory cells to bronchoalveolar compartment. Histological studies demonstrated that GMP markedly inhibits eosinophils

infiltration, goblet cells hyperplasia and collagen deposit in lung tissue. The latter effect was related with an inhibition in transforming growth factor- β expression. In addition, expression of interleukin-5 and -13 were substantially inhibited in lung while that of interleukin-10 was increased. **Conclusion** Our results suggest that administration of GMP may prevent the development of an excessive Th2 response in asthma and effectively ameliorates the progression of the disease.

Keywords Glycomacropeptide · Asthma · Airway inflammation · Airway remodeling · Th2 cytokines

Introduction

Allergic asthma is a chronic respiratory disease characterized by airway inflammation, airway hyper-responsiveness, mucus hypersecretion and airway remodeling, as result of exposition to the allergen [1]. It is classified as a type 1 hypersensitivity reaction that involves an immune-inflammatory response driven by Th2 cells, with the consequent allergen-specific IgE production [2]. Mast cells activation by allergen results in the immediate release of mediators capable of contracting airway smooth muscle cells, inducing edema and mucus secretion, and later generation of chemokines that stimulate the recruitment of inflammatory cells, mainly eosinophils, macrophages, neutrophils and T lymphocytes [1, 3]. The released products from these activated leukocytes mediate the development of airway hyper-responsiveness and airway remodeling. Several mediators of airway inflammation and remodeling have been identified, including the Th2 cytokines: interleukin (IL)-4, IL-5, IL-9 and IL-13; transforming growth factor (TGF)- β ; granulocyte/macrophage colony stimulating

Responsible Editor: John Di Battista.

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factor (GM-CSF); lipid mediators and histamine [4]. IL-13 and IL-5 are of particular importance in airway inflammation because IL-13 induces eosinophilia and immunoglobulin class switching to IgE, as well as goblet cell hyperplasia [5, 6]; and IL-5 is directly involved in eosinophils recruitment, activation and survival in the airways [7–9]. Whereas, TGF- β is one of the main mediators involved in tissue remodeling in the asthmatic lung, as it is implicated in epithelial alterations, subepithelial fibrosis, airway smooth muscle remodeling and microvascular changes [10].

There are approximately 300 million people worldwide who suffer from asthma and in the next 10–15 years it is predicted to rise to more than 400 million [11]. It is estimated that 50 % of this asthmatic population is atopic, affecting mainly the infant and pediatric population [12]. Onset of allergic asthma is influenced by environmental and genetic factors, and cytokines are believed to mediate and perpetuate the disease, becoming a chronic pathology [13, 14]. So, novel therapeutic strategies are needed to address this pathology.

Glycomacropeptide (GMP) is a biologically active peptide derived from the hydrolysis of milk κ -casein, both physiologically as the result of κ -casein digestion in the stomach of neonates, and in the industry as a part of the whey produced during cheese-making process. It is composed of 64 amino acids and contains several units of *N*-acetylneuraminic (sialic) acid [15]. Most of the studies about nutritive properties and biological activities of GMP have been done with bovine GMP. In contrast to its parent protein, GMP has an excellent safety record and is not immunogenic [16]. As a nutrient, GMP is used in the production of infant formulas and in nutritional formulations for people with phenylketonuria, as it contains no phenylalanine in its amino acidic composition [17]. In vivo studies have demonstrated that GMP has prebiotic, anti-inflammatory and immunoregulatory properties, modulating T- and B-lymphocytes response. When orally administrated to mice, GMP significantly increases the number of *Lactobacillus* and *Bifidobacteria* [18]. GMP is found to be anti-inflammatory in rat models of colitis and ileitis, acting at least in part on lymphocytes differentiation [19–21]. In a model of colonic damage caused by dimethyl hydrazine, oral administration of GMP prevents an extensive damage in colon, down-regulating the levels of Th2 cytokines [22]. In relation to B lymphocytes, administration of GMP via oral to mice inhibits the level of serum IgG antibodies specific to an injected antigen [23] and significantly increases the number of IgA + plasma cells in the lamina propria [24]. In a recent study carried out in our laboratory a prophylactic effect of orally administrated GMP on the development of immune

response associated with allergic sensitization was demonstrated in rats [25]. This effect was associated to down-regulation of the specific IgE levels and inhibition of proliferative response and IL-13 production by splenocytes in response to allergen. In consequence, GMP treatment reduced the inflammatory reaction associated to immediate cutaneous hypersensitivity and the severity of anaphylaxis.

The aim of this work was to evaluate whether oral treatment with GMP can influence the development of airway inflammation and remodeling, as well as Th2-immune response associated to allergic asthma, to determine the potential therapeutic effect of GMP.

Materials and methods

Reagents

GMP (Lacprodan[®] CGMP-10) was a gift of Arla Foods Amba (Viby, DE). Ovalbumin (OVA, Grade V) and 3,3-diaminobenzidine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aluminum hydroxide was purchased from Thermo Scientific (Rockford, IL, USA). *Bordetella pertussis* vaccine was purchased from Zurivac (Mexico DF, MX). Wright, hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and Masson's trichrome kits were purchased from Hycel (Mexico DF, MX). Rabbit anti-TGF- β antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). LSAB kit was purchased from Dako Corporation (Carpinteria, CA, USA). E-2 Mount medium was purchased from Shandon lab (Pittsburgh, PA, USA). The SV Total RNA Isolation System and GoTaq qPCR Master Mix were purchased from Promega (Madison, WI, USA). The Retroscript kit was purchased from Ambion (Austin, TX, USA).

Animals

Male Wistar rats (100–150 g) obtained from the Laboratory Animal Service of the Autonomous University of Aguascalientes were used throughout the study. Rats were housed under controlled conditions of temperature (22–24 °C) and illumination (12 h light cycle), and maintained with Rodent Laboratory Chow 5001 and tap water ad libitum. Rats were randomly assigned into three groups: control of sensitization (CTRL, $n = 10$), OVA-induced asthma (OVA, $n = 8$) and OVA-induced asthma with GMP treatment (GMP, $n = 10$). All experiments were carried out with strict adherence to ethical guidelines approved by the Institutional Normative Welfare Standards.

Asthma model and treatment

As previously described a rat model of asthma was developed by OVA sensitization and inhalation [26]. Briefly, animals from OVA and GMP groups were immunized on day 0 by intramuscular (i.m.) injection of 1 mg of OVA in 7.8 mg of aluminum hydroxide dissolved in 1 mL of saline solution. Simultaneously, and as adjuvant, 0.5 mL of *B. pertussis* vaccine containing $10\text{--}15 \times 10^9$ heat-killed bacilli/mL was subcutaneously (s.c.) injected. A booster sensitization was given 7 days later. From days 14 to 19, rats were daily challenged for 30 min with an aerosolized 1 % OVA solution using an ultrasonic nebulizer into a closed acrylic container (60 cm $\times$ 50 cm $\times$ 35 cm). On day 29, animals were exposed to aerosolized 5 % OVA solution for 30 min. CTRL rats underwent an identical schedule for sham sensitization and challenge, but received saline solution instead of OVA. GMP group animals were orally administered, using an esophageal catheter, with 500 mg/kg/day of GMP prepared in tap water from 3 days before sensitization to the final of the study. CTRL and OVA groups received an equal volume of tap water during the same days. On day 30 animals were intraperitoneal injected with ketamine/xylazine to anesthetize them and samples of blood, bronchoalveolar lavage fluid (BALF) and lung were collected. The protocol for sensitization, challenge and treatment is summarized in Fig. 1.

Cell count in peripheral blood and OVA-specific IgE measure in serum

A blood sample was taken from each rat 24 h after the last OVA challenge and a drop was immediately smeared on slides. Serum was obtained and stored at -20°C until used to titrate IgE anti-OVA. Blood slides were Wright-stained

and differential cell counts were manually performed, in a blinded fashion, of three slides/sample counting 100 cells/slide and using standard morphologic criteria and an immersion objective microscope (1000 $\times$ magnification). The serum from each rat was individually analyzed by passive cutaneous anaphylaxis (PCA) as previously described [25].

Collection and analysis of BALF

To perform bronchoalveolar lavage, the thoracic cavity was opened. Before carrying out the lavage of the left lung, the right main bronchus was clamped. Immediately, secondary bronchi of cranial and accessory lobes were clamped and the lobes excised to RNA analysis. Then, 1 mL of neutral formalin (10 % v/v) was introduced using a polyethylene cannula inserted below the clip of the right main bronchus, until the middle and caudal lobes were inflated and both lobes were excised to further histological assays. Then, a total volume of 5 mL (1 mL $\times$ 5 times) PBS was injected into left lung using a cannula introduced into the trachea. BALF was recovered from individual rats, placed on ice and cells were harvested by centrifugation at $500\times g$ for 5 min at 4°C , followed by suspension of the cell pellet in 1 mL PBS. Total cells count and cell viability were estimated using a Neubauer chamber and trypan blue stain. To differential cell counts, cells were smeared on a glass slide and Wright-stained. Leukocytes were counted as indicated for blood samples and expressed as cells/mL average from all the slides per group.

Histology

As previously indicated right lung tissue was obtained from each animal for histological assays. Lungs were immersed in neutral formalin for at least 24 h, after which a sample

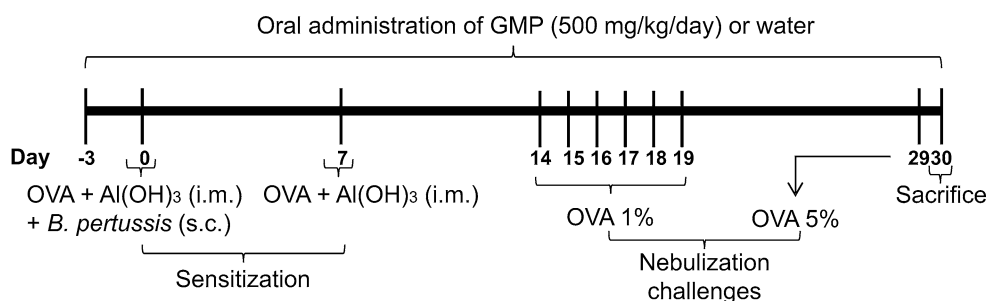


Fig. 1 Schematic diagram of the asthma induction protocol and schedule of GMP treatment. Rats were sensitized on day 0 with an intramuscular (i.m.) injection of OVA mixed with aluminum hydroxide (Al(OH)₃) gel in saline solution. Simultaneously, *Bordetella pertussis* vaccine was injected subcutaneously (s.c.). A booster sensitization of OVA/Al(OH)₃ was injected 7 days later. Animals received inhalational exposure with OVA on days 14–19 and the final

inhalational exposure on day 29. Rats from CTRL group were injected with the same adjuvants but without OVA, and nebulized with saline solution. Rats from GMP group received, daily and orally, GMP prepared in tap water and animals from CTRL and OVA groups received an equivalent volume of water. All animals were sacrificed at day 30 and blood, bronchoalveolar lavage fluid and lung samples were obtained

was taken from the mid-zone of the lung. After an additional time in fixative, they were embedded in paraffin, cut into sections (5 μm), rehydrated and stained with: H&E for examining eosinophil infiltration per area of pulmonary interstitium (40,000 μm^2), PAS to identify goblet cells per 100 μm bronchial perimeter and Masson's trichrome to determine the area of collagen deposition in the airways in relation to subepithelial basement membrane perimeter. To each sample, nine bronchus sections were randomly examined with an AxioPlan Carl Zeiss microscope (400 $\times$ magnification to H&E and 100 $\times$ magnification to PAS and Masson's trichrome stain). Morphometry was performed using AxioVision Rel. 4.8 software. The histological analyses were performed by observers who were not aware of the groups of rats from which the samples originated.

Immunohistochemistry

Paraffin embedded lung specimens were cut into 3 μm -thick sections, deparaffinized and rehydrated. Antigen retrieval was performed by immersing the slides in 0.1 M citrate buffer (pH 6.0) for 2 min in a pressure cooker. The endogenous peroxidase activity was blocked by immersing the slides in 3 % H_2O_2 -methanol for 30 min at room temperature and the background non-specific binding was reduced by incubating with 1 % BSA in PBS for 60 min. The slides were incubated overnight at 4 $^\circ\text{C}$ with antibodies against TGF- β diluted 1:20 in PBS-1 % BSA. Slides were washed and incubated with a biotinylated secondary antibody from the LSAB kit for 30 min at room temperature. After washing, the procedure follows by incubating with the streptavidin-HRP conjugate for 30 min at room temperature. Color was developed using 3,3-diaminobenzidine chromogen solution for 1–5 min in the dark. The reaction was arrested with distilled water and the slides were counterstained with hematoxylin. Thereafter, the tissues were washed in tap water, dehydrated and mounted with E-2 Mount medium. Finally, slides were visualized under light microscopy by an AxioPlan Carl Zeiss microscope (400 $\times$ magnification) and the analysis was performed using Image-Pro Plus 5.0[®] software. To determine positive immunoreactivity (as pixel density), three randomly nonoverlapping areas (69,000 μm^2) were evaluated in three separated immunostained lung sections per animal ($n = 5$ per group).

RNA purification and real-time quantitative PCR (qRT-PCR)

Total RNA was isolated from 100 to 150 mg of right lung tissue using the SV Total RNA Isolation System. Purified RNA was quantified with a Spectro UV (Velab, DF, Mexico) and quality of RNA was evaluated with the

Table 1 Oligonucleotides for gene expression quantification

Gene	Accession number	Oligonucleotides
IL-5	NM_021834.1	Fw: CAGTGGTCAAAGAGACCTTG Rv: GTATGTCTAGCCCCTGAAAG
IL-13	NM_053828.1	Fw: ATCGAGGAGCTGAGCAACAT Rv: ATCCGAGGCCTTTTGGTTAC
IL-10	NM_012854.2	Fw: TGGCTCAGCACTGCTATGTT Rv: TTGTCCAGCTGGTCCTTCTT
β -actin	NM_031144.3	Fw: GTCGTACCACTGGCATTGTG Rv: GCTGTGGTGGTGAAGCTGTA

A260/280 ratio. Only samples with ratio >1.8 were employed for cDNA synthesis. Reverse transcriptions of 2 μg of RNA were performed with the Retroscript kit. Then, 2 μL of 1:10 diluted cDNA reaction was used as template for the real-time quantitative PCR reactions for IL-5, IL-13, IL-10 and β -actin (Table 1) with the GoTaq qPCR Master Mix in an Eco Illumina System (Illumina, San Diego, CA, USA). Relative quantification was determined with the $\Delta\Delta\text{Ct}$ method using β -actin as housekeeping gene for normalization.

Data analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). Student's t test was used for statistical analysis using GraphPad Prism 5.01. Significance was set at $p < 0.05$.

Results

GMP administration reduces allergen-specific IgE and eosinophils in blood

First, we investigated whether oral intake of GMP might modify the development of an asthmatic immune response. In allergic asthma, high levels of allergen-specific IgE are present in blood and the cross-linking of this IgE in the surface of mast cells by allergen is crucial to the initiation of the early asthmatic reaction [27]. So, we analyze changes in OVA-specific IgE levels. At day 30, sera from CTLR group animals were negative in PCA, indicating that adjuvants alone did not induce the production of any IgE antibody specific or cross-reacting to OVA that could generate false-positive reactions. In sera of asthmatic rats high levels of OVA-specific IgE were detected (Fig. 2a). However, when asthmatic rats were GMP-treated the titer of OVA-specific IgE was 60 % reduced. Inactivation of immune sera (56 $^\circ\text{C}$ for 1 h) from OVA and GMP rats eliminated PCA reaction, indicating that IgE, but not IgG1, was the antibody mediating PCA reaction (data not shown).

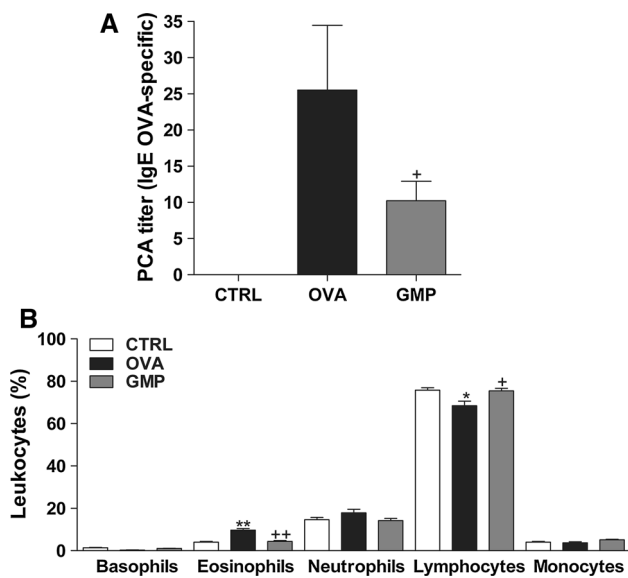


Fig. 2 Effect of GMP treatment on IgE anti-OVA titer and inflammatory cells accumulation in peripheral blood. **a** Titer of OVA-specific serum IgE at 24 h after de last challenge. PCA titers represent the reciprocal of the highest dilution of serum which gave a lesion >5 mm in diameter. **b** Comparison of leukocytes population in peripheral blood of non-asthmatic and asthmatic rats treated or not with GMP. Blood sample was obtained 24 h post-challenge. Wright-stained blood slides were observed and counted under an oil microscope. Each value represents the mean $\pm$ standard error of the mean; $n = 10$ in CTRL and GMP group, $n = 8$ in OVA group; * $p < 0.001$, ** $p < 0.0001$ compared to CTRL; + $p < 0.05$; ++ $p < 0.0001$ compared to OVA

Elevated number of eosinophils in blood is a key feature of allergic asthma [28]. Figure 2b shows the percentage of leukocytes in peripheral blood. In CTRL animals the cells number ranged from 1.36 ± 0.19 % (basophils) to 75.79 ± 1.13 % (lymphocytes). OVA sensitization and nebulization increased by 143 % the number of eosinophils and decreased the lymphocytes by 10 %. There was no change in neutrophils, basophils and monocytes percentage in blood at day 30. Oral treatment with GMP resulted in a significantly inhibition in eosinophil number and an increase in lymphocytes, returning both cell types to control values.

Influence of GMP in the reduction the total cells and leukocytes in bronchoalveolar lavage fluid

To investigate changes in airway inflammation as a result of GMP administration, the total number of cells and percentage of different leukocytes populations in BALF of all animals were analyzed. The number of total cells in OVA rats was 2.14-fold increased as compared to CTRL rats, indicating a significant infiltration of leukocytes, which is a feature of asthmatic inflammation. GMP administration suppressed this infiltration of inflammatory

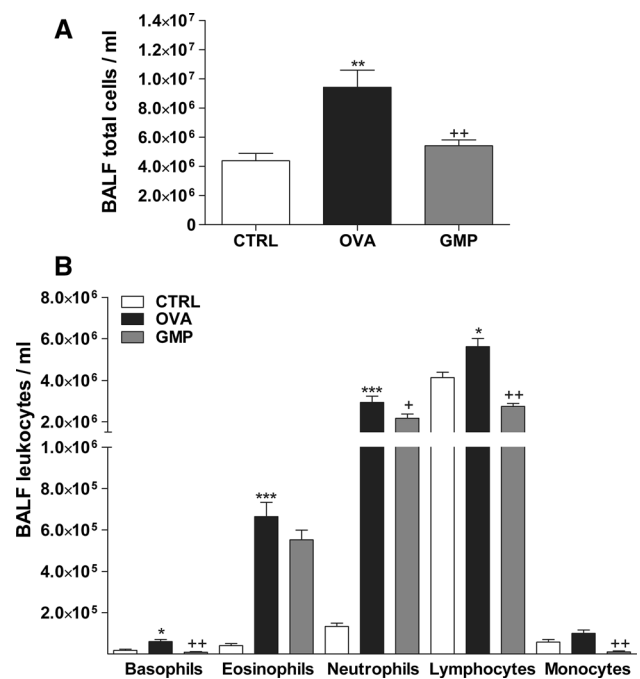


Fig. 3 Characterization of cells in BALF from non-asthmatic and asthmatic rats with and without GMP treatment. **a** Total and **b** leukocyte differential counts from the BALF. BALF was obtained 24 h post-challenge. Total cells were counted in a Neubauer chamber and Wright-stained slides of smeared BALF cells were evaluated by microscopy. Each value represents the mean $\pm$ standard error of the mean; $n = 10$ in CTRL and GMP group, $n = 8$ in OVA group; * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ compared to CTRL; + $p < 0.05$; ++ $p < 0.0001$ compared to OVA

cells in asthmatic animals ($p < 0.0001$) (Fig. 3a). Infiltrating cells in OVA animals included mainly eosinophils and neutrophils ($p < 0.001$), and in a minor number basophils and lymphocytes ($p < 0.05$). Monocytes were also slightly increased in the OVA group. Oral treatment with GMP reduced the percentage of all populations of leukocytes in asthmatic animals. In relation to the most abundant cellular types recruited to lungs, neutrophils were significantly reduced by 26 % with GMP treatment and although eosinophils were numerically lower in the GMP group, the difference to OVA group was not significant (Fig. 3b).

GMP administration alleviates lung eosinophilia and airway remodeling in the rat asthma model

Because GMP inhibited inflammatory cell recruitment to airways, we next examined whether the treatment might modify lung eosinophilia and airway structural changes generated by activated inflammatory cells. Histological analysis revealed typical pathological features of asthma in OVA rats. In lungs of rats from OVA group, an increase in the number of infiltrated eosinophils around bronchioles,

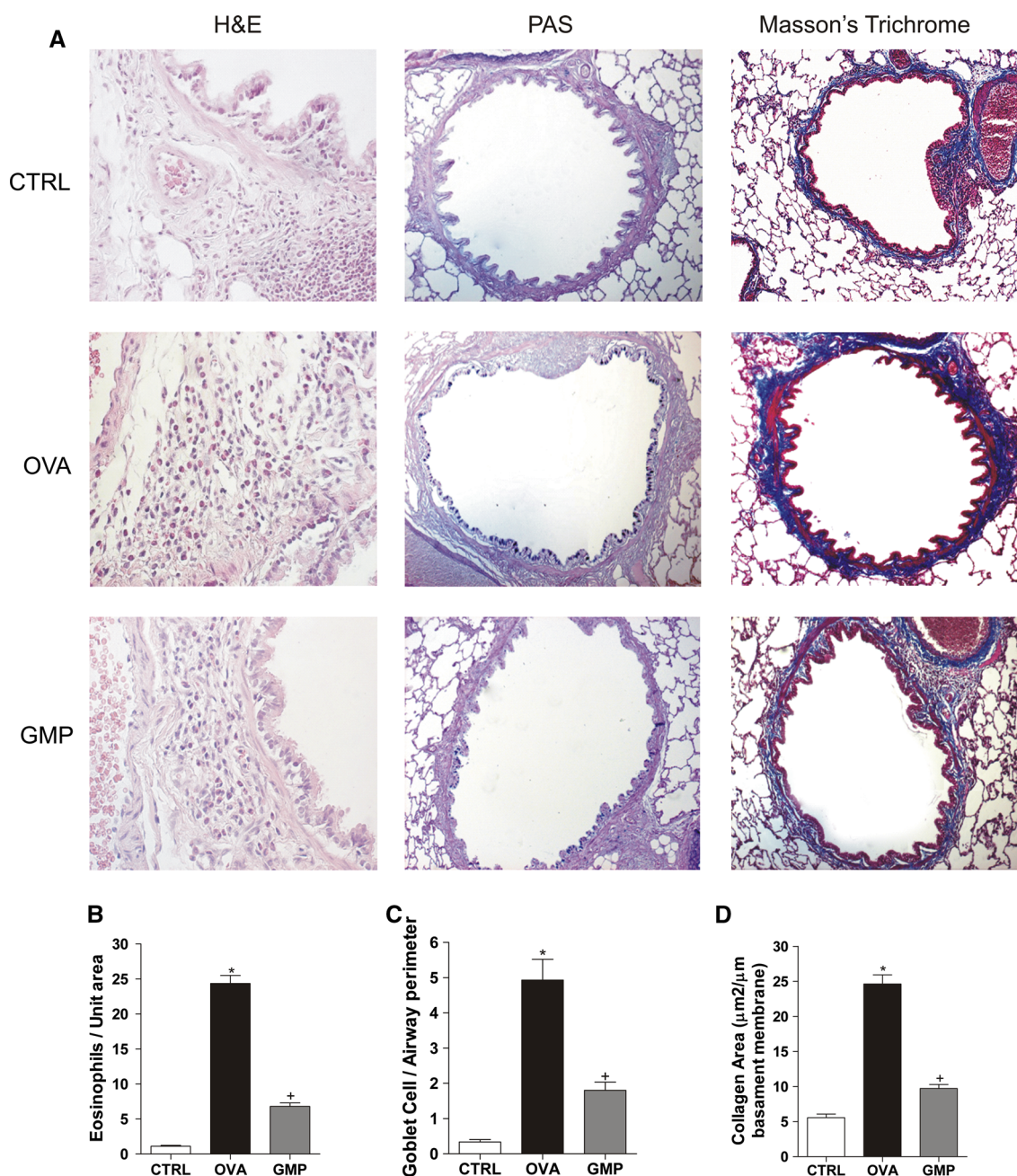


Fig. 4 Effect of GMP treatment on histopathology in the lung. **a** Lung were obtained at day 30 for histological examination and lung sections from each group were stained with hemotoxin and eosin (H&E) for inflammatory cells, periodic acid-Schiff (PAS) for goblet cells and Masson's trichrome for collagen fiber deposits. $\times 400$ magnification to H&E and $\times 100$ magnification to PAS and Masson's trichrome stain. **b** Quantitative analysis of pulmonary inflammation as the extent of eosinophils infiltration per unit area, equal to

40,000 μm^2 of pulmonary interstitium. **c** Quantitative analysis of goblet cell, measure as the number of PAS-positive goblet cells per 100 μm bronchial perimeter. **d** Quantitative analysis of subepithelial collagen deposit, measure as area of collagen deposition in the airways in relation to subepithelial basement membrane perimeter. Each value represents the mean $\pm$ standard error of the mean; $n = 10$ in CTRL and GMP group, $n = 8$ in OVA group; * $p < 0.0001$ compared to CTRL; $^+p < 0.0001$ compared to OVA

hyperplasia of goblet cells in the epithelium of airways and collagen fiber deposits between submucous and muscular layer were observed, as compared with CTRL animals. Treatment with GMP attenuated infiltration of inflammatory cells, goblet cell hyperplasia and subepithelial fibrosis

(Fig. 4a). Morphometry revealed 72 % lower number of eosinophils infiltrated in lung interstitium (Fig. 4b), a reduction of 63.48 % in goblet cells hyperplasia (Fig. 4c) and a 60.53 % lower collagen deposition (Fig. 4d) with GMP treatment as compared to OVA rats. These data

strongly suggest that GMP may inhibit airway inflammation and remodeling in asthma.

A correlation between TGF- β expression and the development of airway remodeling has been demonstrated in asthma [10]. In order to determine the effect of GMP treatment on TGF- β expression in our experimental model of allergic airway inflammation, we performed immunohistochemistry to measure the expression of this cytokine. The results were in line to those observed for collagen deposition and goblet cells hyperplasia. Figure 5a shows an increase in immunoreactive TGF- β localized in lung interstitium of OVA rats as compared to CTRL animals. Treatment with GMP clearly decreased TGF- β expression in the allergen-induced asthmatic lungs. To quantify the expression of TGF- β we measured positive immunoreactivity per area of lung interstitium. In the OVA group the mean of positive immunoreactivity was 2.6-fold higher than that of the CTRL group. When animals were treated with GMP, a 66.97 % reduction in positive immunoreactivity to TGF- β was obtained. So, consistent with the data of collagen deposit and goblet cell analysis, histological evaluation of TGF- β expression revealed that GMP treatment inhibits the development of airway remodeling in asthmatic rats.

Effect of GMP treatment on IL-5, IL-13, and IL-10 expression in pulmonary tissue

During allergic asthmatic inflammation, infiltrated inflammatory cells, lung epithelial cells, and resident lung macrophages are activated and release cytokines promoting a Th2 type immune response in the lungs [29]. To address the question whether GMP administration might modulate this Th2 response, we examined mRNA changes of IL-5 and IL-13 by qRT-PCR in lung tissues, as crucial Th2 cytokines in the pathophysiology of asthma. We found that IL-5 and IL-13 mRNAs were 3.68 and 2.48-fold higher in OVA group when compared to CTRL animals. Interestingly, GMP administration decreased in 59.17 and 47.01 % the expression of IL-5 and IL-13 as compared to OVA group (Fig. 6a and b).

Besides, we analyze mRNA changes of IL-10, one of the most important anti-inflammatory cytokines, which is normally decreased in patients with asthma [30]. As shown in Fig. 6c, the expression of IL-10 was significantly lower in OVA group as compared to CTRL animals but GMP treatment increased in 90.58 % the expression of this anti-inflammatory cytokine in lung. In fact, in animals treated with GMP mRNA levels of IL-5, IL-13, and IL-10 were not significantly different from that of control animals.

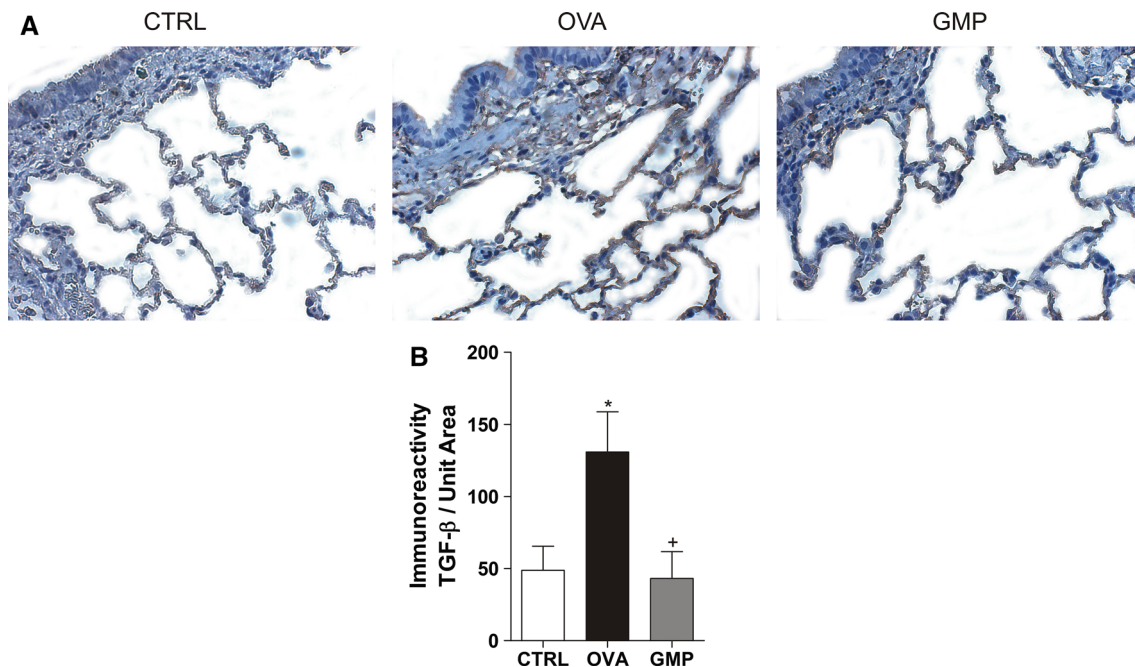


Fig. 5 Effect of GMP treatment on TGF- β expression in lung tissue. **a** Immunohistochemical analysis of TGF- β expression in lung tissues obtained at day 30 from normal and asthmatic rats, treated or not with GMP. Microphotographs are at $\times 400$ magnification. **b** Quantitative analysis of positive immunoreactivity to TGF- β per area (69,000 μm^2) of lung interstitium. Lung sections from five rats from

each experimental group were immunostained with TGF- β antibodies and analyzed under light microscopy ($\times 40$) followed by computer-based quantification using an image analyzing program. Three non-overlapping areas in each lung section were analyzed. Each value represents the mean $\pm$ standard error of the mean; * $p < 0.01$ compared to CTRL; + $p < 0.01$ compared to OVA

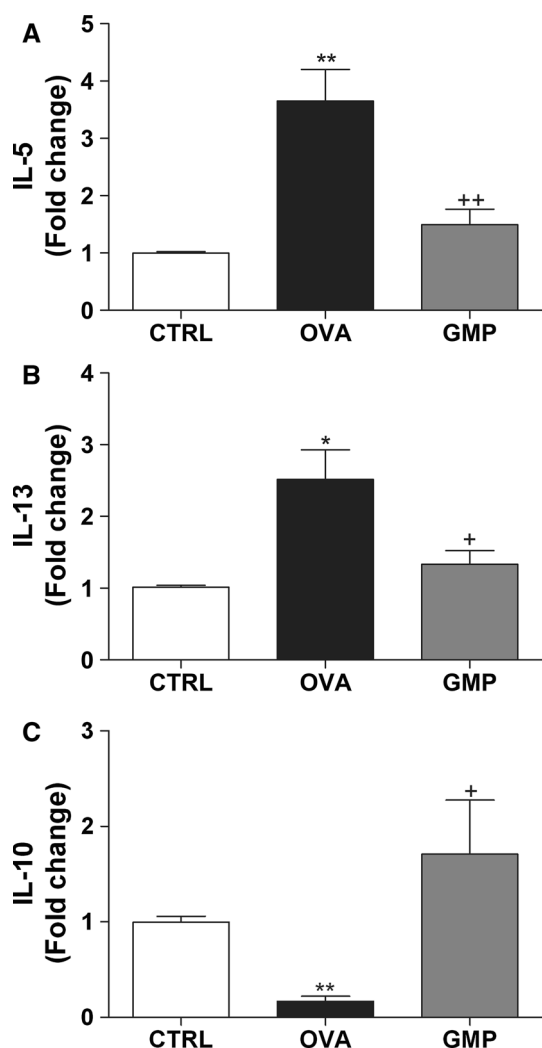


Fig. 6 Effect of GMP treatment on mRNA expression for IL-5, IL-13 and IL-10 in lung tissues. Changes in IL-5 (**a**), IL-13 (**b**) and IL-10 (**c**) mRNA expression relative to values from CTRL rats. Lung tissues were obtained at day 30 from normal and asthmatic rats, treated or not with GMP. Four rats from each experimental group were analyzed. Lung samples from each rat were run (in duplicated) as a single sample for qRT-PCR. Each value represents the mean $\pm$ standard error of the mean; * $p < 0.01$, ** $p < 0.001$ compared to CTRL; + $p < 0.05$, ++ $p < 0.005$ compared to OVA

Discussion

Due to the significant health and economic impact of asthma, new therapies that act on the immunological mechanisms of allergic diseases are required. GMP is a bioactive peptide derived from milk that has been demonstrated to prevent allergen sensitization and reduce the severity of early-phase reaction in cutaneous hypersensitivity and anaphylaxis in rats [25]. In the current study, we demonstrate that oral treatment with GMP decreases airway inflammation by strongly reducing level of IgE-allergen specific in serum, the number of

eosinophils in peripheral blood, total and differential leukocytes in BALF and infiltrating eosinophils in lung tissue. GMP also impacts on airway remodeling as suppresses goblet cells hyperplasia and collagen deposition, by reducing TGF- β expression. These effects are associated with an impaired Th2 response, as the treatment decreases the expression of IL-5 and IL-13 in lung tissue of asthmatic rats.

IgE levels are higher in patients with asthma than in non-atopic individuals [31, 32]. In our experimental model, high levels of OVA-specific IgE were quantified in serum of asthmatic rats. IgE synthesis is the result of Th2 differentiation and in asthmatic patients IgE production is highly dependent of IL-13 [33]. Lebrikizumab, a monoclonal antibody to IL-13, reduces the systemic levels of IgE in asthmatic patients with Th2 inflammation [34]. We show that treatment with GMP significantly reduces the levels of IgE specific to antigen in asthmatic rats. This is consistent with the evidence that GMP administration inhibits IL-13 production by splenocytes from systemically sensitized animals [25]. It is also known that IgE plays important roles in immediate and late phase in asthma, as well as airway remodeling, mainly through mast cells activation and eosinophil recruitment [27]. Histamine and serotonin, two mediators released from mast cells, play crucial roles in eosinophilia in mouse models of allergic asthma [35]. Besides, mice deficient in alpha chain of the high receptor for IgE from mast cells induce less eosinophilia in airway than normal mice when develop asthma [36]. The relation between IgE levels and eosinophilia in airways of asthmatic animals is in line with the reduced number of eosinophil in blood and BALF that we observed in GMP-treated animals, in which the levels of IgE anti-OVA are significantly decreased.

Goblet cells hyperplasia and collagen fiber deposits in airways are two features of remodeling in asthma [4]. These changes in airways are due to an ongoing chronic inflammatory process that involves activation of inflammatory cells. Eosinophils and neutrophils are crucial inflammatory cells related to tissue damage in asthma. Eosinophils secrete major basic protein, eosinophil cationic protein, eosinophil peroxidase and eosinophil-derived neurotoxin that cause tissue inflammation and damage to the lung tissue [37]. They are also one of the main cellular sources of TGF- β , a major mediator involved in fibrotic tissue remodeling within the asthmatic lung [38, 39]. Besides, cationic protein derived from eosinophils induces the synthesis by bronchial epithelial cells of a whole range of remodeling molecules, i.e. TGFs, matrix metalloproteinases, platelet-derived growth factor [40]. Neutrophils also release key mediators in the remodeling of asthmatic airways, as matrix metalloproteinase, elastase and myeloperoxidase [41]. We demonstrate that GMP

treatment reduces goblet cells hyperplasia and collagen deposition, which is in concordance with the decrease in number of eosinophils infiltrated in lung interstitium, in neutrophil percentage in BALF and in TGF- β expression in lung of treated animals.

Although several drugs have been developed to treat asthma, such as steroids, leukotriene inhibitors, mast cells stabilizers and β 2 adrenergic agonists, they are able to control symptoms although they do not modulate immunological dysregulation that characterizes allergic diseases [42]. Allergic asthma is caused by an immune response to allergens primarily mediated by Th2 cells. In our experimental model of asthma high mRNA levels of IL-5 and IL-13 are present in lung tissue. IL-5 is essential for the development of blood and pulmonary eosinophilia, as well as the subsequent onset of airways hyperreactivity and lung damage [7]. IL-13 is the major effector cytokine of goblet cells hyperplasia, airway hyper-responsiveness and inflammation in asthma [43]. We show that GMP administration induces a significant reduction in IL-5 and IL-13 lung expression, which is consistent with the decreased eosinophilia in blood and airway, as well as the attenuated hyperplasia of lung goblet cells observed in the animals. Previous studies show that GMP administration in rats treated with dimethyl hydrazine protects from the colonic damage, which is associated with an inhibition in the levels of IL-5 and other Th2 cytokines in serum and colonic tissue [22]. We have recently demonstrated that GMP treatment in allergen-sensitized animals attenuates the intensity of the immediate cutaneous reaction induced by allergen and protects animals from severe anaphylaxis; effects that are associated with a decrease in IL-13 secretion by splenocytes [25]. All together, these results indicate that GMP may ameliorate the progression of Th2-polarized pathologies by down-regulating the production of Th2 cytokines.

It is known the important regulatory role of IL-10 in airway inflammation [44]. Dendritic cells from patients suffering from allergic asthma show an impaired level of IL-10 [45]. Induction of functional IL-10 producing Tr1 cells or IL-10 secreting B regulatory cells is related to the tolerance to allergens [46, 47]. Besides, IL-10 together with TGF- β are implicated in the suppressive activity of naturally occurring lung CD4⁺ CD25⁺ T cells in lung allergic responses [48]. Our results show that GMP treatment to asthmatic animals increases IL-10 expression in lungs. Previous reports showed that basal secretion of IL-10 from splenocytes is greater when cells are incubated with GMP [21], corroborating that GMP may modulate IL-10 production. In addition, it has been demonstrated that IL-10 up-regulates the expression of inhibitory proteins in TGF- β signaling [49]. TGF- β is an anti-inflammatory cytokine that might control airway inflammation by inducing T cell

tolerance, but its excess in the airways can be pathological as promotes airway remodeling [39]. So, the beneficial effect of GMP in asthma might be mediated by the up-regulation of IL-10 expression in lung, which may contribute to suppress the inflammatory response mediated by Th2 cytokines or the pathological damage induced by TGF- β in lung. On the other hand, experimental studies in mice have demonstrated that the intake of some probiotics, such as *L. rhamnosus* GG, *L. casei rhamnosus* Lcr35, *B. lactis* and *L. reuteri*, suppresses the Th2 response and decreases airway hyper-responsiveness, number of inflammatory cells in BAL and lung inflammation in allergic asthma, suggesting a beneficial role of probiotics in asthma treatment and prevention [50–52]. As GMP can be detected in the duodenum shortly after milk or yogurt intake [53, 54], and it increases the number of *Bifidobacterium* and *Lactibacillus* in intestinal microbiota [18], the prophylactic action of GMP in asthma might be due to prebiotic effects. The precise mechanism of GMP protection on OVA-induced asthma is the aim of our current research.

Collectively, in this study, we found that GMP possesses prophylactic effect in the development of allergic asthma. GMP effectively suppresses serum allergen-specific IgE, percentage of eosinophils in blood, number of total and differential leukocytes in BALF and amount of eosinophils infiltrated in lung interstitium. Goblet cells hyperplasia and collagen deposition are also attenuated by GMP treatment. This effect is related to a decrease in TGF- β expression in lung tissue. Beneficial effect of GMP is associated with down-regulation of IL-5 and IL-13 together with up-regulation of IL-10 in asthmatic lung. Our findings support that GMP can prevent airway inflammation and remodeling associated with allergic asthma.

Acknowledgments This work was supported by grants PIBB 14-1 and 240921 from the Autonomous University of Aguascalientes and CONACYT, respectively. Renata Roldán has a master fellowship from CONACYT. The authors wish to thank Istvan Bercezi for reviewing the paper, Berenice Barrón for excellent technical assistance and MVZ José Luis Ponce for providing the animals for the study.

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