Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2016;171:217–226
DOI: 10.1159/000453035

Received: May 30, 2016
Accepted after revision: November 2, 2016
Published online: January 4, 2017

Novel Mechanisms Underlying the Therapeutic Effect of Glycomacropeptide on Allergy: Change in Gut Microbiota, Upregulation of TGF-β, and Inhibition of Mast Cells

Mariela Jiménez Daniel Cervantes-García Yualli Haydee Muñoz
Alejandra García Luis Miguel Haro Jr. Eva Salinas

Department of Microbiology, Basic Science Center, Autonomous University of Aguascalientes, Aguascalientes, Mexico

Keywords
Glycomacropeptide · Prebiotic action · Mast cells · Transforming growth factor · Antiallergic effect

Abstract
Background: The prevalence of allergic diseases is globally increasing. We have previously described that glycomacropeptide (GMP), a bioactive milk peptide, has therapeutic value in experimental models of skin hypersensitivity, anaphylaxis, and asthma, as it prevents an excessive T helper type 2 cell immune response. The aim of this study was to analyze the effect of GMP on key elements directly involved in the development or control of allergy, in order to improve the precise knowledge about its mechanism of action. ***Methods:*** Rats were systemically sensitized with ovalbumin and orally treated with GMP. Levels of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* were analyzed in their feces. Splenocytes were isolated and the production of transforming growth factor (TGF)-β by allergens was measured. Intradermal skin reactions were developed to evaluate in vivo activation of mast cells. Peritoneal mast cells were isolated and activated by the allergen, and histamine secretion was determined. ***Results:*** GMP administration increased the amount of intestinal *Lactobacillus* and *Bifidobacterium* of allergen-sensitized animals after 3 days of treatment. The increase in *Bacteroides* was also significant, but only after 17 days of GMP administration. Ten days after treatment cessation, *Lactobacillus* and *Bacteroides* were still elevated. GMP intake also elevated the production of TGF-β in the splenocytes of sensitized animals. In addition, treatment with GMP attenuated mast cell activation by the allergen and inhibited histamine secretion, without affecting the number of mast cells. ***Conclusions:*** The prebiotic action of GMP on allergy-protective microbiota, an increase in TGF-β production, and a reduction in mast cell response to allergens are novel mechanisms that explain the antiallergic activity of GMP.
© 2017 S. Karger AG, Basel

Introduction

Atopic diseases are inflammatory disorders caused by an aberrant T helper type 2 cell (Th2) immune response against common environmental allergens, which promotes the production of immunoglobulin E (IgE) and

Mariela Jiménez and Daniel Cervantes-García contributed equally to this study.

KARGER

© 2017 S. Karger AG, Basel

E-Mail karger@karger.com
www.karger.com/iaa

Correspondence to: Dr. Eva Salinas
Department of Microbiology, Basic Science Center
Autonomous University of Aguascalientes
Av. Universidad # 940, Aguascalientes 20131 (Mexico)
E-Mail emsalin@correo.uaa.mx

Downloaded by: A. Mari - 31298
Universita degli Studi, Bibl. Area Biomed. Tor Vergata
160.80.2.38 - 1/15/2017 6:02:33 PM

other chemical mediators [1]. We have previously suggested that glycomacropeptide (GMP) is a potential prophylactic agent for the control of allergic diseases [2, 3]. GMP is a peptide derived from the hydrolysis of milk κ-casein through the action of chymosin, physiologically in the stomach of neonates and industrially during the cheese-making process. It is composed of 64 amino acids and contains varying units of N-acetylneuraminic (sialic) acid [4]. GMP has an excellent safety record and is not immunogenic [5]. Our previous studies have shown that GMP administration prevents allergen sensitization by reducing allergen-specific IgE titers and suppressing the proliferation of splenocytes and their production of interleukin (IL)-13 in sensitized animals, which reduces the severity of immediate cutaneous reaction and anaphylaxis induced by allergens [2]. In asthmatic animals, GMP treatment avoids the development of an excessive Th2 response and attenuates airway inflammation and remodeling [3]. Other groups have also demonstrated that GMP may modulate Th2 response. In a model of dimethyl hydrazine-induced colonic damage, GMP prevented extensive injury by downregulating the levels of Th2 cytokines [6]. Although GMP has been reported as a modulator of the Th2 response, like some of the current treatments to allergy, further information about other alternative mechanisms are required to explain its antiallergic activity.

The suppressor cytokines IL-10 and transforming growth factor (TGF)-β play key roles in modulating inappropriate Th2 immune responses in allergic patients [7]. Our previous studies have shown that GMP does not modify IL-10 production in the splenocytes of sensitized animals, although IL-10 and TGF-β were over- and underexpressed in asthmatic lungs [2, 3]. However, it is known that TGF-β is a pleiotropic cytokine with potent regulatory capacity. It plays a very important role in the suppression of immune responses, particularly in the induction and maintenance of peripheral tolerance to allergens. TGF-β induces the expression of FOXP3 in naïve CD4+ T cells and is essential for in vivo expansion and immunosuppressive capacity of inducible regulatory T cells [8]. Besides, anti-inflammatory activity of GMP in experimental rat ileitis has been associated with increased levels of FOXP3 expression [9]. Therefore, it would be important to analyze the effect of GMP on systemic TGF-β production in allergen-sensitized animals.

Allergic etiology has been traditionally associated with genetic and environmental factors; however, the rapid increase in prevalence over the past decades has pointed to considering it to be highly dependent on environmental factors. Emerging evidence indicates that the composition and function of the gut microbiome modulates local and systemic immune activation, suggesting that resident microbial communities may influence the development of allergy [10]. Different studies have shown that members of the genera *Bacteroides, Lactobacillus*, and *Bifidobacterium* play protective roles in allergy [11–13]. GMP has growth-promoting effects on microbiota in vivo [4, 14]; however, the contribution of this prebiotic function in the antiallergic activity of GMP has not been previously examined.

Different studies have shown a direct modulatory action of GMP on the response of some immune cells. This peptide inhibits lipopolysaccharide and phytohemagglutinin-induced proliferation of mouse splenocytes and rabbit Peyer's patch cells [15], upregulates the expression of IL-1 receptor antagonist-like component in mouse lymphocytes [16] and suppresses the expression of IL-2 receptor on mouse CD4+ T cells [17]. It enhances the proliferation and the phagocytic activities of the human macrophage cell line U937 [18] and upregulates the secretion of tumor necrosis factor (TNF)-α, IL-1β, and IL-18 in human monocyte THP-1 cells [19]. However, there is no information about the effect of GMP on the activation of mast cells, which are one of the main effector cells in allergy.

The aim of this study was to investigate whether oral treatment with GMP can modulate gut microbiota composition, TGF-β production and mast cell activation by allergens in sensitized animals, so as to improve the understanding of the mechanisms that underlie antiallergic effects. Our results could make an important contribution to the practical application of GMP for the control of allergic diseases.

Material and Methods

Animals

Male Wistar rats (150–180 g) were used throughout the study, particularly, pathogen-free rats in the microbiota assays and conventionally raised rats in the other experiments. Animals were kept at room temperature (22–24°C) with a 12-h light/dark cycle, and fed with Rodent Laboratory Chow 5001 and tap water ad libitum. All experiments were carried out with strict adherence to ethical guidelines approved by the Institutional Normative Welfare Standards.

Experimental Design

Rats were randomly assigned to 3 different groups: the sham group (SH, control of sensitization), the sensitized group (S), and the sensitized and GMP-treated group (T). T group animals were orally administered with GMP (Lacprodan® CGMP-10; donated

Downloaded by: A. Mari - 31298
Universita degli Studi, Bibl. Area Biomed. Tor Vergata
160.80.2.38 - 1/15/2017 6:02:33 PM

Table 1. Oligonucleotides for real-time PCR reaction determination of microbiota

Target 16S rRNA gene	Oligonucleotides	Amplicon size, pb	Reference
Bacteroides	fwd: GGGGTTCTGAGAGGAAG rev: GCTACTTGGCTGGTTCAG	115	[20]
Lactobacillus	fwd: GCAGCAGTAGGGAATCTTCCA rev: GCATTYCACCGCTACACATG	340	[21]
Bifidobacterium	fwd: GATTCTGGCTCAGGATGAACGC rev: CTGATAGGACGCGACCCCAT	231	[22]

by Arla Foods Amba, Viby, Roskilde, Denmark) at a dose of 500 mg/kg/day, dissolved in tap water. Rats in the SH and S groups received an equivalent volume of tap water. To investigate the effect of GMP on TGF-β production and gut microbiota composition during allergen sensitization, GMP was administered to the animals from 3 days before sensitization to the end of the study. To analyze the effect of GMP on in vivo activation of mast cells, sensitized rats received GMP over 10 days, starting the treatment at day 11 of the sensitization protocol.

Experimental Sensitization Protocol

Ovalbumin (OVA) sensitization of animals was performed using a previously described method [2]. Briefly, at day 0, rats received an intramuscular injection of 1 mg of OVA (grade V; Sigma-Aldrich, St Louis, MO, USA) and 7.8 mg of aluminium hydroxide gel (Inject® Alum Thermo Scientific, Rockford, USA) in 1 mL of saline solution (s.s.) and a subcutaneous injection of 0.5 mL of *Bordetella pertussis* vaccine (1.2×10^{10} heat-killed bacilli/mL; Zurivac, Mexico City, Mexico), as an adjuvant. At day 7, a booster sensitization was given. SH rats were injected at the same time points with aluminium and the vaccine, but no OVA. Serum was collected from each rat at days 0 and 14 of sensitization, and stored at –80°C.

DNA Isolation from Stool Samples and qPCR Analysis

Total DNA from 100–200 mg of fecal samples was isolated using the ZR Fecal DNA Miniprep (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. DNA quantity and quality were determined with the absorbance at 260 nm and 260/280 nm ratio, respectively, with a Jenway 7305 spectrophotometer (Bibby Scientific, Stone, UK); samples with 260/280 ratios >1.8 were employed for quantitative real-time PCR. For microbiota determination, the 16S genes were targeted with the oligonucleotides listed in Table 1. The absolute standard curves were constructed as follows: after PCR with the Master Mix from Thermo Scientific (Waltham, MA, USA) using the following conditions: 95°C for 3 min, 35 cycles of 95°C for 1 min, 58°C for 30 s, and 72°C for 15 s, in a Mastercycler Personal (Eppendorf, Hamburg, Germany), amplicons were cloned with the pGEM T-Easy Vector System (Promega, Madison, WI, USA) in *Escherichia coli* DH5α. Plasmids isolations were performed with the PureYield Plasmid Miniprep System (Promega). DNA quantity and quality were then determined by spectrophotometry, as previously described. Absolute standard curves were constructed with serial dilutions of log 1–10 copies of 16S genes; limit detection was observed at log 3. Determination of number copies was performed using the GoTaq qPCR Master Mix (Promega) in an Eco real-time qPCR system (Illumina, San Diego, CA, USA).

Spleen Cells Isolation and TGF-β Determination

Spleens were removed aseptically from S and T group rats on day 14 of sensitization. Organs were perfused with cold s.s. and the cell suspension was centrifuged at 212 *g* for 10 min at 10°C, as previously described [23]. The cell suspension was depleted of erythrocytes by incubation in hypotonic lysis buffer (0.17 M Tris, 0.15 M NH_4Cl, pH 7.2) for 5 min on ice, washed twice in s.s. by centrifugation, and the pellet then suspended in RPMI-1640 without phenol red (Sigma-Aldrich), and supplemented with 5% fetal calf serum (FCS) (Invitrogen, Grand Island, NY, USA) and 1% penicillin/streptomycin (Sigma-Aldrich). Then 2×10^5 splenocytes/well were cultured in triplicate for 96 h with OVA (2 mg/mL) or concanavalin A (1 mg/mL, as a positive control) in a humidified incubator at 37°C and 5% CO_2. Basal secretion of TGF-β in cultured splenocytes was used as a negative control. Supernatants were collected and TGF-β levels were measured, according to the manufacturer's protocol, with a commercially available rat enzyme-linked immunosorbent assay kit (Bioscience, Vienna, Austria).

Intradermal Skin Reactions

Skin reactions were induced by intradermal challenge with OVA in rats from the SH, S, and T groups, as previously described [2]. Briefly, at the end of each treatment, reactions were elicited in the shaved dorsal skin after an intradermal injection of 31.2 μg of OVA in 50 μL of sterile s.s. in triplicate. As negative and positive controls, a similar volume of s.s. or histamine (2 mg) solution, respectively, was injected. Immediately, an intravenous injection of Evans blue (34 mg/kg) in 3% s.s. was performed. Thirty minutes after OVA challenge, rats were killed and skin samples with equal areas from the injected sites (blue spots) were removed and incubated with 1 mL of 1.0 N KOH at 37°C overnight. Then, 9 mL of a mixture of acetone and 0.6 N phosphoric acid (5:13) was added. The amount of extravasated dye was determined colorimetrically by measuring absorbance at 620 nm, using a standard curve (0.1–5 mg/mL Evans blue). To normalize data, the negative control value obtained from the same animal was subtracted from each experimental value.

Skin Histological Analysis

Dorsal skin samples of rats were obtained and fixed in 10% neutral formalin for 24 h. Paraffin-embedded, 5-μm-thick sections were stained with toluidine blue (0.015 M in 0.2 M citric acid). Three sections of each sample were analyzed. To evaluate the num-

Downloaded by: A. Mari - 31298
Universita degli Studi, Bibl. Area Biomed. Tor Vergata
160.80.2.38 - 1/15/2017 6:02:33 PM

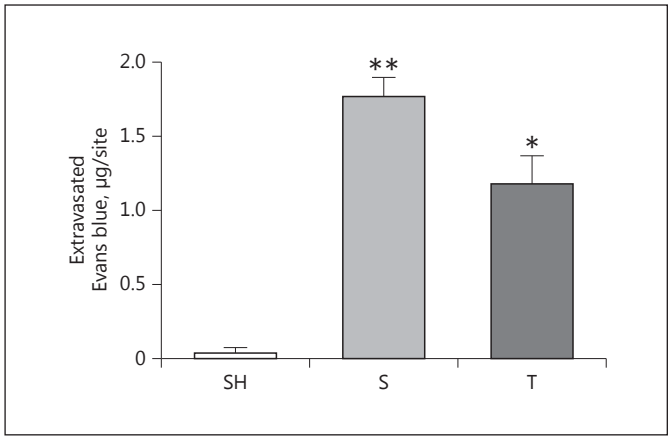

Fig. 1. Allergic skin reaction in experimental animals. Concentration of extravasated Evans blue after intradermal injection of 31.2 µg of OVA. $n = 3$, in triplicate. * $p < 0.05$, T versus S animals; ** $p < 0.0001$ S versus SH animals.

ber of mast cells, 2 randomly selected areas were observed at ×400 under light microscopy (Carl Zeiss AxioPlan, Göttingen, Germany) and analyzed using the program AxioVision Release v4.8. Results were reported as the number of mast cells/40,000 µm^2.

Purification and Activation of Peritoneal Mast Cells

Peritoneal mast cells were obtained from naïve rats by peritoneal lavage with 20 mL of HEPES-buffered Tyrode solution (HBT) at pH 7.4, supplemented with 1% bovine serum albumin (BSA). Mast cells were purified using a (30–80%) discontinuous Percoll gradient and those preparations with of 95–100% purity and viability >98% were used in further studies. Mast cells were suspended in RPMI-1640 without phenol red (Sigma-Aldrich) and supplemented with 5% FCS and 1% penicillin/streptomycin. To evaluate histamine secretion, 2 assays were developed. In 1 assay, 2×10^6 cells (in 2 mL) were incubated with IgE anti-dinitrophenol (DNP, 0.1 µg/mL) for 12 h at 37°C and 5% CO_2, and then preincubated with GMP (10, 50, or 100 µg/mL) or an equal volume of medium alone (used as a negative control) for 30 min. After 2 washes, cells were suspended at 4×10^5 cells/tube in HBT and stimulated (or not) with DNP-BSA (0.01 µg/mL) for 30 min at 37°C. In the other assay, 1×10^5 cells (in 500 µL) were stimulated with GMP (10, 50 and 100 µg/mL), media alone (basal secretion), or C48/80 (5 µg/mL, Sigma-Aldrich; as a positive control of cell response) at 37°C during 30 min. For both assays, cells were separated from the released histamine by centrifugation at 250 *g* for 3 min. After trichloroacetic acid precipitation, the histamine content in the supernatant and the pellet was measured by the o-phthaldialdehyde reaction [24] using an LS 50B luminescence spectrometer (PerkinElmer Co., Beaconsfield, UK). Histamine release was expressed as percentage of the total cellular histamine content calculated by the following formula: ([histamine in supernatant]/[histamine in supernatant + histamine in pellet]) × 100.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Student *t* test, except for the fecal microbiota analysis, where the Bonferroni multiple-comparisons test was used to analyze changes among groups at each time point. Differences were considered statistically significant when $p < 0.05$.

Results

Effect of GMP on Cutaneous Allergic Reaction

To demonstrate OVA sensitization in the experimental groups, an intradermal skin reaction was developed in the SH, S, and T animals. As shown in Figure 1, high levels of colorant were extravasated in sensitized animals after OVA injection. Besides, GMP treatment decreased significantly in 33% the amount of Evans blue extravasated after allergen challenge, corroborating the antiallergic effect of GMP that we previously described [2].

Effect of GMP on the Amount of Lactobacillus, Bifidobacterium, and Bacteroides in the Fecal Microbiota in Sensitized Animals

We analyzed changes in intestinal concentrations of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* in sensitized rats treated with GMP, as these genera of microbiota are related to the suppression of allergic diseases [11–13]. Differences in the amount of this fecal microbiota at different times before and during allergic sensitization are shown in Figure 2a–c. No significant differences in their counts were detected across groups before the beginning of the GMP treatment (day –3). *Lactobacillus* was present in a higher concentration than *Bifidobacterium* and *Bacteroides* in the fecal microbiota of free-pathogen Wistar rats (4.88×10^8 vs. 1.00×10^7 and 1.17×10^7 copies of 16S/100 mg of stool, as the mean of the amount obtained in all analyzed animals, $n = 18$). SH and S animals showed similar concentrations of the 3 bacterial genera in the fecal samples from day 0 to day 14, with no significant differences. However, after 3 days of GMP treatment, *Lactobacillus* and *Bifidobacterium* in the samples increased significantly, and this increase was maintained during the time that the animals received GMP. After 17 days of GMP administration (day 14 of sensitization), *Lactobacillus* and *Bifidobacterium* were 63.13-fold and 14.27-fold higher in GMP-treated animals than in sensitized but nontreated ones (S group) (Fig. 2a, b). The amount of *Bacteroides* remained unchanged during the first 10 days of GMP treatment, but after 17 days of administration (day 14) it was 104-fold higher than in the sensitized, nontreated animals (Fig. 2c). Ten days after treatment cessation, the amount of *Lactobacillus* and *Bac-*

Downloaded by: A. Mari - 31298
Universita degli Studi, Bibl. Area Biomed. Tor Vergata
160.80.2.38 - 1/15/2017 6:02:33 PM

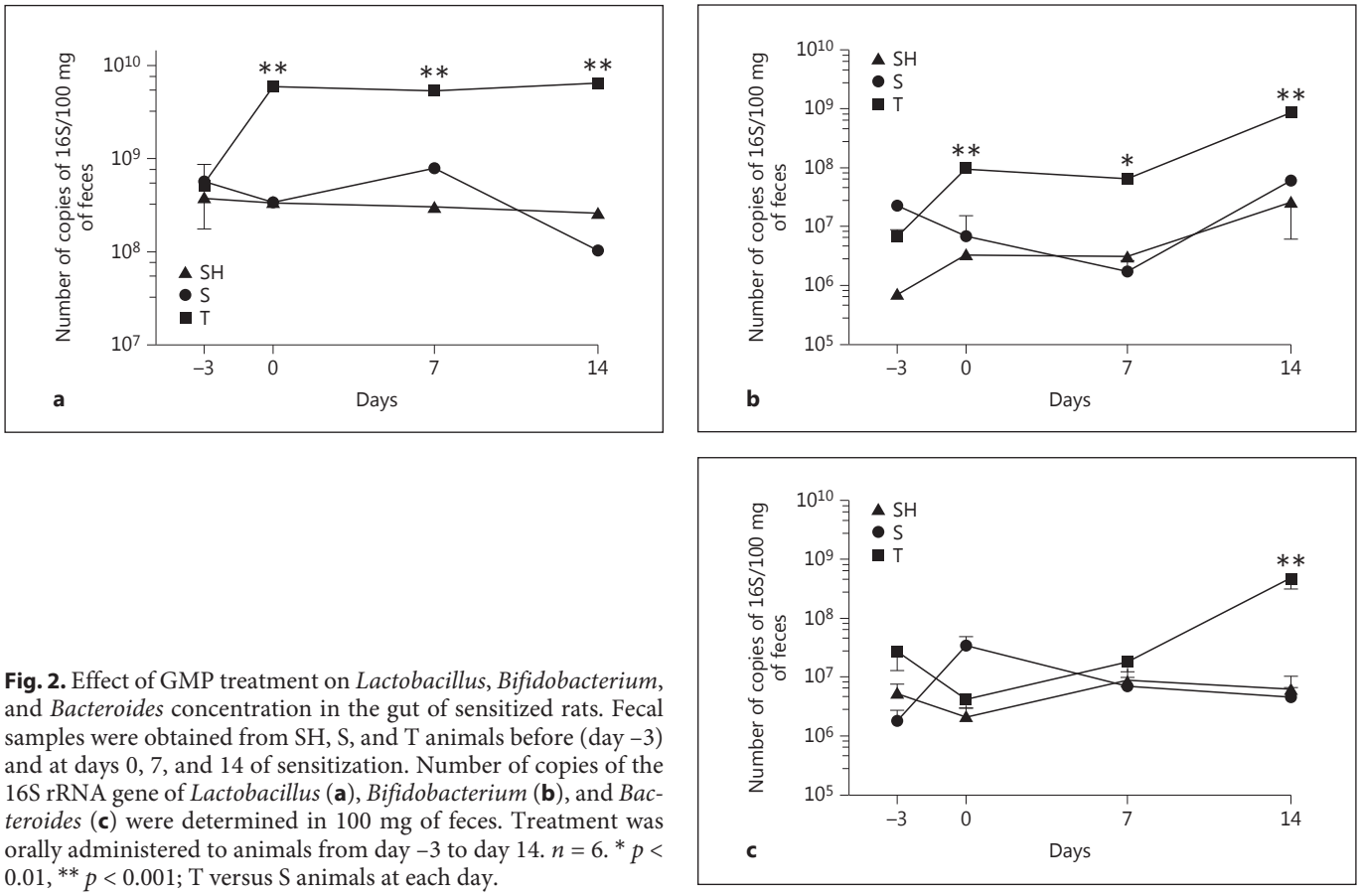

Fig. 2. Effect of GMP treatment on *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* concentration in the gut of sensitized rats. Fecal samples were obtained from SH, S, and T animals before (day –3) and at days 0, 7, and 14 of sensitization. Number of copies of the 16S rRNA gene of *Lactobacillus* (**a**), *Bifidobacterium* (**b**), and *Bacteroides* (**c**) were determined in 100 mg of feces. Treatment was orally administered to animals from day –3 to day 14. $n = 6$. * $p < 0.01$, ** $p < 0.001$; T versus S animals at each day.

teroides in the T group animals remained high with respect to the amount before treatment (3.8-fold and 4.29-fold higher, respectively), although with *Bacteroides*, this increase was not significant. However, the *Bifidobacterium* concentration in the fecal samples decreased profoundly, reaching values 3-fold lower than before GMP administration (Table 2).

Effect of GMP on TGF-β Production

In order to further elucidate the possible mechanisms responsible for the effects of GMP on systemic sensitization, spleen cells were obtained from sensitized rats with or without GMP treatment, and were stimulated with OVA or mitogen to detect TGF-β production. Oral treatment with GMP resulted in a significant increase in TGF-β levels with allergen-specific stimulation, but without changing TGF-β levels stimulated by mitogen (Fig. 3).

Effect of GMP on in vivo Activation of Mast Cells

To determine whether oral intake of GMP can modify in vivo activation of mast cells by allergens, rats were

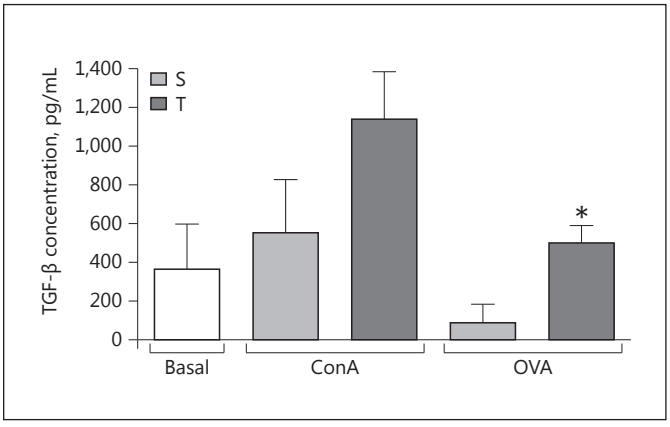

Fig. 3. Effect of GMP treatment on allergen-specific TGF-β production in spleen cell cultures from sensitized animals. Treatment was orally administered to animals from day –3 to day 14. Splenocytes from S or T animals were stimulated with OVA (2 mg/mL) or mitogen concanavalin A (ConA, 1 μg/mL). Basal represents TGF-β production in splenocytes without any stimulus. TGF-β was determined in supernatants after 96 h of stimulation by enzyme-linked immunosorbent assay. $n = 4$ spleens. * $p < 0.05$ T versus S stimulated with OVA.

Int Arch Allergy Immunol 2016;171:217–226
DOI: 10.1159/000453035

Downloaded by: A. Mari - 31298
Universita degli Studi, Bibl. Area Biomed. Tor Vergata
160.80.2.38 - 1/15/2017 6:02:33 PM

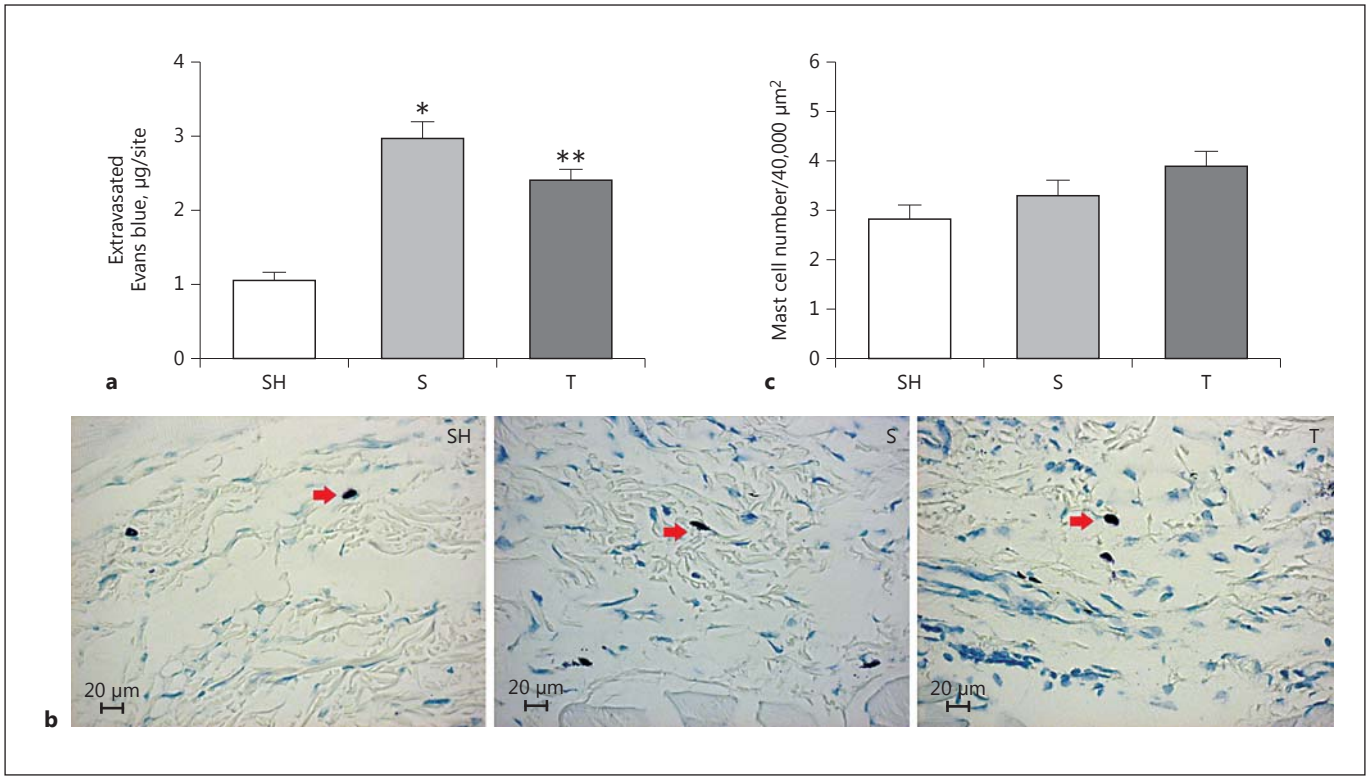

Fig. 4. Effect of GMP administration on mast cell activation (**a**) and number (**b**, **c**) in the skin of sensitized rats. Treatment was orally administered to animals over 10 days, beginning at day 11 of the sensitization protocol. **a** Mast cell activation was measured by intradermal injection of the allergen. Vascular extravasation induced by cell activation was measured by the Evans blue concentration in each cutaneous reaction. $n = 6$. * $p < 0.0001$ with respect to the SH group; ** $p < 0.05$ with respect to the S group. **b** Skin samples were obtained after 10 days of treatment, and sections from each group were stained with Toluidine blue for the visualization of mast cells. ×400. **c** Quantitative analysis of the number of mast cells/40,000 µm^2 of tissue. $n = 6$, in triplicate.

Table 2. Amount of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* (number of copies of 16S) in 100 mg of fecal samples obtained from sensitized animals before the GMP treatment and 10 days after cessation

Time point	*Lactobacillus*	*Bifidobacterium*	*Bacteroides*
Before treatment	1.21×10^9 ±0.21	4.63×10^6 ±2.36	5.82×10^7 ±2.64
Ten days after treatment cessation	4.60×10^9 ±0.01*	1.5×10^6 ±0.04	2.5×10^8 ±2.03

Values are presented as mean (±SEM). $n = 3$. * $p < 0.005$ at day 10 after cessation versus before treatment.

first sensitized and then GMP administration was started at day 11 of the sensitization protocol. At the end of the treatment, skin mast cells were activated by an intradermal skin reaction with OVA. High levels of colorant were extravasated in the sensitized animals in comparison to in the control (SH) rats (Fig. 4a). However, after receiving GMP over 10 days, there was a significant blockade in mast cell activation in response to the allergen, as extravasated Evans blue in treated animals was reduced by 20% compared to the sensitized nontreated animals. The effect of GMP on allergen-induced intradermal skin reaction in sensitized rats was not due to a reduction in the number of mast cells, because after 10 days of treatment there was no change in the number of these cells in the dermis compared to the sensitized nontreated animals (Fig. 4b, c).

Effect of GMP on Histamine Secretion from Mast Cells

Histamine is one of the main preformed substances released by mast cells after allergen activation, and is the most potent vasoactive mediator related to acute signs and symptoms of the early phase of allergy [25]. We first evaluated histamine secretion from resting peritoneal mast cells in the presence or absence of GMP. No change

Downloaded by: A. Mari - 31298
Universita degli Studi, Bibl. Area Biomed. Tor Vergata
160.80.2.38 - 1/15/2017 6:02:33 PM

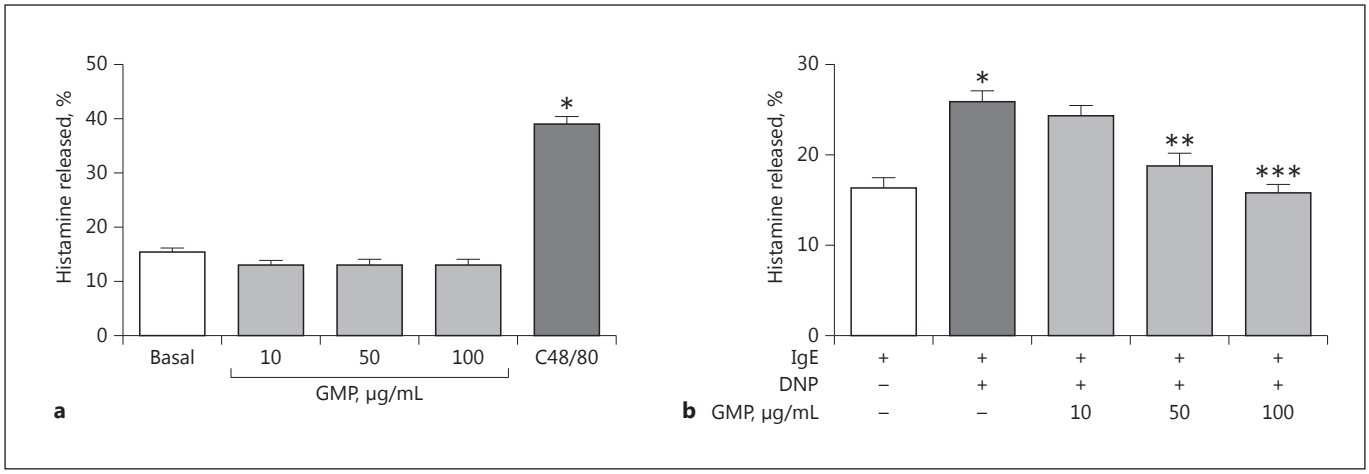

Fig. 5. Effect of GMP on in vitro mast cell histamine secretion. **a** Resting mast cells were incubated with GMP (10, 50, and 100 μg/mL). Basal represents histamine secretion without any stimulus. For a positive control of cell response, mast cells were stimulated with C48/80 (5 μg/mL). $n = 3$, assays in triplicate. * $p < 0.0001$ C48/80 versus control. **b** Sensitized (IgE anti-DNP, 0.1 μg/mL) peritoneal mast cells were preincubated with or without GMP (10, 50, and 100 μg/mL) and stimulated with or without the allergen (DNP, 0.01 μg/mL). $n = 3$, assays in triplicate. * $p < 0.0001$ $IgE^+DNP^+GMP^-$ versus $IgE^+DNP^-GMP^-$; ** $p < 0.01$ IgE^+DNP^+ GMP 50 μg/mL versus $IgE^+DNP^+GMP^-$; *** $p < 0.0001$ IgE^+DNP^+ GMP 100 μg/mL versus $IgE^+DNP^+GMP^-$.

was observed in basal histamine secretion (Fig. 5a), indicating that GMP does not directly activate mast cells or modify its basal secretion of histamine. Histamine secretion was then induced by the stimulation of sensitized IgE anti-DNP mast cells with DNP-BSA, in the presence or absence of GMP. In a dose-dependent manner, the IgE-induced histamine release from mast cells was inhibited by GMP at concentrations of 10–100 μg/mL (Fig. 5b), with the effect being significant at the 50- and 100-μg/mL concentrations, achieving a reduction in histamine release of 27.51 and 38.95%, respectively.

Discussion

GMP has been reported to have prophylactic effects in allergic sensitization and asthma development [2, 3]. Its administration downregulates allergen-specific IgE levels and IL-13 production by splenocytes, protecting animals from severe early-phase reactions in cutaneous reactions and anaphylaxis [2]. It also attenuates the airway inflammation and remodeling associated with asthma, as GMP intake reduces the infiltration of eosinophils, goblet cell hyperplasia and collagen deposition in the lungs of asthmatic rats. This effect goes with the downregulation of IL-5 and IL-13 expression and the upregulation of IL-10 expression in lung tissue [3]. Allergic disorders are multifactorial diseases and involve many humoral and cellular components, so we analyzed other factors and key elements directly involved in the development or control of allergy, in order to gain more precise knowledge about GMP antiallergic effects. In this study, we demonstrated that GMP administration prior and during sensitization induces a significant increase in the amount of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* in the gut of sensitized animals. Treatment with GMP also increased the production of TGF-β by splenocytes. In addition, GMP impacted mast cell function, inhibiting its activation and also the release of histamine in response to allergens, but it did not alter the number of mast cells.

There is considerable evidence indicating that dietary prebiotics modulate the intestinal microbiota with potential therapeutic applications to conditions such as allergy [10]. Growth-promoting effects of GMP on *Bifidobacterium* and *Lactobacillus* spp. have long been demonstrated in vitro [26, 27]; this was recently corroborated by Robitaille [28]. We showed that when sensitized animals were administered with GMP at a dose that has antiallergic effects, this increased the amount of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* in the gut. Our results are consistent with the study of Chen et al. [14], who used a small amount of GMP (0.1 mg/day), where the amount of *Lactobacillus* and *Bifidobacterium* was significantly increased in the fecal samples of mice. In addition, piglets receiving a diet supplemented with GMP over 15 days show in-

Downloaded by: A. Mari - 31298
Universita degli Studi, Bibl. Area Biomed. Tor Vergata
160.80.2.38 - 1/15/2017 6:02:33 PM

creased lactobacilli numbers in the ileum and proximal colon [29], and mice chronically fed a GMP-enriched diet have a significant increase in *Bacteroides* in their feces compared to those fed a casein-enriched diet [30]. In a series of prospective studies analyzing differences in the composition of the intestinal flora between allergic and nonallergic infants, colonization with *Bifidobacterium* and *Bacteroides* during the first years of life appeared to be associated with protection against allergy [11–13]. When *Lactobacillus rhamnosus* was administered prenatally to atopic mothers and postnatally to their infants over 6 months, the frequency of atopic eczema was reduced by half in the infants [31]. As GMP significantly increases concentrations of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides*, 3 bacterial genera related to protection in allergy, we suggest that GMP may exert its antiallergic effect, at least in part, by a prebiotic action. It is important to highlight that when treatment was stopped, the concentrations of *Lactobacillus* and *Bacteroides* remained elevated in the gut, indicating that the effect might be maintained for a time. Further studies are necessary to analyze the duration of this effect when GMP administration is interrupted.

TGF-β is a pleiotropic cytokine with a potent regulatory action on immune response. Specifically, it has a very important role in the induction and maintenance of peripheral tolerance to allergens [7]. Overexpression of TGF-β in OVA-specific CD4+ cells abolished the airway hyperresponsiveness and airway inflammation induced by OVA-specific Th2 cells in a murine model of allergic asthma [32]. Administration of anti-TGF-β antibody to sensitized mice before the transfer of CD4+ CD25+ T regulatory cells countered the suppressor effect of these cells on allergen-induced airway hyperresponsiveness [33], corroborating previous reports in which soluble and membrane-bound TGF-β is involved in airway tolerance induced by CD4+ T cells [34]. We demonstrated that the oral administration of GMP prior to and during sensitization increases the production of TGF-β by splenocytes in response to allergens, indicating that this cytokine may be participating in the immunoregulatory activity of GMP in allergy. Previous studies have demonstrated that supplementation with *Lactobacillus* and *Bifidobacterium* increase the amount of TGF-β detected in the gut [35–37], and also the expression of this regulatory cytokine in the spleens of allergen-sensitized mice [38] and the blood of allergic patients [39]. So, the increase in TGF-β production by allergen-specific splenocytes induced by GMP might be a consequence of its prebiotic action. As TGF-β is mainly produced by T and B regulatory cells, and both of these are involved in tolerance to allergens and suppression of the allergic process [7, 40], the effect of GMP treatment on regulatory cell subtypes is the aim of our current research.

Intradermal skin reaction is a valuable tool to analyze mast cell activation by allergens directly in the animal [41]. In this study, we demonstrated that GMP negatively modulates mast cell activation by allergens, as the intensity of the skin reaction was significantly decreased when animals received GMP orally. A smaller number of skin mast cells in GMP-treated animals might also cause this decrease, but we demonstrated that there was no change in the number of dermal mast cells. IgE levels may also modify the intensity of the skin reaction, but we administered GMP to animals after the last allergen challenge, i.e., once IgE production had been established [42]. Nevertheless, it has been reported that TGF-β can decrease skin mast cell degranulation induced by FcεRI cross-linking and also suppresses FcεRI and c-Kit expression [43, 44]. On the other hand, *L. rhamnosus* orally administered to rats has a systemic inhibitory action on cells, inhibits ex vivo peritoneal mast cell activation, and attenuates passive cutaneous anaphylaxis [45]. The prebiotic action of GMP on *Lactobacillus* and the upregulation of TGF-β production therefore coincide with its inhibitory action on mast cell activation by allergens. However, we cannot exclude a direct action of GMP on mast cell activation, as we demonstrated that GMP significantly inhibits histamine secretion from in vitro allergen-activated mast cells. It has been reported that mast cells express different sialic acid-binding immunoglobulin-type lectin receptors that can inhibit IgE degranulation and histamine release [46]. As GMP is a highly sialylated peptide [4] and can be detected in the bloodstream in significant amounts after ingestion [47], its modulatory action on mast cells may also be induced directly through sialic acid-binding immunoglobulin-type lectin receptors.

In summary, we found that GMP can regulate some key elements that protect from allergy. GMP administration prior and during sensitization induces prebiotic actions on beneficial microbiota in allergy and a higher production of TGF-β. It also negatively modulates mast cell activation and histamine secretion in response to allergens. These new findings provide more information about the mechanisms involved in the antiallergic activity of GMP.

Downloaded by: A. Mari - 31298
Universita degli Studi, Bibl. Area Biomed. Tor Vergata
160.80.2.38 - 1/15/2017 6:02:33 PM

Acknowledgements

This work was supported by grants PIBB 14-1 and PIBB 15-9N from the Autonomous University of Aguascalientes and 240921 from CONACYT. Cervantes-García is a Conacyt Research Fellow at the Autonomous University of Aguascalientes. We wish to thank Claudia Berenice Barrón for excellent technical assistance and Dr. Leopoldo Flores and MVZ José Luis Ponce for providing the animals for the study.

Disclosure Statement

The authors confirm that there are no conflicts of interest.

References

1 Galli SJ, Tsai M, Piliponsky AM: The development of allergic inflammation. Nature 2008; 454:445–454.
2 Jiménez M, Chávez NA, Salinas E: Pretreatment with glycomacropeptide reduces allergen sensitization, alleviates immediate cutaneous hypersensitivity and protects from anaphylaxis. Clin Exp Immunol 2012;170:18–27.
3 Roldán NR, Jiménez M, Cervantes-García D, Marín E, Salinas E: Glycomacropeptide administration attenuates airway inflammation and remodeling associated to allergic asthma in rat. Inflamm Res 2016;65:273–283.
4 Brody P: Biological activities of bovine glycomacropeptide. Br J Nutr 2000;841:S39–S46.
5 Mikkelsen TL, Rasmussen E, Olsen A, Barkholt V, Frøkiaer H: Immunogenicity of kappa-casein and glycomacropeptide. J Dairy Sci 2006;89:824–830.
6 Chen QS, Wang JF, Yan YL, Pang GC: Effects of casein glycomacropeptide on dimethyl hydrazine-induced alteration of cytokine network in rats. Food Science 2014;35:192–198.
7 Palomares O, Martín-Fontecha M, Lauener R, Traidl-Hoffmann C, Cavkaytar O, Akdis M, Akdis CA: Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-β. Genes Immun 2014;15:511–520.
8 Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM: Conversion of peripheral CD4+CD25– naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 2003;198:1875–1886.
9 Requena P, Daddaoua A, Martínez-Plata E, González M, Zarzuelo A, Suárez MD, de Sánchez Medina F, Martínez-Augustin O: Bovine glycomacropeptide ameliorates experimental rat ileitis by mechanisms involving downregulation of interleukin 17. Br J Pharmacol 2008;154:825–832.
10 Fujimura KE, Lynch SV: Microbiota in allergy and asthma and the emerging relationship with the gut microbiome. Cell Host Microbe 2015;17:592–602.
11 Björkstén B, Sepp E, Julge K, Voor T, Mikelsaar M: Allergy development and the intestinal microflora during the first year of life. J Allergy Clin Immunol 2001;108:516–520.
12 Kalliomäki M, Kirjavainen P, Eerola E, Kero P, Salminen S, Isolauri E: Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. J Allergy Clin Immunol 2001;107:129–134.
13 Penders J, Stobberingh EE, van den Brandt PA, Thijs C: The role of the intestinal microbiota in the development of atopic disorders. Allergy 2007;62:1223–1236.
14 Chen Q, Cao J, Jia Y, Liu X, Yan Y, Pang G: Modulation of mice fecal microbiota by administration of casein glycomacropeptide. Microbiology Res 2012;3:8–12.
15 Otani H, Monnai M: Induction of an interleukin-1 receptor antagonist-like component produced from mouse spleen cells by bovine kappa-caseinoglycopeptide. Biosci Biotechnol Biochem 1995;59:1166–1168.
16 Monnai M, Otani H: Effect of bovine kappa-caseinoglycopeptide on secretion of interleukin-1 family cytokines by P388D1 cells, a line derived from mouse monocyte/macrophage. Milchwissenschaft 1997;52:192–196.
17 Otani H, Horimoto Y, Monnai M: Supression of interleukin-2 receptor expression on mouse CD4+ T cells by bovine kappa-caseinoglycopepetide. Biosci Biotechnol Biochem 1996;60:1017–1019.
18 Li EW, Mine Y: Immunoenhancing effects of bovine glycomacropeptide and its derivatives on the proliferative response and phagocytic activities of human macrophagelike cells, U937. J Agric Food Chem 2004;52:2704–2708.
19 Requena P, Daddaoua A, Guadix E, Zarzuelo A, Suárez MD, Sánchez de Medina F, Martínez-Augustin O: Bovine glycomacropeptide induces cytokine production in human monocytes through the stimulation of the MAPK and the NF-κB signal transduction pathways. Br J Pharmacol 2009;157:1232–1240.
20 Verma R, Verma AK, Ahuja V, Paul J: Real-time analysis of mucosal flora in patients with inflammatory bowel disease in India. J Clin Microbiol 2010;48:4279–4282.
21 Walter J, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP: Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol 2001;67:2578–2585.
22 Gueimonde M, Tölkkö S, Korpimäki T, Salminen S: New real-time quantitative PCR procedure for quantification of bifidobacteria in human fecal samples. Appl Environ Microbiol 2004;70:4165–4179.
23 Loomis RJ, Marshall LA, Johnston PV: Sera fatty acid effects on cultured rat splenocytes. J Nutr 1983;113:1292–1298.
24 Shore PA, Burkhalter A, Cohn VH: A method for the fluorometric assay of histamine in tissues. J Pharmacol Exp Ther 1959;127:182–186.
25 Ohtsu H: Pathophysiologic role of histamine: evidence clarified by histidine decarboxylase gene knockout mice. Int Arch Allergy Immunol 2012;158:2–6.
26 Azuma N, Yamauchi K, Mitsuoka T: Bifidus growth-promoting activity of a glycomacropeptide derived from human κ-casein. Agric Biol Chemi 1984;48:2159–2162.
27 Bezkorovainy A, Grohlich D, Nichols JH: Isolation of a glycopolypeptide fraction with *Lactobacillus bifidus* subspecies *Pennsylvanicus* growth-promoting activity from whole human milk casein. Am J Clin Nutr 1979;32: 1428–1432.
28 Robitaille G: Growth-promoting effects of caseinomacropeptide from cow and goat milk on probiotics. J Dairy Res 2013;80:58–63.
29 Hermes R, Molist F, Francisco Pérez J, Gómez de Segura A, Ywazaki M, Davin R, Nofrarías M, Korhonen TK, Virkola R, Martín-Orúe SM: Casein glycomacropeptide in the diet may reduce *Escherichia coli* attachment to the intestinal mucosa and increase the intestinal lactobacilli of early weaned piglets after an enterotoxigenic *E. coli* K88 challenge. Br J Nutr 2013;109:1001–1012.
30 Sawin EA, De Wolfe TJ, Aktas B, Stroup BM, Murali SG, Steele JL, Ney DM: Glycomacropeptide is a prebiotic that reduces *Desulfovibrio* bacteria, increases cecal short-chain fatty acids, and is anti-inflammatory in mice. Am J Physiol Gastrointest Liver Physiol 2015; 309:G590–G601.
31 Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E: Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet 2001;357:1076–1079.

Downloaded by: A. Mari - 31298
Universita degli Studi, Bibl. Area Biomed. Tor Vergata
160.80.2.38 - 1/15/2017 6:02:33 PM

32 Hansen G, McIntire JJ, Yeung VP, Berry G, Thorbecke GJ, Chen L, DeKruyff RH, Umetsu DT: CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. J Clin Invest 2014;105:61–70.

33 Joetham A, Takeda K, Taube C, Miyahara N, Matsubara S, Koya T, Rha YH, Dakhama A, Gelfand EW: Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta. J Immunol 2007;178:1433–1442.

34 Ostroukhova M, Seguin-Devaux C, Oriss TB, Dixon-McCarthy B, Yang L, Ameredes BT, Corcoran TE, Ray A: Tolerance induced by inhaled antigen involves CD4(+)T cells expressing membrane-bound TGF-beta and FOXP3. J Clin Invest 2004;114:28–38.

35 Chen CC, Louie S, Shi HN, Walker WA: Preinoculation with the probiotic *Lactobacillus acidophilus* early in life effectively inhibits murine *Citrobacter rodentium* colitis. Pediatr Res 2005:58;1185–1191.

36 Foye OT, Huang IF, Chiou CC, Walker WA, Shi HN: Early administration of probiotic *Lactobacillus acidophilus* and/or prebiotic inulin attenuates pathogen-mediated intestinal inflammation and Smad 7 cell signaling. FEMS Immunol Med Microbiol 2012;65:467–480.

37 Schiavi E, Barletta B, Butteroni C, Corinti S, Boirivant M, Di Felice G: Oral therapeutic administration of a probiotic mixture suppresses established Th2 responses and systemic anaphylaxis in a murine model of food allergy. Allergy 2011;66:499–508.

38 Toomer OT, Ferguson M, Pereira M, Do A, Bigley E, Gaines D, Williams K: Maternal and postnatal dietary probiotic supplementation enhances splenic regulatory T helper cell population and reduces ovalbumin allergen-induced hypersensitivity responses in mice. Immunobiology 2014;21:367–376.

39 Inoue Y, Kambara T, Murata N, Komori-Yamaguchi J, Matsukura S, Takahashi Y, Ikezawa Z, Aihara M: Effects of oral administration of *Lactobacillus acidophilus* L-92 on the symptoms and serum cytokines of atopic dermatitis in Japanese adults: a double-blind, randomized, clinical trial. Int Arch Allergy Immunol 2014;165:247–254.

40 Braza F, Chesne J, Castagnet S, Magnan A, Brouard S: Regulatory functions of B cells in allergic diseases. Allergy 2014;69:1454–1463.

41 Inagaki N, Nagai H: Analysis of the mechanism for the development of allergic skin inflammation and the application for its treatment: mouse models for the development of remedies for human allergic dermatitis. J Pharmacol Sci 2009;110:251–259.

42 Salinas E, Quintanar JL, Ramírez-Celis NA, Quintanar-Stephano A: Allergen-sensitization in vivo enhances mast cell-induced inflammatory responses and supports innate immunity. Immunol Lett 2009;127:48–54.

43 Zhao W, Gomez G, Yu SH, Ryan JJ, Schwartz LB: TGF-beta1 attenuates mediator release and de novo Kit expression by human skin mast cells through a Smad-dependent pathway. J Immunol 2008;181:7263–7272.

44 Yamazaki S, Nakano N, Honjo A, Hara M, Maeda K, Nishiyama C, Kitaura J, Ohtsuka Y, Okumura K, Ogawa H, Shimizu T: The transcription factor Ehf is involved in TGF-β-induced suppression of FcεRI and c-Kit expression and FcεRI-mediated activation in mast cells. J Immunol 2015;195:3427–3435.

45 Forsythe P, Wang B, Khambati I, Kunze WA: Systemic effects of ingested *Lactobacillus rhamnosus*: inhibition of mast cell membrane potassium (IKCa) current and degranulation. PLoS One 2012;7:e41234.

46 Yokoi H, Choi OH, Hubbard W, Lee HS, Canning BJ, Lee HH, Ryu SD, von Gunten S, Bickel CA, Hudson SA, Macglashan DW Jr, Bochner BS: Inhibition of FcεRI-dependent mediator release and calcium flux from human mast cells by sialic acid-binding immunoglobulin-like lectin 8 engagement. J Allergy Clin Immunol 2008;121:499–505.

47 Chabance B, Marteau P, Rambaud JC, Migliore-Samour D, Boynard M, Perrotin P, Guillet R, Jollès P, Fiat AM: Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. Biochimie 1998;80:155–165.

Downloaded by: A. Mari - 31298
Universita degli Studi, Bibl. Area Biomed. Tor Vergata
160.80.2.38 - 1/15/2017 6:02:33 PM