Biochemical Pharmacology 79 (2010) 1797–1804

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The intestinal antiinflammatory agent glycomacropeptide has immunomodulatory actions on rat splenocytes

Pilar Requena [a], Raquel González [b], Rocío López-Posadas [b], Ana Abadía-Molina [c], María Dolores Suárez [a], Antonio Zarzuelo [b], Fermín Sánchez de Medina [b], Olga Martínez-Augustin [a,*]

[a] Department of Biochemistry and Molecular Biology II, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), University of Granada, Campus de Cartuja s/n, 18071 Granada, Spain
[b] Department of Pharmacology, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), University of Granada, Campus de Cartuja s/n, 18071 Granada, Spain
[c] Department of Biochemistry and Molecular Biology I, University of Granada, 18071 Granada, Spain

ARTICLE INFO

Article history:
Received 13 November 2009
Accepted 16 February 2010

Keywords:
Glycomacropeptide
STAT4
Inflammatory bowel disease
Th1
Treg
Foxp3

ABSTRACT

Bovine glycomacropeptide (GMP) is an immunologically active milk peptide that is a part of the normal human diet. GMP has therapeutic value in preclinical models of intestinal inflammation, and its mechanism may be related to effects on lymphocytes. This study focuses on the actions of GMP on rat splenocytes in vitro and in vivo. Bovine serum albumin and lactoferrin were used for comparative purposes. GMP (0.01–0.1 mg mL^{-1}) enhanced Concanavalin A (ConA) evoked but not basal splenocyte proliferation. At 1 mg mL^{-1} GMP lost this effect but augmented basal TNF-α secretion and also iNOS and COX2 expression. IFN-γ, IL-2 and IL-17 were not affected by GMP in quiescent splenocytes, but IL-10 was augmented at all concentrations tested. On the other hand, GMP produced a marked inhibitory effect (70%) on IFN-γ secretion and to a lower extent (50%) also on TNF-α. GMP was shown to block STAT4 but not IκB-α phosphorylation. The Treg marker Foxp3 was markedly upregulated by GMP. Bovine serum albumin had some effects on splenocyte function which were of lower magnitude and not entirely coincidental, while lactoferrin had a strong antiproliferative effect, as expected, indicating a specific effect of GMP. When administered for 3 days to normal Wistar rats, GMP reproduced the Foxp3 induction effect observed previously in vitro. This was observed in splenocytes but not in thymocytes, and only when administered by the oral rather than the intraperitoneal route. Thus our results support the hypothesis that GMP may limit intestinal inflammation acting at least in part on lymphocytes.

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1. Introduction

Glycomacropeptide (GMP), also known as κ-caseinglycopeptide, is one of the biologically active components of milk [1,2]. GMP is the N-acetylneuraminic (sialic) acid containing, N-terminal 64-aminoacid peptide of κ-casein. GMP is a native component of milk, but it is produced mainly by chymosin/pepsin mediated proteolysis of the parent protein during the digestion of milk. Thus GMP is normally released in the newborn and adult human gastrointestinal tract after milk ingestion [3,4]. In addition, GMP is obtained in large quantities as a byproduct of the cheese making process, as part of the resulting milk whey [2]. GMP (of bovine origin) has nutritional and industrial value, as it is currently added to infant formulas and, due to its low content on aromatic aminoacids, including an absolute absence of phenylalanine, it has been proposed to be useful in the elaboration of products for individuals with phenylketonuria [22]. In addition, GMP is included in tooth pastes because of its anticariogenic properties [23].

In the last decades it has become clear that milk proteins have more than just nutritional value, and the biological effects of GMP have been studied by several research groups [5]. GMP has been reported to bind cholera and *Escherichia coli* enterotoxins, to inhibit bacterial and viral epithelial adhesion, to promote bifidobacterial growth and to modulate the immune system response [2,6,7]. We have found that oral administration of GMP results in substantial antiinflammatory effects in experimental colitis and ileitis [8,9]. The mechanism of action has not been unequivocally established, although inhibition of IL-17 expression was found in the ileitis study [9]. Some in vitro studies have described that GMP inhibits mouse splenocyte proliferation induced by LPS and phytohemagglutinin [11], suppresses the IL-2 receptor expression on mouse $CD4^{+}$ T cells [5], induces the expression of an interleukin-1 receptor

* Corresponding author at: Department of Biochemistry and Molecular Biology II, CIBERehd, School of Pharmacy, University of Granada, Campus de Cartuja s/n, 18071 Granada, Spain. Tel.: +34 958 242335; fax: +34 958 248960.
E-mail address: omartine@ugr.es (O. Martínez-Augustin).

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doi:10.1016/j.bcp.2010.02.008

antagonist-like component in mouse spleen cells [12] and suppresses serum IgG antibody production in mouse lymphocytes [13]. In macrophages, it has been shown that GMP modulates the secretion of IL-1 family cytokines in a mouse monocytic cell line [14] and that it enhances proliferation and phagocytic activities of human macrophagelike cells [15]. Also we recently found that GMP enhances TNF-α, IL-8 and IL-1β production in the human monocytic cell line THP-1 and in primary monocytes, via activation of the NF-κB and MAP kinase pathways [16]. The contribution of these actions on macrophages to the intestinal antiinflammatory effect of GMP is unclear at the moment. Conversely, our in vivo data and previous results obtained in vitro by other groups suggest that lymphocytes may be involved. Thus we set out to further explore this possible mechanism of action by studying in vitro and in vivo effects of GMP on rat splenocytes.

This study demonstrates that GMP inhibits the expression of TNF-α and IFN-γ in Concanavalin A (ConA) stimulated splenocytes while enhancing the expression of Foxp3 and IL-10 secretion in quiescent cells.

2. Materials and methods

2.1. Reagents

Except where indicated, all reagents were obtained from Sigma (Barcelona, Spain). The phosho-IκB-α (Ser32) antibody was purchased from Cell Signaling Technology (Boston, MA, USA); the JLA20 antibody against actin developed by Dr. Lin [17] was obtained from the Development Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA); the COX2 antibody was purchased from Cayman Chemical Company (Ann Arbor, MI, USA); the iNOS and phosphospecific STAT4 antibodies were purchased from BD Biosciences Pharmingen (San Jose, CA, USA). GMP (BioPURE-GMP™) was the kind gift of Davisco Foods International (Eden Prairie, MN). According to the manufacturer, the GMP content is 93% while fat and lactose contents comprise 0.2 and <1%, respectively. Casoplatelin (CAS, NH_2–MAIPPKKNQDK–COOH) was synthesized with a purity >95% by Innovagen (Lund, Sweden).

2.2. Spleen and thymus mononuclear cell isolation

Female Wistar rats were obtained from the Laboratory Animal Service of the University of Granada, sacrificed by cervical dislocation and the spleen and thymus were extracted aseptically. Cell suspensions were obtained by disrupting the tissues between dissecting forceps in medium. After centrifuging, cells were cleared of erythrocytes (spleen only) by suspension on hypotonic lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA·2H_2O$, pH = 7.3) for 30 min on ice. Mononuclear cells were washed and suspended in RPMI medium supplemented with 10% FBS, 100 mg l^{-1} streptomycin, 100,000 U l^{-1} penicillin, 2.5 mg l^{-1} amphotericin B and 0.05 mM of mercaptoethanol.

2.3. Trypan blue assay

Cell viability was quantitated with the Trypan blue exclusion assay. Briefly, cells were analyzed as suspensions of 10^6 cells mL^{-1} in RPMI medium in the presence or absence of GMP, bovine serum albumin (BSA) or lactoferrin (LF) 0.01, 0.1 or 1 mg mL^{-1} or equivalent CAS concentrations for 48 h. Cell suspensions were then diluted 1:2 in 0.4% Trypan blue in PBS, incubated 2 min while shaking, and viable (not blue) and total cells were counted.

2.4. Cell proliferation assay

For the thymidine uptake experiments, 2×10^6 cells mL^{-1} were incubated in RPMI medium for 48 h with GMP, BSA or LF 0.01, 0.1 or 1 mg mL^{-1} and [^{3}H]-thymidine (1 μCi mL^{-1}; GE Healthcare, Spain) in the presence of ConA (5 μg mL^{-1}). The cells were then harvested, washed 3 times with trichloroacetic acid (10%, v v^{-1}), suspended in lysis buffer (1%, w v^{-1} SDS, 0.3 N NaOH) for 30 min at room temperature and collected into plastic vials. Then 4 mL of scintillation liquid (Beckman Coulter, Madrid, Spain) per vial were added and the amount of [^{3}H]-thymidine incorporated was measured with a Tri-Carb liquid scintillation analyzer (Packard Instrument, Meriden, CT).

2.5. Cytokine determination

For cytokine determinations cell suspensions (10^6 cells mL^{-1} in RPMI medium) were cultured as above (without [^{3}H]-thymidine) for 48 h. Cells were also incubated with equivalent casoplatelin (CAS) concentrations. The supernatants collected after gentle centrifugation (3000 × g, 5 min, 4 °C) were kept at −80 °C until cytokine concentration was measured by ELISA kits (Biosource Europe, Nivelles, Belgium and BD Biosciences, Erembodegem, Belgium), following the protocols recommended by the manufacturers.

2.6. Western blot

For cytoplasmic protein expression experiments, 5×10^6 cells mL^{-1} were harvested, washed once with PBS and total protein content was extracted with RIPA buffer (0.1% sodium dodecylsulfate, 0.1% sodium deoxycholate, 1% Triton X-100 in phosphate buffered saline, with freshly added protease inhibitors—phenylmethylsulfonyl fluoride, aprotinin, leupeptin, 1,10-phenanthroline). For STAT4 detection, nuclear proteins were extracted using the Nuclear Extract kit (Active Motif Europe, Rixensart, Belgium) following the kit instructions. Protein was quantitated with the bincinchoninic acid method using bovine serum albumin as standard, and samples were boiled for 5 min in Laemmli buffer, separated by SDS-PAGE, electroblotted to PVDF membranes (Millipore, Madrid, Spain), and probed with the corresponding antibodies. The bands were detected by enhanced chemiluminescence (PerkinElmer, Waltham, MA) and quantitated with NIH software (Scion Image). The composition of the Laemmli buffer (5×) was: 312 nM SDS, 50% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 22.5 mM EDTA trisodium salt, 220 mM Tris and traces of bromphenol blue (pH = 6.8).

2.7. RT-qPCR

The expression of IFN-γ and TNF-α was examined by reverse transcriptase (RT)-qPCR. For the RT-qPCR analysis of total RNA was extracted with Trizol (Invitrogen, Barcelona, Spain). 1 μg of RNA per sample was subjected to reverse transcription using the First-strand cDNA synthesis kit (GE Healthcare, Barcelona, Spain). Real time qPCR was performed using 2 μl of cDNA for a final PCR reaction volume of 25 μl (Sybr-green, Biorad). The expression of the ribosomal 18 S unit was examined as a loading standard. The primers used were: TNF-α (sense 5′-TAC TGA ACT TCG GGG TGA TTG GTC C-3′; antisense 5′-CAG CCT TGT CCC TTG AAG AGA ACC-3′); IFN-γ (sense 5′-TTC ATT GAC AGC TTT GTG CTG G-3′; antisense 5′-AAC AGT AAA GCA AAA AAG GAT GCA TT-3′); ribosomal 18 S unit (sense 5′-CCA TTG GAG GGC AAG TCT GGT G-3′; antisense 5′-CGC CGG TCC AAG AAT TTC ACC-3′).

2.8. In vivo animal treatment

Female Wistar rats (185–215 g) obtained from the Laboratory Animal Service of the University of Granada were used, housed in

makrolon cages and maintained in our laboratory in air conditioned animal quarters with a 12 h light–dark cycle. Animals were provided with free access to tap water and food (Panlab A04, Panlab, Barcelona, Spain). Rats were randomly assigned to 4 different groups ($n = 3$): the control oral (Cor) and intraperitoneal (Cip) groups, and the oral (Gor) and intraperitoneal (Gip) GMP groups. The GMP groups received GMP (500 mg kg^{-1} day^{-1}) either in 1% methylcellulose p.o. or in saline solution i.p., while the control groups received the vehicle every day. Animals received the treatments for 3 days and then were sacrificed by cervical dislocation. Then their splenic and thymus cells were examined for cytokine secretion (after 48 h) and Foxp3 expression (without incubation). In a separate experiment, rats were treated as above and splenocytes examined for cytokine/Foxp3 expression at the mRNA level after only 5 h of incubation. Cells were studied in the presence or absence of ConA (5 μg mL^{-1}). This study was carried out in accordance with the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of the European Union (86/609/EEC).

2.9. Statistical analysis

All results are expressed as mean ± SEM. Differences among means were tested for statistical significance by one way ANOVA and a posteriori least significance tests on preselected pairs. All analyses were carried out with the SigmaStat 2.03 program (Jandel Corporation, San Rafael, CA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. GMP has proliferative effects on ConA-stimulated but not unstimulated splenocytes

GMP was initially tested for effects on cell viability and proliferation in the rat splenocyte population. As shown in Fig. 1, GMP (0.01–1 mg mL^{-1}) had no effect on splenocyte thymidine uptake in basal conditions. As expected, ConA evoked a robust proliferative response, which was significantly enhanced by GMP at 0.01–0.1 mg mL^{-1} but not with 1 mg mL^{-1}. As reported before [27], LF strongly inhibited ConA-stimulated proliferation, but it also had no effect in basal conditions. BSA, used as a control, was inactive. In addition, GMP had no significant impact on splenocyte viability (not shown).

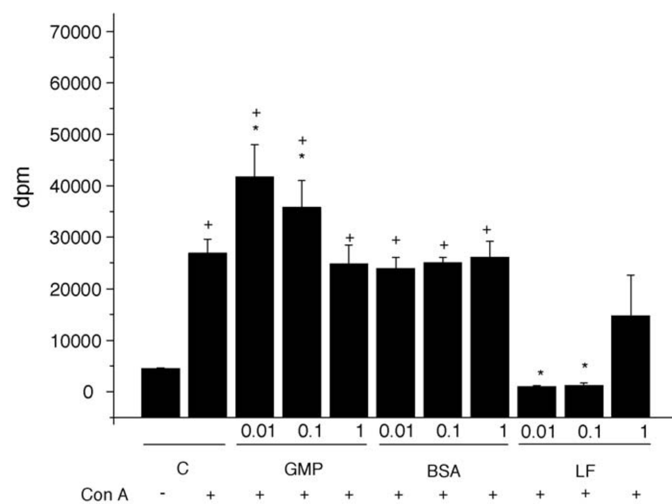

Fig. 1. Effect of glycomacropeptide (GMP), casoplateline (CAS) bovine serum albumin (BSA) and lactoferrin (LF) on splenocyte proliferation, assessed by the thymidine uptake assay. Cells were incubated with GMP, BSA or LF 0.01, 0.1 and 1 mg mL^{-1} in absence or presence of Concanavalin A (ConA, 5 μg mL^{-1}). [^{3}H]-Thymidine was added at the same time and uptake was measured 48 h after. Data are expressed as mean ± SEM; all ConA-stimulated cells were different from the corresponding ConA free cells. $^*p < 0.05$ vs. ConA-stimulated cells. The pooled (normalized) results from 2 different experiments are shown.

3.2. GMP modulates cytokine production by primary splenocytes

GMP did not change basal IL-2 or IFN-γ release in naive splenocytes, but it increased TNF-α production markedly at the highest concentration assayed, 1 mg mL^{-1} and, interestingly, it also augmented IL-10 production at all concentrations tested (data not shown and Fig. 3). There was no significant effect on IL-17 expression at the mRNA level (data not shown). The effect of GMP was higher than that of BSA, which enhanced IL-10 production weakly, and differed from that of LF, which increased TNF-α and IL-2 release at the concentration of 0.01 and 1 mg mL^{-1}, respectively. The levels of the 4 cytokines were upregulated markedly under ConA stimulation, as expected (Figs. 2 and 3). GMP had a 50/80% inhibitory effect on TNF-α and IFN-γ release, respectively, while IL-2 and IL-10 were unchanged. Inhibition was achieved mostly at the concentrations of 0.01 and 0.1 mg mL^{-1}, and it was greatly diminished (IFN-γ) or completely gone (TNF-α) at 1 mg mL^{-1}. BSA was not inactive, as it lowered IFN-γ at the highest concentration assayed. LF inhibited all proinflammatory cytokines markedly, although IL-2 and IFN-γ were somewhat less sensitive than TNF-α. The latter was an expected result, since LF is a strong inhibitor of lymphocyte proliferation [30].

3.3. Effect of GMP on COX2 and iNOS expression in primary splenocytes

Western blot analysis of rat naive splenocytes treated with 1 or 0.1 mg mL^{-1} of GMP in vitro showed a marked stimulatory effect on the expression of both COX2 and iNOS at the highest concentration assayed (Fig. 4). BSA had a less marked effect. In keeping with these effects, GMP augmented the phosphorylation of IκB-α at the same concentrations assayed, indicating the activation of the NF-κB canonical pathway (Fig. 4).

3.4. Effect of GMP on STAT4 phosphorylation in primary splenocytes

The results obtained so far suggest a possible inhibition of Th1 lymphocytes by GMP. In order to examine this effect with more detail we tested the hypothesis that GMP acts at the level of STAT4 activation, a key step in IFN-γ production [19]. The results, shown in Fig. 5, confirm that this step of the pathway is inhibited in ConA-stimulated spleen cells by GMP 0.1 mg mL^{-1} by approximately 75%. However, GMP did not abolish the phosphorylation of IκB-α when cells were stimulated with ConA (Fig. 5).

3.5. Effect of casoplatelin

Casoplatelin (CAS), a 11-aminoacid subpeptide of GMP (aminoacids 106–116 of bovine k-casein), has been described to exert various biological effects [28,29] and thus it might act as the active moiety of GMP. In order to test this hypothesis we assessed the possible effects of CAS on primary rat splenocytes, using GMP equimolar concentrations. In these conditions CAS did not affect cytokine production (Figs. 2 and 3), with two exceptions: CAS enhanced IL-2 production and showed an increase in ConA-stimulated TNF-α secretion at low concentrations. Thus while this peptide may retain some of GMP activity, it cannot account for its effects in our experimental system.

3.6. Effect of GMP on FoxP3

Finally, we set out to verify whether GMP has an effect on Treg cells. The expression of Foxp3 measured by Western

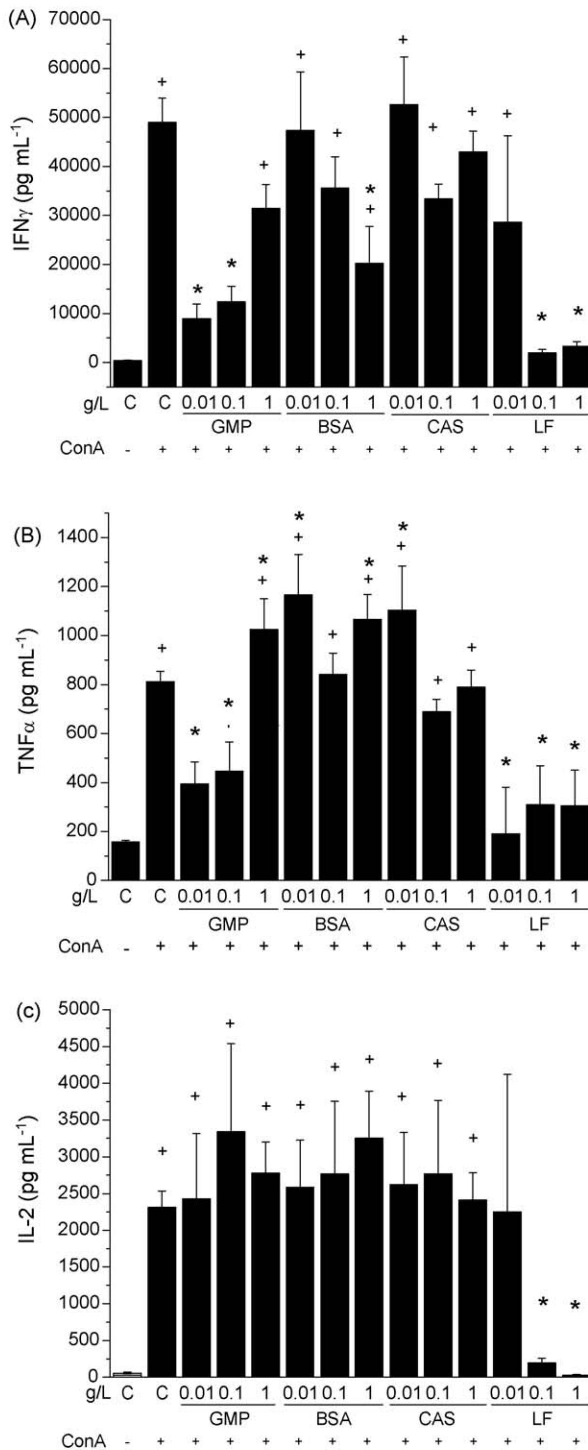

Fig. 2. Effect of glycomacropeptide (GMP), bovine serum albumin (BSA), casoplateline (CAS) and lactoferrin (LF) on IFN-γ (A), TNF-α (B) and IL-2 (C) secretion. Splenocytes were plated in 24-well plates (5×10^5 cells/well) and cultured with GMP, BSA or LF 0.01, 0.1 and 1 mg mL^{-1}, or CAS 0.01, 0.1 and 1 (equivalent molar concentration) in presence of Concanavalin A (ConA, 5 μg mL^{-1}). After 48 h of incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Results are expressed as mean ± S.E.M. of cytokine concentration (pg/mL). $^{+}p < 0.05$ vs. control. The pooled (normalized) results from 3 different experiments are shown.

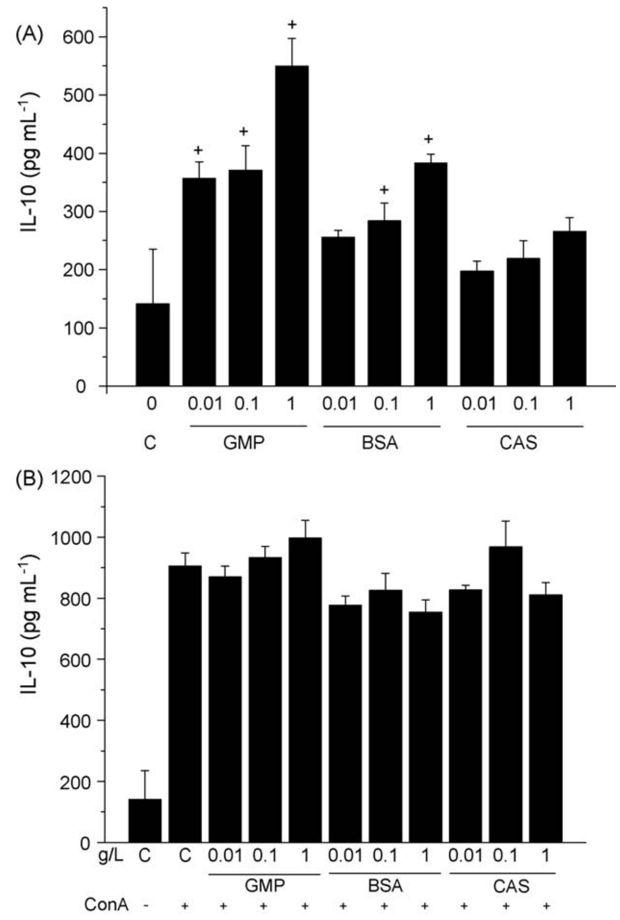

Fig. 3. Effect of glycomacropeptide (GMP), bovine serum albumin (BSA) and casoplateline (CAS) on IL-10 secretion. Splenocytes were plated in 24-well plates (5×10^5 cells/well) and cultured with GMP or BSA 0.01, 0.1 and 1 mg mL^{-1}, or CAS 0.01, 0.1 and 1 (equivalent molar concentration) in absence (A) or presence (B) of Concanavalin A (ConA, 5 μg mL^{-1}). After 48 h of incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Results are expressed as mean ± S.E.M. of cytokine concentration (pg/mL). $^{+}p < 0.05$ vs. control, all ConA-stimulated were different vs. control. The pooled normalized results from 3 different experiments are shown.

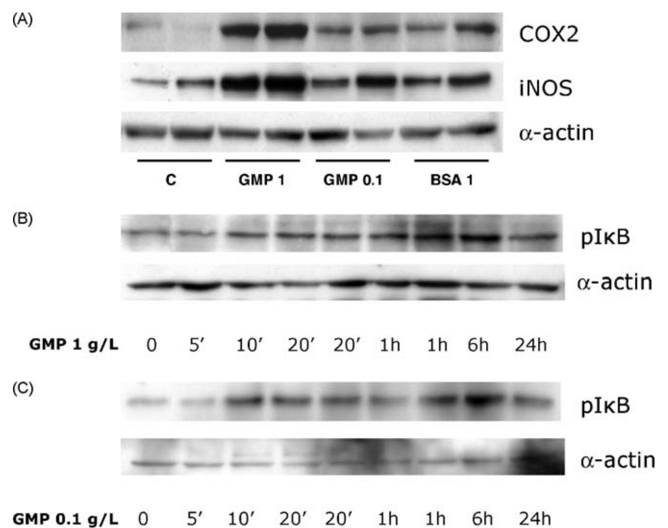

Fig. 4. Effect of GMP on COX2 and iNOS expression and NF-κB activation. (A) Rat splenocytes were incubated with GMP 1 and 0.1 mg mL^{-1} and BSA 1 mg mL^{-1} for 24 h. Protein samples were analyzed by Western blot, and COX2 and iNOS were determined. (B and C) Rat splenocytes were incubated with GMP 1 and 0.1 mg mL^{-1}, respectively for 24 h. Total protein samples were analyzed by Western blot and pIκB was determined. Blots are representative of 3 different experiments.

blotting was used as a suitable marker of this cell population. Fig. 6 shows that in vitro treatment of primary rat splenocytes with 1 mg mL^{-1} of GMP resulted in a 3-fold increase in Foxp3 expression.

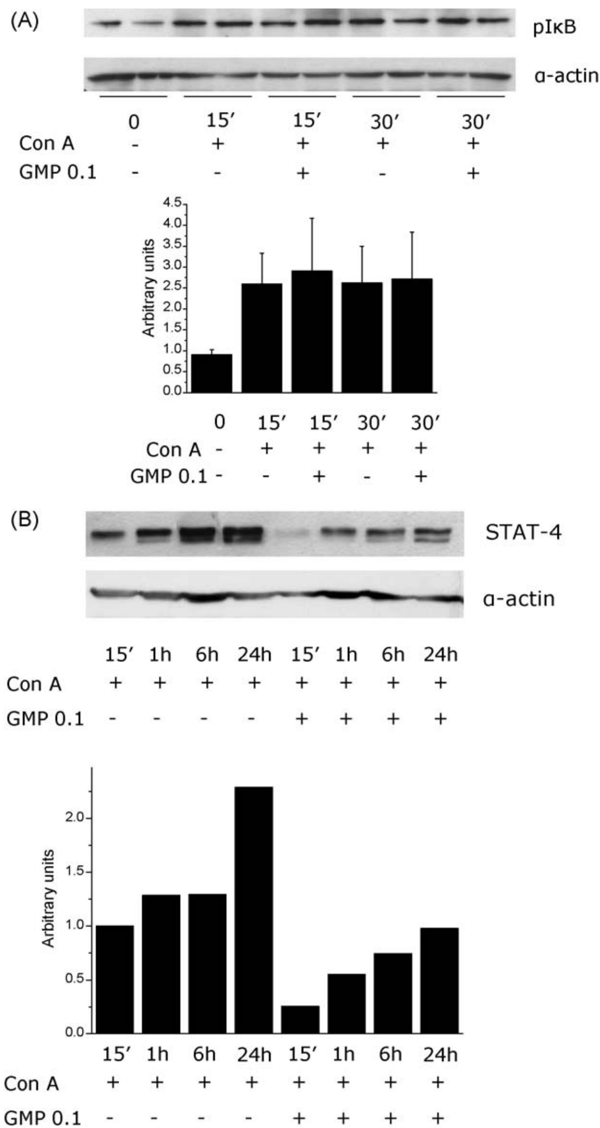

Fig. 5. Effect of GMP on NFκB and STAT-4 activation. (A) Splenocytes were incubated with ConA (5 μg mL^{-1}) in the absence or presence of GMP 0.1 mg mL^{-1} during 15 and 30 min. Total protein samples were analyzed by Western blot and pIκB was determined. (B) Splenocytes were incubated with ConA (5 μg mL^{-1}) in the absence or presence of GMP 0.1 mg mL^{-1} up to 24 h. Nuclei protein samples were analyzed by Western blot and total STAT-4 protein was determined. Blots and quantifications are representative of 2 different experiments. No significant differences were observed in the IκB phosphorylation Western blot.

3.7. Effect of GMP in vivo

Since our ultimate aim was to elucidate the mechanism of the intestinal antiinflammatory effects of GMP in vivo, we carried out an experiment in normal rats in which GMP was administered at the previously established effective dose in experimental intestinal inflammation either by oral gavage or by intraperitoneal injection. The animals were treated daily with GMP or vehicle for 3 days and then the behaviour of primary splenocytes and thymocytes assessed as above. In a first experiment cytokine and Foxp3 expression was measured (Figs. 6B and 7). Oral GMP increased Foxp3 expression in splenocytes, as determined by Western blot. The magnitude of the effect was comparable to that of the in vitro experiments. Interestingly, intraperitoneal GMP administration failed to produce this effect (data not shown). Furthermore, no effect was observed in the thymus by either route, indicating a specific effect on the spleen cells (Fig. 6C). The same results were observed in two such different experiments. GMP had no effect however on the ex vivo splenocyte production of TNF-α or IFN-γ after treatment by either route, although IFN-γ was generally higher with oral GMP, and it only increased IL-2 when administered by intraperitoneal route (Fig. 7). We performed 2 additional experiments in which splenocytes were incubated for only 5 h, in order to be able to detect any effect that may have been missed after long deprivation of GMP (because of the 48 h incubation applied for cytokine measurement). Since protein levels may not be altered at this early time point, mRNA was measured in this case. The results obtained are shown in Fig. 8, and confirm that cytokines are not significantly modulated by GMP in vivo treatment.

4. Discussion

The aim of this study was to evaluate the possible role of lymphocytes in the intestinal antiinflammatory activity of GMP, using primary rat splenocytes as an initial approach. Our results confirm that GMP modulates the activity of these cells and, specifically, they suggest that GMP hampers the activation of Th1 cells while favoring the differentiation of Treg cells, although this may be a direct or an indirect effect (via macrophages). Thus the predominant effect of GMP on primary splenocytes in vitro was: (1) a substantial increase in Foxp3 and IL-10 expression in basal conditions; (2) a marked inhibition of IFN-γ and, to a lower extent, TNF-α, in ConA-stimulated cells, with no changes in IL-2 release. However, GMP also produced a mild stimulation of TNF-α production in quiescent splenocytes, increased cell proliferation under ConA activation, and even upregulated COX2 and iNOS in basal conditions. It should be noted that these latter effects were observed mainly at the highest concentration assayed, i.e. 1 mg mL^{-1} of GMP, at which the inhibitory effects mentioned are already greatly diminished. Because we have previously established that GMP activates monocytes at 1 mg mL^{-1} [16], it is likely that these represent actions on spleen macrophages. Moreover, we have shown an activation of the NF-κB pathway in unstimulated spleen cells after culturing with GMP 0.1 and 1 mg mL^{-1}, a pathway reported previously to be activated by GMP in macrophages [16]. Thus the inhibitory effect would be exerted probably on lymphocytes. It is also possible that the proliferation of Treg lymphocytes balances the putative antiproliferative effect on Th1 cells to a certain extent, but this action is clearly overwhelmed under ConA stimulation, since GMP cannot increase Foxp3 (or IL-10) expression any further in these conditions. This question cannot be clarified until further experiments with isolated cell populations (which are underway) are performed. Thus our conclusions must be considered with caution. A likely source of IL-10 in this study was Treg cells, since both IL-10 and Foxp3 were maximally increased at 1 mg mL^{-1} of GMP, but it could be macrophages or even Th2 cells as well, since we did not measure Th2 cytokines.

It appears therefore that actions on lymphocytes predominate at concentrations below 1 mg mL^{-1}. The mechanism of action of GMP is not known but clearly is located at, or upstream of, STAT4 translocation, which is exquisitely inhibited by the peptide. The effect on TNF-α production could be just the consequence of the impaired IFN-γ production, as macrophages lacked this stimulus. There are two main pathways to stimulate IFN-γ production in lymphocytes: IL-12/IL-18 and T cell receptor (TCR). STAT4 has been found to be essential for IFN-γ production in CD4^{+} T cells, and to account for CD8^{+} T cell IFN-γ production. Both lineages require STAT4 activation for IFN-γ production induced by IL-12/IL-18 signaling, but only CD4^{+} T cells require STAT4 for IFN-γ induction via the TCR pathway [18,19]. ConA acts by binding TCR independently of clone specificity and thereby producing a steady

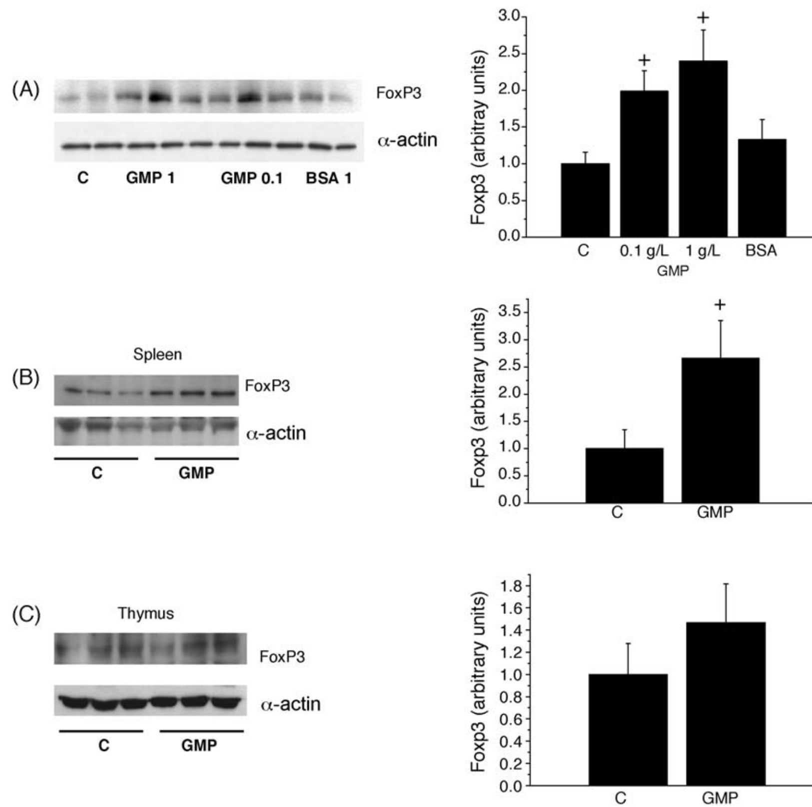

Fig. 6. Effect of GMP on FoxP3 expression. (A) Spleen cells were incubated with GMP 1 and 0.1 mg mL^{-1} and BSA 1 mg mL^{-1} for 24 h. Total protein samples were analyzed by Western blot, and FoxP3 was determined. (B and C) Female Wistar rats were fed for 3 days with GMP (500 mg kg day^{-1}) or vehicle and then killed and spleen (B) and thymus cells (C) were obtained and analyzed for Foxp3 expression. Total protein samples were analyzed by Western blot and FoxP3 was determined. Blots and quantifications are representative of 3 different experiments. $^{*}p < 0.05$ vs. control.

and powerful stimulation, bypassing the interaction with the MHC-peptide complex. STAT4 is phosphorylated as part of the signaling cascade that is thus activated, forming homodimers that regulate IFN-γ expression at the transcriptional level in the nucleus [24]. Of course, this is only one of STAT4 regulated genes, which include CD25 (IL-2 receptor alpha), among others [25,26]. In addition, STAT4 has a role in chromatin remodeling in the context of CD25 regulation [21]. Interestingly, Otani et al. showed that GMP suppresses IL-2 receptor expression on mouse $CD4^{+}$ T cells [5]. Thus we have at least two STAT4 dependent genes that are down-regulated by GMP. Otani et al. found also that bovine GMP binds the mouse $CD4^{+}$ T cell membrane, suggesting a surface site of action. Unfortunately our own efforts to locate the site of GMP actions have been hampered so far by the lack of quality antibodies, but obviously this is an important point to pursue. Certainly the effect of GMP is not due to casoplatelin, as this subpeptide shows a greatly diminished activity.

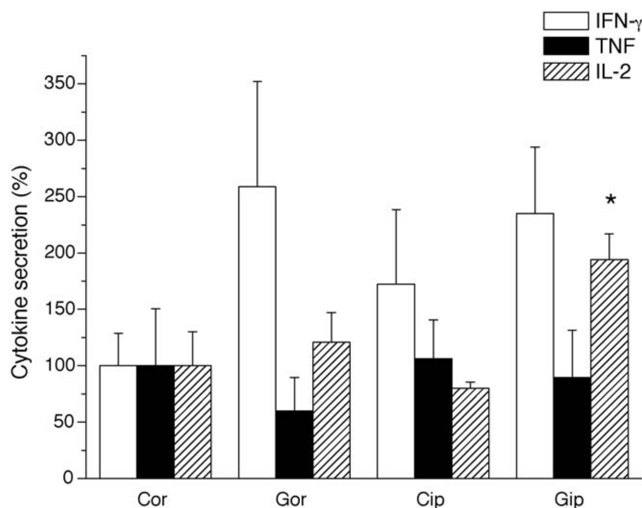

Fig. 7. Effect of in vivo oral or intraperitoneal GMP treatment on ex vivo splenocyte production of IFN-γ, TNF and IL-2. The cells were stimulated with ConA as described in Section 2. The production of cytokines in the Cor group was: 4777.8 ± 1375.6 ng mL^{-1}. The pooled (normalized) results from 2 different experiments are shown. Cor: control, oral route; Gor: GMP, oral route; Cip: control, i.p. route; Gip: GMP, i.p. route. $^{*}p < 0.05$ vs. Cip.

BSA was used in our study as a control for any nonspecific effects of protein on splenocytes. Unexpectedly, BSA had some effects on splenocytes, which were in part similar to those of GMP, since they both reduce IFN-γ in ConA-stimulated splenocytes, but with several important differences. At any rate, our data suggest that BSA has immunological effects that may have been overlooked, or that protein exerts some effect at this level in a nonspecific fashion [31]. On the other hand, lactoferrin, included here for comparison purposes, has well characterized effects on lymphocyte proliferation and, accordingly, reduced dramatically thymidine uptake as well as TNF-α, IFN-γ and IL-2 production in ConA-stimulated splenocytes. These effects are thus substantially different from those of GMP and indicate a different mechanism of action.

The other important issue in our study is the observation that GMP retains the ability to increase Foxp3 expression in splenocytes, but not thymocytes, in vivo, suggesting a peripheral Treg differentiation effect of GMP in which the oral route of administration plays a major role. Perhaps the reported prebiotic properties of GMP may partially account for this dependence of the

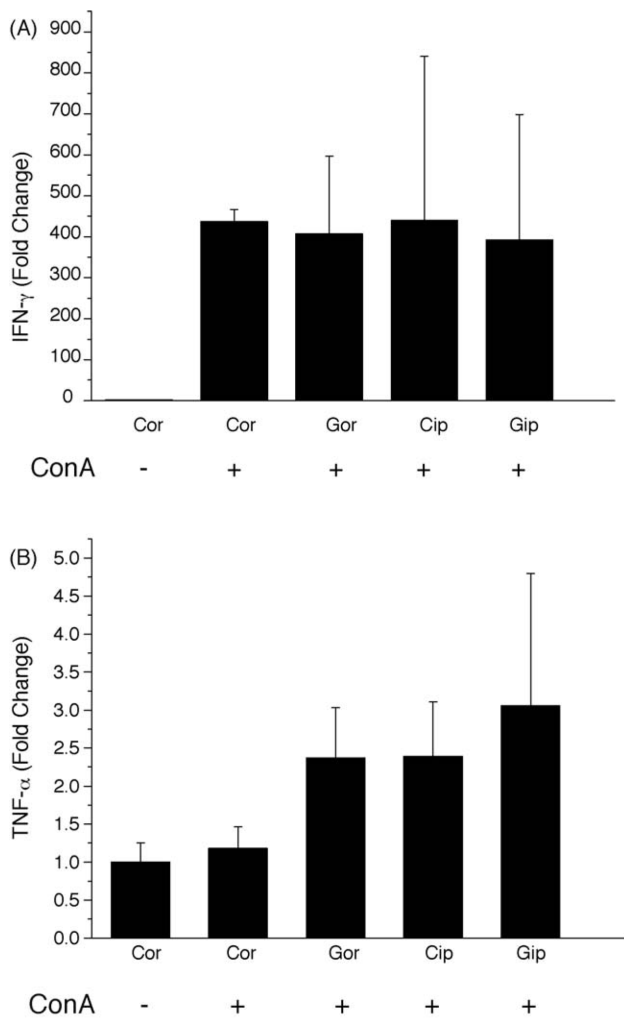

Fig. 8. Effect of in vivo oral or intraperitoneal GMP treatment on ex vivo splenocyte mRNA expression of IFN-γ and TNF-α after short incubation. No significant differences were observed. The pooled (normalized) results from 2 different experiments are shown. Cor: control, oral route; Gor: GMP, oral route; Cip: control, i.p. route; Gip: GMP, i.p. route.

oral route of administration [32,33]. It should be noted that although it was believed originally that Foxp3$^+$ Treg cells were generated solely in the thymus, it has now become clear that peripheral CD4$^+$ cells can differentiate into Foxp3$^+$ Treg cells as well under selective conditions. These appear to involve the inhibition of Th1/Th2 differentiation; thus neutralizing IFN-γ and IL-4 not only potentiates TGF-β-mediated Foxp3 induction in vitro but can also enhance antigen-specific Foxp3$^+$ Treg differentiation in vivo [20]. However, in the in vivo experiments IFN-γ was not inhibited, but rather showed a certain tendency to increase, and IL-2 expression was increased after intraperitoneal treatment, respectively. It has been described that IL-2 is required for the maintenance of the expression of genes involved in the regulation of Treg cell growth and metabolism, but it also stimulates Th1/Th2 cell differentiation. Thus it is possible that the effect on IL-2 observed after i.p. treatment offsets the effect of GMP on Treg cells.

Because of the lack of inhibitory effect on IFN-γ, we speculated that the effect on Th1 cells/IFN-γ may require continuous GMP contact, and that cell isolation would therefore cancel it. In order to check this possibility, the same experiment was repeated (twice) but examining cells after only 5 h after ConA stimulation, a short incubation time yet one that ought to reveal any existing differences. Indeed, IFN-γ mRNA was induced robustly in the cultured splenocytes, but no effect of GMP could be detected. The reason for this important discrepancy between the in vitro and in vivo settings cannot be explained at this time. It is likely that GMP exerts direct inhibitory effects on Th1 lymphocytes and long term effects on Tregs. However, it suggests that IFN-γ downregulation may not be the primary mechanism responsible for Treg induction. Further experiments will be needed to elucidate the mechanism of these effects. Nevertheless, our results are consistent with a relevant role of lymphocyte actions in the intestinal antiinflammatory effect of GMP. The balancing effects of Treg (or Th2) cells and/or the direct inhibition of Th1 cells is expected not only to reduce inflammation in the intestine, but also to contribute to homeostasis and perhaps even intestinal maturation in the neonate. Experiments are underway to examine this possibility.

Acknowledgements

The authors are thankful to Dr. Mercedes González for technical assistance. We are also grateful to Davisco Foods International, Inc. (Eden Prairie, MN). CIBERehd is funded by the Instituto de Salud Carlos III.

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