Validation of bovine glycomacropeptide as intestinal antiinflammatory nutraceutical in the lymphocyte transfer model of colitis

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ABSTRACT

Milk κ-casein derived bovine glycomacropeptide (GMP) has immunomodulatory effects. It exerts intestinal antiinflammatory activity in chemically induced models of colitis. However, in order to validate its clinical usefulness as a nutraceutical, it is important to assess its effects in a model with a closer pathophysiological connection with human inflammatory bowel disease. Hence we used the lymphocyte transfer model of colitis in mice and compared the effects with those obtained in mice with dextran sulfate sodium (DSS). GMP (15 mg/d) resulted in higher body weight gain and a reduction of colonic damage score and myeloperoxidase activity in Rag1-/- mice with colitis induced by transfer of naïve T cells. The colonic and ileal weight:length ratio was decreased ~25%, albeit nonsignificantly. GMP treatment reduced the percent of CD4+ IFN-γ+ cells in mesenteric lymph nodes. Basal production of IL-6 by mesenteric lymph nodes obtained from GMP treated mice *ex vivo* was augmented. However, concanavalin A evoked production was similar. Colonic expression of REG3γ, S100A8, CXCL1 and IL-1β was unaffected by GMP, while that of TNF-α and especially IFN-γ was paradoxically increased. In the DSS model GMP also reduced colonic MPO activity but failed to alter weight gain or intestinal weight:length ratio. GMP augmented IL-10 production by mesenteric lymph node cells and was neutral toward other cytokines, except for a trend to increase IL-6. The lower effect was attributed to the lack of effect of GMP on epithelial cells. We conclude that GMP exerts intestinal antiinflammatory effects in lymphocyte driven colitis.

Introduction

Nutraceuticals are food related products that produce health benefits to the consumer beyond their basic nutritional value. In the last few years there has been a great interest in nutraceuticals and functional foods, since they open up the possibility to deter disease (to a certain extent) as an add-on to regular diet. Although their properties are partly drug-like, their origin as part of foodstuffs and their extremely low toxicity are especially attractive to the general public. Not surprisingly, the food industry has been particularly active in the search and marketing of new products of this type. However, there is an obvious risk of overselling the claimed virtues of a given nutraceutical. Current regulations in Europe (EC 432/2012) require demonstration of specific qualities in terms of physiological benefit or prevention of disease.

One of the conditions where nutraceuticals and functional foods may play a role is inflammatory bowel disease (IBD). Comprising ulcerative colitis and Crohn's disease, IBD is characterized by chronic and relapsing inflammation of the intestine, resulting in a significant deterioration of the quality of life of patients. Furthermore, IBD prevalence is slowly increasing[1,2]. IBD is considered to develop as the result of an insufficiently characterized interplay of genetic, enviromental, microbial and immunological factors, involving an uncontrolled response to luminal antigens that are innocuous for the normal population. These processes have been long thought to be related to augmented adaptive immunity responses, but it has also been proposed that a defect in innate immunity may paradoxically underlie the etiology of IBD[3-6]. Whatever the exact mechanism, IBD is regularly managed pharmacologically with drugs that downregulate the immune system such as corticoids, infliximab, aminosalicylates or azathioprine. All of these agents have a plethora of serious adverse effects which limit their application and they are not effective in all patients. Hence the search for new treatments with a low profile of adverse effects is much warranted[2].

Bovine glycomacropeptide[1] (GMP), also referred to as casein macropeptide, is a 64-aminoacid peptide that contains varying amounts (0 to 5 units) of N-acetylneuraminic (sialic) acid. This peptide

[1] Abbreviations used in this paper: AP, alkaline phosphatase; DSS, dextran sulfate sodium; CXCL1, Chemokine (C-X-C motif) ligand 1; DAI, Disease activity index; GFP, green fluorescent protein; GMP, glycomacropeptide;

results from the enzymatic hydrolysis of milk κ-casein in the bovine stomach due to the action of chymosin (pepsin in humans)[7]. In addition, GMP is present at 10-15% in milk whey as a result of the action of the same enzyme during the cheese making process. Therefore, there is a substantial natural exposure to this peptide. GMP has nutritional interest because its aminoacid profile is high in branched chain aminoacids and lacks aromatic aminoacids, being therefore one of the few naturally occurring proteins safe for individuals with phenylketonuria and perhaps useful in the management of some liver diseases[8,9]. On the other hand, a number of biological activities have been ascribed to GMP. We have previously established that GMP has intestinal antiinflammatory activity in experimental models of IBD[10-13]. However, this is probably insufficient evidence to support the use of GMP as a nutraceutical, because the trinitrobenzenesulfonic acid (TNBS) and dextran sulfate sodium (DSS) rat models used previously are not strictly chronic (i.e. they heal with time) and they are not lymphocyte driven as in human disease. Some authors have advocated the use of other IBD models to achieve a better prediction of human bioactivity[14], such as colitis induced by transfer of naïve T lymphocytes to immunodeficient mice, anticipating that validation of nutritional or pharmacological treatments of IBD should include one such model. Therefore we set out to test the activity of GMP in lymphocyte transfer colitis in mice. Among the parameters evaluated is myeloperoxidase (MPO) activity, a neutrophil marker that is widely used as an index of colitis activity in preclinical models of IBD. Since tissue damage in IBD is the result of an exacerbated inflammatory response, a normalization of this parameter is considered an improvement in disease evolution. Some other mediators related with the innate immune system such as REG3γ (an antimicrobial peptide expressed by intestinal epithelial cells), S100A8 (also a prominent neutrophil marker, a component of calprotectin), CXCL1 (a chemokine with neutrophil chemoattractant activity) and IL-1β (a proinflammatory cytokine mainly produced by macrophages), were also evaluated. The results obtained are consistent with a beneficial effect of the peptide.

IBD, inflammatory bowel disease; LB, lysogenic broth; MLN, mesenteric lymph node; MPO, myeloperoxidase; REG3γ, Regenerating islet-derived protein 3 gamma; TNBS, trinitrobenzenesulfonic acid.

Materials and methods

Reagents

Except where indicated, all reagents and primers were obtained from Sigma (Barcelona, Spain). Retrotranscription was achieved with the iScriptTM cDNA Synthesis Kit and iTM Sybr Green Supermix was used for amplification (Biorad, Alcobendas, Madrid, Spain). Antibodies were purchased from Cayman Technologies (Pickerington, OH, USA) and Sigma (Barcelona, Spain). Mouse ELISA kits were obtained from eBioscience (San Diego, CA, USA). Dextran sulfate sodium (DSS) was obtained from ICN Biomedicals (Costa Mesa, CA, USA). GMP (BioPURE-GMP^{TM}) was the kind gift of Davisco Foods International, Inc. (Eden Prairie, MN). According to the manufacturer the GMP content was 93%, while fat and lactose contents accounted for 0.2% and less that 1%, respectively.

Animals

Female C57/BL6 Wild type and Rag1-/- mice were obtained from Jackson (Jackson Laboratory, CA, USA), housed in makrolon cages and maintained in the animal facilities from the University of Granada in air conditioned animal quarters with a 12 h light-dark cycle. Animals were provided with free access to tap water and food. All animal procedures in this study were 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) and were approved by the Animal Welfare Committee of the University of Granada (reference 710).

Induction of transfer colitis and experimental design

Female C57/BL6 mice were sacrificed by cervical dislocation and the spleen was extracted aseptically. Cell suspensions were obtained by disrupting the tissues between dissecting forceps in medium. After centrifuging, cells were cleared of erythrocytes by suspension on hypotonic lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA{\cdot}2H_2O$, pH=7.3) for 30 min on ice. Cells were filtered using a 70 µM filter (cell strainer BD Falcon^{TM}) to obtain a mononuclear suspension. Mononuclear cells were washed and suspended in MACs buffer. CD4+ CD62L+ T cells isolation from spleen cells was performed using CD4+ CD62L+ T Cell Isolation Kit II (Miltenyi Biotec, Madrid, Spain). First, non-CD4+T cells were indirectly magnetically labeled with a cocktail of biotin-

conjugated antibodies and AntiBiotin MicroBeads. The labeled cells were subsequently depleted by separation over a MACS® Column. In the second step, CD4+CD62L+ T cells were directly labeled with CD62L (L-selectin) MicroBeads and isolated by positive selection from the pre-enriched CD4+ T cell fraction. CD4+ CD62L T cells were eluted in 100 µl of sterile PBS and administered via the intraperitoneal route to C57/BL6 Rag1-/- mice (10^6 cells per mouse). Rag1-/- control mice were administered sterile PBS.

The status of the animals was monitored by general examination and specifically controlling body weight evolution, beginning the experiment after a 10% of body weight loss (about 8 weeks after the transfer). Colitic mice were randomly assigned to 2 different groups. The GMP group (n=7) received by gavage 15 mg/d of GMP while the transfer colitis group (n=8) was administered vehicule (distilled water). A noncolitic control group (Rag1-/- mice administered 100 µl sterile PBS) was also included in the experiment (n=6). Treatment was maintained until animals were sacrificed after 13 d by cervical dislocation.

Induction of colitis by DSS and experimental design

22 female C57/BL6 mice were used. Colitis was induced by adding DSS to drinking water for 8 d[10,15]. We selected the conditions in order to achieve a mild to moderate degree of colitis by using 2% w/v of DSS. The status of the animals was monitored by general examination and specifically by means of the DAI (disease activity index), a combined score for weight loss, diarrhea and hematochezia, which are 3 main signs of pathology in this model[16]. Food intake, water intake, and body weight were measured every day.

Mice were randomly assigned to 3 different groups. The control (C) group (n=6) did not receive DSS and was administered water daily by means of a gastroesophageal catheter. The remainder mice drank DSS supplemented water, and received by gavage either 15 mg/d of GMP (GMP group, n=8) or vehicle (distilled water, DSS group, n=8). Treatment started 2 days before DSS supplementation and was maintained until animals were sacrificed after 10 days by cervical dislocation.

Assessment of colonic damage

The entire colon was removed, gently flushed with saline and placed on an ice-cold plate, cleaned of fat and mesentery, and blotted on filter paper. Each specimen was weighed and its length measured under a constant load (2 g). The large intestine was longitudinally opened and scored for visible damage by a blinded observer. Transfer colitis goes on a 0 to 15 scale. The score was assigned as follows: adhesions (0-3), obstruction (0-2), hyperaemia (0-3), thickness (0-5) and ulceration (0-2). DSS colitis goes on a 0 to 13 scale according to the following criteria: adhesions (0-3), hyperaemia (0-3), fibrosis (rigidity, 0-3), deformation (0-2) and thickening (0-2). A small segment was dissected from the intestine and used for RNA isolation. The colon was subsequently divided longitudinally in several pieces for biochemical determinations. The fragments were immediately frozen in liquid nitrogen and kept at –80ºC until used. Formalin-fixed colon tissue was cut and stained with hematoxylin and eosin. Myeloperoxidase (MPO) and alkaline phoshatase (AP) activities were measured spectrophotometrically as described previously[17,18] and expressed as mU/mg protein. In addition, the sensitivity to the AP inhibitor levamisole was assessed and expressed as % inhibition.

Analysis of gene expression by reverse transcriptase-PCR analysis

Total RNA was obtained by the Trizol method (Invitrogen, Barcelona, Spain) and 1 µg retrotranscribed and specific RNA sequences amplified with a Stratagene MX3005P real time PCR device using the following primers: (18S sense: ACA CGG ACA GGA TTG ACA GAT TG, antisense: GCC AGA GTC TCG TTC GTT ATC G); (S100A8 sense: GCC CTC TAC AAG AAT GAC TTC AAG, antisense: ATC ACC ATC GCA AGG AAC TCC); (IL1-β sense: AAG GGC TGC TTC CAA ACC TTT GAC, antisense: TGC CTG AAG CTC TTG TTG ATG TGC); (TNF-α sense: CGT GGA ACT GGC AGA AGA GG, antisense: CAG GAA TGA GAA GAG GCT GAG AC); (IFN-γ sense: GCT CTG AGA CAA TGA ACG CTA CAC, antisense: TTC TTC CAC ATC TAT GCC ACT TGA G); (CXCL1 sense: CCG AAG TCA TAG CCA CAC TCA AG, antisense: ACC AGA CAG GTG CCA TCA GAC); (REG3γ sense: CAG AGG TGG ATG GGA GTG GAG, antisense: CAC AGT GAT TGC CTG AGG AAG AAG AG).

Mesenteric lymph node (MLN) cells cytokine secretion

Mesenteric lymph nodes were extracted from the mice in the study using sterile technique and dissected mechanically. Cells were washed once with fresh medium and were filtered using a 70 µM

filter (cell strainer BD Falcon™, Ref. 352350) to obtain a mononuclear suspension, mostly T cells. The cells were incubated in RPMI-1640 medium containing fetal bovine serum (10%), 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 mg/ml amphotericin B and 0.05 mM mercaptoethanol. The cells were cultured at 10^6 cells/ml and stimulated with concanavalin A (ConA) at a final concentration of 5 µg/ml. ConA is polyclonal T cell stimulant that evokes a surge in cytokine secretion by MLN cells, which is typically enhanced in colitic animals. Cell culture medium was collected after 48 h and assayed for cytokine content by commercial ELISA. The cytokines determined were IL-6, IL-10, IL-17, IFN-γ and TNF-α. Plates (Nunc™ Inmuno plate, Roskilde, Denmark) were read at 450 nm using a plate reader (Tecan, model Sunrise-basic, Austria).

Epithelial cell experiments

Three different experiments were carried out with GMP and intestinal epithelial cells in vitro. First the effect of GMP (0.01-1 g/l) on IL-8 secretion by concluent HT29 cell monolayers was assessed by ELISA (R&D Systems, Minneapolis, MN). Second, the effect of GMP on bacterial invasion in IEC18 cells was studied. *Escherichia coli* K12 and LF82 strains (gently provided by Dr. Arlette Darfeuille Michaud) expressing green fluorescent protein (GFP) were used. Bacteria were grown routinely in lysogeny broth (LB) supplemented with 20 µg/ml of gentamicin overnight at 37°C with shaking. For every experiment bacteria were freshly grown overnight. The invasion assays were carried out in IEC18 cell monolayers cultured in 12 well plates. GMP (5-20 g/l) was added to the medium 24 hours before and during the invasion assay. Each monolayer was infected with 1 ml of the cell culture medium without antibiotics at a multiplicity of infection of 100 bacteria per epithelial cell. After a 4 h incubation, infected monolayers were washed three times with HBSS and fresh cell culture medium containing 100 mg/l of kanamycin, 500 mg/l streptomycin and 500,000 UI/l of penicillin was added to kill extracellular bacteria. After incubation for an additional hour, monolayers were washed three times again with HBSS. The cells were collected by trypsinization and analyzed by flow cytometry (FACSCalibur, BD, Madrid, Spain) and expressed as percentage of FL1-GFP positive cells, each positive event representing a cell containing GFP expressing bacteria.

Third, the effect of GMP on wound was assessed. Rat epithelial IEC18 cells were grown to confluence in 24-well dishes and were mechanically wounded by parallel scratches using a sterile

pipet tip. Medium on wounded monolayers was replaced with normal medium, or normal medium containing GMP 1 g/ml and incubated for 24 h. Photomicrographs were taken of the lineal wounds using the 4x objective on an Olympus IX71S8F-3 microscope at the moment of wounding, at 6, 11 and 24 h, and the area of each wound was quantified with Image J software.

IEC18 (ECACC 88011801) and HT29 (ECACC 91072201) cells were supplied by the Cell Culture Facility of the University of Granada and were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Boehringer Mannheim, Barcelona, Spain), 2 mM L-glutamine, 100 mg/l streptomycin, 100,000 UI/l penicillin, and 2.5 mg/l amphotericin B, in a humidified 5% CO_2 atmosphere at 37ºC.

Data and statistical analysis

In all the experiments, samples were run in triplicate and results are expressed as mean ± standard error of the mean (SEM). Differences among means were tested for statistical significance by one way ANOVA and *a posteriori* Fisher LSD tests on preselected pairs. All analyses were carried out with the SigmaStat 3.5 program (Jandel Corporation, San Rafael, CA, USA). Differences were considered significant at $P<0.05$.

238 **Results**

239

240 *Lymphocyte transfer colitis*

241 Rag1-/- mice were monitored for 8 weeks after transfer for body weight evolution and overall status.
242 The animals selected for this study showed body weight loss (10% average) and were randomized for
243 treatment with GMP or vehicle. Hence this is a postreatment protocol. Mice administered vehicle
244 maintained a relatively stable body weight, which was slightly increased after 13 days (Table 1). Food
245 and water intake were essentially normal (data not shown). The colon appeared thickened, with
246 hyperemia but no overt signs of ulceration, obstruction or adhesion (Table 1). The resulting damage
247 score was significantly increased. At the microscopic level there was intense infiltration of the
248 mucosa, crypt elongation and occasional epithelial erosions (not shown). The ileum was also
249 thickened (29.6±4.9 vs. 21.9±4.9 mg/cm, $P<0.05$) and there was splenomegaly (5.4±3.5 vs. 1.3±0.1
250 spleen:body weight ratio x1000, $P<0.05$).

251

252 At the biochemical level, colitis was characterized by a 2-fold increase in MPO and 4-fold increase in
253 AP activity (Figs. 1 and 2A). The sensitivity to the specific AP inhibitor levamisole was augmented
254 (Fig. 2B). The mRNA level of REG3γ, S100A8, CXCL1, IL-1β, TNF-α and IFN-γ was significantly
255 increased (Fig. 3). MLN cells were enriched in CD4+ IFN-γ+ cells (Fig. 4) and exhibited an augmented
256 production of cytokines.

257

258 GMP resulted in higher body weight gain (despite a similar food intake) and a reduction of colonic
259 damage score and the ileal (26.5±4.3 vs. 29.6±4.9 mg/cm, $P>0.05$) and colonic weight:length ratio
260 (Table 1), although these did not reach statistical significance. Histological analysis showed similar
261 features as in the control animals (not shown). MPO activity was virtually normalized (Fig. 1), while AP
262 activity was reduced by 17% but without reaching significance (Fig. 2A). The sensitivity of AP to
263 levamisole was unaffected (Fig. 2B). The colonic expression of REG3γ , S100A8, CXCL1, and IL-1β
264 was unaffected by GMP. Conversely, TNF-α and especially IFN-γ were upregulated (Fig. 3). GMP
265 treatment reduced the percent of CD4+ IFN-γ+ cells in mesenteric lymph nodes (Fig. 4A). Basal
266 production of IL-6 by MLNC obtained from GMP treated mice *ex vivo* was markedly increased (Fig.
267 4D). IL-17, IFN-γ and TNF-α displayed a similar but much weaker trend, significant only for IL-17.

However concanavalin A evoked production was similar in all cases (Fig. 4). There was no effect on spleen size (not shown).

DSS colitis

Administration of 2% DSS in drinking water to mice resulted in diarrhea, hematochezia and loss of body weight (Fig. 5), with increased DAI (not shown). The colon was thickened and shortened, resulting in an increased weight:length ratio (Table 2). The mucosa showed intense hyperemia and fibrosis, which gave rise to a substantially augmented damage score (Table 2). Colitic animals also had splenomegalia (2.8±0.15 vs 4.0±0.32, spleen:body weight ratio x1000, $P<0.05$, control vs. DSS). Colonic myeloperoxidase activity was greatly increased (Fig. 6), while AP activity was augmented approximately 3-fold (Fig. 7A). The sensitivity of AP to levamisole was not significantly affected (Fig. 7B). Basal production of MLN cells *ex vivo* was negligible (not shown). Upon ConA stimulation there was a surge of IL-17 and IL-10 release, while IL-6, TNF-α and IFN-γ showed only a nonsignificant trend to increase (Fig. 8).

Pretreatment with GMP reduced colonic MPO activity by ~50% (Fig. 6) but failed to alter weight gain/DAI, colonic weight:length ratio and damage score, AP activity or splenomegalia (Table 2 and Figs. 5-7). Interestingly, GMP augmented IL-10 production by MLN cells *ex vivo* (Fig. 8) while it was essentially neutral toward other cytokines. It also showed a clear trend to increase IL-6 (Fig. 8A), although without reaching statistical significance.

Effect of GMP on intestinal epithelial cells

The difference observed between the effect of GMP in lymphocyte transfer and DSS induced colitis prompted us to assess the effects of the peptide on intestinal epithelial cells. As shown in Fig. 9, there was no effect of GMP on epithelial IL-8 secretion, wound repair or bacterial invasion.

Discussion

The main goal of this study was to validate the intestinal antiinflammatory activity of GMP using a truly chronic, lymphocyte driven model of IBD[14,19]. This model is characterized by progressive expansion of the transferred T lymphocyte population, with a predominance of Th1/Th17 cells and a paucity of Treg cells, resulting in intestinal inflammation, which affects mainly the colon. The process is chronic and may remain relatively stable for weeks or deteriorate slowly until animal death (spontaneous or by euthanasia due to ethical reasons). Despite the obvious advantages of using a model more closely resembling human IBD[14,19], it poses practical problems for the investigation of bioactive products, compared with chemical models. In particular, there is an extended lag period until the disease develops, which significant variation among mice even within a single experiment. The onset of the disease is insidious, making it difficult to judge when the animal is 'ready' for testing. In this study we transferred a relatively large number of Rag1-/- mice and grouped them for further experimentation based on body weight evolution and other signs of disease such as reduced movement, huddling or diarrhea (other groups were used for unrelated treatments). Thus the mice used for this experiment exhibited similar characteristics prior randomization to receive GMP gavage or vehicle. It is important to note that the use of this model represents a leap forward in the state of the art in the development of nutraceuticals for intestinal inflammatory conditions.

GMP was administered at the dose of 15 mg/d, which is equivalent to that used successfully as a pretreatment in rats (500 mg/kg), based on body surface. This dose is roughly equivalent to 5 g for an adult human (again on a body surface basis), an amount that cannot be easily achieved by milk consumption but that is easily attainable as a functional food or a drug. Because of the reasons stated above the protocol applied in this case is a postreatment, which is expected to produce less pronounced effects in general. Another possible source of variation is the use of a different species (mouse vs. rat). In these conditions GMP exerted significant protective effects. A positive response was anticipated before animal sacrifice based on a higher body weight gain (albeit nonsignificant), which was achieved despite a similar food intake compared to control mice. Body weight loss in acute and semichronic models of colitis is mainly a consequence of anorexia, which is part of the acute

response. In this model food intake was within normal boundaries in both control and GMP treated mice, suggesting that differences are due to absorptive problems and/or enhanced catabolism.

The large intestine showed signs of a relatively mild colitis compared with the harsh impact of TNBS or DSS colitis, with loss of visible vascular pattern (edema), hyperemia and thickening, but no necrosis, major adhesions or strictures. This was better evidenced at the histological level, which showed an intense infiltration and crypt enlargement but with only minor areas of epithelial erosion and no overt ulceration. Intestinal thickening was a major sign of colitis, and it was improved by GMP treatment in both colon and ileum, but without reaching statistical significance. Thus GMP appears to ameliorate edema and crypt elongation but this effect probably requires a higher group size to be picked up by statistical analysis. The effect of GMP was better evidenced by biochemical analysis. Thus colonic MPO activity was increased 2-fold in the colitic group, consistent with a relatively mild degree of inflammation, and this was fully normalized by GMP treatment, indicating inhibition of neutrophil infiltration. Colonic AP activity, on the other hand, showed only a nonsignificant effect of GMP. AP activity is increased in colitis as a result of the combination of leukocyte infiltration and a change of isoform expressed at the epithelial level[18]. Thus colonic inflammation is characterized not only by augmented AP activity, but also by a higher inhibition by the specific inhibitor levamisole in vitro, as in the present study. The fact that MPO was reduced by GMP while AP activity was not suggests that it failed to modulate the epithelial component, in line with its documented lack of activity on epithelial cells. In fact, our in vitro experiments indicate that GMP is inactive in modulating epithelial repair, antibacterial defense or cytokine secretion, in line with this hypothesis. However, we cannot completely exclude the possibility of epithelial actions because of the limitations imposed by the use of intestinal epithelial cell lines. Thus it remains possible that GMP exerts significant effects on the epithelium in vivo. This is very difficult to study directly because primary enterocytes have generally an extremely limited lifespan.

Other biochemical inflammatory markers indicative of ongoing inflammation are the upregulated parameters measured by RT-PCR in colonic tissue and the cytokines released by MLN cells *ex vivo*[20]. The effects of GMP at this level are complex, but they are generally consistent with the dual action previously observed with this peptide, boosting innate immunity but blocking adaptive

353 immunity. It is interesting in this regard that GMP is associated with an increase in IL-6. We[21] and
354 others[22] have noted the stimulatory effect of GMP on macrophages, in terms of cytokine production
355 and phagocytic activity. GMP may interfere with interleukin 1ß (IL-1) receptor binding[23]. Conversely,
356 GMP has been described to inhibit the proliferation of splenocytes and Peyer's patch cells[24]. We
357 have recently observed that GMP inhibits IFN-γ release by rat splenocytes by blocking STAT4
358 activation (unpublished observations). However, the production of IgG by mouse B-lymphocytes
359 seems to be increased by GMP[25]. Therefore, it is possible that GMP acts modulating lymphocyte and
360 macrophage function so as to enhance intestinal barrier function and dampen colitis. At any rate, the
361 mechanism of action of GMP must involve the MLN cell population or monocytes and lymphocytes in
362 the lamina propria, since it has no effect on epithelial cells. IL-6 may be a common pathway in both
363 models, as mentioned above.
364
365 It is interesting to compare these data with those obtained in the mouse DSS model. We undertook
366 this experiment originally as a preliminary stage to study the involvement of macrophages vs.
367 lymphocytes in GMP effect, namely applying the DSS model to Rag1-/- mice. Our results indicate that
368 while GMP is beneficial in regular C57/BL6 mice, the magnitude of the effect is certainly diminished
369 compared to that obtained in rat colitis or ileitis. Clinical improvement was evidenced by amelioration
370 of colonic MPO activity and increased IL-10 production by MLN cells *ex vivo*. Taken together, our
371 studies indicate that GMP is active in several different models of intestinal inflammation, but the
372 results differ depending on animal species and experimental model, with rats being more sensitive
373 than mice, and TNBS more than DSS. DSS is thought to elicit intestinal inflammation by slowly
374 altering the epithelial integrity, augmenting permeability and ultimately resulting in an immune reaction
375 against luminal antigens[26]. In contrast, TNBS acts as a hapten by a delayed hypersensitivity
376 mechanism involving the reaction with mucosal proteins[27]. Oxidative stress may also play a role[28].
377 The epithelium therefore plays a more important role in DSS than in TNBS colitis, and since, as
378 noted, GMP has no effect on the epithelium it is logical to obtain less pronounced effects in the former
379 model. Another example of this disparate behaviour is the effect of the flavonoid luteolin in DSS and
380 IL-10 -/- colitis in mice, being deleterious in the former and protective in the latter[29], a discrepancy
381 explained by the inhibitory action of the flavonoid on the NF-κB pathway in the epithelium, which may
382 compromise the defense of the mucosa toward epithelial disruption by DSS.

383

384 In conclusion, our data validate the intestinal antiinflammatory activity of GMP in chronic, lymphocyte
385 driven colitis, considered one of the best models of colitis because of resemblance to human IBD.
386

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORSHIP

MOG, FCC, PR, BO, IRC and CA carried out the experiments. MDS, AZZ, FSM and OMA designed the experiments. FSM and OMA wrote the manuscript. All authors contributed to the discussion and overall structure of the manuscript.

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483
484
485

FIGURE LEGENDS

Fig. 1. Colonic myeloperoxidase activity in mice with lymphocyte driven colitis. Chronic colitis was induced in mice and they were then treated with GMP (15 mg/kg) or vehicle for 13 d. MPO was measured spectrophotometrically.

Fig. 2. Colonic alkaline phosphatase activity in mice with lymphocyte driven colitis. Chronic colitis was induced in mice and they were then treated with GMP (15 mg/kg) or vehicle for 13 d. AP was measured spectrophotometrically. A. AP activity. B. Inhibition by levamisole in vitro. $^{+}P<0.05$ vs. control group.

Fig. 3. Colonic expression of inflammatory markers in mice with lymphocyte driven colitis. Chronic colitis was induced in mice and they were then treated with GMP (15 mg/kg) or vehicle for 13 d. mRNA levels were measured by RT-PCR. A. REG3γ. B. S100A8. C. CXCL1. D. IL-1β. E. TNF-α. F. IFN-γ. $^{+}P<0.05$ vs. control group.

Fig. 4. Cytokine secretion by mesenteric lymph node cells *ex vivo* in mice with lymphocyte driven colitis. Chronic colitis was induced in mice and they were then treated with GMP (15 mg/kg) or vehicle for 13 d. MLN cells were isolated and cultured, with or without concanavalin A and the cytokine levels in the supernatant measured by ELISA. A. Percent of CD4+ IFN-γ+ (assessed by flow cytometry). B. IFN-γ. C. TNF-α. D. IL-6. E. IL-10. F. IL-17. $^{+}P<0.05$ vs. control group.

Fig. 5. Body weight evolution in mice with DSS colitis. Colitis was induced in mice with 2% DSS in drinking water. GMP (15 mg/kg) or vehicle were administered 2 d prior to DSS and for an additional 7 d. $^{+}P<0.05$ vs. control group.

Fig. 6. Colonic myeloperoxidase activity in mice with DSS colitis. Colitis was induced in mice with 2% DSS in drinking water. GMP (15 mg/kg) or vehicle were administered 2 d prior to DSS and for an additional 7 d. $^{+}P<0.05$ vs. control group.

Fig. 7. Alkaline phosphatase activity in mice with DSS colitis. Colitis was induced in mice with 2% DSS in drinking water. GMP (15 mg/kg) or vehicle were administered 2 d prior to DSS and for an additional 7 d. A. AP activity. B. Inhibition by levamisole in vitro. $^{+}P<0.05$ vs. control group.

Fig. 8. Cytokine secretion by mesenteric lymph node cells *ex vivo* in mice with DSS colitis. Colitis was induced in mice with 2% DSS in drinking water. GMP (15 mg/kg) or vehicle were administered 2 d prior to DSS and for an additional 7 d. $^{+}P<0.05$ vs. control group.

Fig. 9. Effect of GMP in intestinal epithelial cells. A. Effect on IL-8 secretion by HT29 cells (GMP 0.01-1 g/l). B. Effect on bacterial invasion in IEC18 cells. Cells containing GFP expressing bacteria are detected as GFP positive events and represented as percent of the total cells. C. Effect on wound healing in IEC18 cells. There was no significant effect of GMP in any case.

Table 1. Macroscopic damage parameters values of transfer colitic mice and body weight.

(Mean values with their standard errors)

	Body weight (% initial weight)	Damage score	Colon weight/length ratio (mg/cm)	Colon length (cm)
Control	116.0 ± 1.7	0 ± 0	21.6 ± 1.4	7.1 ± 0.2
Transfer colitis	101.9 ± 2.5	1.3 ± 0.5	54.3 ± 6.3^{+}	9.1 ± 0.2
GMP	104.0 ± 3.4	1.0 ± 0.5	46.4 ± 4.8^{+}	9.0 ± 0.4

GMP, glycomacropeptide.

^{+}P<0.05 as compared with the control group.

Table 2. Macroscopic damage parameters values of DSS colitic mice and body weight.

(Mean values with their standard errors)

	Body weight (% initial weight)	Damage score	Colon weight/length ratio (mg/cm)	Colon length (cm)
Control	104.7 ± 1.6	0 ± 0	21.1 ± 0.6	7.9 ± 0.3
DSS	80.8 ± 1.6^{+}	3.0 ± 0.5^{+}	30.8 ± 1.4^{+}	5.4 ± 0.3^{+}
GMP	78.7 ± 1.6^{+}	2.8 ± 0.5^{+}	31.7 ± 2.2^{+}	5.4 ± 0.3^{+}

DSS, dextran sulfate sodium; GMP, glycomacropeptide.

$^{+}P<0.05$ vs. control

Fig. 1

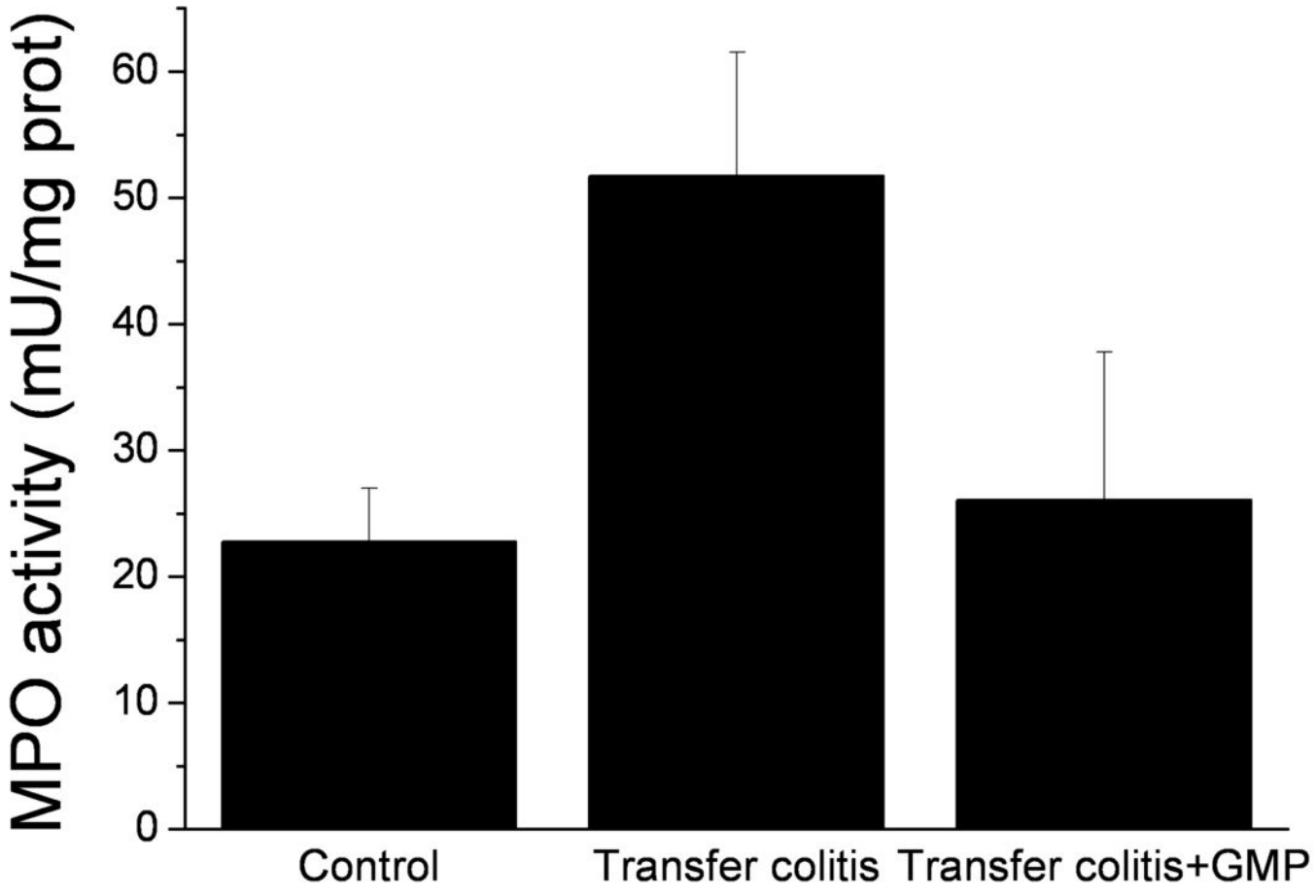
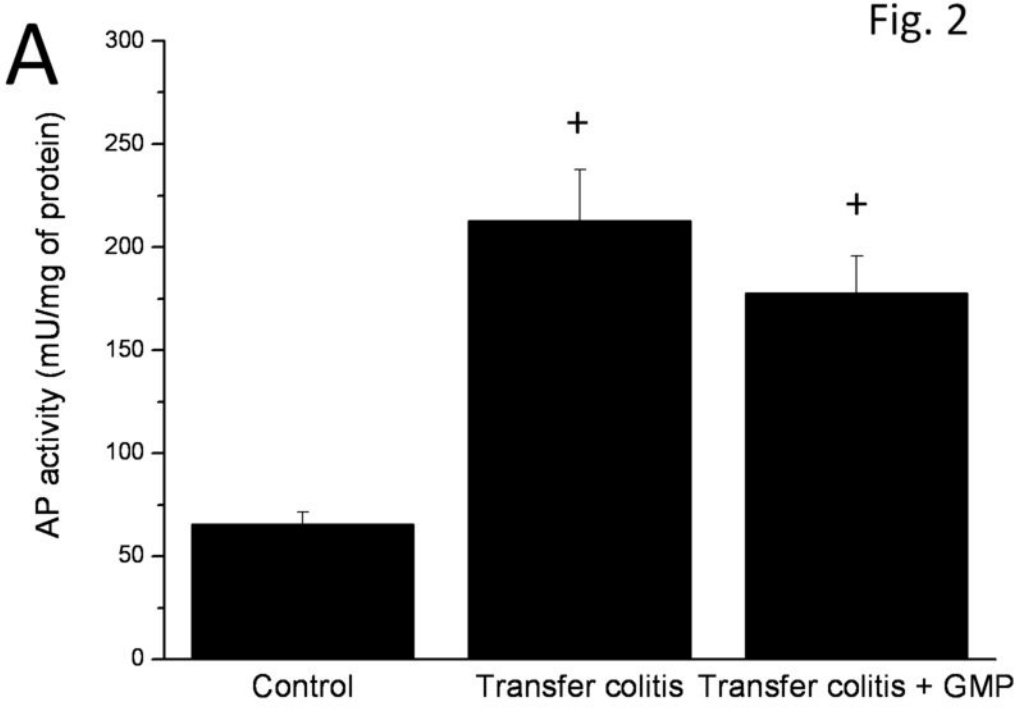

MPO activity (mU/mg prot)
0
10
20
30
40
50
60
Control
Transfer colitis
Transfer colitis+GMP

Fig. 2


A
AP activity (mU/mg of protein)
300
250
200
150
100
50
0
+
+
Control
Transfer colitis
Transfer colitis + GMP

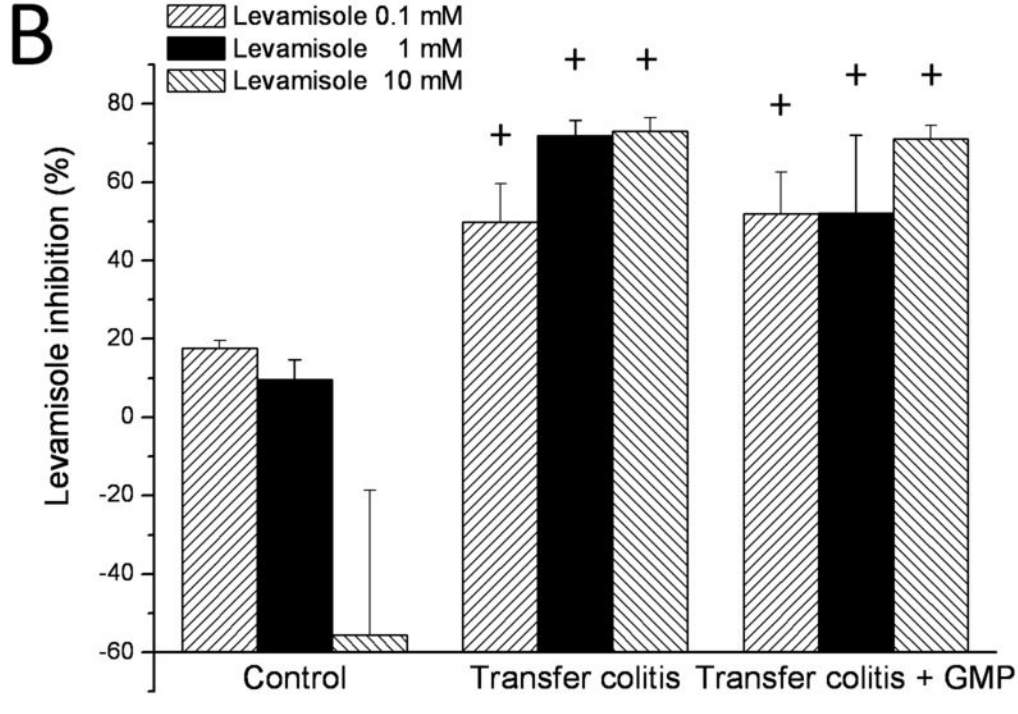
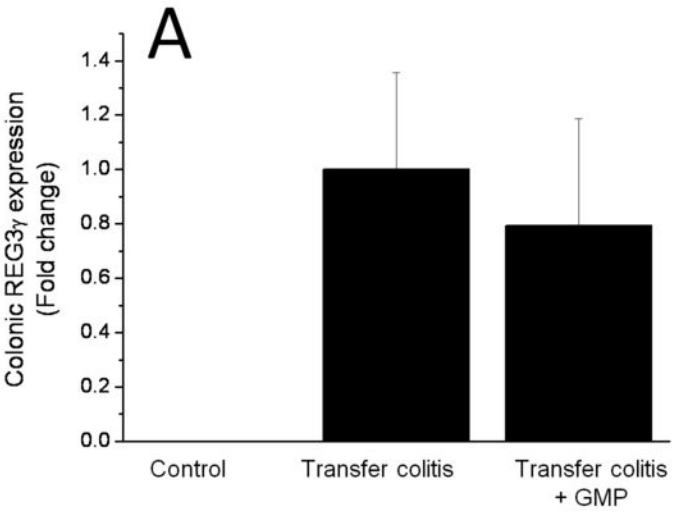
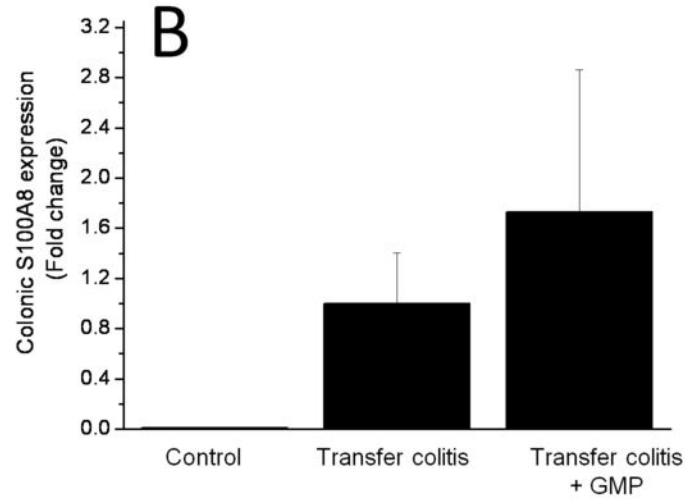
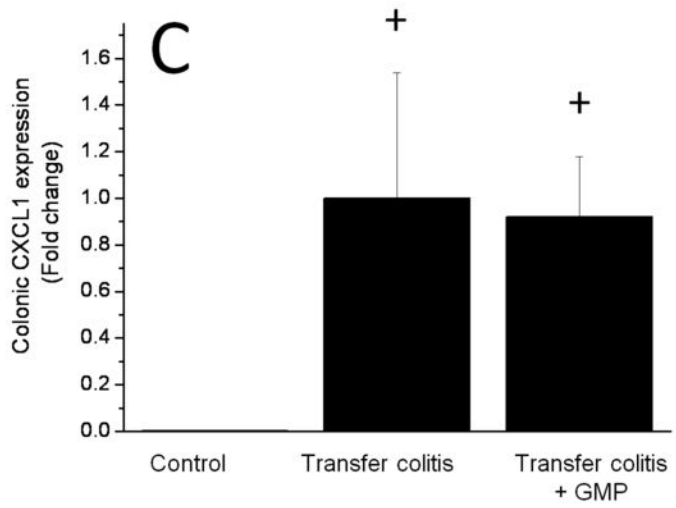
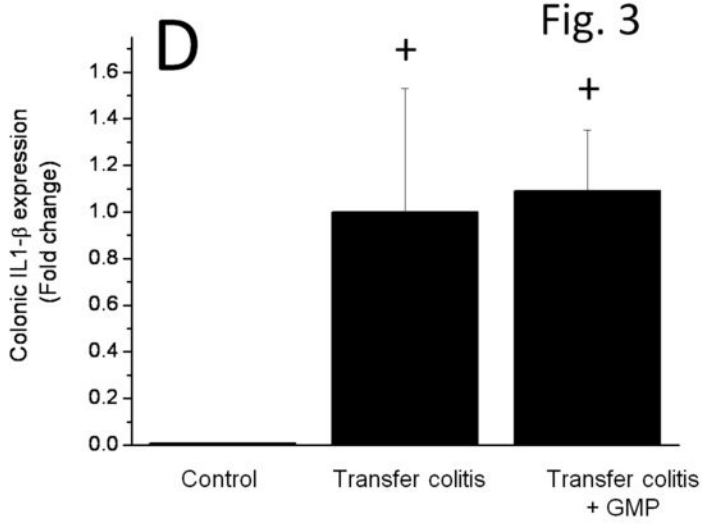

B
Levamisole 0.1 mM
Levamisole 1 mM
Levamisole 10 mM
Levamisole inhibition (%)
80
60
40
20
0
-20
-40
-60
+
+
+
+
+
+
Control
Transfer colitis
Transfer colitis + GMP

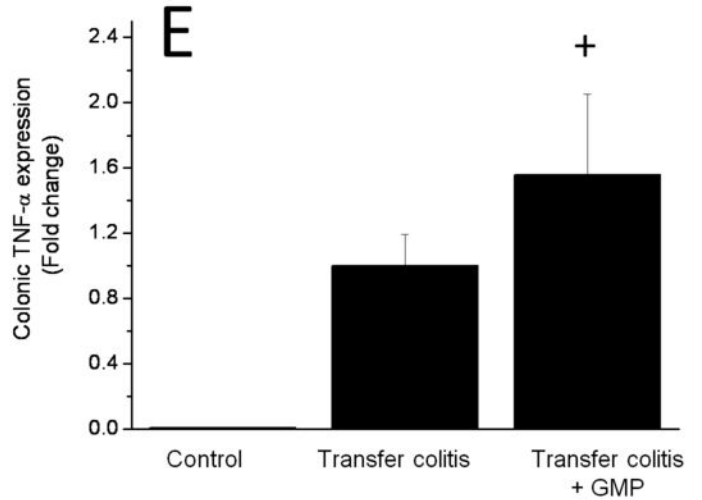
Fig. 3

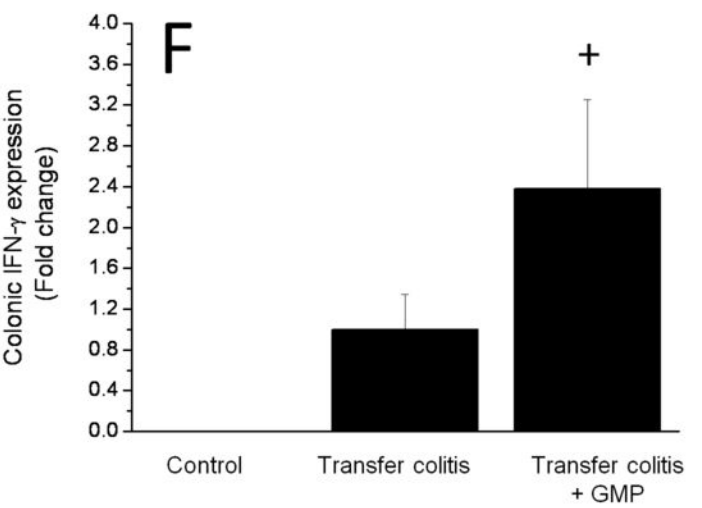

A
Colonic REG3γ expression (Fold change)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Transfer colitis
Transfer colitis + GMP


B
Colonic S100A8 expression (Fold change)
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Control
Transfer colitis
Transfer colitis + GMP


C
Colonic CXCL1 expression (Fold change)
+
+
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Transfer colitis
Transfer colitis + GMP


D
Colonic IL1-β expression (Fold change)
+
+
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Transfer colitis
Transfer colitis + GMP


E
Colonic TNF-α expression (Fold change)
+
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Control
Transfer colitis
Transfer colitis + GMP


F
Colonic IFN-γ expression (Fold change)
+
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Control
Transfer colitis
Transfer colitis + GMP

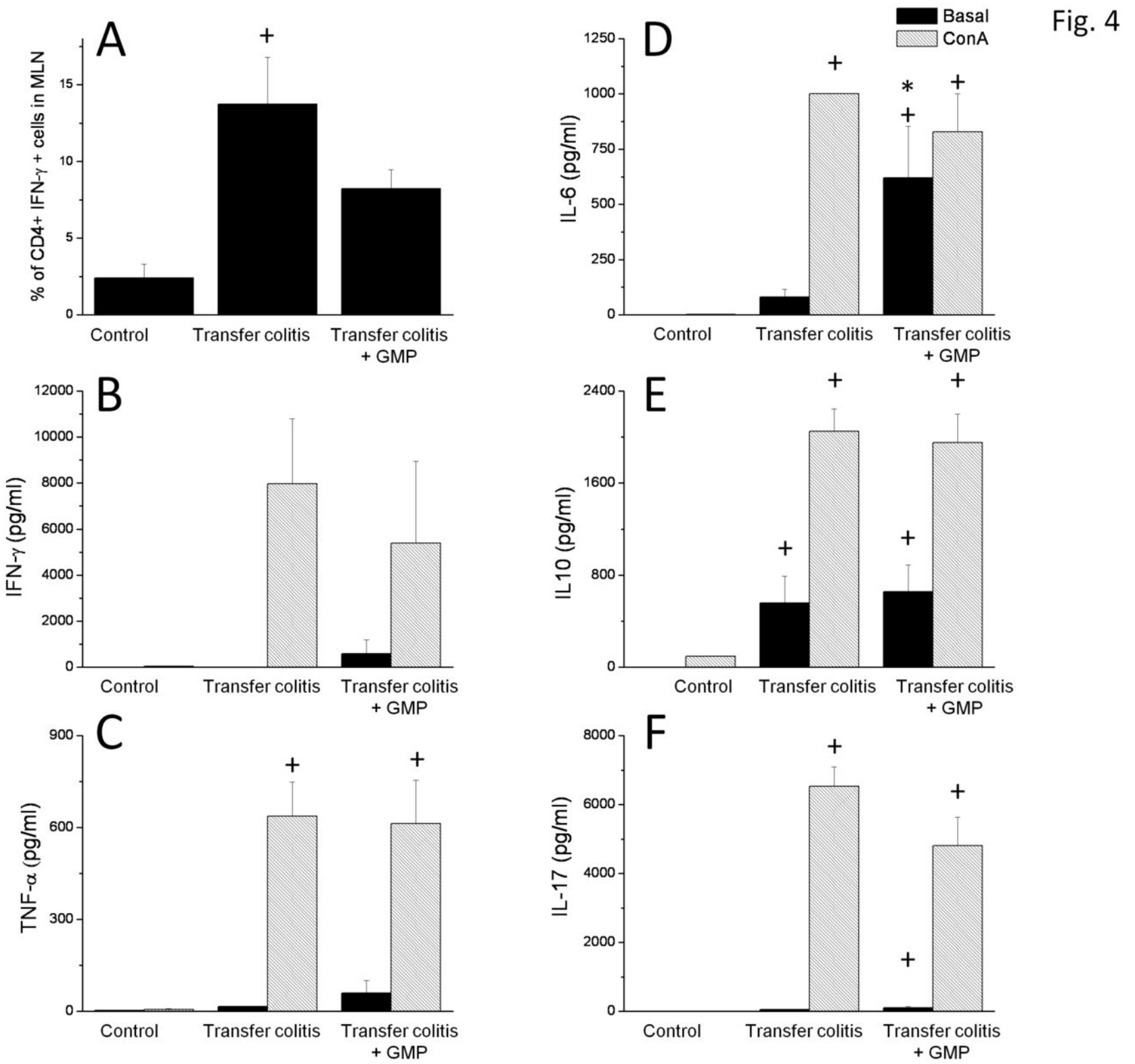

A
% of CD4+ IFN-γ + cells in MLN
0
5
10
15
+
Control
Transfer colitis
Transfer colitis + GMP
B
IFN-γ (pg/ml)
0
2000
4000
6000
8000
10000
12000
Control
Transfer colitis
Transfer colitis + GMP
C
TNF-α (pg/ml)
0
300
600
900
+
+
Control
Transfer colitis
Transfer colitis + GMP
D
Basal
ConA
IL-6 (pg/ml)
0
250
500
750
1000
1250
+
*
+
+
Control
Transfer colitis
Transfer colitis + GMP
E
IL10 (pg/ml)
0
800
1600
2400
+
+
+
+
Control
Transfer colitis
Transfer colitis + GMP
F
IL-17 (pg/ml)
0
2000
4000
6000
8000
+
+
+
Control
Transfer colitis
Transfer colitis + GMP

Fig. 4

Fig. 5

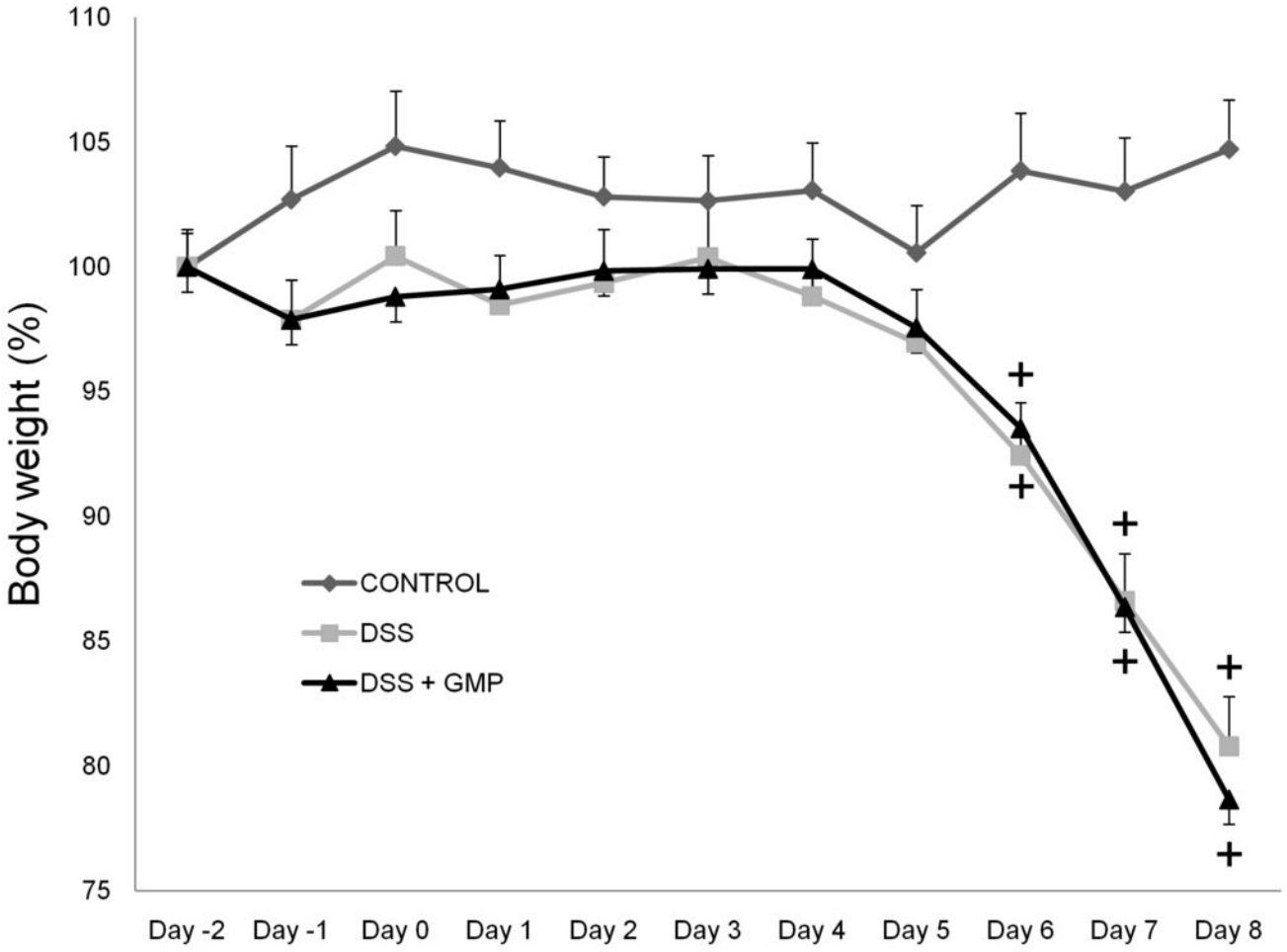

110
105
100
95
90
85
80
75
Body weight (%)
CONTROL
DSS
DSS + GMP
Day -2
Day -1
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8

Fig. 6

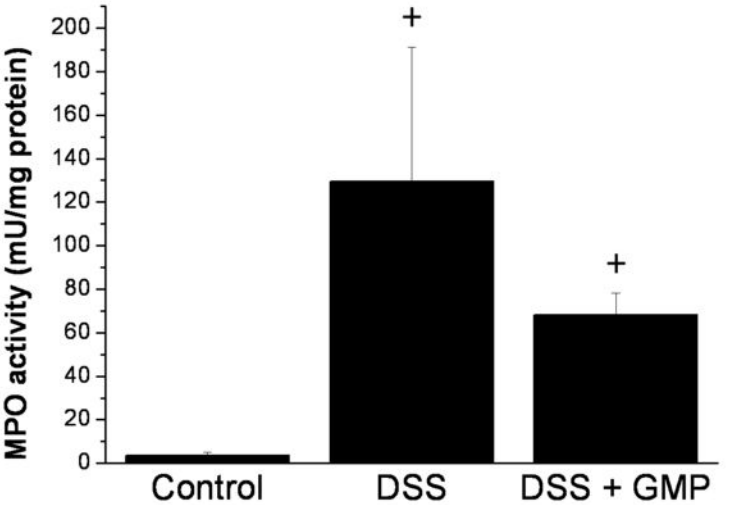

MPO activity (mU/mg protein)
200
180
160
140
120
100
80
60
40
20
0
+
+
Control
DSS
DSS + GMP

Fig. 7

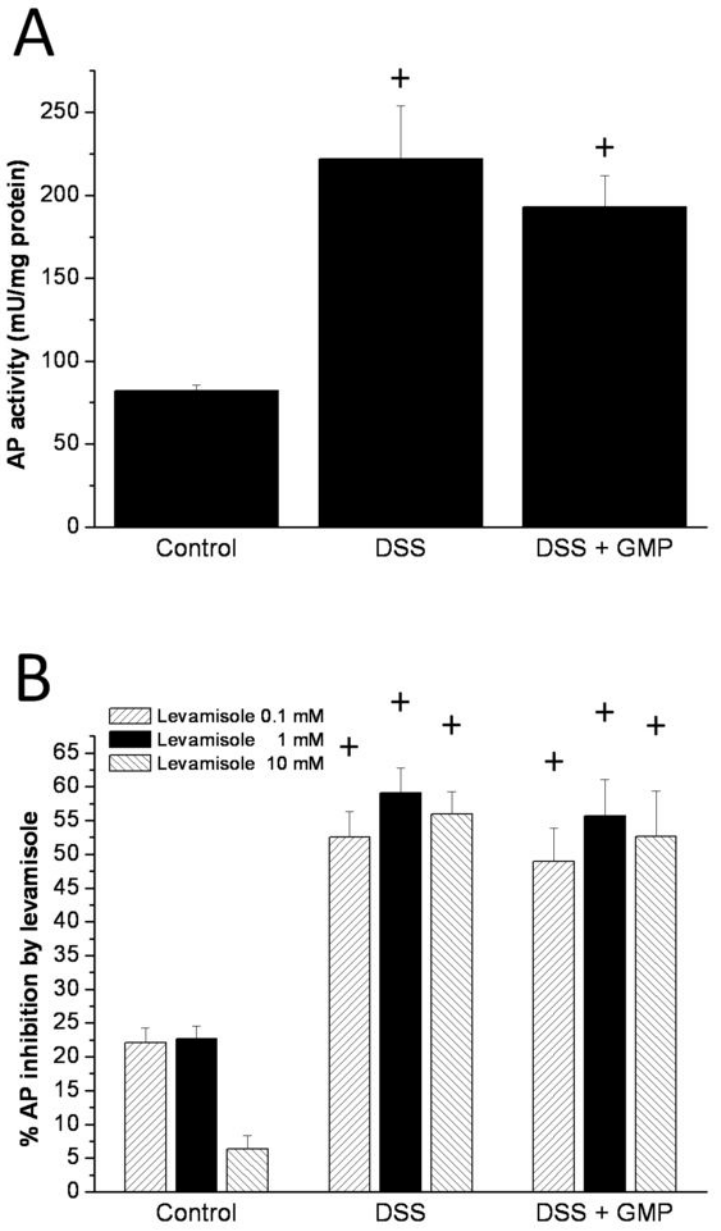

A
AP activity (mU/mg protein)
0
50
100
150
200
250
+
+
Control
DSS
DSS + GMP
B
Levamisole 0.1 mM
Levamisole 1 mM
Levamisole 10 mM
% AP inhibition by levamisole
0
5
10
15
20
25
30
35
40
45
50
55
60
65
+
+
+
+
+
Control
DSS
DSS + GMP

Fig. 8

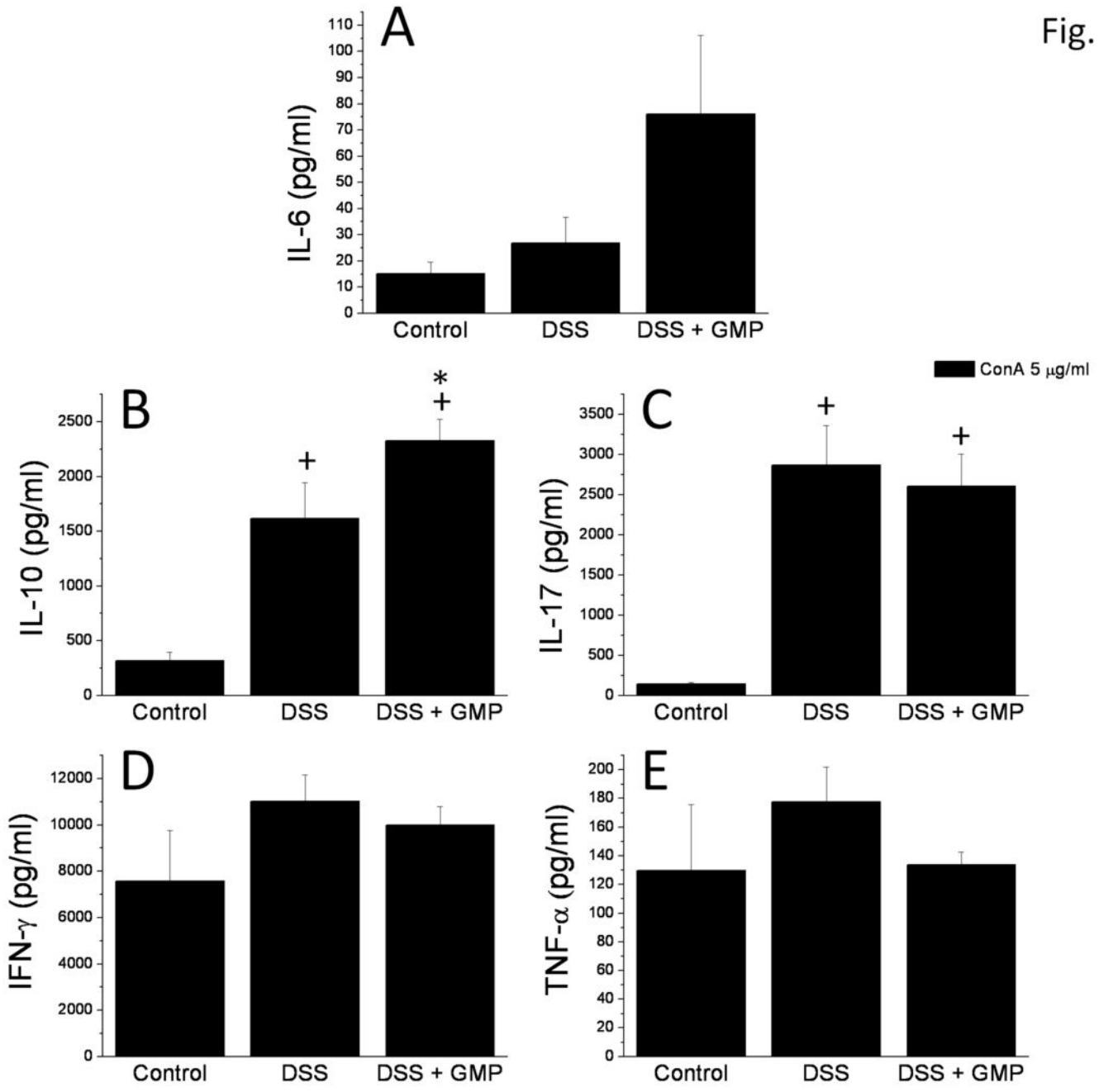
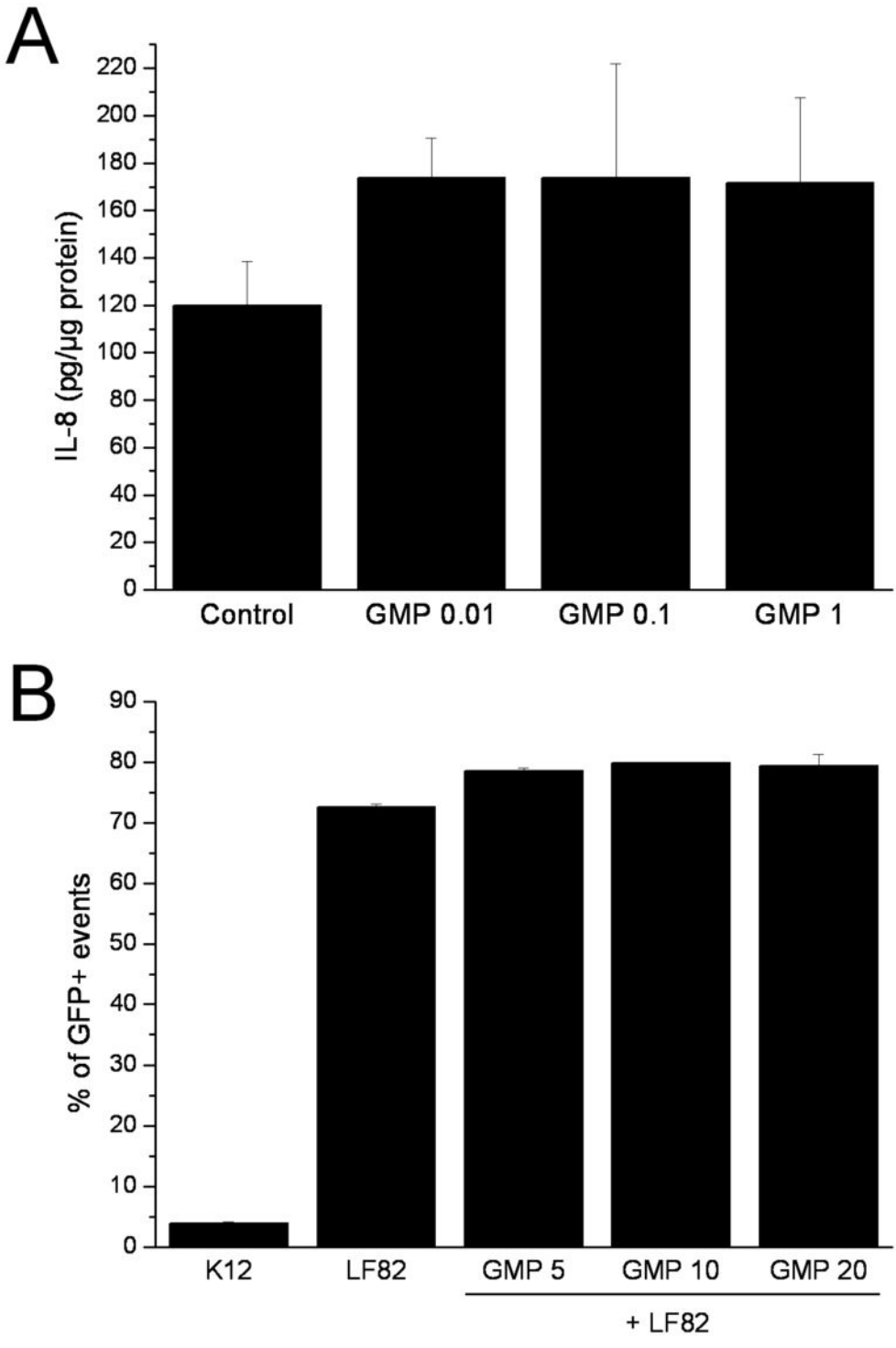

A
IL-6 (pg/ml)
110
100
90
80
70
60
50
40
30
20
10
0
Control
DSS
DSS + GMP
ConA 5 μg/ml
B
IL-10 (pg/ml)
2500
2000
1500
1000
500
0
+
*
+
Control
DSS
DSS + GMP
C
IL-17 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
+
+
Control
DSS
DSS + GMP
D
IFN-γ (pg/ml)
12000
10000
8000
6000
4000
2000
0
Control
DSS
DSS + GMP
E
TNF-α (pg/ml)
200
180
160
140
120
100
80
60
40
20
0
Control
DSS
DSS + GMP

Fig. 9


A
IL-8 (pg/µg protein)
0
20
40
60
80
100
120
140
160
180
200
220
Control
GMP 0.01
GMP 0.1
GMP 1
B
% of GFP+ events
0
10
20
30
40
50
60
70
80
90
K12
LF82
GMP 5
GMP 10
GMP 20
+ LF82
C
Control
GMP 1g/L
% wound area
0
10
20
30
40
50
60
70
80
90
100
0
6
11
24
Time (hours)