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## 3rd International Immunonutrition Workshop

# Session 9: Food ingredients, immunity and inflammation: animal and *in vitro* models
# New insights into the immunological effects of food bioactive peptides in animal models of intestinal inflammation

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Bioactive peptides have proven to be active in several conditions, including inflammatory bowel disease (IBD). This is a chronic and relapsing condition of unknown aetiology that comprises chiefly ulcerative colitis and Crohn's disease. Although there are treatments for IBD, they have frequent side effects and they are not always effective; therefore there is a need for new therapies that could alleviate this condition. Two bioactive peptides present in milk (transforming growth factor-β (TGF-β) and casein macropeptide, also named glycomacropeptide) have been shown to have intestinal anti-inflammatory activities. In fact, TGF-β is currently added to formulas intended for patients with IBD, and several studies indicate that these formulas could induce clinical remission. In this paper, evidence supporting the anti-inflammatory effect of TGF-β and bovine glycomacropeptide, as well as their mechanisms of action, is reviewed, focusing on the evidence obtained in animal models.

**Milk: Biopeptides: Inflammatory bowel disease: Bovine glycomacropeptides: Transforming growth factor-β**



## Food bioactive peptides

There is a series of specific food peptides referred to as bioactive peptides that, besides their nutritional value, modulate biological processes. Bioactive peptides can be present in food as such or can result from *in vivo* or *in vitro* protein digestion[1]. To obtain the latter, proteolytic enzymes from animal or bacterial origin can be used. Many of the bioactive peptide enriched functional foods currently marketed are obtained by bacterial fermentation. The low cost of these products and the positive image associated with fermented drinks and foods make them very attractive[1,2]. Any source of food proteins can produce bioactive peptides. Thus, they have been isolated from sardine, corn, soya, egg, gelatin, etc.[3]. Nevertheless, the main source of food bioactive peptides is milk[1]. Several studies have demonstrated that food bioactive peptides can reach the intestine, resisting digestion, and go through the intestinal barrier, and hence exert effects both at the intestinal and systemic levels[2]. Accordingly, intestinal[1,4,5], cardiovascular[6,7] and immunological[4] effects have been

**Abbreviations:** IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; Th, T helper; IFN, interferon; TGF, transforming growth factor; BGMP, bovine glycomacropeptide; TNBS, trinitrobenzene sulfonic acid; DSS, dextran sodium sulphate.

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described[1,3,8]. In addition, a group of peptides can also act as biocarriers for Ca and other minerals, increasing their bioavailability[8,9]. The activities of milk peptides and products containing them have been recently reviewed elsewhere[1,2].

## Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic and relapsing condition that severely affects the quality of life of patients. The term IBD refers to a group of diseases such as microscopic colitis, collagenous colitis, pseudomembranous colitis, ulcerative colitis (UC) and Crohn's disease (CD). UC and CD are the most relevant conditions, both in incidence and prevalence[10]. They share histological and clinical similarities, but there are clear differences between them; whereas in UC only the colonic mucosa is damaged, in CD any part of the gastrointestinal tract from the mouth to the anus can be affected (although mostly the ileum and colon), and damage involves not only the intestinal mucosa but the whole intestinal wall[10]. On the other hand, differences in the pathophysiology of these diseases have been observed that indicate a preponderance of T helper (Th) 1 response in CD patients, with an increased production of IL-12, interferon (IFN)-γ and IL-18, whereas a Th2 response seems to be operating in UC patients[11].

The aetiology of IBD is unknown but an interplay of genetic[12], environmental and lifestyle factors (dietary habits, smoking and stress) has been associated with this condition[10,13–15]. Genetic variants have been linked to intestinal microflora and intestinal permeability alterations in IBD patients, and currently the most accepted theory proposes that intestinal inflammation results from an inappropriate immune response by the host (driven by genetic and environmental factors) to the intestinal microflora.

IBD can be currently treated with drugs, among which immunosuppressors, like azathioprine and corticoids, and aminosalicylates, together with anti-TNF antibodies, are the treatment of choice in acute episodes and/or for delaying relapses of the disease[10]. Unfortunately, most of these treatments can lead to severe side effects[16]. Therefore, the search for new treatments that could alleviate or treat the disease is in progress.

## Pathophysiology of inflammatory bowel disease

Every type of intestinal cell is involved in the pathophysiology of IBD. Epithelial cells constitute the first line of defence. Studies in which the NF-κB response in the intestinal epithelium of mice was specifically ablated showed that colonic inflammation and pancolitis developed at 3 weeks of age[17]. The authors of this study indicated that the inhibition of the NF-κB response favours the disruption of the intestinal barrier integrity, resulting in bacterial translocation and intestinal inflammation[17]. In accordance with this observation, low levels of defensins in intestinal epithelial cells have been observed in IBD patients[18].

Macrophages and neutrophils are the first cells attracted to the focus of inflammation. There macrophages produce mainly TNF, IL-1β, IL-6 and IL-12. These cytokines attract neutrophils, T and B lymphocytes and regulate the inflammatory response. At least four different types of $CD4^+$ Th lymphocytes (Th1, Th2, Th17 and Treg) are involved in the immune response in IBD. These are the result of naive Th cell (Th0) differentiation that ultimately depends on the cytokines produced in the inflammatory site (Fig. 1). Differentiated Th cells in turn produce cytokines that regulate the immune response activating or directing B lymphocytes, macrophages, neutrophils and cytotoxic cells.

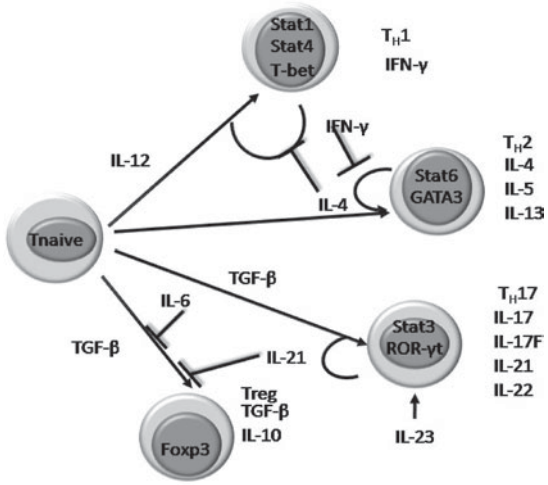


**Fig. 1.** Molecular requirements for T helper (Th) cell differentiation. Adapted from Dennick and Tangye[76]. IFN, interferon; ROR, retinoic acid-related orphan receptor; Stat, signal transducers and activator of transcription; T-bet, T-cell specific T-box transcription factor; Treg, regulatory T cells.

Treg are $CD4^+Foxp3^+$ regulatory T cells that play an essential role in intestinal homoeostasis. Treg are characterized by the expression of Foxp3, which is considered to confer their regulatory activity[19]. The expression of Foxp3 in peripheral Th0 is dependent on transforming growth factor (TGF)-β. Treg appear in the intestine after the oral administration of antigens and produce IL-10 and TGF-β1, which, in general terms, down-regulate inflammation. Several pro-inflammatory cytokines inhibit Treg induction, including IL-6, IL-21, IL-23 and IL-27. Among them, IL-23 is the key for the inhibition of Treg during inflammation[20]. IBD patients appear to have lower numbers of Treg both in blood and colon; nevertheless it is important to bear in mind that Treg isolated from these patients are functionally active *in vitro*[20].

Th17 cells are a newly described subpopulation of Th cells involved in the pathogenesis of IBD. These pro-inflammatory lymphocytes are characterized by the expression of the master transcription factor retinoic acid-related orphan receptor-γt as well as IL-17A, IL-17F, IL-21, IL-22 and IL-26[21]. Th17 cells differentiate under the influence of IL-1β, IL-6, IL-21, IL-23 and TGF-β. The latter has been shown to be essential for differentiation[21]. Th17 cells appear to be involved in the pathophysiology of IBD, particularly in CD, in which Th1 cells are also implicated[22–24].

Th1 cells express STAT-4 and produce IL-2, IL-12, IL-18 and IFN-γ. While IFN-γ, IL-12 and IL-18 drive Th1 differentiation, IL-10, TGF-β and IL-4 inhibit it. IL-2

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acting in an autocrine fashion induces Th1 proliferation. The Th1 response implicates mainly the activation of macrophages, increasing the production of IFN-γ by lymphocytes and in turn the production of IL-12 by macrophages and dendritic cells. Th2 cells express GATA-3 and produce IL-4, IL-5, IL-6 and IL-13. Th2 cells proliferate in response to IL-4, while IFN-γ inhibits them. The activation of the Th2 response implicates B cells, increasing antibody production. As indicated above, the Th1 response is predominant in CD patients, while in UC colitis the Th2 response seems to dominate[10].

## Animal models of inflammatory bowel disease

In general, animals do not suffer from chronic intestinal inflammation spontaneously. Therefore, several animal models that mimic different aspects of the disease have been developed, including gene knockout, transgenic, chemical and adoptive transfer models[25]. Chemical models use different substances that disrupt the intestinal barrier or induce an immune reaction to elicit colonic inflammation[26]. Among these the models of murine colitis induced by the administration of trinitrobenzene sulfonic acid (TNBS) or dextran sodium sulfate (DSS) are the most widely used[27,28]. The first is a simple and convenient model based on one single rectal administration of TNBS dissolved in ethanol. TNBS is a hapten that elicits an immune response when bound with high-molecular-weight tissue proteins, while ethanol contributes by the disruption of the intestinal barrier. The result is a severe and prolonged degenerative inflammation of large parts of the colon that shares several clinical and molecular characteristics with CD. In particular, the inflammation produced by the administration of TNBS-ethanol involves all the layers of the intestinal mucosa and produces long-lasting damage, with cell infiltration and ulcers[29]. Furthermore, the administration of TNBS-ethanol to mice, as observed in human CD, produces a Th1-driven inflammation characterized at the initial stage by the infiltration of macrophages and neutrophils, releasing high levels of pro-inflammatory cytokines such as TNF, IL-1β or IL-6, followed by infiltration of T cells, mainly of the $CD4^+$ phenotype, that produce IL-2 and IFN-γ[27,29]. It is interesting to note that, when administered directly into the ileum of rats, TNBS also induces ileitis[30]. The administration to mice or rats of DSS in the drinking water gives rise to an inflammation characterized by bloody diarrhoea, ulcerations and granulocyte infiltration. DSS is thought to be directly toxic to epithelial cells, disrupting the intestinal mucosal barrier. Both the TNBS and DSS models can be reproduced successfully in the absence of adaptive immunity, although this does not mean that the latter does not contribute to pathology in normal conditions[27]. The involvement of Th17 cells in these models is unclear or controversial at this time. Besides their similarities to multiple aspects of human IBD, the DSS and the TNBS murine models have several outstanding characteristics: the onset and duration of inflammation are immediate and controllable and there are no artificial genetic deletions or manipulations that are not found in human IBD[31].

The most used knockout model of IBD is the $Il10^{-/-}$ mice. These animals develop a spontaneous caecal inflammation and colitis at 2–4 months of age that features many characteristics observed in human IBD. The inflammation in this model is Th1 driven and is similar, therefore, to CD inflammation in human subjects. Transgenic models of IBD are well represented by the HLA-B27 model of rat colitis. In this model human HLA-B27/microglobulin 2 transgenic rats develop chronic colitis that, among other characteristics, is accompanied by some extraintestinal complications resembling those seen in IBD patients, like spondyloarthropathy involving peripheral and axial joints, dermatological complications and male genital inflammation[32]. Finally, in the transference model $CD4^+$ $CD45RB^{high}$ T cells (or equivalent) from healthy wild-type mice are transferred to mice lacking functional B and T cells[33]. After 5–8 weeks, pancolitis and intestinal inflammation are observed in recipient mice with features that are similar to those of human CD. This model presents the advantages that both early symptoms of inflammation and the perpetuation of the disease can be studied[33].

## Bioactive peptides and inflammatory bowel disease

So far, the evidence indicates that at least two milk bioactive peptides could be useful in the treatment of IBD: TGF-β and bovine glycomacropeptide (BGMP), also known as bovine casein macropeptide.

TGF-β is a growth factor present in high concentrations in the milk of several species, including human milk, and it is also produced in small amounts in the intestine of newborn infants. There are at least three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3). These isoforms have high homology among themselves (70% TGF-β1 *v.* TGF-β2 and 74% TGF-β3 *v.* TGF-β2), and the amino acid sequence is also highly conserved in different species (<94%)[34]. It has been described that TGF-β1 and TGF-β2 are generally equivalent in their functionality both *in vivo* and *in vitro*[35]. Human milk contains mainly TGF-β1 and TGF-β2, the last being the major isoform (95%)[34,36]. Recent studies indicate that TGF-β is expressed as a pre-pro-factor that gives rise to the secretion of the pro-factor after intracellular hydrolysis, included with other proteins in a latent complex. The latent form in milk is finally activated by gastric acid[37].

TGF-β has pleiotropic functions, including the regulation of the immune function as well as functions related to cellular growth and differentiation. TGF-β1 transferred from the mother via placenta or milk is so biologically relevant that mice in which this gene is disrupted survive until weaning and then succumb to a wasting syndrome accompanied by a multifocal, mixed inflammatory cell response and tissue necrosis, leading to organ failure and death[38]. It has been widely demonstrated in rodents that the oral administration of TGF-β induces oral tolerance and inhibits allergic reactions[35]. When TGF-β is orally administered to TGF-β-null mice it prevents their death and can be localized in internal organs such as the lung, indicating that it can be active at extraintestinal levels[38,39]. The fast absorption in newborns together with

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the detection of high TGF-β levels in the plasma of healthy human volunteers reinforces this hypothesis[35,40].

Alterations in TGF-β signalling can play an important role in IBD. In fact, in a study carried out in mice in which a dominant-negative mutant form of the TGF-β type II receptor was overexpressed specifically in the intestine, the animals spontaneously developed diarrhoea and under specific pathogen-free conditions they were more susceptible to the induction of colonic inflammation by DSS[41]. In these mice, treatment with DSS produced an increased expression of MHC class II, an exacerbated generation of autoantibodies against intestinal goblet cells, and an augmented activity of matrix metalloproteinase in intestinal epithelial cells, compared with wild-type littermates[41]. On the other hand, there is strong evidence that indicates the importance of TGF-β in the development of IBD. The induction of TGF-β brought about by the oral administration of haptenated colonic proteins (to induce oral toleration) protects mice from the induction of colitis by the administration of TNBS. This preventive effect is also obtained after the systemic administration of TGF-β. Conversely, the administration of anti-TGF-β antibodies reverses the protective effect of oral haptenated colonic proteins[42]. These studies indicate that TGF-β is a major regulator of intestinal mucosal homoeostasis, acting in fact by regulating suppressive cells in an autocrine fashion, preventing the expression of IFN-γ and therefore inhibiting the activation of T (mainly Th1) and B cells, resulting in protection of the epithelial monolayer from the permeability-enhancing effects of TNF[40].

Several authors have shown that the oral administration of TGF-β has anti-inflammatory effects at the intestinal level in animal models of colitis. Recently, a study has been published in which animals were fed cow's milk (500 µl) containing TGF-β (3 µg/l) daily for 2 weeks, before the induction of colitis by the administration of DSS. The authors observed that the animals that received TGF-β lost less weight and had a decreased degree of intestinal inflammation[35]. Furthermore, in 2005 Schiffrin *et al*. carried out a study to determine the effect of a casein-based formula containing TGF-β (1 ng/mg protein) to treat inflammation in human leucocyte antigen (HLA)-B27 transgenic rats[16]. These authors described that the administration of TGF-β reduced leucocytes and the acute phase reactants fibrinogen and orosomucoid. In addition, colonic weight and wall thickness as well as the mRNA for IFN-γ were reduced. Finally, there was an increase in mucin-2 production in the caecum of the animals that received the TGF-β containing formula and a normalization of the muscle proteolytic activity. A formula with the same protein composition has been proven to be anti-inflammatory in paediatric patients with CD, associated with a reduction in the expression of IFN-γ[43]. In this study, seven children with CD received a TGF-β2-enriched formula for 8 weeks. In spite of the small number of patients, the results were quite clear since all the children showed a significant improvement of the disease activity, with C-reactive protein returning to normal, an increase in serum albumin and a substantial weight gain. Furthermore, ileal biopsies of these children showed reduced mucosal inflammation in six of seven children, with complete healing in two[43]. However, the lack of a control group limits the usefulness of this study. This was followed by another study with 29 children with CD in which a 79% remission rate was achieved, with complete healing in ten cases, after 8 weeks administration of the TGF-β-enriched formula[44]. In this study, Fell *et al*. described a fall in ileal IFN-γ, indicating a decrease in Th1 response, and a strong fall in ileal and colonic IL-1β and in colonic IL-8 mRNA[44].

These and several other studies have indicated that formulas enriched in TGF-β could induce clinical remission associated with mucosal healing in IBD patients[43–46]. These formulas are casein-based, lactose-free formulas that are enriched in TGF-β and are currently in the market. Although several studies have shown that the formulas are less effective than corticosteroids, they have proven to be more active in paediatric patients than in adults[45]. As a result, TGF-β-enriched formulas are used in paediatric patients in which the administration of corticosteroids could have deleterious effects on linear growth[45].

The other bioactive peptide potentially useful in IBD is BGMP, a 64-amino-acid peptide derived from the digestion of milk k-casein. This peptide is produced physiologically *in vivo*, as a result of k-casein hydrolysis, and *in vitro*. Actually, BGMP is one of the components that elute with the whey that results after chymosin digestion during the cheese-making process. This peptide can be easily purified and is found in the market with a high degree of purity. BGMP is heavily glycosylated and many of its actions depend on glycosylation[47].

A wide variety of actions and applications have been described for BGMP. Because it does not contain phenylalanine in its sequence, BGMP has been proposed to be useful in the making of products for individuals with phenylketonuria[48–50]. On the other hand, this peptide has anticarcinogenic properties and has been proposed to be used in tooth pastes[47]. Its mechanism of action is related to the inhibition of bacterial growth, the prevention of tooth demineralization and the promotion of enamel remineralization[47]. In addition, BGMP may combat infection since it has been reported to bind to cholera and *Escherichia coli* enterotoxins, to inhibit bacterial and viral adhesion, to promote bifidobacteria growth and to modulate the immune system response[2,6,7]. Because of its facilitation of mineral absorption[51] and its possible antimicrobial and prebiotic effects, BGMP is added to infant formulas[52]. It is important to indicate that, although as indicated earlier there are some studies showing that this peptide promotes the growth of bifidobacteria *in vitro*[53,54], so far there is not definitive evidence of the prebiotic effect of BGMP, since the studies carried out with rhesus monkeys and human infants were hampered by a high initial level of bifidobacteria[51,55].

BGMP has been shown to affect both innate and adaptive immunity. Thus BGMP increases the proliferation of concanavalin A-stimulated rat splenocytes[56] and induces the expression and production of inducible nitric oxide synthase, cyclooxygenase, IL-10 and Foxp3 at the concentration of 1 g/l. However, there is also evidence that BGMP inhibits mouse splenocyte proliferation induced by lipopolysaccharide and phytohaemagglutinin[57], while an

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inhibition in the production of IFN-γ has been observed in concanavalin A-stimulated splenocytes (P Requena, A Daddaoua, A Zarzuelo, MD Suárez, F Sanchez de Medina and O Martinez-Augustin, unpublished results).

In macrophages BGMP seems to have also a stimulatory effect. For instance, BGMP increased the proliferation and phagocytic activity in a human macrophage cell line[58]. Our research group has shown, both in THP-1 cells (a monocyte/macrophage cell line) and in human peripheral blood macrophages, that BGMP stimulates the production of several cytokines (TNF, IL-1β and IL-8)[59]. In these experiments the mechanism of action of this peptide was further studied, showing that the whole peptide is needed, since it was active when protease inhibitors were added to the culture medium[59], and no effect was observed when it was hydrolysed (P Requena, A Daddaoua, A Zarzuelo, MD Suárez, F Sanchez de Medina and O Martinez-Augustin, unpublished results). The effect of BGMP was prevented by the addition of mitogen-activated protein kinase or NF-κB inhibitors, and BGMP induced the phosphorylation of NFκB inhibitor-α. Therefore, an involvement of these signal transduction pathways in the stimulatory effect of BGMP on cytokine production by macrophages was hypothesized[59]. In addition, studies by Monnai and Otani[60] indicated that BGMP increases the expression of an IL-1 receptor antagonist-like component in mouse spleen cells, involving probably monocytes.

In general, these results suggest that BGMP modulates the immune/inflammatory response by the activation of macrophages, favouring the differentiation of Treg cells and hampering the activation of Th1 cells.

As stated above, an exacerbated immune response and an imbalance in the intestinal flora have been blamed, among other factors, for the aetiology of IBD. The fact that BGMP exerts immunomodulatory, antimicrobial and possibly prebiotic activities makes it a very good candidate to modulate intestinal inflammation. In fact, our research group has described that the oral administration of BGMP results in substantial anti-inflammatory effects in experimental colitis and ileitis[30,61].

In a first study we used the model of colitis induced by the administration of TNBS to rats. The animals were administered BGMP daily starting either 2 d before (pre-treatment) or 3 h after (post-treatment) colitis induction[61]. The effect of BGMP (500 mg/kg per d) was compared with that of sulfasalazine, an established drug used in the treatment of IBD. We found that pre-treatment with glycomacropeptide had a dose-dependent anti-inflammatory effect, characterized by lower body-weight loss, decreased anorexia (57 %), colonic damage (65 %) and weight; length ratio (32 %), as well as a reduction in colonic alkaline phosphatase activity (42 %) and IL-1β, trefoil factor 3 and inducible nitric oxide synthase mRNA levels ($P<0{\cdot}05$)[61]. The magnitude of the anti-inflammatory effect was generally comparable with that of sulfasalazine[61].

To further ascertain the mechanism involved in the anti-inflammatory effect of BGMP and to find new beneficial applications for this peptide, we studied its effect in a model of ileitis induced by the injection of TNBS in the ileum of rats[30]. Since CD affects any part of the intestine, ileitis frequently affects CD patients. Furthermore, ileitis can be also the result of ileal infections[62,63] and it is a frequent complication of the ileal pouch-anal anastomosis interventions practised to treat UC[64]. While lymphocyte Th1 and Th2 responses are predominant in colonic CD and UC patients, respectively, it seems that at least in Crohn's ileitis both types of T cells are involved in the inflammatory response. Our results indicated that BGMP pre-treatment (500 mg/kg per d) results in a marked reduction of inflammatory injury, as assessed by lower extension of necrosis and diminished damage score, myeloperoxidase, alkaline phosphatase, inducible nitric oxide synthase, IL-1β and IL-17. Again, the effects of BGMP were similar to those observed in the same experiment for 5-aminosalicylic acid (200 mg/kg per d). 5-Aminosalicylic acid is the active part of sulfasalazine, which is released from the prodrug by colonic bacteria[65].

From the above-mentioned studies with BGMP, we can conclude that BGMP has an anti-inflammatory effect in animal models of ileitis and colitis induced by TNBS and that this effect is comparable with that of drugs frequently used to treat IBD. We have shown that BGMP decreases the expression of IL-1β, IL-1ra, TNF and IL-17 in ileitis/colitis and therefore we can conclude that the mechanism of action of BGMP probably involves Th17 cells, macrophages and T cells but not Th1 cells[30,61].

In a third study, we obtained mRNA from the colon of control and TNBS colitic rats administered vehicle or BGMP (500 mg/kg per d) as described earlier and carried out microarrays, allowing the simultaneous measurement of over 30 000 genes and real-time PCR for 96 selected genes (P Requena, A Daddaoua, A Zarzuelo, MD Suárez, F Sanchez de Medina and O Martinez-Augustin, unpublished results). The use of powerful genomic and post-genomic techniques gave us further insight into the mechanism of action of this peptide. The processing of our results from microarrays and real-time PCR with Ingenuity Pathway Analysis software indicated that the Il1b (IL-1β) and Il6 (IL-6) genes, both of which are down-regulated by BGMP, are key points in its mechanism of action. We observed also a decrease in IL-17, in neutrophil and macrophage infiltration and in tissue remodelling genes, as well as an increase in the *Il10* gene and in a set of genes related to lymphocyte infiltration.

IL-6 is a key factor in the development of inflammation and specifically in the uncontrolled inflammatory process in IBD[66–68]. In fact, increased blood and mucosal levels of this cytokine have been described in UC and CD patients, correlated with the severity of the disease[66]. Accordingly, antibodies against IL-6 or its soluble receptor have been shown to be useful in the treatment of IBD in human subjects[67]. There is growing evidence indicating that this cytokine, produced mainly by macrophages and $CD4^+$ T cells, is one of the main ones in the chronic phase of colitis[66]. IL-6 has three main roles in inflammation: (i) the recruitment of neutrophils to the inflammatory site and their activation to produce matrix metalloproteinases, thus contributing to severe tissue damage; (ii) the regulation of $CD4^+$ leucocyte apoptosis: IL-6, together with IL-12 and TNF, is an important anti-apoptotic cytokine involved in the pathogenesis of CD, whose inflammatory response, like that of the TNBS model

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of colitis, is driven by Th1 (and possibly Th17) cells; and (iii) the induction of Th17 cell differentiation[66–68].

From our results we may hypothesize that down-regulation of *Il6* gene expression could: (1) specifically decrease Th17 cell populations, (2) reduce neutrophil infiltration at the site of inflammation, (3) lower the expression of tissue remodelation genes, probably as a consequence of the decreased neutrophil infiltration, and (4) probably allow the expression of Il10, which could also contribute to the resolution of colitis. In this study, control rats were fed with BGMP and *Il6* gene expression was also down-regulated in these rats, reinforcing the idea that IL-6 down-regulation could be a key point in the anti-inflammatory effect of BGMP.

It is known that in acute inflammation, leucocyte recruitment to the site of inflammation is characterized by an initial infiltration of neutrophils, which are later replaced by a more sustained influx of mononuclear cells, initially macrophages and then lymphocytes[15]. Therefore, the presence of increased amounts of lymphocytes together with a decrease in neutrophils and macrophages may be interpreted as indicative of an advanced stage of recovery from inflammation. Our results show just that, i.e. a decrease in the expression of genes related to macrophage and granulocyte activation, movement, recruitment and/or accumulation, with BGMP treatment, together with an up-regulation in both B and T cell marker genes, indicating that the inflammatory state of BGMP-treated animals was closer to resolution (P Requena, A Daddaoua, A Zarzuelo, MD Suárez, F Sanchez de Medina and O Martinez-Augustin, unpublished results). These data are further sustained by the finding that *Il1b* (mainly produced by macrophages)[59] and *Il6* (a neutrophil recruitment cytokine)[18] are key genes in the effect of BGMP, as indicated by the analysis of microarray data using Ingenuity Pathway Analysis software.

Because of the nature of our studies we cannot ascertain whether BGMP prevents the inflammatory damage induced by TNBS administration or accelerates the recovery from injury. Nevertheless, when BGMP was administered to TNBS rats before (pre-treatment) or after (post-treatment) the TNBS challenge, we found that the pre-treatment was more effective, stressing the importance of preventive actions. As alluded to earlier, studies carried out with splenocytes have shown that the addition of BGMP to culture medium increases the production of macrophage-produced cytokines (IL-1β, TNF and IL-8) in a fashion that seems to be dependent on the activation of mitogen-activated protein kinase and NF-κB[59]. These results are apparently at odds with the anti-inflammatory effect of BGMP. However, the very complexity of IBD pathology makes it possible that monocyte activation may be involved in the anti-inflammatory activity of BGMP. Thus several studies suggest that defective response of the mucosa to harmful stimuli may actually worsen the outcome, at least in some cases[17,69]. We have already described the study of Nenci *et al.*[17] in which reduced epithelial activation of the NF-κB pathway results in spontaneous severe colonic inflammation. Similarly, the absence of monocytes and dendritic cells aggravates rather than ameliorates experimental colitis[69]. Because it is well established that intestinal inflammation is strongly dependent on the presence of non-pathogenic bacteria, it may be concluded that the microbiota constitutes the challenge that must be met by the intestinal mucosa in just adequate terms: not too soft, not too hard[70]. Thus, defects in immune function may impair a prompt resolution of intestinal injury, triggering a more robust reaction to a normally trivial challenge. If so, monocyte stimulation would result in a more efficient and prompt response to luminal antigens as they gain access to the subepithelial milieu. In this regard, it is tempting to speculate that BGMP may work at least in part via monocyte/macrophage-stimulating actions.

## Other biopeptides with anti-inflammatory potential

As indicated above, a number of milk-derived peptides with antimicrobial and immunomodulatory effects have been described. These properties could be very useful in the amelioration of intestinal inflammation. The properties of these peptides have been discussed in several reviews[1,8]. Recent studies have shown in the HLA-B27 model of intestinal inflammation that Ca-supplemented diets ameliorate several important aspects of colitis, reducing IL-1β expression and histological scores and increasing the expression of extracellular matrix remodelling genes[71]. Since many casein phosphopeptides have been shown to enhance $Ca^{2+}$ absorption *in vivo*[8,9], these could be good anti-inflammatory candidates. On the other hand, several peptides and fermented milk products have been reported to have antioxidant effects. Antioxidants including glutathione and flavonoids[72–75] have proven to be effective in the treatment of IBD in several animal models, indicating that perhaps antioxidant peptides derived from milk could also be beneficial.

## Conclusions

Research is demonstrating that milk-derived peptides offer a wide variety of actions and some of them are good candidates to attenuate intestinal inflammation. Among these peptides TGF-β and BGMP have proven to be useful in animal models of intestinal inflammation and TGF-β has successfully been used in the treatment of CD patients. While TGF-β is a factor involved naturally in intestinal inflammation, its anti-inflammatory mechanism of action, related at least in part to the inhibition of the Th1 response, needs to be better studied. The mechanism of action of BGMP remains to be fully described. Nevertheless, evidence indicates that it could have preventive effects, activating macrophages and, in inflammatory conditions, it could directly or indirectly down-regulate IL-6 and up-regulate IL-10. No clinical studies have been carried out with BGMP although it is known to be a safe food ingredient and is currently being added to infant formulas.

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## References

1. Hayes M, Stanton C, Fitzgerald GF *et al*. (2007) Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part II: bioactive peptide functions. *Biotechnol J* **2**, 435–449.
2. Hayes M, Ross RP, Fitzgerald GF *et al*. (2007) Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part I: overview. *Biotechnol J* **2**, 426–434.
3. Korhonen H & Pihlanto A (2003) Food-derived bioactive peptides – opportunities for designing future foods. *Curr Pharm Des* **9**, 1297–1308.
4. Politis I & Chronopoulou R (2008) Milk peptides and immune response in the neonate. *Adv Exp Med Biol* **606**, 253–269.
5. Baldi A, Ioannis P, Chiara P *et al*. (2005) Biological effects of milk proteins and their peptides with emphasis on those related to the gastrointestinal ecosystem. *J Dairy Res* **72** Special No., 66–72.
6. Erdmann K, Cheung BW & Schroder H (2008) The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *J Nutr Biochem* **19**, 643–654.
7. Saito T (2008) Antihypertensive peptides derived from bovine casein and whey proteins. *Adv Exp Med Biol* **606**, 295–317.
8. Martinez Augustin O & Martinez de Victoria Munoz E (2006) Proteins and peptides in enteral nutrition. *Nutr Hosp* **21** Suppl. 2, 1–13, 11–14.
9. Shimizu M (2004) Food-derived peptides and intestinal functions. *Biofactors* **21**, 43–47.
10. Bernstein CN, Fried M, Krabshuis JH *et al*. (2009) World gastroenterology organisation practice guidelines for the diagnosis and management of IBD in 2010. *Inflamm Bowel Dis* **16**, 112–124.
11. Baumgart DC & Carding SR (2007) Inflammatory bowel disease: cause and immunobiology. *Lancet* **369**, 1627–1640.
12. Achkar JP & Fiocchi C (2009) Gene–gene interactions in inflammatory bowel disease: biological and clinical implications. *Am J Gastroenterol* **104**, 1734–1736.
13. Gassull MA, Mane J, Pedrosa E *et al.* (2005) Macronutrients and bioactive molecules: is there a specific role in the management of inflammatory bowel disease? *JPEN J Parenter Enteral Nutr* **29**, S179–S182; discussion S182–S173, S184–S178.
14. Regueiro M, Kip KE, Cheung O *et al*. (2005) Cigarette smoking and age at diagnosis of inflammatory bowel disease. *Inflamm Bowel Dis* **11**, 42–47.
15. Levenstein S, Prantera C, Varvo V *et al*. (2000) Stress and exacerbation in ulcerative colitis: a prospective study of patients enrolled in remission. *Am J Gastroenterol* **95**, 1213–1220.
16. Schiffrin EJ, El Yousfi M, Faure M *et al*. (2005) Milk casein-based diet containing TGF-beta controls the inflammatory reaction in the HLA-B27 transgenic rat model. *JPEN J Parenter Enteral Nutr* **29**, S141–S148; discussion S149–S150, S184–S148.
17. Nenci A, Becker C, Wullaert A *et al*. (2007) Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* **446**, 557–561.
18. Ramasundara M, Leach ST, Lemberg DA *et al.* (2009) Defensins and inflammation: the role of defensins in inflammatory bowel disease. *J Gastroenterol Hepatol* **24**, 202–208.
19. Gupta S, Shang W & Sun Z (2008) Mechanisms regulating the development and function of natural regulatory T cells. *Arch Immunol Ther Exp (Warsz)* **56**, 85–102.
20. Boden EK & Snapper SB (2008) Regulatory T cells in inflammatory bowel disease. *Curr Opin Gastroenterol* **24**, 733–741.
21. Korn T, Bettelli E, Oukka M *et al*. (2009) IL-17 and Th17 Cells. *Annu Rev Immunol* **27**, 485–517.
22. Brand S (2009) Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. *Gut* **58**, 1152–1167.
23. Sakuraba A, Sato T, Kamada N *et al*. (2009) Th1/Th17 immune response is induced by mesenteric lymph node dendritic cells in Crohn's disease. *Gastroenterology* **137**, 1566–1570.
24. Leppkes M, Becker C, Ivanov, II, *et al.* (2009) RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology* **136**, 257–267.
25. Jurjus AR, Khoury NN & Reimund JM (2004) Animal models of inflammatory bowel disease. *J Pharmacol Toxicol Methods* **50**, 81–92.
26. Izcue A, Coombes JL & Powrie F (2009) Regulatory lymphocytes and intestinal inflammation. *Annu Rev Immunol* **27**, 313–338.
27. Wirtz S, Neufert C, Weigmann B *et al*. (2007) Chemically induced mouse models of intestinal inflammation. *Nat Protoc* **2**, 541–546.
28. Sanchez de Medina F, Martinez-Augustin O, Gonzalez R *et al*. (2004) Induction of alkaline phosphatase in the inflamed intestine: a novel pharmacological target for inflammatory bowel disease. *Biochem Pharmacol* **68**, 2317–2326.
29. Martinez-Augustin O, Merlos M, Zarzuelo A *et al*. (2008) Disturbances in metabolic, transport and structural genes in experimental colonic inflammation in the rat: a longitudinal genomic analysis. *BMC Genomics* **9**, 490.
30. Requena P, Daddaoua A, Martinez-Plata E *et al*. (2008) Bovine glycomacropeptide ameliorates experimental rat ileitis by mechanisms involving downregulation of interleukin 17. *Br J Pharmacol* **154**, 825–832.
31. Alex P, Zachos NC, Nguyen T *et al*. (2009) Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inflamm Bowel Dis* **15**, 341–352.
32. Sartor RB (2000) Colitis in HLA-B27/beta 2 microglobulin transgenic rats. *Int Rev Immunol* **19**, 39–50.
33. Ostanin DV, Bao J, Koboziev I *et al*. (2009) T cell transfer model of chronic colitis: concepts, considerations, and tricks of the trade. *Am J Physiol Gastrointest Liver Physiol* **296**, G135–G146.
34. Massague J (1990) The transforming growth factor-beta family. *Annu Rev Cell Biol* **6**, 597–641.
35. Ozawa T, Miyata M, Nishimura M *et al*. (2009) Transforming growth factor-beta activity in commercially available pasteurized cow milk provides protection against inflammation in mice. *J Nutr* **139**, 69–75.

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36. Cheifetz S, Hernandez H, Laiho M *et al*. (1990) Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. *J Biol Chem* **265**, 20533–20538.
37. Nakamura Y, Miyata M, Ando T *et al*. (2009) The latent form of transforming growth factor-beta administered orally is activated by gastric acid in mice. *J Nutr* **139**, 1463–1468.
38. Letterio JJ, Geiser AG, Kulkarni AB *et al*. (1994) Maternal rescue of transforming growth factor-beta 1 null mice. *Science* **264**, 1936–1938.
39. Letterio JJ (2000) Murine models define the role of TGF-beta as a master regulator of immune cell function. *Cytokine Growth Factor Rev* **11**, 81–87.
40. Donnet-Hughes A, Duc N, Serrant P *et al*. (2000) Bioactive molecules in milk and their role in health and disease: the role of transforming growth factor-beta. *Immunol Cell Biol* **78**, 74–79.
41. Hahm KB, Im YH, Parks TW *et al*. (2001) Loss of transforming growth factor beta signalling in the intestine contributes to tissue injury in inflammatory bowel disease. *Gut* **49**, 190–198.
42. Neurath MF, Fuss I, Kelsall BL *et al*. (1996) Experimental granulomatous colitis in mice is abrogated by induction of TGF-beta-mediated oral tolerance. *J Exp Med* **183**, 2605–2616.
43. Beattie RM, Schiffrin EJ, Donnet-Hughes A *et al*. (1994) Polymeric nutrition as the primary therapy in children with small bowel Crohn's disease. *Aliment Pharmacol Ther* **8**, 609–615.
44. Fell JM, Paintin M, Arnaud-Battandier F *et al*. (2000) Mucosal healing and a fall in mucosal pro-inflammatory cytokine mRNA induced by a specific oral polymeric diet in paediatric Crohn's disease. *Aliment Pharmacol Ther* **14**, 281–289.
45. Fell JM (2005) Control of systemic and local inflammation with transforming growth factor beta containing formulas. *JPEN J Parenter Enteral Nutr* **29**, S126–S128; discussion S129–S133, S184–S128.
46. Afzal NA, Van Der Zaag-Loonen HJ, Arnaud-Battandier F *et al*. (2004) Improvement in quality of life of children with acute Crohn's disease does not parallel mucosal healing after treatment with exclusive enteral nutrition. *Aliment Pharmacol Ther* **20**, 167–172.
47. Aimutis WR (2004) Bioactive properties of milk proteins with particular focus on anticariogenesis. *J Nutr* **134**, 989S–995S.
48. Laclair CE, Ney DM, MacLeod EL *et al*. (2009) Purification and use of glycomacropeptide for nutritional management of phenylketonuria. *J Food Sci* **74**, E199–206.
49. van Calcar SC, MacLeod EL, Gleason ST *et al*. (2009) Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids. *Am J Clin Nutr* **89**, 1068–1077.
50. Ney DM, Hull AK, van Calcar SC *et al*. (2008) Dietary glycomacropeptide supports growth and reduces the concentrations of phenylalanine in plasma and brain in a murine model of phenylketonuria. *J Nutr* **138**, 316–322.
51. Bruck WM, Kelleher SL, Gibson GR *et al*. (2003) rRNA probes used to quantify the effects of glycomacropeptide and alpha-lactalbumin supplementation on the predominant groups of intestinal bacteria of infant rhesus monkeys challenged with enteropathogenic *Escherichia coli*. *J Pediatr Gastroenterol Nutr* **37**, 273–280.
52. Sandstrom O, Lonnerdal B, Graverholt G *et al*. (2008) Effects of alpha-lactalbumin-enriched formula containing different concentrations of glycomacropeptide on infant nutrition. *Am J Clin Nutr* **87**, 921–928.
53. Idota TKH & Nakajima I (1994) Growth-promoting effects of *N*-acetylneuraminic acid-containing substances on Bifidobacteria. *Biosci Biotech Biochem* **58**, 1720–1722.
54. Yakabe TKH & Idota T (1994) Growth stimulation agent for bifidus and lactobacillus. Japanese patent 07-267866.
55. Bruck WM, Redgrave M, Tuohy KM *et al*. (2006) Effects of bovine alpha-lactalbumin and casein glycomacropeptide-enriched infant formulae on faecal microbiota in healthy term infants. *J Pediatr Gastroenterol Nutr* **43**, 673–679.
56. Requena P, Gonzalez R, Lopez-Posadas R *et al*. (2010) The intestinal anti-inflammatory agent glycomacropeptide has immunomodulatory actions on rat splenocytes. *Biochem Pharmacol* **79**, 1797–1804.
57. Otani HMM, Kawasaki Y, Kawakami H *et al*. (1995) Inhibition of mitogen-induced proliferative responses of lymphocytes by bovine kappa-caseinoglycopeptides having different carbohydrate chains. *J Dairy Res* **62**, 349–357.
58. Li EW & Mine Y (2004) Immunoenhancing effects of bovine glycomacropeptide and its derivatives on the proliferative response and phagocytic activities of human macrophagelike cells, U937. *J Agric Food Chem* **52**, 2704–2708.
59. Requena P, Daddaoua A, Guadix E *et al*. (2009) Bovine glycomacropeptide induces cytokine production in human monocytes through the stimulation of the MAPK and the NF-kappaB signal transduction pathways. *Br J Pharmacol* **157**, 232–240.
60. Monnai M & Otani H (1997) Effect of bovine k-caseinoglycopeptide on secretion of interleukin-1 family cytokines by P388D1 cells, a line derived from mouse monocyte/macrophage. *Milchwissenschaft* **52**, 192–196.
61. Daddaoua A, Puerta V, Zarzuelo A *et al*. (2005) Bovine glycomacropeptide is anti-inflammatory in rats with hapten-induced colitis. *J Nutr* **135**, 1164–1170.
62. Sands BE (2004) From symptom to diagnosis: clinical distinctions among various forms of intestinal inflammation. *Gastroenterology* **126**, 1518–1532.
63. Navarro-Llavat M, Domenech E, Masnou H *et al*. (2007) Collagenous duodeno-ileo-colitis with transient IgG deficiency preceded by *Yersinia enterocolitica* intestinal infection: case report and review of literature. *Gastroenterol Hepatol* **30**, 219–221.
64. Alexander F (2007) Complications of ileal pouch anal anastomosis. *Semin Pediatr Surg* **16**, 200–204.
65. Nielsen OH & Munck LK (2007) Drug insight: aminosalicylates for the treatment of IBD. *Nat Clin Pract Gastroenterol Hepatol* **4**, 160–170.
66. Mudter J & Neurath MF (2007) Il-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. *Inflamm Bowel Dis* **13**, 1016–1023.
67. Mitsuyama K, Sata M & Rose-John S (2006) Interleukin-6 trans-signaling in inflammatory bowel disease. *Cytokine Growth Factor Rev* **17**, 451–461.
68. Carey R, Jurickova I, Ballard E *et al*. (2008) Activation of an IL-6:STAT3-dependent transcriptome in pediatric-onset inflammatory bowel disease. *Inflamm Bowel Dis* **14**, 446–457.
69. Qualls JE, Kaplan AM, van Rooijen N *et al*. (2006) Suppression of experimental colitis by intestinal mononuclear phagocytes. *J Leukoc Biol* **80**, 802–815.
70. Seksik P, Sokol H, Lepage P *et al*. (2006) Review article: the role of bacteria in onset and perpetuation of inflammatory bowel disease. *Aliment Pharmacol Ther* **24** Suppl. 3, 11–18.
71. Schepens MA, Schonewille AJ, Vink C *et al*. (2009) Supplemental calcium attenuates the colitis-related increase in diarrhea, intestinal permeability, and extracellular matrix


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breakdown in HLA-B27 transgenic rats. *J Nutr* **139**, 1525–1533.
72. Mane J, Loren V, Pedrosa E *et al*. (2009) *Lactobacillus fermentum* CECT 5716 prevents and reverts intestinal damage on TNBS-induced colitis in mice. *Inflamm Bowel Dis* **15**, 1155–1163.
73. Sanchez de Medina F, Galvez J, Gonzalez M *et al*. (1997) Effects of quercetin on epithelial chloride secretion. *Life Sci* **61**, 2049–2055.
74. Sanchez de Medina F, Galvez J, Romero JA *et al*. (1996) Effect of quercitrin on acute and chronic experimental colitis in the rat. *J Pharmacol Exp Ther* **278**, 771–779.
75. Sanchez de Medina F, Vera B, Galvez J *et al*. (2002) Effect of quercitrin on the early stages of hapten induced colonic inflammation in the rat. *Life Sci* **70**, 3097–3108.
76. Deenick EK & Tangye SG (2007) Autoimmunity: IL-21: a new player in Th17-cell differentiation. *Immunol Cell Biol* **85**, 503–505.



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