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(54) Title: CHROMOGRANIN-A-DERIVED POLYPEPTIDES AND METHODS OF USE

(57) Abstract: Provided herein are methods for treating an inflammatory bowel disease in a subject. In one embodiment the method includes administering to a subject in need thereof an effective amount of a composition that includes a Chromogranin A (CgA)-derived protein, or a polynucleotide that encodes a CgA protein. The subject may have, or be at risk of having, an inflammatory bowel disease. Examples of inflammatory bowel diseases that may be treated using the methods disclosed herein include Crohn's disease, ulcerative colitis, collagenous colitis, lymphocytic colitis, ischaemic colitis, diversion colitis, Behcet's disease, and indeterminate colitis. Also provided herein are methods for evaluating treatment options for a subject having inflammatory bowel disease.



CHROMOGRANIN-A-DERIVED PROTEINS AND METHODS OF USE

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 61/827,127, filed May 24, 2013, which is incorporated by reference herein.

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BACKGROUND

Inflammatory bowel diseases (IBD) are idiopathic chronic, recurrent intestinal disorders of complex pathogenesis, which include Crohn's disease (CD) and ulcerative colitis (UC). The estimated prevalence in Canada is around 500/100,000 persons (Bernstein et al., 2000, Am J Gastroenterol 95:677-83). These diseases often present in adolescence or young adulthood and hence affected individuals have a long burden of disease with significant psychosocial, physical and economic impacts. In Canada, IBD represents a public health issue due to their impact on patient quality of life (estimated costs: \$1.7 billions) (Bernstein et al., 2000, Am J Gastroenterol 95:677-83, CCFC. Annual Report. CCFC 2008).

The etiopathogenesis of IBD is multifactorial, involving an aberrant immune response to some environmental antigen in genetically predisposed individuals. The apparent therapeutic beneficial effect of biological therapy (tumor necrosis factor-(TNF- α -neutralizing antibody) (Han et al., 2007, Gut 2007;56:73-81), corticosteroids and thiopurines underscores the importance of the dysregulated immune response. However, some patients are resistant to these drugs, and all of these therapeutic agents have adverse side effects (Domenech, 2006, Digestion 73 Suppl 1:67-76, Ananthakrishnan et al., 2010, J Clin Gastroenterol 44:272-9). For the most ill patients monoclonal antibodies to TNF- α are used. These agents are expensive, require indefinite use, and the concern for infectious and potentially even malignant complications (Vermeire et al., 2007, Aliment Pharmacol Ther 25:3-12) limit the enthusiasm for introducing these agents earlier in the treatment paradigm.

A complex network of events at molecular, cellular, and tissue levels underlie inflammation and remodeling that eventually lead to development of IBD symptoms. Cell proliferation and migration are cardinal cellular events that are tightly regulated by various mediators and mechanisms in physiological conditions. Also, environmental antigen exposure impairs regulatory mechanisms in IBD patients leading to pathological features including gut inflammation. As the first line of defense, the mucosa plays a major role in innate immunity and a large number of endocrine cells (*e.g.* enterochromaffin (EC) cells) reside among the epithelial cells and react to changes in luminal content by releasing hormones that modify gut physiology. Mucosal changes in IBD are characterized by mucosal and transmural inflammation accompanied by a prominent infiltrate of activated cells from both the innate and adaptive immune systems.

An alteration in the prohormone chromogranin-A (CgA) within the gut is associated with inflammation of the gut (El-Salhy et al., 1997, *J Intern Med* 242:413-9). CgA is an enteric mucosal signaling molecule influencing gut physiology (Ghia et al., 2004, *Life Sci* 2004;75:1787-99, Ghia et al., 2004, *Regul Pept* 121:31-9). CgA of the granin family of proteins is stored in secretory granules (large dense core vesicles) of some CNS and enteric neurons, as well as specific types of endocrine, immune and neuroendocrine cells. It is co-released along with catecholamines, hormones, neurotransmitters and neuropeptides (Taupenot et al., 2003, *N Engl J Med* 348:1134-49) that are also stored in these granules.

Human CHGA gene includes eight exons separated by seven introns and has been mapped into chromosome 14q32.16. It translates to a 457 amino acid protein containing a signal peptide of 18 amino acids. The mature human CHGA protein, also referred to as CgA, is 439 amino acids. The overall homology for CgA in different vertebrates is around 40%, but the most highly conserved regions occur at the N and C-termini, which show up to 88% sequence homology. The sequence of CgA include numerous post-translational modifications such as phosphorylation and O-glycosylation (Gadroy et al., 1998, *J Biol Chem.* 273(51):34087-97) that modulate the natural proteolytic degradation. Cell and tissue specific processing of CgA has been described in the rat, mouse and human GI tract (Curry et al., 1991, *Histochemistry* 96:531-8, Portela-Gomes et al., 2001, *J Histochem Cytochem* 49:483-90, Portela-Gomes et al., 2002, *J Histochem Cytochem* 50:1487-92). The presence of numerous pairs of basic amino acids indicate potential sites for cleavage by prohormone convertases (PC) 1/3 or 2, carboxypeptidase

E/H (Seidah et al., 1999, Brain Res 848:45-62), consistent with evidence that CgA may serve as a prohormone for shorter bioactive fragments, (Eiden, 1987, Nature 325:301, and see Figure 9), as also suggested by the high sequence conservation of proteolytic fragments of CgA.

Proteolytic fragments of CgA, referred to herein as CgA-derived peptides, CgA-derived polypeptides, or CgDPs, exert a broad spectrum of regulatory activities on the cardiovascular, endocrine and immune systems. Among them, pancreastatin (human CgA₂₅₀₋₃₀₁) inhibits insulin release from pancreatic-islet B cells, promotes hepatic glycogenolysis, and regulates lipid metabolism; vasostatin-I (human/bovine CgA₁₋₇₆, chromofungin (human/bovine CgA₄₇₋₆₆), prochromacin (bovine CgA₇₉₋₄₃₁), chromacin (human CgA₁₇₆₋₁₉₇) and catestatin (human CgA₃₅₂₋₃₇₂) display antibacterial and antifungal effects at the micromolar range, but are not hemolytic as these peptides are also able to activate immune cells such as polynuclear neutrophils; vasostatin 2 (human CgA₁₋₁₁₃) has regulatory roles in the heart and the vascular system (Helle et al., 2007, Cell Mol Life Sci 64:2863-86). Among its highly conserved C terminal regions, CgA gives rise to main peptides of biological importance: the antihypertensive peptide catestatin (CTS; CgA₃₅₂₋₃₇₂) (Mahapatra et al., 2005, J Clin Invest 115:1942-52, Mahata et al., 2010, Regul Pept 162:33-43, Mahata et al., 1997, J Clin Invest 100:1623-33) and its short version cateslytin (CTL; CgA_{corriger364-378}), which have antimicrobial activity (Briolat et al., 2005, Cell Mol Life Sci 62:377-85), activate neutrophils (Zhang et al., 2009, PLoS One. 4(2):e4501. doi:10.1371/journal.pone.0004501), and regulate smooth muscle cell proliferation (Guo et al., 2011, Biochem Biophys Res Commun 407:807-12). CTS stimulates chemotaxis of human peripheral blood monocytes, exhibiting its maximal effect at a concentration of 1nM comparable to the established chemoattractant formylated peptide Met-Leu-Phe (fMLP) (Egger et al., 2008, Eur J Pharmacol 598:104-111). More recently CTL, a shorter version of CTS, has been described with better physiological efficacy as compared to CTS, has been discovered, and new data demonstrated the potential use of CTL when inserted on a polysaccharide multilayer film (Cado et al., 2013 DOI: 10.1002/adfm.201300416, Advanced functional materials).

In addition to gut inflammation, the relation between TNF- α and CgA has also been demonstrated in rheumatoid arthritis (RA), a disease that shares some common features with IBD. Correlation between CgA and tumor necrosis factor receptor (TNFR)-I and TNFR-II has been evaluated in patients before the initiation of treatment with infliximab® compared to during treatment (di Comite et al., 2006, Ann N Y Acad Sci 1069:428-37). The authors observed a high

correlation between CgA and both receptors. Moreover, they found that treatment with anti-TNF- α mAbs abrogated the correlation between CgA, TNFR-I and TNFR-II. Subsequently, the same group described that patients with RA had significantly higher serum levels of CgA and TNFRs compared to controls and that the highest levels of CgA identified the population of patients with extra-articular manifestations (di Comite et al., 2006, Ann N Y Acad Sci 1069:428-37).

A link has been observed between serum concentration of CgA and outcome in patients admitted with or without systemic inflammatory response syndrome. CgA concentrations were positively correlated with inflammatory markers like procalcitonin and C-reactive protein, but also with simplified acute physiological score (SAPS) (Zhang et al., 2009, Ann Med 41:38-44, Zhang et al., 2008, Clin Chem 54:1497-503). More recently it has been reported that a plasma VS-I concentration above 3.97 ng/ml is associated with poor outcome (Schneider et al., 2012, Intensive Care Med. 38(9):1514-22. doi: 10.1007/s00134-012-2611-3). In addition, a significant association between CgA level and periodontitis has been reported (Hironaka et al., 2008, Biomed Res 29:125-30). Finally, a study was conducted to evaluate the presence and processing of the CgA in the vitreous of patients with diabetic retinopathy DR (DV), compared with nondiabetic vitreous (NDV). This study showed differences in the presence and endogenous processing of CgA, from DV vs. NDV. In DV, the increase of complete granins and the attenuation of their endogenous proteolytic processing (*via* PC1/2) participated in DR progression by reducing the presence of regulatory peptides, being important for the pro-/anti-angiogenic balance in the eye (Fournier et al., 2011, Regul. Pept. 167(1):118-24).

Receptors for CgDPs appear not to exist. Rather, sequence similarity of CgDPs with cell penetrating peptides (Henriques et al., 2006, Biochem J 399:1-7) appears to allow CgDPs to enter cells and induce calcium influx via a calmodulin (CaM)-regulated calcium-independent phospholipase (iPL) A2 pathway (Zhang et al., 2009, PLoS One 4:e4501). In addition, CgDPs regulate endothelial barrier function and protect against plasma leakage (Ferrero et al., 2002, Ann N Y Acad Sci 971:355-8) by an action on the cytoskeleton.

Macrophages play a critical role in intestinal inflammatory responses through the secretion of chemokines and cytokines, and through antigen presentation to T lymphocytes. Macrophages also play a key role in host defense against bacterial pathogens, which stimulate macrophages via the activation of toll-like receptors. Macrophage activation by pathogens results

in the secretion of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin-1b (IL-1b) and interleukin-6 (IL-6) and in the induction of a Th-1 cytokine response, with the production of interleukin-12 (IL-12) and interferon- γ (INF- γ). In this context, macrophages are considered to be classical pro-inflammatory effector cells (Watanabe et al., 2003, Dig Dis Sci 48:408-14). Macrophage Colony-Stimulating Factor (M-CSF) is a critical trophic and differentiation factor for macrophages and osteoclasts (Sweet et al., 2003, Arch Immunol Ther Exp (Warsz) 51:169-77). Mice with a defect in the M-CSF encoding region exhibit osteopetrosis and have been shown to be deficient in osteoclasts, monocytes and tissue macrophages (Naito et al., 1997, Mol Reprod Dev 46:85-91). A single nucleotide (T) insertion 262 bp downstream from the initiation codon resulted in a frameshift and the creation of a stop 21bp downstream of the insertion. M-CSF deficient (op/op) mice have provided a useful tool in the investigation of the role macrophages in a variety of conditions. Macrophages are derived into several subsets, based on exposure to panels of chemokines and growth factors such as Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and M-CSF (Akagawa, 2002, Int J Hematol 76:27-34, Mantovani et al., 2004, Trends Immunol 25:677-86). Both M-CSF and GM-CSF have been implicated in the differentiation of macrophages with counter-inflammatory properties, and that M-CSF enhances the IL-10 dependent suppressor activity of macrophages (Mochida-Nishimura et al., 2001, Cell Immunol 214:81-8). The role of M-CSF dependent macrophages in the inflammatory processes was recently examined using M-CSF-deficient op/op mice (Ghia et al., 2008, Am J Physiol 294:770-77). The results indicated that the absence of these cells reduced the severity of DSS colitis.

SUMMARY OF THE APPLICATION

Provided herein are uses of a CgA-derived polypeptide. In one embodiment, the use is in the preparation of a medicament for an inflammatory bowel disease. In one embodiment, the use is for treating an inflammatory bowel disease. In one embodiment, the use is in the preparation of a medicament for an inflammatory bowel disease, wherein the CgA-derived polynucleotide encodes a CgA-derived polypeptide. In one embodiment, the use is for treating an inflammatory bowel disease, wherein the CgA-derived polynucleotide encodes a CgA-derived polypeptide. In

one embodiment, the CgA-derived polypeptide may be combined with a pharmaceutically acceptable carrier.

Also provided herein are methods for using a CgA-derived polypeptide described herein. In one embodiment, the method includes treating an inflammatory bowel disease in a subject, including administering to a subject in need thereof an effective amount of a composition that includes a CgA-derived polypeptide. In one embodiment, the method includes treating a subject having, or at risk of having, inflammatory bowel disease, including administering to a subject in need thereof a composition that includes a CgA-derived polypeptide, wherein the subject has decreased abdominal pain, vomiting, diarrhea, rectal bleeding, intestinal cramps, weight loss, anemia, or a combination thereof, when compared to the subject before the administering. In one embodiment, the method includes treating an inflammatory bowel disease in a subject, including administering to a subject in need thereof an effective amount of a composition that includes a CgA-derived polynucleotide, wherein the CgA-derived polynucleotide encodes a CgA-derived polypeptide. In one embodiment, the method includes treating a subject having, or at risk of having, inflammatory bowel disease, including administering to a subject in need thereof a composition that includes a CgA-derived polynucleotide, wherein the subject has decreased abdominal pain, vomiting, diarrhea, rectal bleeding, intestinal cramps, weight loss, anemia, or a combination thereof, when compared to the subject before the administering, wherein the CgA-derived polynucleotide encodes a CgA-derived polypeptide. In one embodiment, the subject may be a human. In one embodiment, the method also includes administering a therapeutic compound. The therapeutic compound may be, for instance, an aminosalicylate, a corticoid, an immunosuppressive compound, a therapeutic antibody, an antibiotic, a thiopurine, a methotrexate, or a combination thereof.

In one embodiment, the CgA-derived polypeptide includes an amino acid sequence SEQ ID NO:1 or no greater than 3 substitutions compared to SEQ ID NO:1, and optionally the CgA-derived polypeptide includes no greater than 438 amino acids. In one embodiment, the CgA-derived polypeptide includes an amino acid sequence having at least 80% identity with SEQ ID NO:2, 3, or 4, and optionally the CgA-derived polypeptide includes no greater than 438 amino acids. In one embodiment, the CgA-derived polypeptide includes an amino acid sequence including SEQ ID NO:2, 3, or 4, and optionally the CgA-derived polypeptide includes no greater than 438 amino acids. The inflammatory bowel disease may be selected from Crohn's disease

and ulcerative colitis. In one embodiment, the CgA-derived polypeptide is a fusion polypeptide. The CgA-derived polynucleotide may be present in a vector, such as a viral vector.

A CgA-derived polypeptide may include heterologous amino acids. For instance, a CgA-derived protein that includes SEQ ID NO:1, or an amino acid sequence having no greater than 3
5 substitutions compared to SEQ ID NO:1, may further include at least one heterologous amino acid flanking SEQ ID NO:1 or an amino acid sequence having no greater than 3 substitutions compared to SEQ ID NO:1. Whether the at least one amino acid is heterologous is determined by comparison to the CgA amino acid sequence depicted at SEQ ID NO:6. In another
10 embodiment, a CgA-derived protein that includes an amino acid sequence having at least 80% identity with SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, may further include at least one heterologous amino acid flanking the amino acid sequence having at least 80% identity with SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4. Whether the at least one amino acid is
15 heterologous is determined by comparison to the CgA amino acid sequence depicted at SEQ ID NO:6. The at least one heterologous amino acid may be present at the amino-terminal end and/or the carboxy-terminal end of the CgA-derived protein.

Also provided herein are methods for evaluating treatment options for a subject having inflammatory bowel disease. In one embodiment, the method includes obtaining a biological sample from the subject, such as a human, measuring the level of CgA-derived polypeptide in the biological sample, and comparing the level of CgA-derived polypeptide in the biological
20 sample with the level of CgA-derived polypeptide in a control biological sample obtained from a healthy subject, wherein the presence of a decreased level of CgA-derived polypeptide compared to the control biological sample indicates the subject may be treated with a CgA-derived polypeptide. In one embodiment, the biological sample includes tissue from the gastrointestinal tract of the subject. In one embodiment, the method includes administering to the subject a CgA-
25 derived polypeptide or a fragment thereof.

The term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements.

The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be
30 preferred, under the same or other circumstances. Furthermore, the recitation of one or more

preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

5 Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

Also herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

10 For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application,
15 guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

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Figure 1: Differential expression of mouse (m) and human (h) CgA and CgA352-372 (catestatin, CTS) in colonic inflammatory conditions. *A, B*: The presence of the mCgA and mCTS was detected at the distal part of the colon in dextran sulfate sodium (DSS)-induced colitis. *C, D*: The presence of the mCgA and mCTS was detected at the distal part of the colon in
25 2, 4 dinitrobenzene sulfonic acid (DNBS)-induced colitis. Values are shown as the mean $\pm$ SEM. Samples were collected on day 3 or 5 post- DNBS or DSS induction; mice per group n^3 6. *E, F*: The presence of hCgA and hCTS was detected in the serum from persons with non-inflamed colon (control, $n=15$), with ulcerative colitis (UC, $n=15$) or Crohn's disease (CD, $n=15$). ^a $p<0.05$ compared to control groups, ANOVA followed by the Dunnett multiple
30 comparisons *post hoc* analysis. m or hCgA or CTS were measured using commercially available ELISA kits.

Figure 2: Human (h)CgA352-372, hCgA360-372 and hCgA352-366 treatment alleviate the disease activity index in dextran sulphate sodium (DSS)-induced colitis. Treatments (6 days, intra-rectal) were started one day prior to colitis induction. *A*: Disease activity index; dose-dependent effect of hCgA352-372 peptide (0.5, 1, 1.5 mg/kg/day, 6 days) *B*: Disease activity index; hCgA352-372 hCgA360-372, hCgA352-366 peptide (1.5 mg/kg/day, 6 days). Values are shown as the mean $\pm$ SEM, $n \geq 8$ mice per group. ^a $p < 0.05$ compared to saline DSS-treated group, ^b $p < 0.05$ compared to hCgA352-372 peptide (1 mg/kg/day, 6 days) DSS-treated group. ANOVA followed by the Tukey multiple comparisons *post hoc* analysis. Control represents data obtained in non-colitic non-treated mice, because no significant differences were determined between this group and any other non-colitic treated group of animals. sh represents the modified hCgA352-372 peptide.

Figure 3. Human (h)CgA352-372, hCgA360-372 and hCgA352-366 treatment alleviate disease activity, colon length and colonic myeloperoxidase (MPO) activity in dextran sulfate sodium (DSS)-induced colitis. Treatments (6 days, intra-rectal 0.5, 1, 1.5 mg/kg/day, 6 days) were started one day prior to colitis induction. *A*: Macroscopic score; *B*: Colon length; *C*: MPO activity. Values are shown as the mean $\pm$ SEM. Samples were collected on day 5 post-DSS; $n \geq 8$ mice per group. ^a $P < 0.05$ compared to control group, ^b $P < 0.05$ compared to saline DSS-treated group. [#] $P < 0.05$. ANOVA followed by Tukey multiple comparisons *post hoc* analysis. sh represents the modified hCgA352-372 peptide.

Figure 4. Human (h)CgA352-372, hCgA360-372 and hCgA352-366 treatment alleviate histological score in dextran sulfate sodium (DSS)-induced colitis. *A*: Appearance of the colon *a*: in mice with DSS-induced colitis; *b*: in hCgA352-372, *c*: in hCgA360-372 *d*: in hCgA352-366, and *e*: in shCgA352-372 (1.5 mg/kg/day, intra-rectal, 6 days)-treated mice with DSS-induced colitis *B*: Histological score; values are shown as the mean $\pm$ SEM. Samples were collected on day 5 post-DSS; $n \geq 8$ mice per group. ^a $P < 0.05$ compared to control group, ^b $P < 0.05$ compared to saline DSS-treated group. Hematoxylin and eosin staining, 100X magnifications. sh represents the modified hCgA352-372 peptide.

Figure 5. Human (h)CgA352-372, hCgA360-372 and hCgA352-366 decrease serum C-reactive protein level and pro-inflammatory colonic cytokines in dextran sulfate sodium (DSS)-induced colitis. Treatments (6 days, intra-rectal, 0.5, 1, 1.5 mg/kg/day, 6 days) were started one day prior to colitis induction *A*: Serum C-reactive protein (CRP); *B*: Colonic interleukin (IL)-1 β

amount; *C*: Colonic IL-6 amount; *D*: Colonic tumor necrosis factor (TNF)- α amount. Values are shown as the mean $\pm$ SEM. Samples were collected on day 5 post-DSS; $n \geq 8$ mice per group. ^a $P < 0.05$ compared to control group, ^b $P < 0.05$ compared to saline DSS-treated group, [#] $P < 0.05$. ANOVA followed by the Tukey multiple comparisons *post hoc* analysis. CRP, IL-1 β , IL-6 and TNF- α were measured using commercially available ELISA kits. sh represents the modified hCgA352-372 peptide.

Figure 6. Functional role of peritoneal macrophages in the amelioration of colitis induced by hCgA352-372, hCgA360-372 or hCgA352-366 (1.5 mg/kg/day, intra-rectal, 6 days) treatments. Peritoneal macrophages were isolated from *in vivo* colitic hCgA352-372, hCgA360-372 or hCgA352-366-treated mice with dextran sulfate sodium (DSS)-induced colitis. Interleukin (IL)-1 β (*A*), IL-6 (*B*) and tumor necrosis factor (TNF)- α (*C*) were measured in conditioned media (24h) using commercially available ELISA kits. ^a $P < 0.05$ compared to control group respectively, ^b $P < 0.05$ compared to saline DSS-treated group, ANOVA followed by the Tukey multiple comparisons *post hoc* analysis, $n \geq 8$. Values are shown as the mean $\pm$ SEM of four separate experiments. sh represents the modified hCgA352-372 peptide.

Figure 7: Functional role of naive peritoneal and bone marrow-derived macrophages (BMDMs). Lipopolysaccharide (LPS)-stimulated (100 ng/ml^{-1}) peritoneal mouse macrophages and BMDMs cultures from naive control mouse treated *in vitro* with hCgA352-372, hCgA360-372 or hCgA352-366 (10^{-7}M or 10^{-5}M). Interleukin (IL)-6 (*A*, *B*), IL-1 β (*C*, *D*) and TNF- α (*E*, *F*) were measured in conditioned medium (24h) using commercially available ELISA kits. BMDMs and naive peritoneal macrophages isolated from naive mouse are less potent producers of pro-inflammatory cytokines when treated with hCgA352-372, hCgA360-372 or hCgA352-366 (10^{-5}M). The modified peptides (sh) did not have any effects. ^a $P < 0.05$ compared to control non-LPS treated group, ^b $P < 0.05$ compared to medium LPS-treated group, ANOVA followed by the Tukey multiple comparisons *post hoc* analysis, $n \geq 5$. [#] $P < 0.05$. The values are shown as the mean $\pm$ SEM of four separate experiments.

Figure 8. p-STAT3 expression in colonic tissues and peritoneal macrophages. *A*: Colonic tissue isolated from *in vivo* colitic and non-colitic hCgA352-372, hCgA360-372 or hCgA352-366-treated mice (6 days, intra-rectal, 0.5, 1, 1.5 mg/kg/day, 6 days); *B*: Peritoneal macrophages isolated from *in vivo* colitic and non-colitic hCgA352-372, hCgA360-372 or hCgA352-366-treated mice (6 days, intra-rectal, 0.5, 1, 1.5 mg/kg/day, 6 days); *C*: Naive peritoneal

macrophages isolated from non-colitic mice and treated *in vitro* with hCgA352-372, hCgA360-372 or hCgA352-366 (10^{-7} M or 10^{-5} M) and stimulated with lipopolysaccharide (LPS) (100 ng/ml). p-STAT3 was measured using commercially available ELISA kits. ^aP<0.05 compared to non-colitic or in the absence of LPS, ^bP<0.05 compared to saline DSS-treated or medium LPS-treated groups, [#]P<0.05. ANOVA followed by the Tukey multiple comparisons *post hoc* analysis, n≥5. The values are shown as the mean ± SEM of four separate experiments. sh represents the modified hCgA352-372 peptide.

Figure 9. A. Schematic representation of human chromogranin A gene, protein, and some of its biologically active peptides. Human chromogranin A gene spans 12 194 bp in chromosome 14q32 and has eight exons giving rise to a 2043 nucleotide transcript, of which 1374 nucleotide is processed for translation. UTR: untranslated region. Chromogranin A protein consists of 457 amino acid residues, which matures to a 439 amino acid residue protein after removal of the signal peptide. The sequences are described as without the signal peptide sequence. B. Amino acid sequence of a human chromogranin A protein (SEQ ID NO:6). Amino acids 1-18 are the signal peptide; amino acids 370-390 are italicized and in bold and correspond to SEQ ID NO:2; amino acids 378-383 are italicized, in bold, and underlined and correspond to SEQ ID NO:1. C. An amino acid alignment of a human chromogranin A protein (SEQ ID NO:6) and a murine chromogranin A protein (available at Genbank accession number NP_031719.1, SEQ ID NO:7). Identical amino acids are marked with an asterisk (“*”), strongly conserved amino acids are marked with a colon (“:”), and weakly conserved amino acids are marked with a period (“.”).

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Provided herein are isolated chromogranin-A derived proteins. As used herein, the term “protein” refers broadly to a polymer of two or more amino acids joined together by peptide bonds. The term “protein” also includes molecules which contain more than one protein joined by a disulfide bond, or complexes of proteins that are joined together, covalently or noncovalently, as multimers (e.g., dimers, tetramers). Thus, the terms polypeptide, oligopeptide, enzyme, and peptide are all included within the definition of protein and these terms are used interchangeably. It should be understood that these terms do not connote a specific length of a polymer of amino acids, nor are they intended to imply or distinguish whether the protein is

produced using recombinant techniques, chemical or enzymatic synthesis, or is naturally occurring. An “isolated” protein is one that has been removed from a cell. For instance, an isolated protein is a protein that has been removed from the cytoplasm of a cell, and many of the proteins, nucleic acids, and other cellular material of its natural environment are no longer

present. A “purified” protein is one that is at least 60% free, preferably at least 75% free, and most preferably at least 90% free from other components of a cell. Proteins that are produced by recombinant, enzymatic, or chemical techniques are considered to be isolated and purified by definition, since they were never present in a cell.

As used herein, a “chromogranin-A derived protein” or “CgA-derived protein” refers to a polypeptide that includes an amino acid sequence present in the carboxy-terminal end of a chromogranin-A protein. An example of a human chromogranin-A polypeptide is available at Genbank accession number NP_001266 (SEQ ID NO:6). In one embodiment, a “CgA-derived protein” includes at least 7 amino acids present in a catestatin peptide. A “CgA-derived protein” includes the amino acid sequence ARAYGFR (SEQ ID NO:1). A “CgA-derived protein” also includes a polypeptide that has structural similarity with ARAYGFR (SEQ ID NO:1).

A CgA-derived protein may include other amino acid residues. In one embodiment, a CgA-derived polypeptide includes additional amino acids at the amino-terminal end, the carboxy-terminal end, or both the amino- and carboxy-terminal ends, of ARAYGFR (SEQ ID NO:1) or a polypeptide having structural similarity with ARAYGFR (SEQ ID NO:1). The number of amino acids at the amino-terminal end, the carboxy-terminal end, or both the amino- and carboxy-terminal ends may be, may be at least, or may be no greater than, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, or 70 amino acids. In one embodiment, the additional amino acids may be those present in a CgA polypeptide, such as the one available at Genbank accession number NP_001266, and flanking the sequence ARAYGFR (SEQ ID NO:1).

Examples of such CgA-derived polypeptides include SSMKLSFRARAYGFRGPGPQL (SEQ ID NO:2), also referred to in the art as catestatin; SSMKLSFRARAYGFR (SEQ ID NO:3), also referred to in the art as cateslytin, and ARAYGFRGPGPQL (SEQ ID NO:4). The relationship between SEQ ID NOs:1-4 are shown in Table 1.

Table 1. Relationship between SEQ ID NO:1, 2, 3, 4, and 6.

<u>ARAYGFR</u>	SEQ ID NO:1	Amino acids 378-383 of SEQ ID NO:6
<u>SSMKLSFRARAYGFRGPGPQL</u>	SEQ ID NO:2	Amino acids 370-390 of SEQ ID NO:6
<u>SSMKLSFRARAYGFR</u>	SEQ ID NO:3	Amino acids 370-383 of SEQ ID NO:6
<u>ARAYGFRGPGPQL</u>	SEQ ID NO:4	Amino acids 378-390 of SEQ ID NO:6

5 Structural similarity of two polypeptides can be determined by aligning the residues of the two polypeptides (for example, a candidate polypeptide and a reference polypeptide described herein) to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must
10 nonetheless remain in their proper order. A reference polypeptide may be a polypeptide described herein, such as SEQ ID NO:1, 2, 3, 4 or 6. A candidate polypeptide is the polypeptide being compared to the reference polypeptide. A candidate polypeptide may be isolated, for example, from a cell of an animal, such as a mouse, a rat, or a primate, such as a human, or can be produced using recombinant techniques, or chemically or enzymatically synthesized. A
15 candidate polypeptide may be inferred from a nucleotide sequence present in the genome of an animal cell.

In some embodiments, such as those embodiments where the reference amino acid sequence has 10 or fewer residues, such as ARAYGFR (SEQ ID NO:1), a pair-wise comparison analysis of amino acid sequences can be carried out by hand, e.g., the candidate and reference
20 sequences can be lined up and the differences between the two sequences easily determined.

In some embodiments, such as those embodiments where the reference has more than 10 residues, a pair-wise comparison analysis of amino acid sequences can be carried out using the Blastp program of the blastp suite-2sequences search algorithm, as described by Tatiana et al., (*FEMS Microbiol Lett*, 174, 247-250 (1999)), and available on the National Center for
25 Biotechnology Information (NCBI) website. The default values for all blastp suite-2sequences search parameters may be used, including general parameters: expect threshold=10, word size=3, short queries=on; scoring parameters: matrix = BLOSUM62, gap costs=existence:11 extension:1, compositional adjustments=conditional compositional score matrix adjustment.

Alternatively, polypeptides may be compared using the BESTFIT algorithm in the GCG package (version 10.2, Madison WI).

In the comparison of two amino acid sequences, structural similarity may be referred to by percent “identity” or may be referred to by percent “similarity.” “Identity” refers to the presence of identical amino acids. “Similarity” refers to the presence of not only identical amino acids but also the presence of conservative substitutions. A conservative substitution for an amino acid in a polypeptide described herein may be selected from other members of the class to which the amino acid belongs. For example, it is known in the art of protein biochemistry that an amino acid belonging to a grouping of amino acids having a particular size or characteristic (such as charge, hydrophobicity and hydrophilicity) can be substituted for another amino acid without altering the activity of a protein, particularly in regions of the protein that are not directly associated with biological activity. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Conservative substitutions include, for example, Lys for Arg and vice versa to maintain a positive charge; Glu for Asp and vice versa to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free -NH₂. The overall structural similarity for CgA in different vertebrates is around 40%, but the most highly conserved regions occur at the N and C-termini, which show much higher levels of structural similarity.

Guidance on how to modify the amino acid sequences of a protein disclosed herein includes the information provided at Figure 9C. This figure shows the amino acid sequences of a human CgA and a mouse CgA in a protein alignment. Identical amino acids are marked with an asterisk (“*”), strongly conserved amino acids are marked with a colon (“:”), and weakly conserved amino acids are marked with a period (“.”). By reference to this figure, the skilled person can predict which alterations to an amino acid sequence are likely to modify the biological activity of a CgA-derived protein, as well as which alterations are unlikely to modify biological activity.

Thus, as used herein, in one embodiment a CgA-derived polypeptide includes those having, having at least, or having no greater than, 1, 2 or 3 amino acid substitutions, for instance

a conservative substitution, compared to a reference amino acid sequence, such as SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:6. In one embodiment, a CgA-derived polypeptide includes those with at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% amino acid sequence similarity to a reference amino acid sequence, such as SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:6.

A CgA-derived polypeptide has biological activity. Whether a polypeptide has biological activity may be determined by *in vitro* or *in vivo* assays. In one embodiment, biological activity refers to the ability to stimulate chemotaxis of human peripheral blood monocytes, exhibiting its maximal effect at a concentration of 1nM comparable to the established chemoattractant formylated peptide Met-Leu-Phe (fMLP) (Egger et al., 2008, Eur J Pharmacol 598:104-111).

In one embodiment, a CgA-derived polypeptide, such as one that includes SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4 or having structural similarity with SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, includes no greater than 435, 436, 437, or 438 amino acids (the full length processed CgA polypeptide is typically 439 amino acids).

As discussed above, a CgA-derived polypeptide may include other amino acid residues. In one embodiment, the additional amino acids are heterologous amino acids. As used herein, “heterologous amino acids” refers to amino acids that are not normally or naturally found flanking the sequence depicted at, for instance, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4 in a natural CgA protein. In one embodiment, the natural CgA is SEQ ID NO:6, and whether an amino acid flanking SEQ ID NO:1, 2, 3, or 4, or a sequence having structural similarity is heterologous is determined by comparing the flanking sequence to SEQ ID NO:6.

For instance, whether amino acids flanking the carboxy terminal end of SEQ ID NO:4 are heterologous is determined by comparing the amino acids SEQ ID NO:4 to amino acids beginning at residue 391 of SEQ ID NO:6. The number of heterologous amino acids at the amino-terminal end, the carboxy-terminal end, or both the amino- and carboxy-terminal ends may be, may be at least, or may be no greater than, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67,

68, 69, or 70 amino acids, and so on. Such a polypeptide that includes, for instance, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, and heterologous amino acids may be referred to as a fusion polypeptide.

In one embodiment, the additional amino acid sequence may be useful for purification of the fusion polypeptide by affinity chromatography. Various methods are available for the addition of such affinity purification moieties to proteins. Representative examples include, for instance, polyhistidine-tag (His-tag) and maltose-binding protein (see, for instance, Hopp et al. (U.S. Pat. No. 4,703,004), Hopp et al. (U.S. Pat. No. 4,782,137), Sgarlato (U.S. Pat. No. 5,935,824), and Sharma (U.S. Pat. No. 5,594,115)). In one embodiment, the additional amino acid sequence may be a carrier polypeptide. The carrier polypeptide may be used to increase the immunogenicity of the fusion polypeptide to increase production of antibodies that specifically bind to a polypeptide of the invention. The invention is not limited by the types of carrier polypeptides that may be used to create fusion polypeptides. Examples of carrier polypeptides include, but are not limited to, keyhole limpet hemacyanin, bovine serum albumin, ovalbumin, mouse serum albumin, rabbit serum albumin, and the like. In another embodiment, the additional amino acid sequence may be a fluorescent polypeptide (e.g., green, yellow, blue, or red fluorescent proteins) or other amino acid sequences that can be detected in a cell, for instance, a cultured cell, or a tissue sample that has been removed from an animal. If a polypeptide described herein includes an additional amino acid sequence not normally or naturally associated with the polypeptide, the additional amino acids are not considered when percent structural similarity to a reference amino acid sequence is determined.

Polypeptides described herein can be produced using recombinant DNA techniques, such as an expression vector present in a cell. Such methods are routine and known in the art. The polypeptides may also be synthesized *in vitro*, e.g., by solid phase peptide synthetic methods. The solid phase peptide synthetic methods are routine and known in the art. A polypeptide produced using recombinant techniques or by solid phase peptide synthetic methods can be further purified by routine methods, such as fractionation on immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC, chromatography on silica or on an anion-exchange resin such as DEAE, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, gel filtration using, for example, Sephadex G-75, or ligand affinity. Such methods may also be used to isolate a CgA-derived polypeptide from a cell.

Also provided are polynucleotides. In one embodiment, a polynucleotide encodes a polypeptide described herein. Also included are the complements of such polynucleotide sequences. A polynucleotide encoding a polypeptide having biological activity is referred to herein as a CgA-derived polynucleotide.

5 As used herein, the term “polynucleotide” refers to a polymeric form of nucleotides of any length, either ribonucleotides, deoxynucleotides, peptide nucleic acids, or a combination thereof, and includes both single-stranded molecules and double-stranded duplexes. A polynucleotide can be obtained directly from a natural source, or can be prepared with the aid of recombinant, enzymatic, or chemical techniques. In one embodiment, a polynucleotide is
10 isolated. An “isolated” polynucleotide is one that has been removed from a cell. For instance, an isolated polynucleotide is a polynucleotide that has been removed from a cell and many of the polypeptides, nucleic acids, and other cellular material of its natural environment are no longer present. A “purified” polynucleotide is one that is at least 60% free, preferably at least 75% free, and most preferably at least 90% free from other components of a cell. Polynucleotides that are
15 produced by recombinant, enzymatic, or chemical techniques are considered to be isolated and purified by definition, since they were never present in a cell.

Given the amino acid sequence of any one of the CgA-derived polypeptides described herein, a person of ordinary skill in the art can determine the full scope of polynucleotides that encode that amino acid sequence using conventional, routine methods. The class of nucleotide
20 sequences encoding a selected polypeptide sequence is large but finite, and the nucleotide sequence of each member of the class may be readily determined by one skilled in the art by reference to the standard genetic code, wherein different nucleotide triplets (codons) are known to encode the same amino acid.

A CgA-derived polynucleotide described herein may include heterologous nucleotides
25 flanking the coding region encoding the CgA-derived polypeptide. As used herein, the terms “coding region” and “coding sequence” are used interchangeably and refer to a nucleotide sequence that encodes a polypeptide and, when placed under the control of appropriate regulatory sequences, expresses the encoded polypeptide. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3'
30 end. A “regulatory sequence” is a nucleotide sequence that regulates expression of a coding sequence to which it is operably linked. Non-limiting examples of regulatory sequences include

promoters, enhancers, transcription initiation sites, translation start sites, translation stop sites, and transcription terminators. The term “operably linked” refers to a juxtaposition of components such that they are in a relationship permitting them to function in their intended manner. A regulatory sequence is “operably linked” to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory sequence.

As used herein, “heterologous nucleotides” refers to a nucleotide sequence that is not normally or naturally found flanking an open reading frame in a cell encoding a CgA-derived polypeptide. Nucleotides normally or naturally found flanking nucleotides encoding a CgA-derived polypeptide include those present in exon VII of the CgA gene (Figure 9). Typically, heterologous nucleotides may be at the 5' end of the coding region, at the 3' end of the coding region, or the combination thereof. Examples of heterologous nucleotides include, but are not limited to, a regulatory sequence. The number of heterologous nucleotides may be, for instance, at least 10, at least 100, or at least 1000.

A polynucleotide described herein can be present in a vector. A vector is a replicating polynucleotide, such as a plasmid, phage, or cosmid, to which another polynucleotide may be attached so as to bring about the replication of the attached polynucleotide. Construction of vectors containing a polynucleotide of the invention employs standard ligation techniques known in the art. See, e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989). A vector can provide for further cloning (amplification of the polynucleotide), i.e., a cloning vector, or for expression of the polynucleotide, i.e., an expression vector. The term vector includes, but is not limited to, plasmid vectors, viral vectors, cosmid vectors, transposon vectors, and artificial chromosome vectors. Examples of viral vectors include, for instance, adenoviral vectors, adeno-associated viral vectors, lentiviral vectors, retroviral vectors, and herpes virus vectors. A vector may be replication-proficient or replication-deficient. A vector may result in integration into a cell's genomic DNA. Typically, a vector is capable of replication in a host cell, for instance a mammalian and/or a bacterial cell, such as *E. coli*.

Selection of a vector depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, vector replication rate, use in gene transfer into cells of the gastrointestinal tract, and the like. Suitable host cells for cloning or expressing the vectors herein

are prokaryotic or eukaryotic cells. Suitable eukaryotic cells include mammalian cells, such as murine cells and human cells. Suitable prokaryotic cells include eubacteria, such as gram-negative organisms, for example, *E. coli*.

5 An expression vector optionally includes regulatory sequences operably linked to the polynucleotide of the present invention. An example of a regulatory sequence is a promoter. A promoter may be functional in a host cell used, for instance, in the construction and/or characterization of a CgA-derived polynucleotide, and/or may be functional in the ultimate recipient of the vector. A promoter may be inducible, repressible, or constitutive, and examples of each type are known in the art. A polynucleotide of the present invention may also include a
10 transcription terminator. Suitable transcription terminators are known in the art.

Polynucleotides described herein can be produced *in vitro* or *in vivo*. For instance, methods for *in vitro* synthesis include, but are not limited to, chemical synthesis with a conventional DNA/RNA synthesizer. Commercial suppliers of synthetic polynucleotides and reagents for *in vitro* synthesis are known. Methods for *in vitro* synthesis also include, for
15 instance, *in vitro* transcription using a circular or linear expression vector in a cell free system. Expression vectors can also be used to produce a polynucleotide of the present invention in a cell, and the polynucleotide may then be isolated from the cell.

Also provided are compositions including one or more polypeptides or polynucleotides described herein. Such compositions typically include a pharmaceutically acceptable carrier. As
20 used herein “pharmaceutically acceptable carrier” includes, but is not limited to, saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Additional compounds can also be incorporated into the compositions.

A composition may be prepared by methods known in the art of pharmacy. In general, a
25 composition can be formulated to be compatible with its intended route of administration. A formulation may be solid or liquid. Administration may be systemic or local. In some aspects local administration may have advantages for site-specific, targeted disease management. Local therapies may provide high, clinically effective concentrations directly to the treatment site, with less likelihood of causing systemic side effects.

30 Examples of routes of administration include parenteral (e.g., intravenous, intradermal, subcutaneous, intraperitoneal, intramuscular), enteral (e.g., oral or rectal), and topical (e.g.,

epicutaneous, inhalational, transmucosal) administration. Appropriate dosage forms for enteral administration of the compound of the present invention may include tablets, capsules or liquids. Appropriate dosage forms for parenteral administration may include intravenous administration. Appropriate dosage forms for topical administration may include nasal sprays, metered dose
5 inhalers, dry-powder inhalers or by nebulization.

Solutions or suspensions can include the following components: a sterile diluent such as water for administration, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as
10 ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates; electrolytes, such as sodium ion, chloride ion, potassium ion, calcium ion, and magnesium ion, and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. A composition can be enclosed in, for instance, ampoules, disposable syringes or multiple dose vials made of glass or plastic.

15 Compositions can include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile solutions or dispersions. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline. A composition is typically sterile and, when suitable for injectable use, should be fluid to the extent that easy syringability exists.
20 It should be stable under the conditions of manufacture and storage and preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. Prevention of the action of microorganisms can be achieved by various antibacterial and
25 antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, and sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

30 Sterile solutions can be prepared by incorporating the active compound (e.g., a polypeptide or polynucleotide described herein) in the required amount in an appropriate solvent

with one or a combination of ingredients such as those enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a dispersion medium and other ingredients such as from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation that may be used include vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

For enteral administration, a composition may be delivered by, for instance, nasogastric tube, enema, colonoscopy, or orally. Oral compositions may include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier. Pharmaceutically compatible binding agents can be included as part of the composition. The tablets, pills, capsules, troches and the like may contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the active compounds may be delivered in the form of an aerosol spray, a nebulizer, or an inhaler, such as a nasal spray, metered dose inhaler, or dry-powder inhaler.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds may be formulated into ointments, salves, gels, or creams as generally known in the art. An example of transdermal administration includes iontophoretic delivery to the dermis or to other relevant tissues.

The active compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

5 The active compounds may be prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Such formulations can be prepared using standard techniques. The materials can also be obtained commercially. Liposomal suspensions can
10 also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art. Delivery reagents such as lipids, cationic lipids, phospholipids, liposomes, and microencapsulation may also be used.

In one embodiment, an active compound may be associated with a targeting group. As used herein, a "targeting group" refers to a chemical species that interacts,
15 either directly or indirectly, with the surface of a cell, for instance with a molecule present on the surface of a cell, e.g., a receptor. The interaction can be, for instance, an ionic bond, a hydrogen bond, a Van der Waals force, or a combination thereof. Examples of targeting groups include, for instance, saccharides, polypeptides (including hormones), polynucleotides, fatty acids, and catecholamines. Another example of a targeting group
20 is an antibody. The interaction between the targeting group and a molecule present on the surface of a cell, e.g., a receptor, may result in the uptake of the targeting group and associated active compound. Cells that may be targeted include, but are not limited to, cell of the gastrointestinal tract such as macrophages, CD4+ and CD8+ T cells, NKT cells, mast cells, intraepithelial T cells, T regulatory cells, granulocyte (neutrophils,
25 basophils, eosinophils), B cells, and dendritic cells.

When a polynucleotide is introduced into cells using a suitable technique, the polynucleotide may be delivered into the cells by, for example, transfection or transduction procedures. Transfection and transduction refer to the acquisition by a cell of new genetic material by incorporation of added polynucleotides. Transfection can
30 occur by physical or chemical methods. Many transfection techniques are known to those of ordinary skill in the art including, without limitation, calcium phosphate DNA co-

precipitation, DEAE-dextrin DNA transfection, electroporation, naked plasmid adsorption, cationic liposome-mediated transfection (commonly known as lipofection), use of glycoconjugates and polyplexes, targeting serpin-enzyme complex receptors, and polyethyleneimine. Transduction refers to the process of transferring nucleic acid into a cell using a DNA or RNA virus.

A polynucleotide described herein may be used in combination with other agents assisting the cellular uptake of polynucleotides, or assisting the release of polynucleotides from endosomes or intracellular compartments into the cytoplasm or cell nuclei by, for instance, conjugation of those to the polynucleotide. The agents may be, but are not limited to, peptides, especially cell penetrating peptides, protein transduction domains, and/or dsRNA-binding domains which enhance the cellular uptake of polynucleotides (Dowdy et al., US Published Patent Application 2009/0093026, Eguchi et al., 2009, *Nature Biotechnology* 27:567-571, Lindsay et al., 2002, *Curr. Opin. Pharmacol.*, 2:587-594, Wadia and Dowdy, 2002, *Curr. Opin. Biotechnol.* 13:52-56. Gait, 2003, *Cell. Mol. Life Sci.*, 60:1-10). The conjugations can be performed at an internal position in the oligonucleotide and/or at a terminal position, such as the 5'-end and/or the 3'-end.

Toxicity and therapeutic efficacy of such active compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the ED₅₀ (the dose therapeutically effective in 50% of the population).

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. Examples of animal models include, but are not limited to, dextran sulfate sodium (DSS)-induced colitis (Neurath et al., 1995, *J Exp Med* 182:1281-90; Ghia et al., 2007, *J Clin Invest* 118:2209-18; Ghia et al., 2007, *Am J Physiol Gastrointest Liver Physiol* 293:G711-8; Ghia et al., 2008, *Am J Physiol Gastrointest Liver Physiol* 294:770-777), the 2,4-dinitrobenzenesulfonic acid (DNBS) model which mimics some features of CD (Elson et al., 1995, *Gastroenterology*, 109:1344-67), and a spontaneous model of ileal inflammation that bears a very close resemblance to CD, known as SAMPI/YitFc (Pizarro et al., 2003, *Trends Mol Med* 9:218-222; Rivera-Nieves et al., 2003, *Gastroenterology* 124:972-982). The dosage of such active compounds lies preferably within a range of concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the

dosage form employed and the route of administration utilized. For an active compound used in the methods of the invention, it may be possible to estimate the therapeutically effective dose initially from cell culture assays. A dose may be formulated in animal models to achieve a concentration range that includes the IC_{50} (i.e., the concentration of the test compound which achieves a half-maximal inhibition of signs and/or symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

The compositions can be administered one or more times per day to one or more times per week, including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with an effective amount of a polynucleotide or a polypeptide can include a single treatment or can include a series of treatments.

The present invention includes methods for using the polypeptides and polynucleotides disclosed herein. In one embodiment, a method includes contacting a cell with an effective amount of a CgA-derived polypeptide. In one embodiment, the contacting is under conditions suitable for allowing the CgA-derived polypeptide to interact with the surface of the cell. In one embodiment, the contacting is under conditions suitable for introduction of a CgA-derived polypeptide into the cell. In another embodiment, a method includes contacting a cell with an effective amount of a CgA-derived polynucleotide. In one embodiment, the contacting is under conditions suitable for introduction of a CgA-derived polynucleotide into the cell.

Conditions that are “suitable” for an event to occur, such as introduction of a polypeptide into a cell, or “suitable” conditions are conditions that do not prevent such events from occurring. Thus, these conditions permit, enhance, facilitate, and/or are conducive to the event. As used herein, an “effective amount” relates to a sufficient amount of a CgA-derived polypeptide or a CgA-derived polynucleotide to provide the desired effect. For instance, in one embodiment an “effective amount” is an amount effective to alter certain characteristics of cells, such as macrophages, including peripheral blood monocytes. Examples of characteristics include, but are not limited to,

altered production of proinflammatory cytokines. The method may result in decreased production of proinflammatory cytokines by a cell. In one embodiment, a cell is considered to have decreased production of proinflammatory cytokines if there is a statistically significant decrease in the production of one or more proinflammatory cytokines compared to a control not contacted with the CgA-derived polypeptide.

Examples of proinflammatory cytokines include, for instance, IL-6, IL-1 β , and TNF- α . In one embodiment, a cell is considered to have a decrease in the production of one or more proinflammatory cytokines if there is a decrease of at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, or at least 80% compared to a control not contacted with the CgA-derived polypeptide.

A cell that may be used in the methods described herein may be *ex vivo* or *in vivo*. As used herein, “*ex vivo*” refers to a cell that has been removed from the body of an animal. *Ex vivo* cells include, for instance, primary cells (e.g., cells that have recently been removed from a subject and are capable of limited growth in tissue culture medium), and cultured cells (e.g., cells that are capable of long term culture in tissue culture medium). Examples of primary cells include cells normally present in an animal’s gastrointestinal tract, including, but not limited to, immune cells (such as neutrophils and macrophages, CD4 and CD8+ T cells, NKT cells, mast cells, intraepithelial T cells, T regulatory cells), and also enterochromaffin cells, paneth cells, and goblet cells. Other examples of primary cells include bone marrow macrophages and peripheral macrophages. Examples of cultured cells include, but are not limited to, CD4+ and CD8+ T cells, NKT cells, mast cells, intraepithelial T cells, T regulatory cells, granulocyte (neutrophils, basophils, eosinophils), B cells, dendritic cells, but also enterochromaffin cells, paneth cells, and goblet cells, glial cells or neurons (including sensitive, inter or moto-neurons). Control cells may be obtained from the ATCC and may be cultured according to methods known in the art. Control cells may also be obtained from tissue samples through, for example, biopsy. As used herein, “*in vivo*” refers to a cell that is present within an animal. A cell that may be used in the methods described herein may be a mammalian cell, such as, for instance, mouse, rat, primate (e.g., monkey, human), or a dog, a sheep, a guinea pig, or a horse.

The present invention also includes methods for treating certain diseases. In one embodiment, a method includes treating a disease in a subject, where a subject in need thereof is administered an effective amount of a composition that includes a CgA-derived polypeptide or a CgA-derived polynucleotide. The subject may be a mammal, such as a member of the family Muridae (a murine animal such as rat or mouse), a primate, (e.g., monkey, human), a dog, a sheep, a guinea pig, or a horse. As used herein, the term “disease” refers to any deviation from or interruption of the normal structure or function of a part, organ, or system, or combination thereof, of a subject that is manifested by a characteristic symptom or clinical sign. Diseases include inflammatory bowel diseases, such as Crohn’s disease, ulcerative colitis, collagenous colitis, lymphocytic colitis, ischaemic colitis, diversion colitis, Behçet’s disease, and indeterminate colitis

As used herein, the term “symptom” refers to subjective evidence of disease or condition experienced by the patient and caused by disease. As used herein, the term “clinical sign,” or simply “sign,” refers to objective evidence of a disease present in a subject. Symptoms and/or signs associated with diseases referred to herein and the evaluation of such signs are routine and known in the art. A symptom and/or sign may be localized to, for instance, a subject’s gastrointestinal tract, or a combination thereof. Whether a subject has a disease, and whether a subject is responding to treatment, may be determined by evaluation of signs associated with the disease.

In one embodiment, the method also includes evaluating a subject for signs associated with the disease after the CgA-derived polypeptide is administered. Such an evaluation may be used to determine the status of disease in the subject, and/or determine if the treatment has resulted in a reduction of the disease. The evaluating may be done at various intervals after the administering, such as 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 2 weeks, 1 month, 2 months, 3 months, and so on, after the administering.

In one embodiment, a disease treated using a method of the present invention is inflammatory bowel disease, such as Crohn’s disease, ulcerative colitis, collagenous colitis, lymphocytic colitis, ischaemic colitis, diversion colitis, Behçet’s disease, and indeterminate colitis. Signs of inflammatory bowel disease, may include, but are not limited to, abdominal pain, vomiting, diarrhea, rectal bleeding, intestinal cramps, weight loss, and/or anemia.

In one embodiment, the methods include contacting cells of a subject's gastrointestinal tract with an effective amount of a composition that includes a CgA-derived polypeptide or a CgA-derived polynucleotide. The method may result in decreased production of proinflammatory cytokines by cells, such as macrophages. In one embodiment, the subject may be suffering from, or at risk of suffering from, inflammatory bowel disease.

Treatment of a disease can be prophylactic or, alternatively, can be initiated after the development of a disease. Treatment that is prophylactic, for instance, initiated before a subject manifests signs of a disease, is referred to herein as treatment of a subject that is "at risk" of developing a disease. An example of a subject that is at risk of developing a disease is a person having a risk factor. Examples of risk factors include genetic susceptibilities including, but not limited to, coding mutations in JTNI, SLC22A4, NDD2, ATGI6L1, XBP1, NOD2, CARD9, FCGR2A, MST1, ERAP2, IL23R, TNFSF15, IL7R, IL27, GPX1, H5PA5, UTS2, PEX13, THADA, AND GCKR. Examples of other risk factors include age, ethnicity, diet, family history, and parasite exposure. Treatment can be performed before, during, or after the occurrence of the diseases described herein. Treatment initiated after the development of a disease may result in decreasing the severity of the signs of the disease, or completely removing the signs. An "effective amount" may be an amount effective to alleviate one or more symptoms and/or signs of the disease. In one embodiment, an effective amount is an amount that is sufficient to effect a reduction in a symptom and/or sign associated with a disease. A reduction in a symptom and/or a sign is, for instance, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 100% in a measured sign as compared to a control, a non-treated subject, or the subject prior to administration of the CgA-derived polypeptide or CgA-derived polynucleotide. It will be understood, however, that the total daily usage of the compositions and formulations as disclosed herein will be decided by the attending physician within the scope of sound medical judgment. The exact amount required will vary depending on factors such as the type of disease being treated.

The polypeptides and/or polynucleotides described herein may also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. Therapeutic compounds useful for the treatment of the diseases described herein are known and used routinely. Therapeutic compounds may include, but are not limited to, Aminosalicylates (such as mesalazine or 5-aminosalicylic acid), corticoids (such as oral

prednisolone, prednisone, budesonide or intravenous hydrocortisone, methylprednisolone or topical suppositories, foam or liquid enemas include hydrocortisone, prednisolone, metasulphobenzoate, betamethasone, budesonide), immunosuppressive compounds (such as ciclosporin or tacrolimus), chimeric monoclonal therapy (an anti-TNF monoclonal antibody such as Infleximab), antibiotics (metronidazole, ciprofloxacin), thiopurines (azathioprine or mercaptopurin), or methotrexate.

Also provided are methods for evaluating treatment options for a subject having inflammatory bowel disease. For instance, such a method may indicate that treatment with a CgA-derived polypeptide or a CgA-derived polynucleotide is appropriate. In one embodiment, the method may include measuring the expression of a CgA-derived polypeptide by a cell present in, or explanted from, the gastrointestinal tract of a subject. In one embodiment, the method may include measuring the amount of CgA-derived polypeptide in the gastrointestinal tract of a subject. A decrease of CgA-derived polypeptide in the cell or in the gastrointestinal tract relative to a control cell indicates that it may be possible to treat the subject by administration of a CgA-derived polypeptide. Optionally, the method also includes obtaining a biological sample from the subject.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a subject, including but not limited to, for example, cells, and tissues such as biopsy samples, from a gastrointestinal tract, such as macrophages, CD4+ and CD8+ T cells, NKT cells, mast cells, intraepithelial T cells, T regulatory cells, granulocyte (neutrophils, basophils, eosinophils), B cells, dendritic cells, but also enterochromaffin cells, Paneth cells, and goblet cells, glial cells or neurons (including sensitive, inter or motor-neurons). Biological samples also include explants and primary and/or transformed cell cultures derived from patient tissues. A biological sample can be provided by removing a sample of cells or a fluid from a subject, but can also be accomplished by using previously isolated cells (e.g., isolated by another person, at another time, and/or for another purpose). Methods for measuring the amount of polypeptides such as a CgA-derived polypeptide are known in the art and are routine. Such methods include, for instance, HPLC-MS/MS.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Example 1

This example shows that catestatin (CTS) is increased during colitis and that human (h)CTS (hCTS) modulates intestinal inflammation *via* the macrophage population and through a STAT-3 dependent pathway in a murine model of colitis. Identification of the molecular mechanism underlying the protective role of this peptide may lead to a novel therapeutic option in IBD.

Colitis was induced by administration of dextran sulfate sodium or 2, 4 dinitrobenzenesulfonic acid to C57BL/6 mice. Treatment with hCTS or its proximal or distal part was started one day before colitis induction and colonic inflammatory markers were determined. Pro-inflammatory cytokines were evaluated in peritoneal isolated and bone marrow derived macrophages (BMDMs); p-STAT3 level was studied. Serum levels of chromogranin A (CgA) and CTS were assessed in experimental colitis and in a separate study in inflammatory bowel disease (IBD) patients and healthy controls.

We show that sera from IBD patients and that in experimental colitis conditions the colonic level of mouse (m)CgA and mCTS are significantly increased. Moreover, *in vivo* treatment with human (h)CTS reduces the disease onset and suppresses exacerbated inflammatory responses in preclinical settings of colitis associated with an increase of p-STAT3. *In vitro*, hCTS treatment decreases pro-inflammatory cytokine release by peritoneal macrophages and BMDMs and increases p-STAT3 levels.

Materials and Methods

Animals. Male C57BL/6 (7–9 weeks old) mice were purchased from Charles Rivers (Canada) and maintained in the animal care facility at the University of Manitoba under specific pathogen-free conditions. All experiments were approved by the University of Manitoba Animal Ethics Committee (10-073) and conducted under the Canadian guidelines for animal research.

Peptides used. hCTS (hCgA₃₅₂₋₃₇₂: SSMKLSFRARAYGFRGPGPQL (SEQ ID NO:2, Mahata et al., Regul Pept, 2010;162:33-43); modified hCTS (shCgA₃₅₂₋₃₇₂: SLPRRQLPSSAGMRGGKFAYF, SEQ ID NO:8); hCTS, proximal sequence (hCgA₃₅₂₋₃₆₆: SSMKLSFRARAYGFR, SEQ ID NO:3), and hCTS, distal sequence (hCgA₃₆₀₋₃₇₂:

5 ARAYGFRGPGPQL, SEQ ID NO:4) were used (obtained from Dr. Metz-Boutigue or purchased from Biopeptide Co., Inc, San Diego, CA). The peptides were used at different doses ranging from 0.5 to 1.5mg/kg/day as reflected by previous published data related to the use of peptide for intra-rectal injection (Dave et al., J Immunol., 2007;179:7852-9).

DSS and 2, 4 DNBS colitis. DSS (molecular weight [MW], 40 kilodaltons: MP
10 Biomedicals, Soho, OH) was added to the drinking water at a final concentration of 5% (wt/vol) for 5 days (Ghia et al., Am J Physiol Gastrointest Liver Physiol, 2008;294:770-7, Okayasu et al., Gastroenterology, 1990;98:694-702). Controls were time-matched and consisted of mice that received normal drinking water only. Mean DSS consumption was noted per cage each day. For the DNBS study, mice were anaesthetized using Isoflurane® (Abbott, Toronto, Canada). PE-90
15 tubing (10 cm long; ClayAdam, Parisppany, NJ) that was attached to a tuberculin syringe (BD, Mississauga, Canada) was inserted 3.5 cm into the colon. Colitis was induced by administration of 100µl of 4mg of DNBS solution (ICN Biomedical Inc. Aurora, OH) in 30% ethanol (Sigma, Mississauga, Canada) and left for 3 days (Ghia et al., J Clin Invest., 2008;118:2209-18). For the DNBS study mice were supplied with 6% sucrose in their drinking water to prevent dehydration.

20 Assessment of colitis severity - disease activity index (DAI). DAI scores have historically correlated well with the pathological findings in a DSS-induced model of IBD (Cooper et al., Lab Invest., 1993;69:238-49). DAI is the combined score of weight loss, stool consistency, and bleeding. Scores were defined as follows: weight: 0, no loss; 1, 5%–10%; 2, 10%–15%; 3, 15%–20%; and 4, 20% weight loss; stool: 0, normal; 2, loose stool; and 4, diarrhoea; and bleeding: 0,
25 no blood; 2, presence of blood; and 4, gross blood. Blood was assessed using the Hemocult II test (Beckman Coulter, Oakville, Canada). DAI was scored from day 0 to day 5 during DSS treatment.

Macrophage isolation. Five or 3 days after the beginning of the DSS or the DNBS induction respectively, resident peritoneal cells were collected as described (Kruisbeek and
30 Vogel, Current Protocol in Immunology, 2002:14.1.1-.2.8) with a slight modification. Sterile PBS (Gibco BRL Life Technologies, Grand Island, NY) (10ml) was injected into the caudal half

of the peritoneal cavity using a 25-gauge needle (BD, Mississauga, Canada) and the body was shaken for 15sec. By inserting a 19-gauge needle (BD, Mississauga, Canada) resident peritoneal cells were slowly withdrawn and resident peritoneal macrophages were purified by adhesion. Resident peritoneal cells were plated in macrophage culture medium (RPMI 1640 supplemented with 10% HI FCS containing 50 IU of penicillin, 50 µg streptomycin, and 2 mM glutamine per milliliter (Gibco BRL Life Technologies, Grand Island, NY)) for 60 mins at 37°C. Non-adherent cells were removed by washing five times with 500 µl of warm PBS. The overall cell viability of the adherent cell before and after treatment was greater than 97%, and more than 94% of the cells were macrophages using DiffQuick (Jorgensen Laboratory, Loveland, CO) staining.

Primary bone marrow-derived macrophages (BMDMs) from naive C57BL/6 mice were generated as previously described (Fortier and Falk, Curr Protoc Immunol 2001;Chapter 14:Unit 14 1) and were cultured in complete RPMI-10 medium. Cells were maintained at 37°C in a humidified incubator containing 5% CO₂.

Lipopolysaccharide (LPS, Sigma, Mississauga, Canada) was added to the cultures at a final concentration of 100ng/ml. In a separated set CgDPs were added to the medium at a final concentration of 10⁻⁷ or 10⁻⁵M one hour before the LPS was added in the presence or absence of STAT3 V blocker (10⁻⁵M, STATTC; Sigma, Mississauga, Canada). Supernatants were collected 24hrs after LPS was either added or not to assess IL-1β, IL-6 and p-STAT3 levels using ELISA.

Macroscopic scores. After macrophage isolation, mice were sacrificed and the abdominal cavity was opened, the colon was located, and observations on distension, fluid content, hyperaemia, and erythema were recorded. The colon was removed and opened longitudinally, and macroscopic damage was immediately assessed on the full section of the colon. Macroscopic scores were performed using a previously described scoring system for DSS colitis (Cooper et al., Lab Invest., 1993;69:238-49) and for DNBS colitis (Appleyard et al., Am J Physiol., 1995;269:G119-25).

C-reactive protein (CRP) assay in serum. At sacrifice date, blood was collected by intracardiac puncture in anaesthetized (Isoflurane®, Abbott, Toronto, Canada) mice. CRP levels were determined using an ELISA commercial kit (Immuno-Consultants, Portland, OR).

Colonic histology and myeloperoxidase (MPO) activity. Formalin (Sigma, Mississauga, Canada) -fixed colon segments coming from the splenic flexure were paraffin (Sigma, Mississauga, Canada) -embedded and 3-µm sections were stained using hematoxylin-eosin

(H&E) (Sigma, Mississauga, Canada). Colonic damage was scored based on a published scoring system that considers architectural derangements, goblet cell depletion, oedema/ulceration, and degree of inflammatory cell infiltrate (Cooper et al., Lab Invest., 1993;69:238-49). MPO activity was determined following an established protocol (Boughton-Smith et al., Agents Actions, 1988;25:115-23). Briefly, MPO activity, used as a marker of granulocyte infiltration, was extracted and the activity was measured using a modified version of the method described by Bradley (Bradley et al., J Invest Dermatol., 1982;78:206-9). Tissue samples were homogenized (50mg/ml) in ice-cold 50mM potassium phosphate buffer (pH 6.0) (Gibco BRL Life Technologies, Grand Island, NY) containing 0.5% hexadecyl trimethyl ammonium bromide (Sigma, Mississauga, Canada). The homogenate was freeze-thawed three times, briefly sonicated, and then centrifuged at 12000 rpm for 12min at 4°C. The supernatant was then added to a solution of O-dianisidine (Sigma, Mississauga, Canada) and hydrogen peroxide (Sigma, Mississauga, Canada). The absorbance of the colorimetric reaction was measured by a spectrophotometer (Biotek, Winooski, VT). MPO is expressed in units per milligram of wet tissue, 1 unit being the quantity of enzyme able to convert 10^{-6} M of hydrogen peroxide to water in 1 minute at room temperature.

CgA, CTS, cytokines and p-STAT3 levels. Colonic samples were homogenized in 700µl of Tris·HCl buffer containing protease inhibitors (Sigma, Mississauga, Canada). Samples were centrifuged for 30 min, and the supernatant was frozen at -80°C until assay. Commercial ELISA were used to determine cytokine levels (IL-1β, IL-6 and TNF-α), (R&D Systems, Minneapolis, MN), human and mouse CgA and CTS level (CUSABIO, Cedarlane, Burlington, Canada).

Cell lysates from colonic samples and macrophages were used to detect p-STAT3 (STAT3 [pY705] ELISA, LifeScience, Burlington, Canada)

Human sera. Sera were collected from persons with Crohn's disease (n=15), ulcerative colitis (n=15) and from healthy controls who did not have any known chronic immune disease or first-degree relatives with known chronic immune diseases (n=15).

Statistical analysis. Results are presented as the mean ± SEM. Statistical analysis was performed using one or two way ANOVA followed by the Tukey-Kramer multiple comparisons *post hoc* analysis, and a P value of <0.05 considered significant with n=8 to 12 depending on the groups tested (Prism 5, GraphPad, La Jolla, CA).

Results

mCgA and mCTS in the context of DSS-induced colitis and IBD.

First, we examined the relation between mCgA and mCTS during the development of experimental colitis using the DSS and DNBS models. In this context, our data demonstrate a significant increase of mCgA during the development of colitis (Figure 1A, C) confirming previously published clinical data (Sciola et al., *Inflamm Bowel Dis.*, 2009;15:867-71, El-Salhy et al., *Mol Med Rep.*, 2011;4:603-5, Wagner et al., *Inflammation* 2013;36(4):855-61). However, for the first time this was correlated with an increase of colonic mCTS when compared to the control group (Figure 1B, D). To determine the translational applicability of our study, we determined the level of hCgA and hCTS in IBD patients. hCgA and hCTS levels were significantly higher in persons with IBD than in healthy subjects. In the IBD group there were no significant differences in mean hCgA and hCTS levels among those with UC and CD (Figure 1E, F). These data demonstrate that in the context of colitis m and hCTS are up regulated and may act as a pro- or anti-inflammatory regulators.

The effect of hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ and hCgA₃₅₂₋₃₆₆ in the absence of colitis.

To determine if the three peptides have some direct effect in the absence of colitis, we injected the peptides for six days (daily i.r. injection). hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ caused no changes in weight gain, colonic appearance or histology, CRP, MPO, or cytokine levels in C57BL/6 without colitis (Figure 3-6, 8).

The effect of hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ and hCgA₃₅₂₋₃₆₆ on DAI.

To determine the potential regulatory effect related to hCTS on colonic inflammation, we studied the effect of the full sequence of hCTS (hCgA₃₅₂₋₃₇₂), its proximal (hCgA₃₅₂₋₃₆₆) and distal (hCgA₃₆₀₋₃₇₂) part using the DSS-induced experimental model of colitis. Colitis was characterized by weight loss and frequent stools; this was evident by day 4 in the saline treated group of mice (Figure 2). In hCgA₃₅₂₋₃₇₂-treated (1.5mg/kg/day, 6 days, i.r.) mice, the onset of colitis was delayed as injury reflected in the DAI was seen within 5 days (Figure 2A, B) and the differences between groups reached statistical significance on day 4 and 5 of DSS regime. This effect was dose-dependent (0.5, 1 and 1.5mg/kg/day, i.r.) and the highest reduction was achieved with a dose of 1.5mg/kg/day; the modified peptides (shCgA₃₅₂₋₃₇₂, 1.5mg/kg/day, 6 days, i.r.) did

not show any significant effects (Figure 2A). Daily administration of hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ (1.5 mg/kg/day, i.r.) showed a significantly lower DAI for the two last days when compared to saline-DSS group (Figure 2B), but no difference were seen between the peptides.

5 The effect of hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ and hCgA₃₅₂₋₃₆₆ on macroscopic score.

At sacrifice day, feces consistency, hyperaemia and presence of blood were assessed. As shown in Figure 3A, B, hCgA₃₅₂₋₃₇₂ treatments (0.5, 1 and 1.5mg/kg/day, 6 days, i.r.) significantly decreased the macroscopic scores and increased the length of colon. The modified peptides (shCgA₃₅₂₋₃₇₂, 1.5mg/kg/day, 6 days, i.r.) did not show any significant effects. The
10 decreased severity of colitis in hCgA₃₆₀₋₃₇₂- or hCgA₃₅₂₋₃₆₆ treated mice (1.5 mg/kg/day, 6 days, i.r.) compared to saline-DSS group was further demonstrated by the 1.72 and 1.81-fold decrease in the macroscopic damage score respectively (Figure 3A).

The effect of hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ and hCgA₃₅₂₋₃₆₆ on the colonic MPO activity.

15 MPO a marker of granulocyte infiltration was studied. DSS treatment increased the MPO activity from 0.6±0.1 U/mg in control mice to 2.23±0.2 U/mg (Figure 3C), but treatment with hCgA₃₅₂₋₃₇₂ (0.5, 1 and 1.5mg/kg/day, 6 days, i.r.) resulted in a significantly lower MPO activity when compared to saline-DSS group. The modified peptides (shCgA₃₅₂₋₃₇₂, 1.5mg/kg/day, 6 days, i.r.) did not show any significant effects. Treatments with hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ (1.5
20 mg/kg/day, 6 days, i.r.) resulted in a significantly lower MPO activity of 1.33±0.4 and 1.37±0.3 U/mg respectively compared to saline-DSS group (Figure 3C).

The effect of hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ and hCgA₃₅₂₋₃₆₆ on the histological score.

Mucosal inflammation and infiltration was assessed through histological scoring. DSS
25 colitis induces a significant increase of cell infiltration (Figure 4A(a)). As shown in Figure 4A, (b) hCgA₃₅₂₋₃₇₂, (c) hCgA₃₆₀₋₃₇₂ or (d) hCgA₃₅₂₋₃₆₆ treatments (1.5mg/kg/day, 6 days, i.r.) significantly decreased the severity of colitis. This was associated with a reduction in the loss of tissue architecture and edema, and a decrease in the mixed immune cell infiltrate (mononuclear cells, neutrophils, and eosinophils). The modified peptides (shCgA₃₅₂₋₃₇₂, 1.5mg/kg/day, 6 days,
30 i.r.) did not show any significant effects (Figure 4A(e)). Treatments with hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂

or hCgA₃₅₂₋₃₆₆ (1.5 mg/kg/day, 6 days, i.r.) decreased the histological score from 2.7 ± 0.1 to 1.3 ± 0.1 , 1.2 ± 0.2 and 1.3 ± 0.1 respectively (Figure 4B).

The effect of hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ and hCgA₃₅₂₋₃₆₆ on serum CRP level.

5 To confirm any potential effect on systemic inflammation, serum CRP levels were studied. CRP decreased from 35.63 ± 0.7 in colitic mice to $25.14 \pm 0.22 \mu\text{g/ml}$ in colitic mice treated with hCgA₃₅₂₋₃₇₂ (1.5mg/kg/day, 6 days, i.r.) and this effect was dose-dependent (Figure 5A). The modified peptides (shCgA₃₅₂₋₃₇₂, 1.5mg/kg/day, 6 days, i.r.) did not show any significant effects (Figure 5A). The decreased severity of colitis in hCgA₃₆₀₋₃₇₂- or hCgA₃₅₂₋₃₆₆-
10 treated mice (1.5 mg/kg/day, 6 days, i.r.) compared to saline-DSS group was further demonstrated by a 1.5 and 1.6-fold decrease in serum CRP level respectively (Figure 5A).

The effect of hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ and hCgA₃₅₂₋₃₆₆ on colonic cytokine levels.

To determine more precisely the mucosal inflammatory states, we studied the colonic
15 pro-inflammatory cytokine levels. We found significantly lower fold increases in the levels of IL-1 β (2-fold), IL-6 (1.8-fold) and TNF- α (1.9-fold) in the colon of colitic mice treated with hCgA₃₅₂₋₃₇₂ (1.5mg/kg/day, 6 days, i.r.) when compared to the saline-DSS group, this effect was dose-dependent (Figure 5 B-D). The modified peptides (shCgA₃₅₂₋₃₇₂, 1.5mg/kg/day, 6 days, i.r.) did not show any significant anti-inflammatory effects on the development of colitis (Figure 4).
20 IL-1 β levels in hCgA₃₆₀₋₃₇₂ and hCgA₃₅₂₋₃₆₆-treated colitic mice (1.5mg/kg/day, 6 days, i.r.) were 2.35 and 2.46-fold lower respectively compared to the saline-DSS group (Figure 5B) and IL-6 and TNF- α levels were 1.79- 17.4 and 2 1.68-fold lower, respectively (Figure 5C, D).

hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ regulate macrophage pro-inflammatory cytokines
25 release.

Monocytes and macrophages are an important component in the development of colitis, and recent data demonstrated that those cells are the main producer of IL-1 β , IL-6 and TNF- α . To elucidate the mechanism by which the hCTS is influencing the development of colitis, we next investigated the role of the three peptides in macrophage function in relation to gut
30 inflammation. This role was further studied by examining the ability of hCTS to inhibit macrophages to produce pro-inflammatory cytokines by isolating macrophages from the

peritoneal cavity of non-colitic or colitic mice treated or not *in vivo* and *in vitro* with the different peptides. Alternatively, BMDMs were isolated from naïve mice and IL-1 β , IL-6 and TNF- α production was assessed in the presence or absence of the three peptides. Peritoneal macrophages isolated from the saline-DSS group revealed an increased release of IL-1 β , IL-6 and TNF- α when compared to the saline-DSS group (Figure 6); however, this was not evident in peritoneal macrophages isolated from the DSS group treated *ex vivo* with hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ (1.5 mg/kg/day, i.r.) (Figure 6).

We next examined whether direct cell culture treatments with the peptides decreased the release of cytokine from peritoneal macrophages and BMDMs isolated from naïve control mouse. Peritoneal macrophages cultured with LPS (100 ng/ml⁻¹) and treated with hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ (10⁻⁷ or 10⁻⁵M) demonstrated a significant decreased release in the three pro-inflammatory cytokines (Figure 7A, C, E); this effect was confirmed using BMDMs (Figure 7B, D, F).

In both experiments, the modified peptides (shCgA₃₅₂₋₃₇₂) (10⁻⁵M) did not show any significant effects on the cytokine release (Figure 6-7).

hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ increase p-STAT3 level

In physiological and pathological conditions, CTS has been described to increase the level of phosphorylated STAT-3 (p-STAT3) (Bandyopadhyay et al., J Biol Chem., 2012;287:23141-51). Therefore, to determine the possible mechanism by which hCTS can affect DSS-induced colitis, we obtained cytosolic and nuclear fractions from macrophages and colon to study the level of p-STAT3. Colitis was associated with an increase of colonic p-STAT3 level. We found a significantly higher level of p-STAT3 in the colon of colitic mice treated with hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ (1.5mg/kg/day, 6 days, i.r.) when compared to saline-DSS group (Figure 8A). Low concentration of hCgA₃₅₂₋₃₇₂ (0.5, 1 mg/kg/day, 6 days, i.r.) or the modified shCgA₃₅₂₋₃₇₂ (1.5 mg/kg/day, 6 days, i.r.) peptides did not show any significant effects.

In parallel, a significant increase of p-STAT3 was observed in peritoneal macrophages isolated from mice treated *in vivo* with hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ (1.5 mg/kg/day, 6 days, i.r.) when compared to saline-DSS group (Figure 8B). Low concentration of hCgA₃₅₂₋₃₇₂ (0.5, 1 mg/kg/day, 6 days, i.r.) or the modified shCgA₃₅₂₋₃₇₂ (1.5 mg/kg/day, 6 days, i.r.) peptides did not show any significant effects.

We described the same effect when naïve peritoneal macrophages were isolated and stimulated with LPS (100ng/ml) in the presence of absence of the peptides (10^{-5} M) (Figure 8C). Low concentration of hCgA₃₅₂₋₃₇₂ or hCgA₃₆₀₋₃₇₂ (10^{-7} M) or the modified shCgA₃₅₂₋₃₇₂ (10^{-5} M) peptides did not show any significant effects.

- 5 In the presence of the STATTIC (STAT3 blocker; 10^{-5} M), the beneficial effect of the treatment on peritoneal macrophages cytokine release was abolished (Table 2).

Table 2.

A

	hCgA ₃₅₂₋₃₇₂ 1.5mg/kg		hCgA ₃₆₀₋₃₇₂ 1.5mg/kg		hCgA ₃₅₂₋₃₆₆ 1.5mg/kg	
	Medium	STATTC	Medium	STATTC	Medium	STATTC
IL-6 pg/ml	93±3.4	104±12	44.3±3 ^a	89.4±9.7 ^b	47.9±6.3 ^a	78.9±8.8 ^b
IL-1β pg/ml	736±55	889±42	412±22 ^a	823±67 ^b	377±30 ^a	745±65 ^b
TNF-α pg/ml	289±10	334±25	167±20 ^a	245±48 ^b	178±15 ^a	274±34 ^b

B

	hCgA ₃₅₂₋₃₇₂ 10 ⁻⁵ M		hCgA ₃₆₀₋₃₇₂ 10 ⁻⁵ M		hCgA ₃₅₂₋₃₆₆ 10 ⁻⁵ M	
	Medium	STATTC	Medium	STATTC	Medium	STATTC
IL-6 pg/ml	111±13	137±12	27.1±5 ^a	99.7±19 ^b	29.5±4 ^a	84.3±12.7 ^b
IL-1β pg/ml	18014±818	19342±921	11024±93 ^a	19354±756 ^b	11387±339 ^a	18649±382 ^b
TNF-α pg/ml	10359±478	10942±921	6644±177 ^a	9892±534 ^b	6239±285 ^a	10374±437 ^b

10

Table 2. Functional role of STAT3 in the release of pro-inflammatory cytokines. *A*: Peritoneal macrophages (10⁺⁶ cells) isolated from colitic *in vivo* hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ -treated mice (1.5mg/kg,day, i.r., 6 days) and treated *in vitro* with STAT3 inhibitor V (STATTC; 10⁻⁵M); *B*: Naive peritoneal macrophages (10⁺⁶ cells) isolated from non-colitic mice stimulated with LPS (100ng/ml) and treated *in vitro* with hCgA₃₅₂₋₃₇₂, hCgA₃₅₂₋₃₆₆ and hCgA₃₆₀₋₃₇₂ (10⁻⁵M) in the presence or absence of STATTC (10⁻⁵M). Interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α levels were measured in conditioned media (24h) using commercially available ELISA kits. ^aP<0.05 compared to colitic medium non-treated group, ^bP<0.05 compared to colitic medium treated group. ANOVA followed by the Dunnett multiple comparisons *post hoc* analysis, n≥6. The values are shown as the mean ± SEM of four separate experiments.

15

The level of mCgA and mCTS and the effect of hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ on DNBS-induced colitis.

To determine whether the above-described changes were restricted to the DSS-based model, we performed studies using the DNBS-based model of experimental colitis. As shown in
5 table 3, hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ treatments significantly decreased the macroscopic scores at day 3 after DNBS induction. DNBS increased MPO activity from 1.3 ± 0.1 U/mg in control mice to 4.3 ± 0.2 U/mg and hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ treatment (1.5mg/kg/day) resulted in significantly lower MPO activity compared to DNBS-treated controls. In addition, we found significantly lower decreases in the IL-1 β , IL-6 and TNF- α levels in the
10 colon of DNBS treated mice with hCgA₃₅₂₋₃₇₂, hCgA₃₅₂₋₃₆₆ and hCgA₃₆₀₋₃₇₂ when compared to DNBS-treated controls (Table 3).

Table 3.

	Saline	Ethanol 30%	DNBS 4mg	DNBS 4mg +Etho 30%	DNBS 4mg +hCgA ₃₅₂₋₃₇₂ 1.5mg/kg/day	DNBS 4mg +Etho 30% +hCgA ₃₆₀₋₃₇₂ 1.5mg/kg/day	DNBS 4mg +Etho 30% +hCgA ₃₅₂₋₃₆₆ 1.5mg/kg/day	DNBS 4mg +Etho 30% +shCgA ₃₅₂₋₃₇₂ 1.5mg/kg/day
Macroscopic Score	0.1±0.4	0.6±0.5	4.3±0.8 ^a	1.6±0.8 ^b	1.8±0.7 ^b	2.3±0.6 ^b	4.7±0.9	
MPO U/mg/tissue	0.5±0.1	1.3±0.1	4.3±0.2 ^a	1.5±0.18 ^b	2.1±0.15 ^b	1.9±0.2 ^b	4.8±0.7	
IL-1β pg/mg protein	BLS	BLS	79.5±8.1 ^a	35.5±5.5 ^b	31.3±4.8 ^b	37.2±6 ^b	83.2±8.9	
IL-6 pg/mg protein	BLS	BLS	166±10 ^a	101±7 ^b	109±6 ^b	95±18 ^b	177.2±11	
TNF-α pg/mg protein	BLS	6±2.7	185±9 ^a	66±12 ^b	57±13 ^b	54±14 ^b	181±18	

Table 3. hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ reduce the severity of 2, 4 dinitrobenzene sulfonic acid (DNBS)–induced colitis. hCgA₃₅₂₋₃₇₂, hCgA₃₆₀₋₃₇₂ or hCgA₃₅₂₋₃₆₆ (1.5 mg/kg/day, 4 days, i.p.) treatment started one day before disease induction. Macroscopic score, myeloperoxidase (MPO) activity and cytokine profile in colonic tissue were determined 3 days post-DNBS induction. ^ap<0.05 vs saline; ^bp<0.05 vs DNBS 4mg + ethanol 30%-saline treated group; Values are shown as the mean ± SEM, n≥8. *BLS (below the lowest standard). sh represents the modified hCgA₃₅₂₋₃₇₂ peptide.

Discussion

It was recently demonstrated that there are significant increases in CgA in several subtype of colitis (Sciola et al., *Inflamm Bowel Dis.*, 2009;15:867-71, Wagner et al., *Inflammation* 2013;36(4):855-61, Sidhu et al., *Inflamm Bowel Dis* 2010;16:361) and that hCTS regulates immune cells and the STAT-3 pathway (Bandyopadhyay et al., *J Biol Chem.*, 2012;287:23141-51). The present study shows that h and mCTS are significantly increased during the development of colitis. Moreover, we showed that hCTS attenuates the inflammatory response during experimental colitis. Colitis induced by DSS and DNBS were less severe in mice treated with hCTS and could be mimicked by the proximal and distal part of the peptide. Conversely, administration of the scramble peptide did not modify the course of colonic inflammation. A protective role for hCTS in macrophage and BMDM cytokine release *via* the STAT-3 pathway implicates a role for macrophages in this anti-inflammatory effect. Taken together, these findings extend the influence of hCTS to intestinal inflammation and immune regulation.

The most widely used and characterized experimental model of UC is the DSS-induced colitis, which was developed by administration of DSS in the drinking water. DSS induces a very reproducible acute colitis characterized by mucosal inflammation with ulcerations, body weight loss, and bloody diarrhoea infiltrations (Neurath et al., *J Exp Med* 1995;182:1281-90), polymorphonuclear cells, macrophages, lymphocytes infiltration and changes regarding the number of EC cells (Linden et al., *Am J Physiol Gastrointest Liver Physiol* 2003;285:G207-16, Oshima et al., *Histochem Cell Biol* 1999;112:257-63).

After chromaffin cells, EC cells are the main source of CgA and its derived peptides in the gut (Waldum et al., *Adv Exp Med Biol* 2000;482:361-7), which are an important enteric mucosal signalling molecules influencing gut physiology (Ghia et al., *Life Sci* 2004;75:1787-99, Ghia et al., *Regul Pept* 2004;121:31-9). Changes in intestinal EC cell numbers and hCgA levels are observed in patients with IBD (Sidhu et al., *Inflamm Bowel Dis* 2010;16:361, Conlon et al., *Regul Pept* 2010;165:5-11, El-Salhy et al., *J Inter Med* 1997;242:413-9, Sidhu et al., *J Gastrointest Liver Dis* 2009;18:23-6) and in diarrhea predominant-irritable bowel syndrome patients (Sciola et al., *Inflamm Bowel Dis.*, 2009;15:867-71). The unifying hypothesis proposed relies on EC cell hyperplasia and the potential neuroendocrine system activation in response to

inflammation (Sciola et al., *Inflamm Bowel Dis.*, 2009;15:867-71) that produces elevated serum CgA levels (Dunlop et al., *Gastroenterology* 2003;125:1651-9). In this study, we have shown that induction of DSS or DNBS colitis was correlated with a significant increase in mCgA and mCTS expression. In parallel, using serum from persons with IBD we have demonstrated that the levels of hCgA and hCTS were significantly higher in IBD than controls, confirming previously published data (Wagner et al., *Inflammation* 2013;36(4):855-61). Since subjects with IBD were drawn from the general population and not a clinic we did not have access to their clinical data; hence it would be important to determine how hCgA and hCTS levels vary by disease activity status. Considering there were similar levels in CD and UC we think it is less likely that levels would vary by phenotype but this remains to be proven. This can be explained by the fact the CgA or its derived peptides may have some anti-inflammatory proprieties that need to be expressed not only during the acute phase but also during the remission phase to keep the inflammation under control *via* some anti-inflammatory mechanisms. Our data demonstrate for the first time an increased in h and mCTS in colitis and suggest that its role should be examined during the development of inflammatory conditions.

To determine the role of hCTS in colitis, we have shown that preventive administration of hCTS not only attenuated the severity of inflammation associated with DSS-induced colitis but also reduced the production of pro-inflammatory mediators in the gut. The attenuation of DSS-induced inflammation in treated mice was observed in all the parameters examined, including disease activity, macroscopic and histologic scores, and MPO and CRP activity.

MPO is an enzyme contained in azurophilic granules of neutrophils and in other myeloid cells, and as such, it is commonly used as an index of inflammation (Smith et al., *Am J Physiol* 1978;234:R72-9). Previous studies reported an extensive accumulation of neutrophils and a significant increase in the serum CRP level and colonic MPO activity in DSS-colitis (Ghia et al., *J Clin Invest.*, 2008;118:2209-18). In this study, we observed significantly lower levels of serum CRP and colonic MPO activity in mice treated with hCTS after induction of colitis. Our data confirm the potential relation between CgDPs and CRP level as described in the context of systemic inflammatory response syndrome (Zhang et al., *Ann Med* 2009;41:38-44, Zhang et al., *Clin Chem* 2008;54:1497-503). It is noteworthy that the modified peptide did not show any effect on colitis, confirming the role of wild-type sequence. These findings, along with the reduction in colonic IL-1 β , IL-6 and TNF- α suggest that hCTS has an important role in the

pathogenesis of colitis by regulating the infiltration of inflammatory cells and production of pro-inflammatory mediators in the colon.

In an attempt to define the role of the proximal and distal part of hCTS, we studied two specific sequences. Preventive treatment with hCgA₃₅₂₋₃₆₆ and hCgA₃₆₀₋₃₇₂ decreased colonic inflammation. There was associated with a significant decrease in disease activity, macroscopic and histological scores in colitic mice treated with the two peptides. Moreover, we demonstrated a significant down-regulation of MPO activity, serum CRP and pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α). These observations further provide evidence in favour of a crucial role of hCTS in regulation of gut inflammation and might define the importance of studying the common sequence shared by the three peptides.

To gain mechanistic insight, it is helpful to identify the cell types involved in the regulation of gut inflammation by hCTS. Gut inflammation is characterized by mucosal recruitment of macrophages which play a critical role in intestinal inflammatory responses through the secretion of chemokines and cytokines, and through antigen presentation to T lymphocytes (Sartor, Am J Gastroenterol 1997;92:5S-11S). Macrophages also play a key role in the host defense against bacterial pathogens, which stimulate macrophages *via* the activation of toll-like receptors (Schenk et al., Semin Immunol 2007;19:84-93). Macrophage activation by pathogens results in the secretion of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α and in the induction of a Th-1 cytokine response. In this context, macrophages are considered to be classical pro-inflammatory effector cells (Watanabe et al., Dig Dis Sci 2003;48:408-14). Due to the strategic location of EC and macrophages, it is likely that CgDPs may play an important role in regulation of macrophages in colitis. In the present study, we observed a lower amount of IL-1 β , IL-6 and TNF- α released in the culture supernatant of macrophages isolated from the peritoneal cavity of colitic mice treated *in vivo* with the three peptides.

Receptors for CgDPs appear not to exist, but the sequence similarity of CgDPs with cell penetrating peptides (Henriques et al., Biochem J 2006;399:1-7) appears to allow the peptides to enter cells (Zhang et al., PLoS One 2009;4:e4501) and could explain the intracellular effect. We confirmed the proinflammatory cytokine regulation on naïve macrophages and BMDMs treated *in vitro* with the peptides. Using an ELISA method of detection, this down-regulation was associated with an increase of p-STAT3 level, corroborating the study of Bandyopadhyay et al. where, using a western blot analysis, they described that *in vitro* hCTS treatment of an adipose

explant from diet-induced obese mice stimulates STAT3 phosphorylation (Bandyopadhyay et al., J Biol Chem., 2012;287:23141-51). In our study, the increase of p-STAT3 and the decrease in pro-inflammatory cytokines confirm the data demonstrating that mice lacking STAT3-deficient macrophages are characterized by excessive cytokine release and develop colitis (Alonzi et al., Cytokine 2004;26:45-56, Takeda et al., Immunity 1999;10:39-49). Conversely, STAT3 activation in Socs3-deficient macrophages triggers a strong anti-inflammatory response (Yasukawa et al., Nat Immunol 2003;4:551-6). Taken together, these observations suggest that hCTS plays an important role in gut inflammation by influencing the macrophage production of cytokines *via* the STAT3 protein and a yet unknown mechanism. This effect is in contrast to the data that demonstrated that hyperactivation of STAT3 results in severe colitis (Suzuki et al., J Exp Med 2001;193:471-81) and that STAT3 is constitutively activated in T cells from a Crohn's patient (Lovato et al., J Biol Chem 2003;278:16777-81). Therefore, the effect presented in our study might be restricted to the macrophages population.

Finally, we investigated the role of hCTS using the DNBS-based model of experimental colitis to see whether the observed role of hCTS using the DSS model was specific to this model. DNBS-colitis considered as a model of CD, is characterized by transmural inflammation (Khan et al., Infect Immun 2002;70:5931-7, Qiu et al., Nat Med 1999;5:1178-82). Similarly, we observed significant attenuation in colonic inflammation in DNBS-treated mice.

It is conceivable that other factors contribute to the protective effect of the treatment in our study. For example, it is known that CgDPs can influence monocyte trafficking (Zhang et al., PLoS One 2009;4:e4501), and thus, it is possible that hCTS may target the macrophages and polymorphonuclear neutrophils in a quantitative manner, correlating with the significant decrease of MPO activity described in our study. Conversely, the bovine CTS sequence stimulated rat mast cell histamine release (Kruger et al., Ann N Y Acad Sci 2002;971:349-51); however, we consider it is unlikely that these mechanisms made a significant contribution because mast cell activation portrays a pro-inflammatory effect not visible in our study. One potentially confounding factor is the antibacterial role of CTS (Aslam et al., Curr Med Chem 2012;19:4115-23). Over the last 5 years, much attention has been given to the gut microbiota in relation to IBD development. It has been shown that a change in gut microbiota could affect the development of experimental colitis (Klimesova et al., Inflamm Bowel Dis 2013;19:1266-77); therefore

intrarectal infusion of the peptides might induce a beneficial gut microbiota dysbiosis, which subsequently can affect the development of colitis.

The direct correlate of our findings and the exact role of the significant hCTS increase in IBD patients are not clear. However, due to its major implication in the development of colorectal carcinoma and transitional mucosa (Mori et al., J Clin Pathol 1995;48:754-8), it is possible to speculate that hCTS may be released to prevent the long-term progression of chronic intestinal inflammation to cancer (Sebastian et al., J Crohns Colitis 2014; 8(1):5-18). Conversely, it needs to be highlighted, that p-STAT3 activation also plays an important role in the development of colitis-associated cancer (Liang et al., Cancer Cell 2013;23:107-20), therefore, additional data using this experimental model are needed. Furthermore, studies of serial serum CgA and CgDPs measurements, or targeting different immune cells (e.g. DC, CD4⁺ T cell, T-reg cells) will strengthen our understanding.

This study, taken together with others, offers potential new therapeutic approaches to the management of IBD. The present study reveals a novel function of hCTS in regulation of gut inflammation in relation to the activation of pro-inflammatory cytokine production. In addition, this study demonstrated a p-STAT3 dependent molecular mechanism of hCTS-mediated activation of immune cells in the context of inflammation. Up-regulation of mCTS signalling in response to chemical stimuli like DSS or DNBS can take part in lowering gut inflammation by influencing immune cell activation, and by decreasing production of inflammatory mediators. In addition to enhancing our understanding of the pathogenesis of experimental colitis, this study provides novel data on CTS in the context of immunoendocrine interactions in the gut and in intestinal homeostasis.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference in their entirety. Supplementary materials referenced in publications (such as supplementary tables, supplementary figures, supplementary materials and methods, and/or supplementary experimental data) are likewise incorporated by reference in their entirety. In the event that any inconsistency exists between the disclosure of the present application and

the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. Use of a CgA-derived protein in the preparation of a medicament for an inflammatory bowel disease.
2. Use of a CgA-derived protein and a pharmaceutically acceptable carrier, for treating an inflammatory bowel disease.
3. Use of a CgA-derived polynucleotide in the preparation of a medicament for an inflammatory bowel disease, wherein the CgA-derived polynucleotide encodes a CgA-derived protein.
4. Use of a CgA-derived polynucleotide and a pharmaceutically acceptable carrier, for treating an inflammatory bowel disease, wherein the CgA-derived polynucleotide encodes a CgA-derived protein.
5. The use of claim 1 or 3 wherein the medicament further comprises a pharmaceutically acceptable carrier.
6. The use of any of claims 1, 2, 3, or 4 wherein the CgA-derived protein comprises an amino acid sequence ARAYGFR (SEQ ID NO:1) or no greater than 3 substitutions compared to SEQ ID NO:1.
7. The use of claim 6 wherein the CgA-derived protein comprises SSMKLSFRARAYGFR (SEQ ID NO:3) or ARAYGFRGPGPQL (SEQ ID NO:4), or an amino acid sequence having at least 80% identity with SEQ ID NO:3 or SEQ ID NO:4.
8. The use of claim 7 wherein the CgA-derived protein comprises SSMKLSFRARAYGFRGPGPQL (SEQ ID NO:2), or an amino acid sequence having at least 80% identity with SEQ ID NO:2.

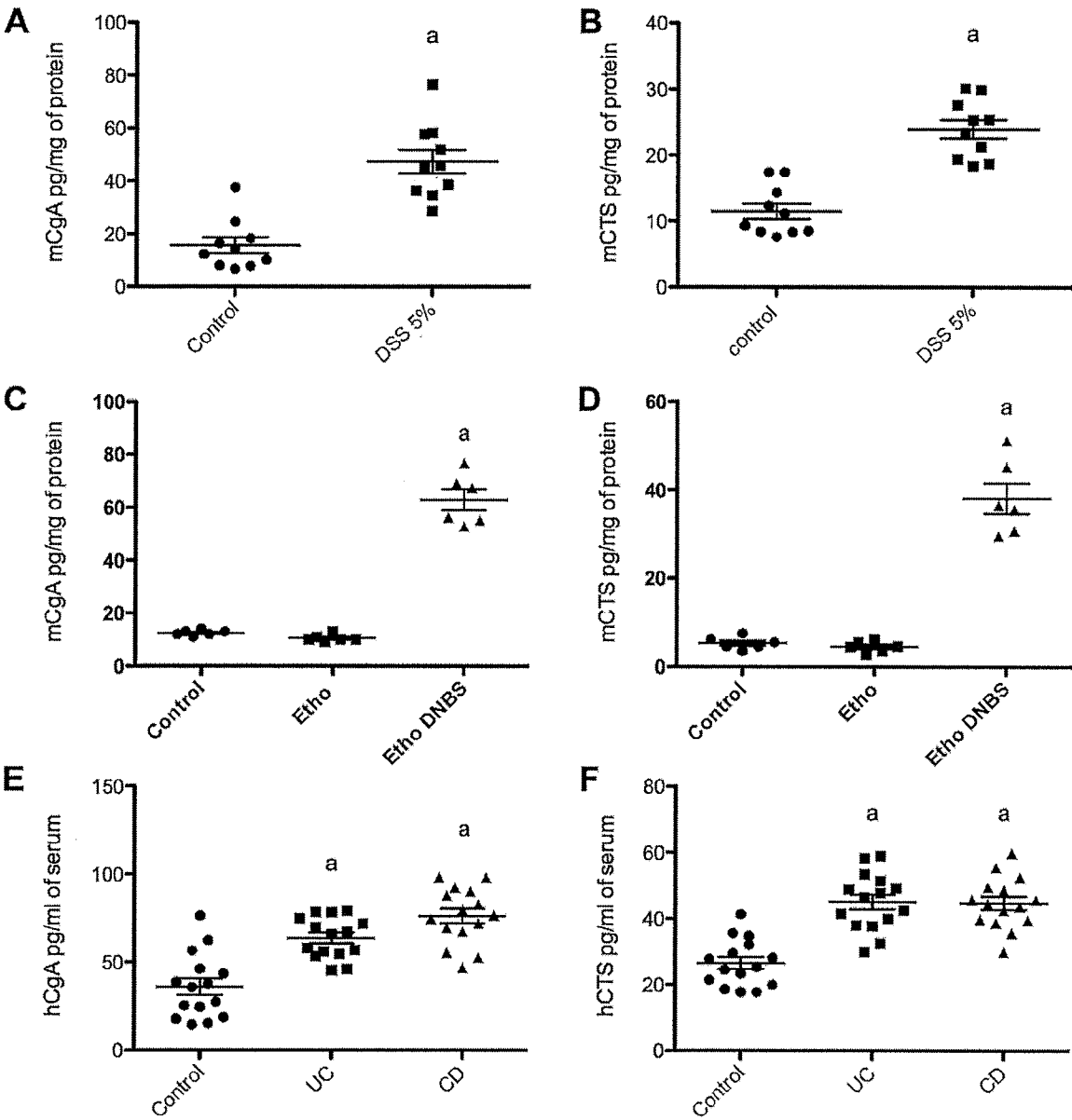
9. The use of any of claims 1 to 5 wherein the CgA-derived protein comprises SEQ ID NO:1 or an amino acid sequence having no greater than 3 substitutions compared to SEQ ID NO:1, wherein the CgA-derived protein further comprises at least one heterologous amino acid flanking SEQ ID NO:1 or an amino acid sequence having no greater than 3 substitutions compared to SEQ ID NO:1, wherein the at least one heterologous amino acid is heterologous compared to the CgA amino acid sequence depicted at SEQ ID NO:6.
10. The use of claim 9 wherein the at least one heterologous amino acid is present at the amino-terminal end or the carboxy-terminal end of the the CgA-derived protein.
11. The use of any of claims 1 to 5 wherein the CgA-derived protein comprises an amino acid sequence having at least 80% identity with SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, wherein the CgA-derived protein further comprises at least one heterologous amino acid flanking the amino acid sequence having at least 80% identity with SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, wherein the at least one heterologous amino acid is heterologous compared to the CgA amino acid sequence depicted at SEQ ID NO:6.
12. The use of claim 11 wherein the at least one heterologous amino acid is present at the amino-terminal end or the carboxy-terminal end of the the CgA-derived protein.
13. The use of any of claims 1, 2, 3, 4, or 5 wherein the inflammatory bowel disease is selected from Crohn's disease and ulcerative colitis.
14. The use of any of claims 1, 2, 3, 4, or 5 wherein the CgA-derived protein is a fusion protein.
15. The use of claim 3 or 4 wherein the CgA-derived polynucleotide is present in a vector.
16. The use of claim 15 wherein the vector is a viral vector.

17. A method for treating an inflammatory bowel disease in a subject, comprising administering to a subject in need thereof an effective amount of a composition comprising a CgA-derived protein.
18. A method for treating a subject having, or at risk of having, inflammatory bowel disease, comprising:
administering to a subject in need thereof an effective amount of a composition comprising a CgA-derived protein, wherein the subject has decreased abdominal pain, vomiting, diarrhea, rectal bleeding, and intestinal cramps, weight loss, anemia, or a combination thereof, when compared to the subject before the administering.
19. A method for treating an inflammatory bowel disease in a subject, comprising administering to a subject in need thereof an effective amount of a composition comprising a CgA-derived polynucleotide encoding a CgA-derived protein.
20. A method for treating a subject having, or at risk of having, inflammatory bowel disease, comprising:
administering to a subject in need thereof an effective amount of a composition comprising CgA-derived polynucleotide encoding a CgA-derived protein, wherein the subject has decreased abdominal pain, vomiting, diarrhea, rectal bleeding, and intestinal cramps, weight loss, anemia, or a combination thereof, when compared to the subject before the administering.
21. The method of any of claims 17, 18, 19, or 20 wherein the subject is a human.
22. The method of any of claims 17, 18, 19, or 20 wherein the method further comprises administering a therapeutic compound.
23. The method of claim 22 wherein the therapeutic compound is selected from an aminosalicylate, a corticoid, an immunosuppressive compound, a therapeutic antibody, an antibiotic, a thiopurine, a methotrexate, or a combination thereof.

24. The method of any of claims 17, 18, 19, or 20 wherein the CgA-derived protein comprises an amino acid sequence ARAYGFR (SEQ ID NO:1) or no greater than 3 substitutions compared to SEQ ID NO:1.
25. The method of claim 24 wherein the CgA-derived protein comprises SSMKLSFRARAYGFR (SEQ ID NO:3) or ARAYGFRGPGPQL (SEQ ID NO:4), or an amino acid sequence having at least 80% identity with SEQ ID NO:3 or SEQ ID NO:4.
26. The method of claim 25 wherein the CgA-derived protein comprises SSMKLSFRARAYGFRGPGPQL (SEQ ID NO:2), or an amino acid sequence having at least 80% identity with SEQ ID NO:2.
27. The method of any of claims 17, 18, 19, or 20 wherein the CgA-derived protein comprises SEQ ID NO:1 or an amino acid sequence having no greater than 3 substitutions compared to SEQ ID NO:1, wherein the CgA-derived protein further comprises at least one heterologous amino acid flanking SEQ ID NO:1 or an amino acid sequence having no greater than 3 substitutions compared to SEQ ID NO:1, wherein the at least one heterologous amino acid is heterologous compared to the CgA amino acid sequence depicted at SEQ ID NO:6.
28. The method of claim 27 wherein the at least one heterologous amino acid is present at the amino-terminal end or the carboxy-terminal end of the the CgA-derived protein.
29. The method of any of claims 17, 18, 19, or 20 wherein the CgA-derived protein comprises an amino acid sequence having at least 80% identity with SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, wherein the CgA-derived protein further comprises at least one heterologous amino acid flanking the amino acid sequence having at least 80% identity with SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, wherein the at least one heterologous amino acid is heterologous compared to the CgA amino acid sequence depicted at SEQ ID NO:6.
30. The method of claim 29 wherein the at least one heterologous amino acid is present at the amino-terminal end or the carboxy-terminal end of the the CgA-derived protein.

31. The method of any of claims 17, 18, 19, or 20 wherein the inflammatory bowel disease is selected from Crohn's disease and ulcerative colitis.
32. The method of any of claims 17, 18, 19, or 20 wherein the CgA-derived protein is a fusion protein.
33. The method of claim 19 or 20 wherein the CgA-derived polynucleotide is present in a vector.
34. The method of claim 33 wherein the vector is a viral vector.
35. A method for evaluating treatment options for a subject having inflammatory bowel disease comprising:
obtaining a biological sample from the subject;
measuring the level of CgA-derived protein in the biological sample; and
comparing the level of CgA-derived protein in the biological sample with the level of CgA-derived protein in a control biological sample obtained from a healthy subject, wherein the presence of a decreased level of CgA-derived protein compared to the control biological sample indicates the subject may be treated with a CgA-derived protein.
36. The method of any of claim 35 wherein the biological sample comprises tissue from the gastrointestinal tract of the subject.
37. The method of any of claim 35 further comprising administering to the subject a CgA-derived protein or a fragment thereof.
38. The method of claim 35 wherein the subject is a human.

Fig. 1



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Fig. 2

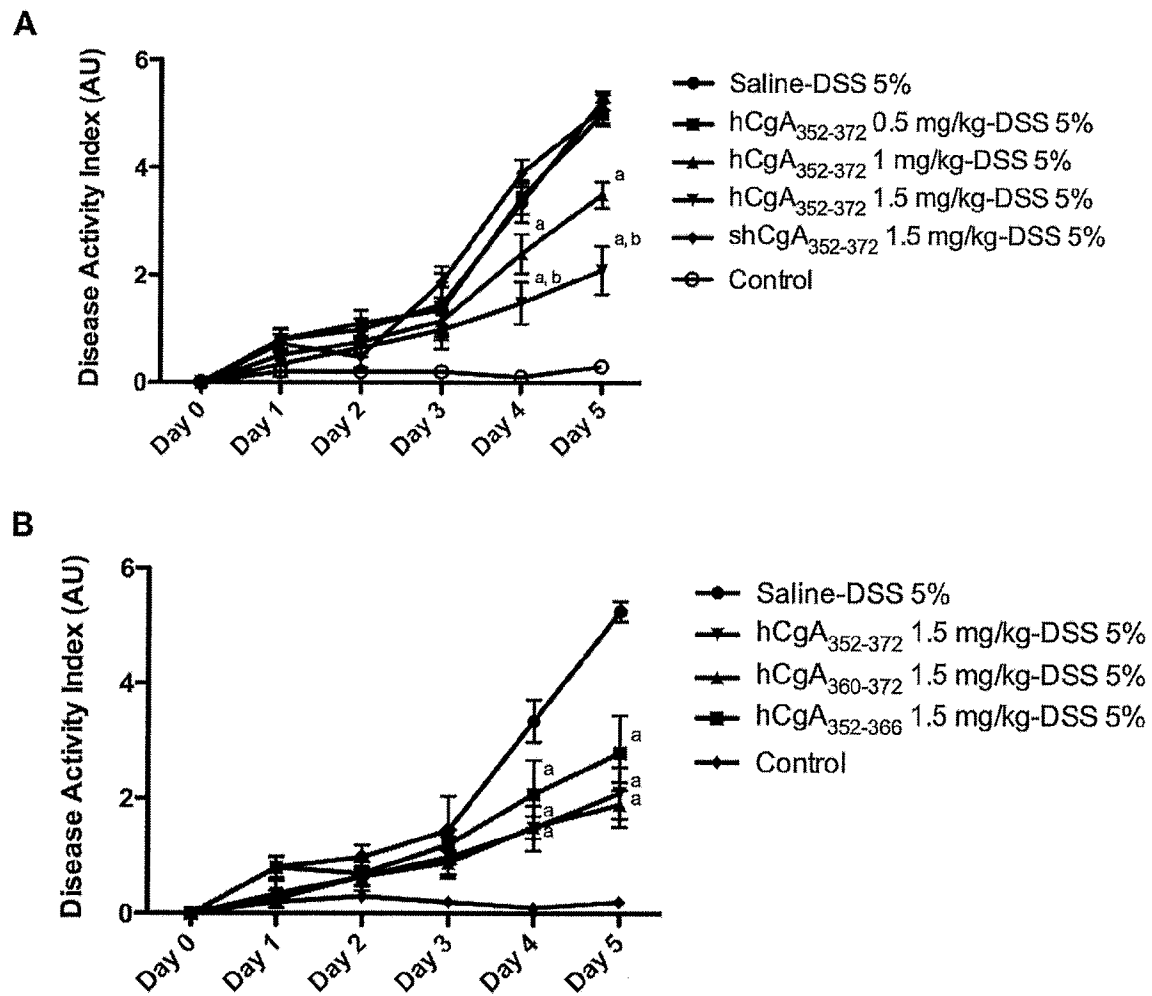


Fig. 3

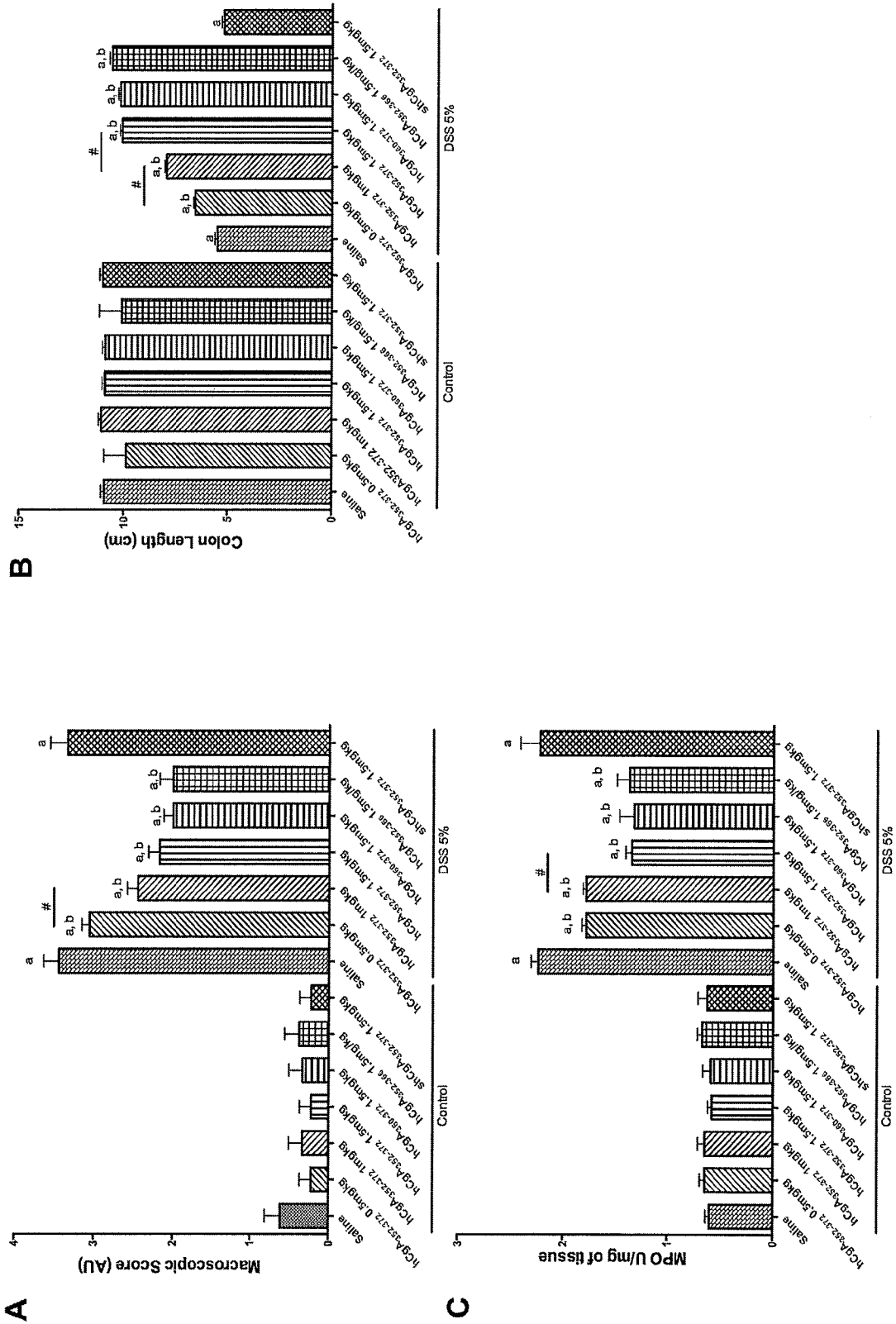


Fig. 4

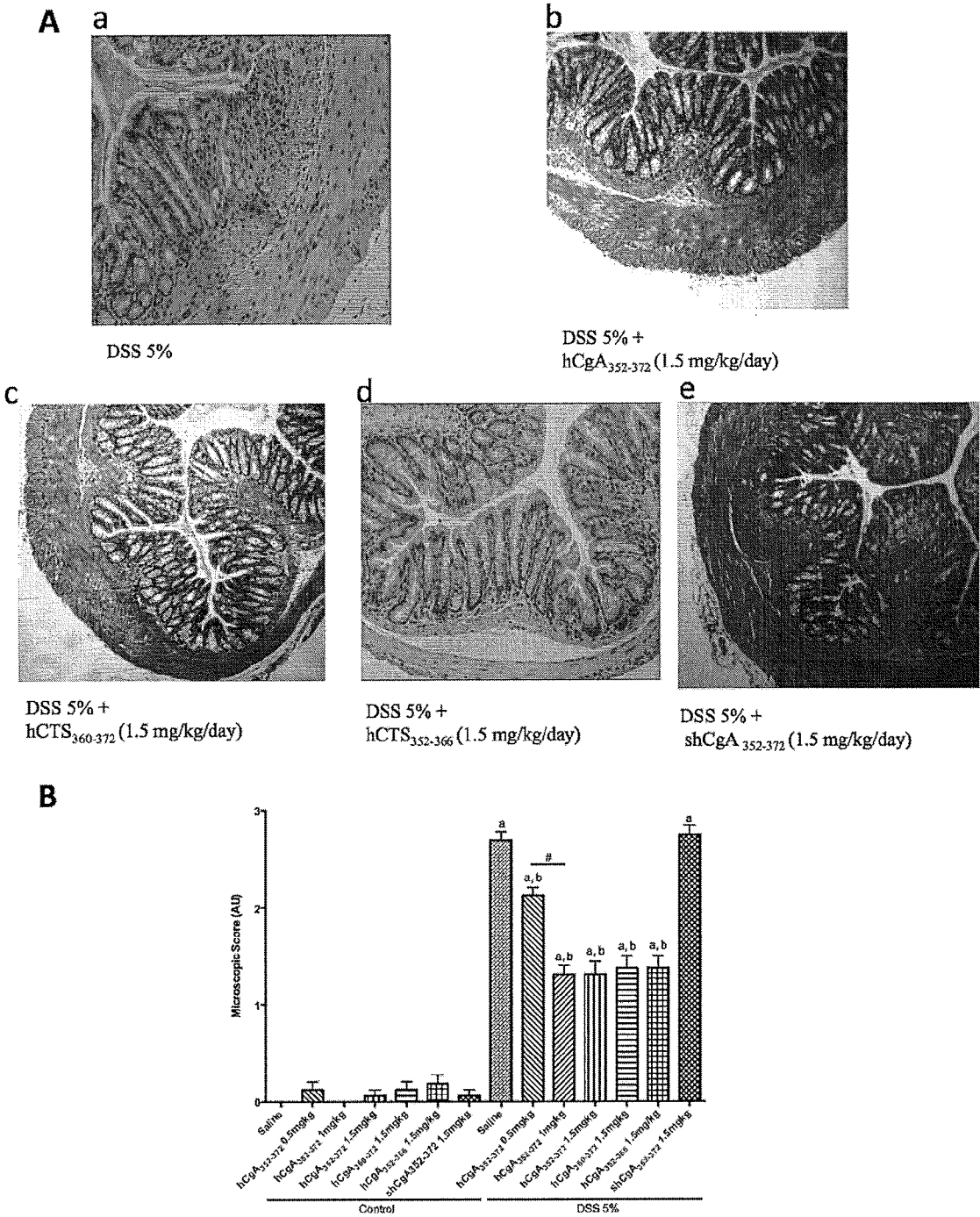


Fig. 5

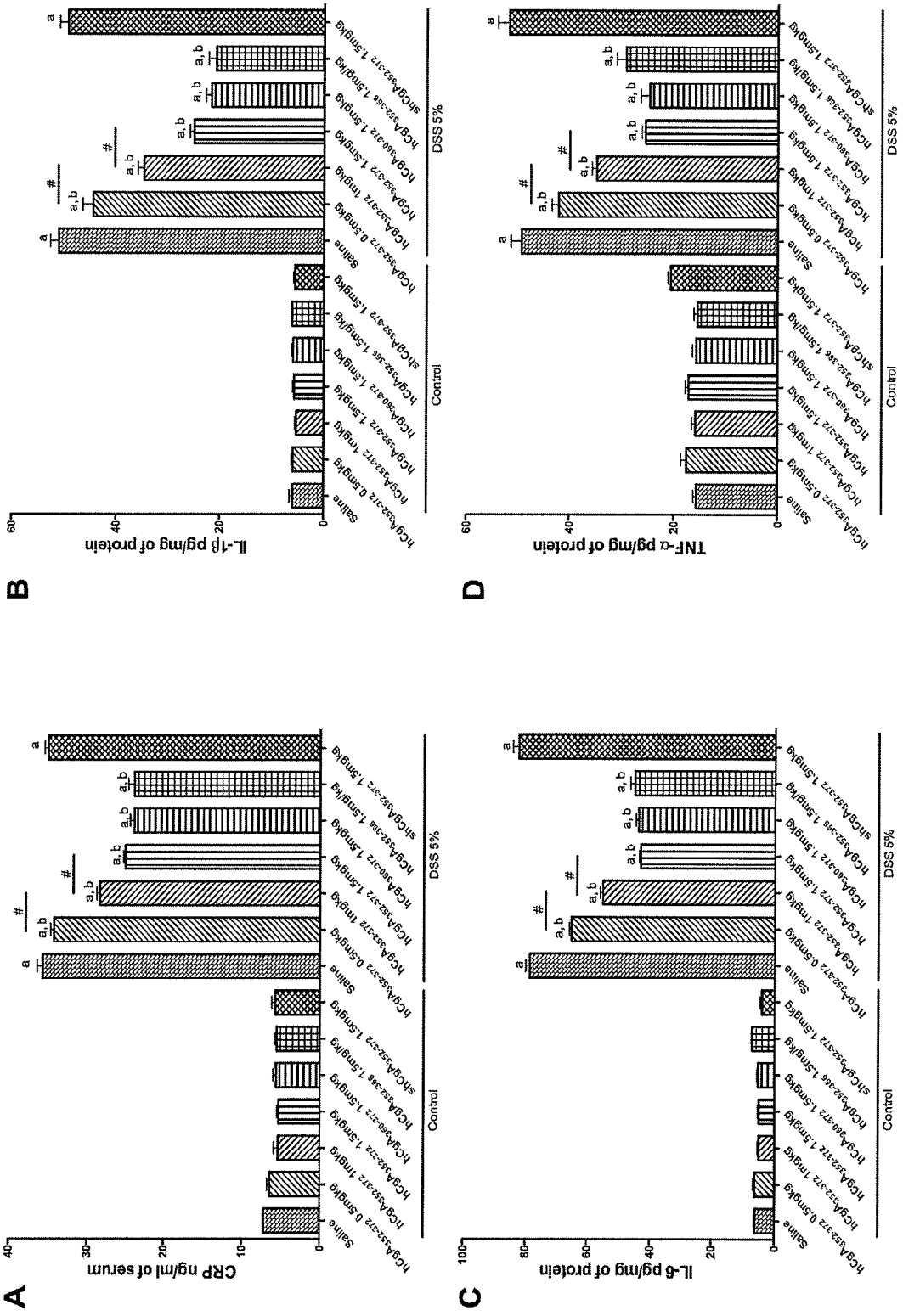
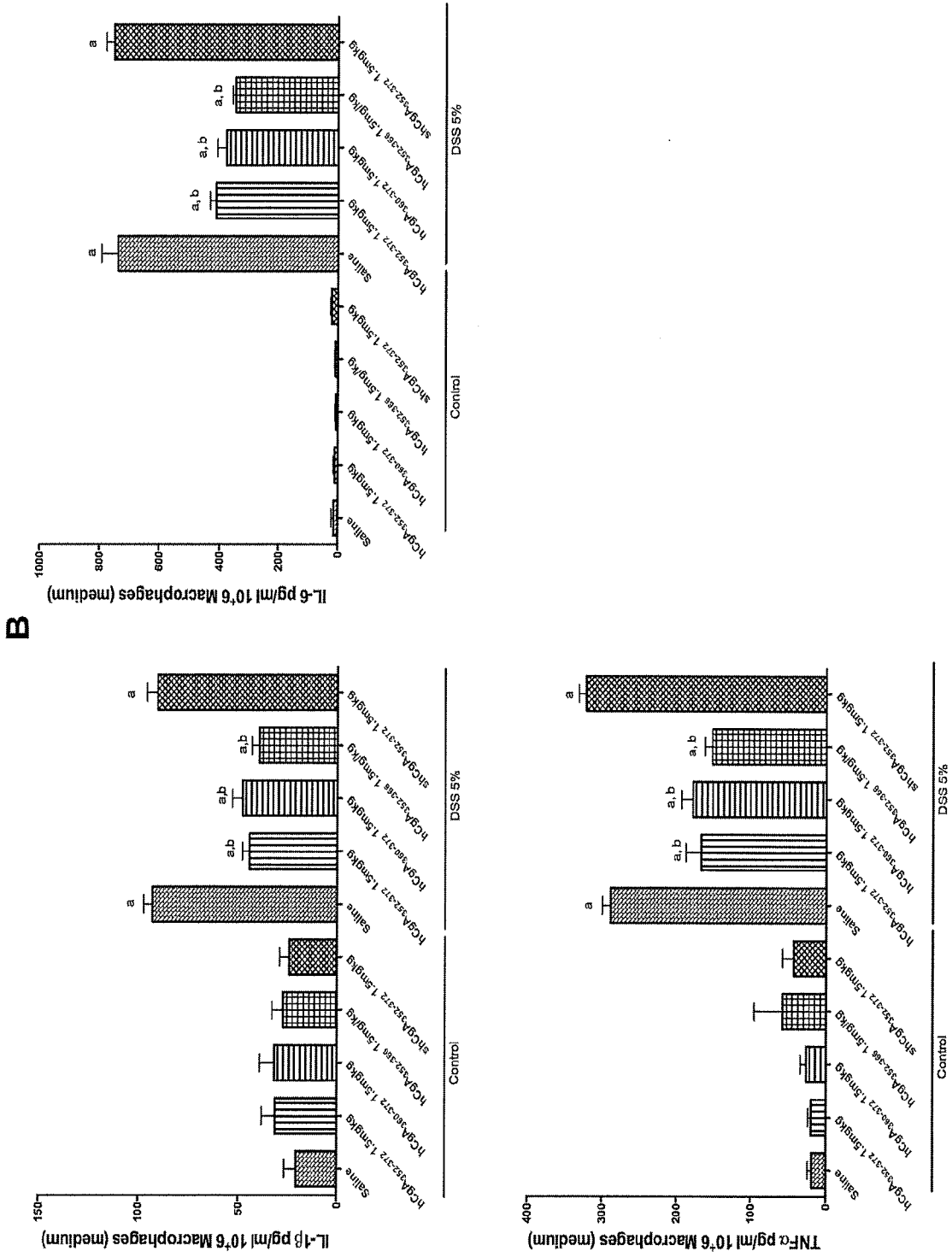


Fig. 6



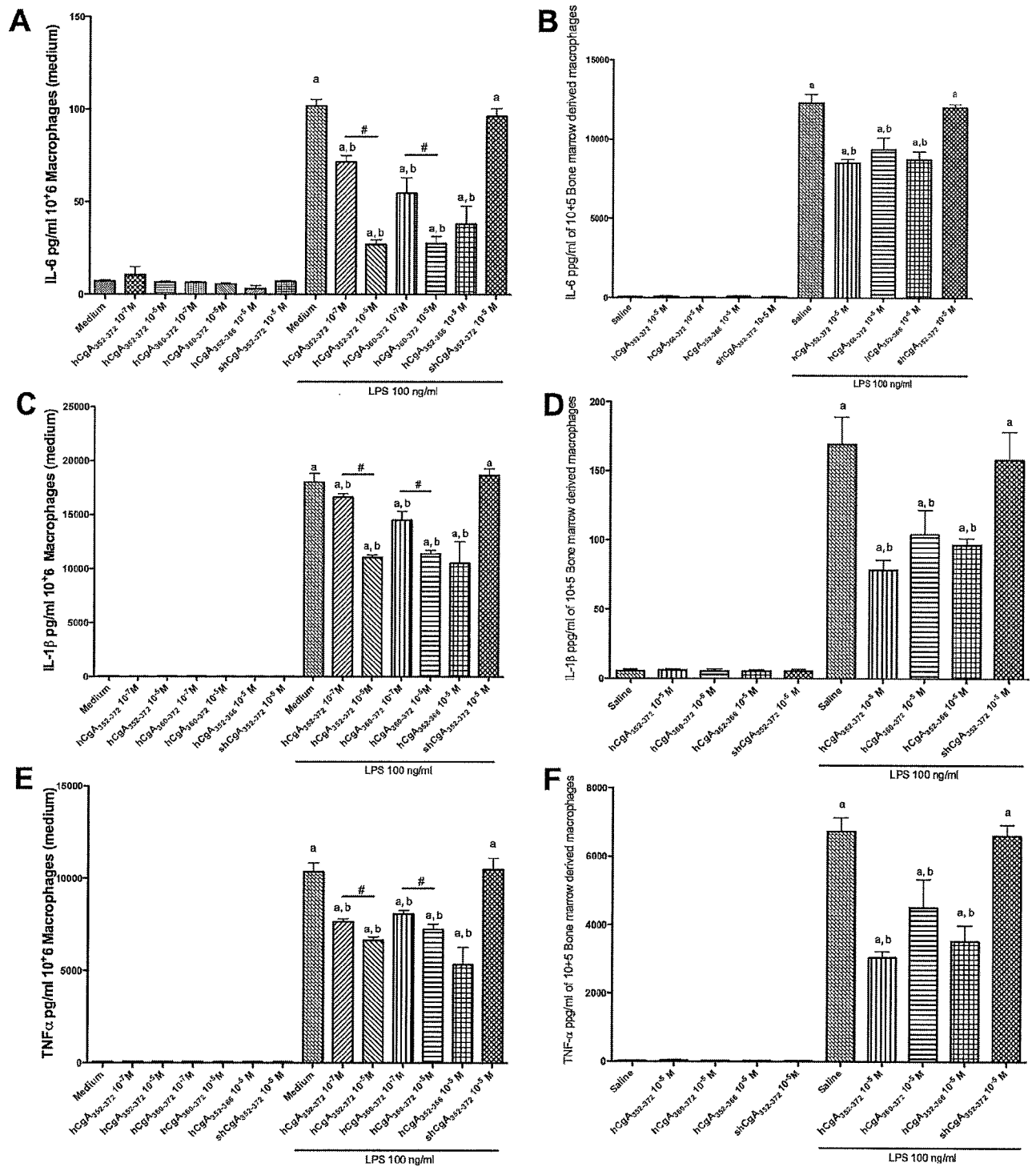
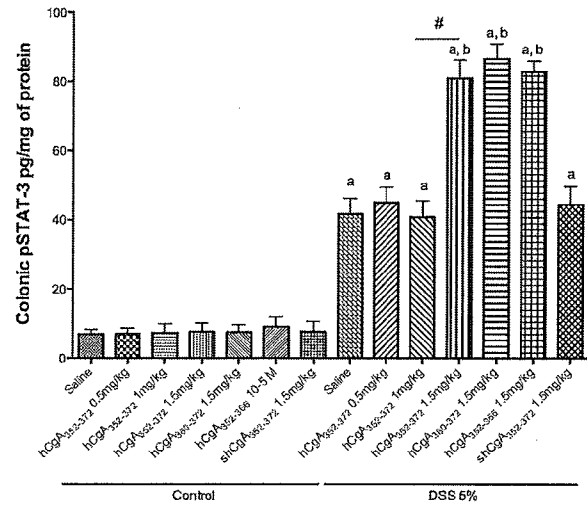
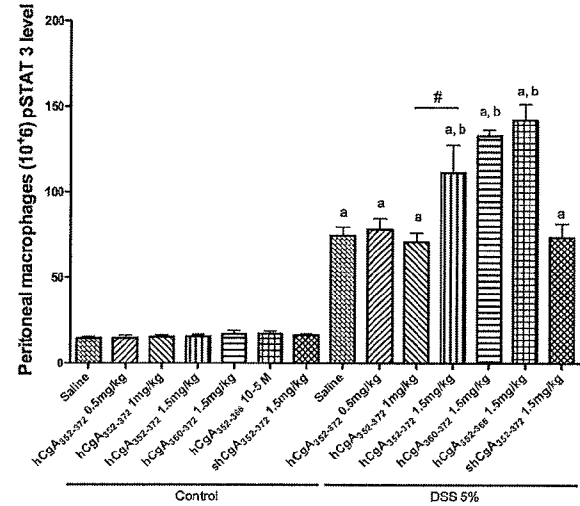


Fig. 8

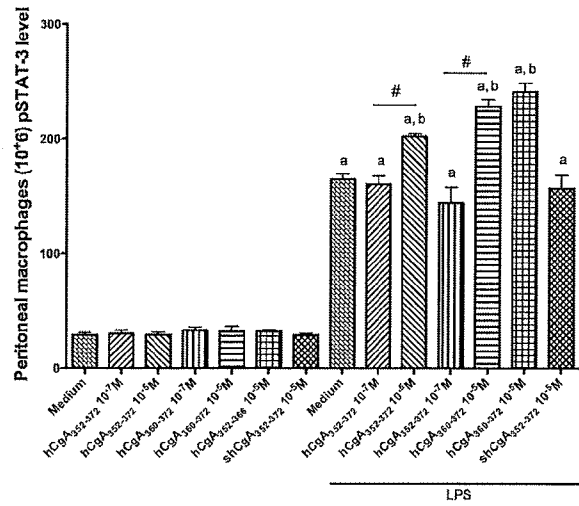
A



B



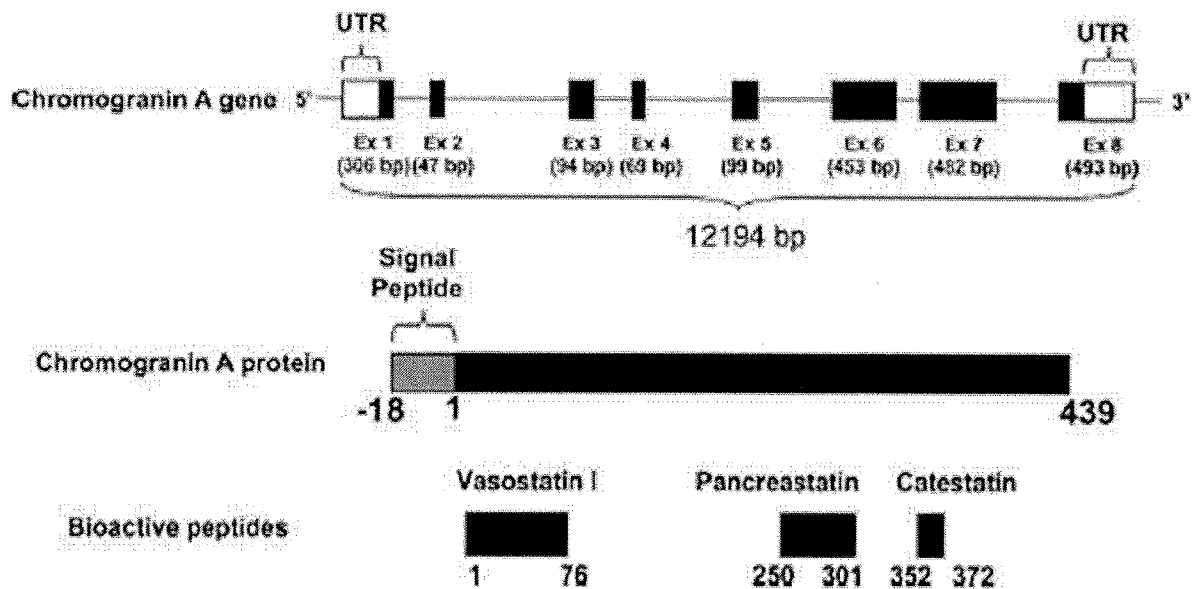
C



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Fig. 9-1

A.



B.

```

1  mrsaavllall lcagqvltalp vnspmnkgdt evmkcivevi sdtlskpspm pvsqecfetl
61  rgderilsil rhqnllkelq dlalggaker ahqqkksghf edelsevlen qssqaelkea
121 veepsskdvm ekredskeae ksgeatdgar pgalpepmqe skaegnngap geeeeeeeea
181 tnthppaslp sqkypgpqae gdseglsqgl vdrekglxae pgwqakreee ceeeeeeaeag
241 eeavpeeeegp tvvlnphpsl gykeirkges rsealavdga gkpgaeaaqd pegkgeqehs
301 qqkeeeema vvpqglfrgg ksgeleqeee rlskewedsk rwskmndlak eltaekrleg
361 qeeeednrds smklsfrara ygfrgpggpql rrgwrpssre dsleaglplq vrgypeekke
421 eegsanrrpe dgeleslsai eaelekvhq lqalrrg

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Fig. 9-2

C.

```
Human      mrsaaviiallllcagqvtaipvnspmnkgdtevmkciveisdtlskpsmpvpvsqecfetl
Mouse      mrstavlallllcagqvfaipvnspmtkgdtkvmkcvlevisdslskpsmpvpvspeclatl
          ***:*****.*****.***:****:*****:***** **:*

Human      rgderilsilrhqnlkkelqdlalqgakerahq-----qkhhsgf
Mouse      qgderilsilrhqnlkkelqdlalqgakeragqplkqqqqppkqqqqqqqqqqqegqhssf
          :*****:*
                                     ::**

Human      edelsevlenqssqaelkeaveepsskdvmekredskeaeksg--eatdgarpqalpepm
Mouse      edelsevfengspdakhrdaaaevpsrdtmekrkdskgqqdgfeattegrpqafpepn
          *****:*: :*. * *.*.****:*:::* :*: * ****:*

Human      qeskaegnngapgeeeeeeeeatnthppaslpqkyppgaegdseglsqglvdrekgls
Mouse      qespmmgdsespged-----tatntqsptslpsqehvdpqatgdser-----gls
          *** *:::***: *****: : *** ****

Human      aepgw-qakreeeeeeeeaaageeavpееegptvlnphpslgykeirkgesrsealav
Mouse      aqqqarkakqeekeeeeeeeavarekagpeevptaassshfhagykaiqkddggsdsqav
          *: :*:*:***** ...* . ** **.. * *** *: :*:*: **

Human      dgagkpgaeeaaqdpqegkgegehsqqkeee-eemavvpqglfrrgksge-----leqe
Mouse      dgdgkteasealpsegkgelehsqqeedgeeamvtpqglfpggkggrelehkqeeeee
          ** * * *.** ***** *****:*: * *. ***** * *. *:*

Human      eerlskewedskrwskmqdlakeltaekrlegqeeeednrdsmsklfraygrfgpp
Mouse      eerlsrwed-krwsrmdqlakeltaekrleged---dpdrsmklfrtraygrfdpp
          *****:*: *****:*****: : : * *****:***** **

Human      qlrrgwrpssredsleaglpqvrqypeekkeeeegsanrrpedqeleslsaieaelekva
Mouse      qlrrgwrpssredsvears-----dfeekkeeeegsanrraedqeleslsaieaelekva
          *****:*: ***** *****

Human      hqlqalrrg
Mouse      hqlqalrrg
          *****
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2014/061629

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 38/22** (2006.01), **A61P 1/00** (2006.01), **A61P 29/00** (2006.01), **G01N 33/48** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 38/22 (2006.01), **A61P 1/00** (2006.01), **A61P 29/00** (2006.01), **G01N 33/48** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Questal Orbit, Total Patent, Canadian Patent Database, Scopus, Pubmed
Keywords: Chromogranin, catestatin, inflammat+, bowel, Crohn's, colitis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PARK, A., et al. "Inhibitory influence of catestatin a chromogranin A-derived peptide in experimental colitis". Abstract 158. Canadian Digestive Diseases Week and the 5 th Annual Canadian Association for the Study of Liver Winter Meeting. February 27-March 2, 2009 (02/03-2009). Banff, Alberta, Canada.	1-8, 13-26 and 31-34
X	KHAN, W.I AND GHIA, J.E. "Gut hormones: emerging role in immune activation and inflammation". CLINICAL AND EXPERIMENTAL IMMUNOLOGY. July 2010 (07-2010), V. 161, p. 19-27. ISSN: 1365-2249. see page 23.	1-8, 13-26 and 31-34
A	GHIA, J-E. et al. "A role for chromogranin A (4-16), a vasostatin-derived peptide, on human colonic motility. An in vitro study". REGULATORY PEPTIDES. September 2004 (09-2004), V. 121, p. 31-39. ISSN: 0167-0115.	

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier application or patent but published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search
26 August 2014 (26-08-2014)Date of mailing of the international search report
02 October 2014 (02-10-2014)Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer

Tera Mosher (819) 994-4248

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2014/061629

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RUMIO, C. et al. "The N-terminal fragment of Chromogranin A, vasostatin-1 protects mice from acute or chronic colitis upon oral administration". DIGESTIVE DISEASES AND SCIENCES. May 2012 (05-2012), V. 57, p. 1227-1237. ISSN: 1573-2568.	
A	WO 2010/150082 A2 (MARCUCCI, F. et al.) 29 December 2010 (29-12-2010).	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2014/061629**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim Nos.: 17-38
because they relate to subject matter not required to be searched by this Authority, namely: Claims 17-34 are directed to and claims 35-38 encompass a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to search under Rule 39.1(iv) of the PCT. However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in claims 17-38.
2. ☒ Claim Nos.: 1-6 and 9-38
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically: Chromogranin-A derived proteins encompass many different proteins with a variety of functions (see for example page 3, lines 4-26 of the instant application). The search of the claims have been limited to the Chromogranin-A derived protein, catestatin (i.e., SEQ ID NO: 2) and its N and C-terminal fragments as defined by SEQ ID NOs: 3 and 4. Further, no search has been carried out on the subject matter of claims 9-12 and 27-30.
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IB2014/061629

Patent Document
Cited in Search Report

Publication
Date

Patent Family
Member(s)

Publication
Date

WO2010150082A2

29 December 2010 (29-12-2010) WO2010150082A3
ITMI20091125A1

19 May 2011 (19-05-2011)
26 December 2010 (26-12-2010)