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γ-Glutamyl cysteine and γ-glutamyl valine inhibit TNF-α signaling in intestinal epithelial cells and reduce inflammation in a mouse model of colitis *via* allosteric activation of the calcium-sensing receptor

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ABSTRACT

Background: The extracellular calcium-sensing receptor (CaSR) is distributed throughout the gastrointestinal tract, and its activation has been shown to promote intestinal homeostasis, suggesting that CaSR may be a promising target for novel therapies to prevent chronic intestinal inflammation such as inflammatory bowel disease (IBD). The γ-glutamyl dipeptides γ-glutamyl cysteine (γ-EC) and γ-glutamyl valine (γ-EV) are dietary flavor enhancing compounds, and have been shown to activate CaSR *via* allosteric ligand binding. The aim of this study was to examine the anti-inflammatory effects of γ-EC and γ-EV *in vitro* in intestinal epithelial cells and in a mouse model of intestinal inflammation.

Results: In vitro, treatment of Caco-2 cells with γ-EC and γ-EV resulted in the CaSR-mediated reduction of TNF-α-stimulated pro-inflammatory cytokines and chemokines including IL-8, IL-6, and IL-1β, and inhibited phosphorylation of JNK and IκBα, while increasing expression of IL-10. *In vivo*, using a mouse model of dextran sodium sulfate (DSS)-induced colitis, γ-EC and γ-EV treatment ameliorated DSS-induced clinical signs, weight loss, colon shortening and histological damage. Moreover, γ-EC and γ-EV reduced the expression of TNF-α, IL-6, INF-γ, IL-1β, and IL-17, and increased the expression of IL-10 in the colon, in a CaSR-dependent manner. The CaSR-mediated anti-inflammatory effects of γ-EC were abrogated in β-arrestin2 knock-down Caco-2 cells, and involvement of β-arrestin2 was found to inhibit TNF-α-dependent signaling *via* cross-talk with the TNF-α receptor (TNFR).

Conclusions: Thus CaSR activation by γ-EC and γ-EV can aid in maintaining intestinal homeostasis and reducing inflammation in chronic inflammatory conditions such as IBD.

1. Introduction

The calcium-sensing receptor (CaSR), a member of the G-protein-coupled receptor (GPCR) family, detects extracellular calcium ions and regulates the release of intracellular calcium, a secondary signaling molecule involved in intracellular signal transduction. CaSR is widely distributed in diverse cell types in various tissues including the parathyroid, brain, kidney, lung and bone marrow, where it regulates cellular activities including secretion, apoptosis, proliferation, differentiation and ion-channel activity [1,2]. CaSR is also present in the gastrointestinal tract, and is expressed on the apical and basolateral membranes of villous and crypt epithelial cells of the small intestine and colon, respectively, where it is involved in the regulation of various processes such as intestinal absorption, secretion and motility [3,4]. Studies have shown that CaSR plays a key role in maintaining and restoring intestinal homeostasis [5,6], and calcium-induced activation of CaSR was found to promote differentiation of colonic myofibroblasts and stimulate regeneration of the intestinal barrier [6], suggesting that CaSR may be a promising target for treating intestinal inflammation.

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic relapsing inflammation of the gastrointestinal tract [7]. Increased expression of inflammatory mediators during IBD results in the recruitment and activation of immune cells, including neutrophils and lymphocytes, perpetuating the inflammatory response. TNF-α plays a predominant role in IBD pathogenesis [8,9], and a number of anti-TNF-α therapies have been shown to successfully reduce pathology and morbidity in IBD patients [10].

GPCR agonists have been shown to exert anti-inflammatory effects and interfere in TNF-α-stimulated signaling pathways [11,12]. The use of exogenous ligands to modulate CaSR signaling is of great therapeutic

Abbreviations: CaSR, calcium-sensing receptor; GPCR, G-protein-coupled receptor; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; γ-EC, γ-glutamyl cysteine; γ-EV, γ-glutamyl valine; GRK, GPCR kinase; TRAF, TNF-receptor-associated factor; TNFR, TNF-α receptor; IEC, intestinal epithelial cell; MCP-1, monocyte chemoattractant protein-1; TAK1, TGF-β-activated kinase 1; TAB, TAK1 binding-protein; TLR, Toll-like receptor

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interest [13], and polyvalent CaSR agonists neomycin sulfate and spermine were recently found to reduce LPS-stimulated TNF-α secretion in mouse macrophage cells [14]. L-amino acids and γ-glutamyl peptides have been identified as CaSR agonists [15–17], however their role in modulating intestinal inflammation has not been addressed. The γ-glutamyl dipeptides γ-glutamyl cysteine (γ-EC) and γ-glutamyl valine (γ-EV) are dietary flavor enhancing compounds that have been isolated from various sources, including edible beans [18] and yeast extracts [19]. γ-Glutamyl dipeptides are also involved in glutathione (GSH) metabolism, which plays an important role in antioxidant defense, and γ-EC in particular is a crucial intermediate in GSH synthesis [20]. Both γ-EC and γ-EV have been shown to activate CaSR *via* allosteric ligand binding [17], and may therefore be useful for reducing inflammation and restoring intestinal homeostasis in IBD.

Upon ligand activation of GPCRs, signal transduction is regulated by β-arrestins, adaptor proteins that bind receptors phosphorylated by G protein-coupled receptor kinases (GRKs) [21]. β-arrestins couple GPCRs to various downstream signaling components including mitogen-activated protein kinases (MAPK) such as JNK, ERK 1/2, and p38 MAPK, as well as IκBα and TNF-receptor-associated factor (TRAF) 6 [22,23]. β-Arrestins can also mediate crosstalk between GPCR-induced signaling and other receptors, such as the TNF-α receptor (TNFR), and lead to the inhibition of inflammatory signaling pathways [11].

In the present study we demonstrated that γ-EC- and γ-EV-mediated activation of CaSR reduces inflammatory mediator expression *in vitro* in intestinal epithelial cells (IECs), by inhibiting JNK and IκBα phosphorylation, and improves clinical and histological parameters and reduces pro-inflammatory cytokine and chemokine responses in a mouse model of experimental colitis. Furthermore, we have shown that CaSR activation by γ-EC can prevent TNF-α-induced pro-inflammatory signaling *via* cross-talk with TNFR in a β-arrestin-dependent manner.

2. Materials and methods

2.1. Cell culture and treatment

Human colorectal adenocarcinoma-derived intestinal epithelial cells (Caco-2) (ATCC, Manassas, VA) were grown in DMEM/F12 (Gibco/Life Technologies, Grand Island, NY) supplemented with 1 mM sodium pyruvate (Gibco/Life Technologies), 20% FBS (HyClone, Logan, UT) and 50 U/mL penicillin–streptomycin (Gibco/Life Technologies). For treatment with CaSR agonists/antagonists, cells between passages 15–45 were seeded at a density of 1×10^5 cells/well in 24- or 48-well plates (Corning, Lowell, MA) and grown for 5–7 days. The CaSR agonists γ-EC and γ-EV, and the CaSR antagonist NPS-2143, were kindly provided by Ajinomoto Co., Ltd. (Kawasaki, Japan). Confluent cell monolayers were rinsed with Hank's buffered salt solution and treated with γ-EC or γ-EV in culture medium containing 5% FBS for 2 h. For dose determination experiments cells were treated with 0.01, 0.1, or 1 mM γ-EC or γ-EV; for all other experiments a concentration of 0.5 mM γ-EC or γ-EV was used. Cells were stimulated with recombinant human TNF-α (2 ng/mL) (Invitrogen/Life Technologies) to induce inflammation. To block CaSR activation, cells were pre-treated with NPS-2143 (1 μM) for 45 min before addition of γ-EC or γ-EV.

2.2. Animals

6–8-week-old female BALB/c mice (16–20 g) (Charles River Laboratories Inc., Montreal, Quebec) were group housed on a 12-h light–dark cycle and allowed unrestricted access to standard mouse chow and water. All animal studies were approved by the University of Guelph Animal Care Committee and carried out in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

2.3. Treatment of mice with CaSR agonists/antagonists and induction of DSS-induced colitis

Mice were administered γ-EC or γ-EV (50 or 150 mg/kg body weight in 100 μL of water) or vehicle, by oral gavage, starting on day 1 and continuing until day 14. On day 7, colitis was induced by the addition of 5% dextran sodium sulfate (DSS) (MW 36–50 kDa, MP Biomedicals, Solon, OH) to drinking water and continued until day 14. Negative control mice received water only, and positive control mice received DSS only. For NPS-2143 treatment, mice were injected i.v. with 1 mg/kg NPS-2143, dissolved in 20% 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO), as a 30 μL bolus into the tail vein. One hour after NPS-2143 injection mice were administered γ-EC or γ-EV (150 mg/kg) by oral gavage. Mice were euthanized on day 14. Colons were removed and measured, and tissue sections were flash frozen or stored in RNAlater® (Ambion/Life Technologies) for further analysis. A section of distal colon was fixed in 10% buffered formalin for histological analysis.

2.4. Clinical analysis of colitis

Mice were weighed daily, and data are expressed as mean percentage change relative to starting body weight. Mice were monitored daily for stool consistency, presence of blood in stool or bleeding and general appearance, and a clinical activity score (ranging from 0 to 7) was calculated as described by Maxwell et al. [24].

2.5. Histological analysis of colitis

Paraffin-embedded sections of distal colon were stained with hematoxylin and eosin (H&E) (Animal Health Laboratory, University of Guelph, Guelph, Ontario), and histological scoring to assess colonic tissue injury and inflammation was performed as described by Maxwell et al. [24].

2.6. Cytokine ELISAs

Measurement of IL-8 in Caco-2 culture supernatants was carried out as previously described [25]. Measurement of TNF-α and IL-6 concentrations in mouse colon tissues, tissues were homogenized in three volumes of ice-cold PBS containing 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin A (Sigma-Aldrich) using a Polytron® homogenizer (PT 1200, Kinematica Inc., Bohemia, NY) and centrifuged at 12,000 ×*g* for 10 min at 4 °C. Protein concentration was measured by DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). TNF-α and IL-6 ELISAs were carried out using anti-mouse IL-6 (MP5-20F3) or anti-mouse/rat TNF-α (TN3-19.12) and biotinylated anti-mouse IL-6 (32C11) or biotinylated anti-mouse TNF-α (C1150-14) (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

2.7. RNA isolation and real-time RT-PCR

Total RNA was extracted from cells using the Aurum™ Total RNA Mini Kit (Bio-Rad) according to the manufacturer's instructions. Mouse colon tissues were homogenized in TRIZOL® Reagent (Invitrogen/Life Technologies) according to the manufacturer's instructions, and total RNA was extracted from the aqueous phase using the Aurum Total RNA Mini Kit. RNA (1 μg) was reverse transcribed using a qScript™ cDNA Synthesis Kit (Quanta Biosciences, Inc., Gaithersburg, MD) and real-time quantitative PCR was carried out as previously described [26] using the primers listed in Supplemental Table S1. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [27] using GAPDH as the reference gene. Results are presented as fold expression change relative to negative control.

2.8. Total GSH assay

Total intracellular GSH in Caco-2 cells was measured according the method of Allen et al. [28], with modifications. Following treatment in 24-well plates, cells were lysed with 400 μL of ice cold 0.1% Triton X-100 (Sigma-Aldrich) and then combined with 200 μL of 5% 5-sulfosalicylic acid (Sigma-Aldrich) for 2 min at room temperature. Samples were centrifuged at 8000 ×*g* for 10 min at 4 °C. Protein concentration of lysates was measured by DC Protein Assay (Bio-Rad). Lysates (25 μL) were mixed with 125 μL of sodium phosphate buffer (143 mM) containing 4 mM EDTA, 0.2 mM NADPH, 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and 100 U/mL of GSH reductase (all from Sigma-Aldrich), and incubated for 5 min at room temperature. Absorbance was measured at 405 nm. GSH concentration was determined from a GSH standard curve.

2.9. Western blot analysis

Following treatment, cells were washed twice with cold PBS and lysed in ice-cold RIPA buffer (ThermoFisher, Waltham, MA) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFisher). Cell lysates were sonicated for 5 min and centrifuged at 10,000 ×*g* for 25 min, and protein concentration was measured by DC Protein Assay. Samples (30–50 μg of protein) were separated by SDS-PAGE (10%) and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked using 5% non-fat milk powder in TBS, and incubated with primary antibody, at a dilution of 1:1000 or 1:2000, overnight at 4 °C. Antibodies used were: anti-p-SAPK/JNK (81E11), anti-SAPK/JNK (56G8), anti-p-IκBα (5A5) (Cell Signaling Technology, Inc., Danvers, MA), anti-IκB-α (H-4), anti-p-p38 (D-8), and anti-p38α/β (A-12) (Santa Cruz Biotechnology, Santa Cruz, CA). HRP-conjugated anti-mouse or anti-rabbit IgG (Promega, Madison, WI) was used at a dilution of 1:10,000. Proteins were detected using ECL Western Blotting Detection Reagent (GE Healthcare) and densitometry was performed using Image J software (Image Processing and Analysis in Java, National Institutes of Health, http://rsbweb.nih.gov/ij/). In order to verify the activation of IκBα, JNK or P38 signaling pathways involved in regulating TNF-α-induced signaling cascades, Caco-2 cells were stimulated by 2 ng/mL TNF-α for 2 h after pretreated with or without inhibitors including SB203580 (25 μM) (CAS 152121-47-6, Santa Cruz Biotechnology, Inc.), Bay11-7082 (50 μM) (CAS 19542-67-7) or SP600125 (50 μM) (CAS129-56-6) for 30 min. The production of IL-8 and phosphorylation of P38, IκB or JNK were assayed by ELISA and Western blot analysis, respectively as described above.

2.10. siRNA transfection

Caco-2 cells were transiently transfected with β-arrestin-2 or negative control siRNA using Lipofectamine® LTX and Plus™ Reagent (Invitrogen/Life Technologies) according to the manufacturer's instructions. Cells were cultured in 24- or 6-well plates until 60–80% confluency was reached. siRNA targeting human β-arrestin-2 (Hs ARRB2-3 siRNA) (QIAGEN, Germantown, MD) or negative control siRNA (Scramble) (QIAGEN) (0.5 μg or 2.5 μg per well for 24- or 6-well plates, respectively) were diluted in 100 μL Opti-MEM® I medium (Invitrogen/Life Technologies) and incubated for 15 min at room temperature with 0.5 or 2.5 μL PLUS Reagent. Lipofectamine LTX (1.5 μL or 6 μL) was diluted in 100 μL Opti-MEM® I medium and added to the siRNA solution, and the mixture was incubated for 25 min at room temperature. The transfection mixture was diluted in 400 or 800 μL Opti-MEM® I, respectively, and added to the cells in 24- or 6-well plates. After 6–8 h, 0.5 or 1 mL of DMEM/F12 medium containing 2× FBS, 1 mM sodium pyruvate, and 50 U/mL penicillin–streptomycin was added directly to each well of a 24- or 6-well plate. After 48 h, cells were treated with γ-EC and TNF-α as previously described. Culture supernatants were collected for ELISA and cells were harvested for Western blotting.

2.11. Co-immunoprecipitation analysis

Cells were harvested 30 min after TNF-α stimulation and immunoprecipitation was carried out according to the manufacturer's instructions using a Pierce Crosslink Immunoprecipitation Kit (ThermoFisher). Briefly, anti-β-arrestin-2 (C16D9)(Cell Signaling) or anti-TAK1 (H-5) antibodies (Santa Cruz Biotechnologies) (2 μg–10 μg) were cross-linked to protein A/G-agarose beads, and incubated with pre-cleared lysate (550 μg of total protein) for 2 h at room temperature. The eluted antigen was analyzed by Western blot analysis using anti-TAB1 (25E9) (Cell Signaling) or anti-CaSR (H-100) (Santa Cruz Biotechnology) antibodies.

2.12. Statistical analysis

Data are expressed as means ± SEM. Statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad, San Diego, CA) using one-way ANOVA followed by Dunnett's test to compare groups to a control, or two-way ANOVA followed by Tukey's post test, as indicated. Unpaired Student's *t* test was used when comparisons between only two groups were carried out. Differences were considered significant when $p < 0.05$.

3. Results

3.1. γ-EC and γ-EV Reduce Expression of TNF-α-induced Inflammatory Mediators in Intestinal Epithelial Cells

We first examined the potential anti-inflammatory properties of γ-EC and γ-EV using an *in vitro* model of TNF-α-induced inflammation in Caco-2 IECs. Cells were treated with γ-EC or γ-EV, and TNF-α was added to induce inflammation. Stimulation of Caco-2 cells with TNF-α increases the expression of inflammatory mediators, including IL-8 [29], which was used here as a marker of inflammation. Pre-treatment with γ-EC or γ-EV significantly reduced TNF-α-induced IL-8 secretion from Caco-2 cells in a dose-dependent manner, reducing IL-8 concentrations up to 6-fold when used at the highest dose (1 mM) (Fig. 1A). Likewise, treatment with γ-EC or γ-EV significantly reduced mRNA levels of IL-8, as well as pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, when compared to cells treated with TNF-α alone (Fig. 2B–E). A significant increase in the expression of the anti-inflammatory cytokine IL-10 was also observed in response to γ-EC and γ-EV treatment (Fig. 1F). The expression of CaSR in Caco-2 cells following treatment with TNF-α, γ-EC and γ-EV was also measured, and no significant effect of treatment was observed on CaSR expression at both the gene and protein levels when compared to untreated control cells (data not shown). Serial concentrations of $CaCl_2$ (0.1, 0.5, 1.0, 2.5 and 5 mM) were supplemented to DMEM/F12 10% FBS medium (Ca^{2+} level is 1.05 mM) with or without 0.5 mM γ-EC or γ-EV, and incubated with Caco-2 cells for 2 h before adding 2 ng/mL TNF-α (Fig. 2). A significant decrease of IL-8 level was detected in 2.5 mM or 5 mM $CaCl_2$ treated Caco-2 cells after sole TNF-α stimulation as shown in Fig. 2. The result firstly confirmed that calcium as orthosteric agonist for CaSR activation has anti-inflammatory activity to reduce TNF-α-induced inflammation in Caco-2 cells at a relatively high concentration compared with allosteric agonists γ-EC or γ-EV. However, the inhibitory activity of γ-EC or γ-EV on IL-8 secretion was not significantly affected by an increase of calcium concentrations.

Since γ-glutamyl dipeptides, in particular γ-EC, are precursors in the synthesis of GSH, a major antioxidant in mammalian cells, we next examined whether the addition of γ-EC and γ-EV may have led to an increase in cellular GSH levels, thereby reducing inflammatory responses. Treatment of Caco-2 cells with TNF-α, γ-EC or γ-EV did not significantly affect intracellular GSH concentrations (data not shown), suggesting that increased GSH synthesis was not a major contributor to the anti-inflammatory effects of γ-EC and γ-EV observed here.

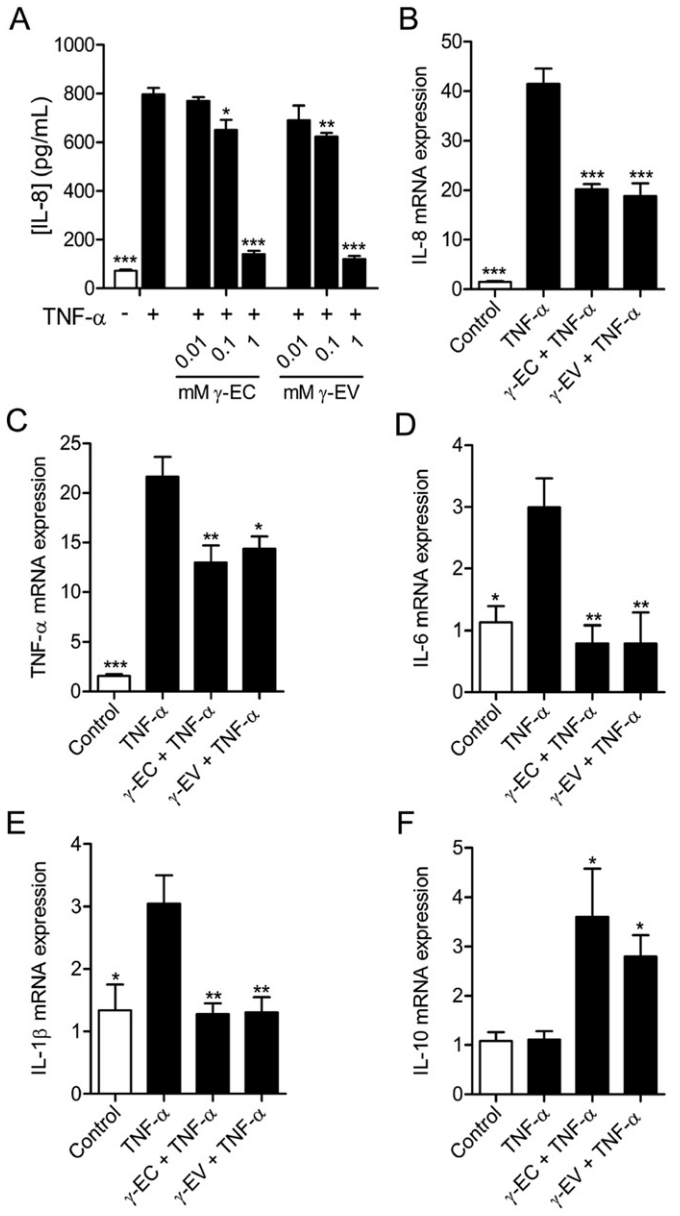

Fig. 1. γ-EC and γ-EV reduce TNF-α-stimulated pro-inflammatory cytokine expression and increase IL-10 expression in Caco-2 cells. A, Caco-2 cells were pre-treated for 2 h with γ-EC or γ-EV at indicated doses, followed by stimulation with 2 ng/mL TNF-α for 4 h. IL-8 concentration in supernatants was measured by ELISA. Data are presented as mean ± SEM ($n = 4$ independent determinations). B–F, Caco-2 cells were pre-treated for 2 h with 0.5 mM of γ-EC or γ-EV followed by stimulation with 2 ng/mL TNF-α for 4 h. mRNA expression was measured by real-time quantitative RT-PCR using GAPDH as the reference gene, and results are expressed as mRNA level relative to control. Data are presented as mean ± SEM ($n = 6$ independent determinations). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* TNF-α alone using one-way ANOVA with Dunnett's post test.

3.2. γ-EC inhibits TNF-α-induced JNK and IκB phosphorylation in Caco-2 IECs

At first in order to verify the activation of IκBα, JNK or P38 signaling pathways involved in regulating TNF-α-induced signaling cascades, a series of pharmacologic inhibitors, including Bay11-7082, SP600125 and SB203580 were subjected in this experiment to block the TNF-α-mediated activations of IκBα, JNK or P38 in Caco-2 cells, respectively. The secretion of IL-8 from TNF-α-treated Caco-2 cells was completely abrogated by Bay11-7082 and markedly inhibited by SP600125, but not SB203580 as shown in Fig. 3. The TNF-α-induced phosphorylation of IκBα or JNK was abrogated by Bay11-7082 or SP600125. However,

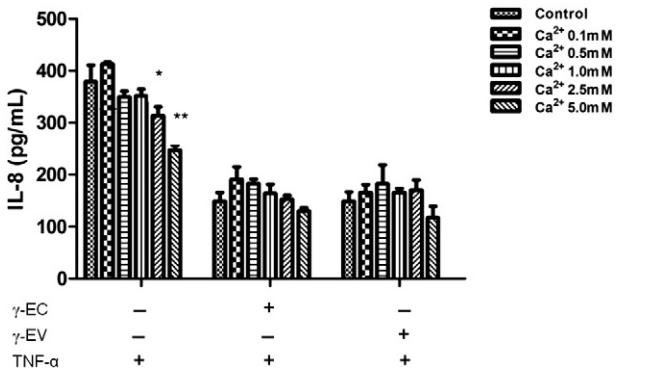

Fig. 2. Calcium, γ-EC and γ-EV reduce TNF-α-stimulated IL-8 expression in Caco-2 cells. Caco-2 cells were pre-treated for 2 h with supplemental calcium with or without γ-EC or γ-EV at indicated doses, followed by stimulation with 2 ng/mL TNF-α for 4 h. IL-8 concentration in supernatants was measured by ELISA. Data are analyzed by one-way ANOVA within each group and presented as mean ± SEM ($n = 3$ independent experiments). $^{*}p < 0.05$; $^{**}p < 0.01$ *versus* TNF-α alone.

pretreatment with SB203580 inhibitor did not demonstrate similar inhibitory effects on phosphorylation of P38. The results suggest that IκBα and JNK were dominantly involved in TNF-α-initiated signaling cascades. Since both peptides exhibited similar effects on cytokine expression as shown in Fig. 1, and γ-EC has been shown to be a stronger CaSR agonist than γ-EV *in vitro* [17,18], it was selected for use in further cell signaling experiments. To examine the effects of g-glutamyl peptides on TNF-α-stimulated MAPK and NF-κB activation, Caco-2 cells were first treated

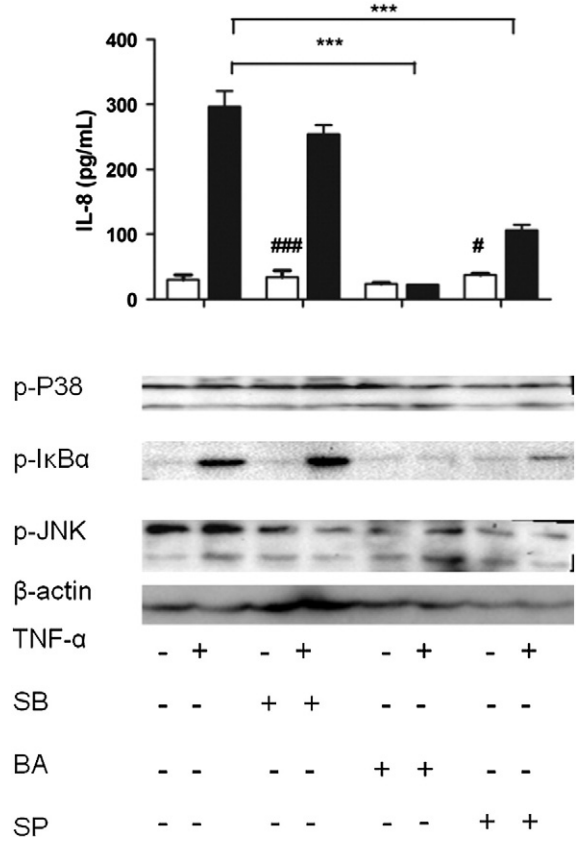

Fig. 3. TNF-α-induced IL-8 secretion in Caco-2 cell *via* activation of NF-κB and MAPK pathways. Caco-2 cells were stimulated by 2 ng/mL TNF-α for 2 h after being pretreated with or without inhibitors including SB203580 (25 μM), Bay11-7082 (50 μM) or SP600125 (50 μM) for 30 min. The production of IL-8 and phosphorylation of P38, IκB or JNK were assayed by ELISA and Western blot analysis, respectively. Data are presented as means ± SEM (n = 3), $^{***}p < 0.001$ *versus* sample without inhibitors. $^{###}p < 0.001$ or $^{#}p < 0.5$ *versus* TNF-α alone.

with γ-EC, followed by stimulation with TNF-α. Western blot analysis of total cell lysates using phospho-specific antibodies revealed that TNF-α-induced phosphorylation of JNK and IκBα was significantly reduced by γ-EC (γ-EC + TNF-α) when compared to cells treated with TNF-α alone (TNF-α) (Fig. 4A and B). p38 phosphorylation was low in all treatments, and only a slight decrease was observed following γ-EC treatment. There was no effect of peptide alone (γ-EC) on JNK, IκBα, or p38 phosphorylation. Taken together with the cytokine data, these results suggest that treatment of Caco-2 cells with γ-EC or γ-EV may prevent TNF-α-induced activation of the JNK pathway and NF-κB transcriptional activity, thereby reducing the production of inflammatory mediators.

3.3. The CaSR antagonist NPS-2143 abrogates the anti-inflammatory effects of γ-EC and γ-EV in Caco-2 IECs

Since γ-EC and γ-EV are known CaSR agonists, we then sought to confirm that the observed anti-inflammatory effects were mediated *via* CaSR. To this end, pre-treatment with NPS-2143, a CaSR-specific antagonist, was used to block CaSR activation by γ-EC and γ-EV. NPS-2143, a negative allosteric regulator of CaSR, been used widely in *in vitro* studies to inhibit CaSR agonist-induced signaling cascades [30,31]. Here, we observed that pre-treatment of Caco-2 cells with NPS-2143 prior to the addition of γ-EC or γ-EV abrogated the anti-inflammatory effect of the peptides, and restored TNF-α-induced IL-8 secretion in cells treated with γ-EC or γ-EV (Fig. 5A). The significant difference between NPS-2143 pretreatment and TNF-α treatment alone was not observed, which suggests that CaSR-mediated signaling cascade is primarily involved in blocking TNF-α-initiated inflammatory response (data not shown). Likewise, NPS-2143 prevented the inhibitory effect of γ-EC on the TNF-α-stimulated phosphorylation of JNK and IκBα (Fig. 5B and C) in Caco-2 cells.

3.4. γ-EC and γ-EV reduce the severity of inflammation in a mouse model of DSS-induced colitis

To investigate whether the γ-glutamyl peptides γ-EC and γ-EV could also prevent inflammation *in vivo*, a mouse model of DSS-induced colitis was used. Mice were orally administered γ-EC or γ-EV for 14 days, and DSS was introduced into the drinking water on day 7 to induce colitis (Fig. 6A). On day 14, tissues were collected for analysis. After receiving DSS for 7 days, positive control mice (Pos) displayed characteristic signs of colitis including weight loss, diarrhea and rectal bleeding when compared to untreated control mice (Neg). Mice receiving γ-EC or γ-EV (50 or 150 mg/kg) for 14 days showed significantly reduced clinical signs when compared to Pos mice (Fig. 4B). Treatment with γ-EC and γ-EV also attenuated DSS-induced weight loss (in all groups except 150 mg/kg γ-EC) (Fig. 6C) and colon shortening (Fig. 6D and E). Histopathological evaluation of H&E-stained colon sections revealed loss of mucosal architecture, infiltration of inflammatory cells into the mucosa and submucosa, and intense cellular inflammation in all layers of colonic tissue in DSS-treated positive control mice. Significantly less tissue damage, cellular infiltration and mucosal ulceration was observed in mice treated with γ-EC (150 mg/kg) or γ-EV (50 and 150 mg/kg) as compared to the Pos control group (Fig. 6F and G).

There was no significant difference of clinical signs identified between two dosages treated groups of mice in Fig. 6B, but a significant difference of weight changes, colon shortening and histological score in the group of mice treated with 150 mg/kg BW γ-EV was only observed in this study.

The effect of γ-EC and γ-EV administration on DSS-induced inflammatory cytokines in the colon was also examined. Concentrations of TNF-α and IL-6 in colon tissue homogenates were measured by ELISA. Both doses of γ-EC and γ-EV significantly reduced TNF-α production when compared to Pos mice (Fig. 7A). While IL-6 concentrations were reduced in all groups, only the high dose of γ-EC (150 mg/kg) resulted in a significant reduction in IL-6, despite marked weight loss observed in this group. The anti-inflammatory effect of γ-EC and γ-EV on DSS-induced inflammation in the colon was most evident in the mRNA expression levels of key inflammatory mediators. Analysis using qRT-PCR revealed that relative mRNA expression of TNF-α, IL-6, IFN-γ, IL-1β, and IL-17A was significantly reduced in mice treated with either dose of γ-EC or γ-EV (Fig. 7B-F), as was the expression of the chemokine monocyte chemoattractant protein (MCP)-1 (Fig. 7G). Moreover, both γ-EC and γ-EV significantly increased mRNA expression of IL-10 (Fig. 7H). There was no effect of the peptides alone on any of the parameters measured (data not shown). There was no dose–response existing in current study except MCP-1 and IL-10 gene expression treated with γ-EC. These findings are in line with our *in vitro* results in IECs,

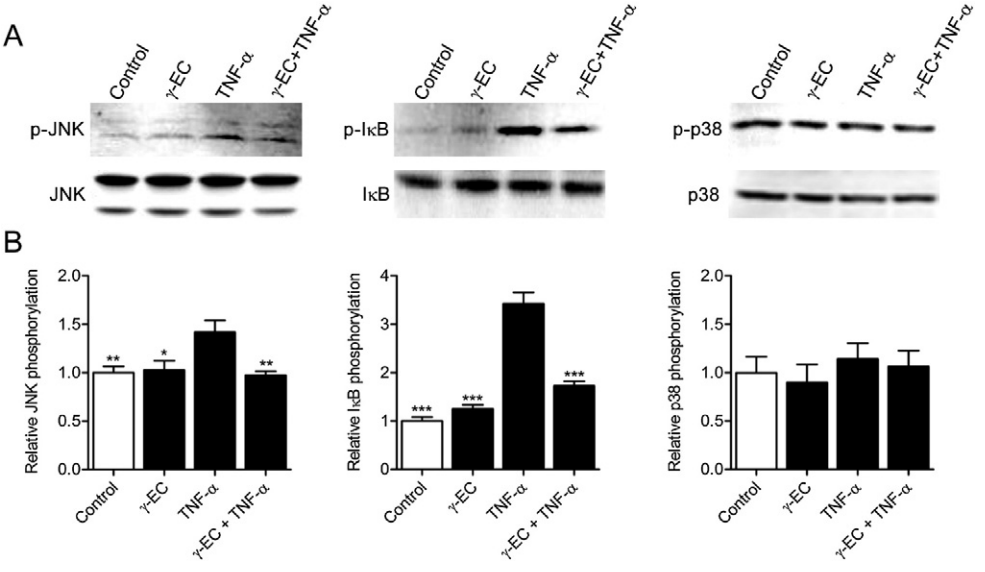

Fig. 4. TNF-α-stimulated phosphorylation of JNK and IκB is reduced by γ-EC. Caco-2 cells were pre-treated for 2 h with 0.5 mM γ-EC, followed by stimulation with 2 ng/mL TNF-α for 30 min. A, Western blot analysis of cell lysates was performed using antibodies against phospho-JNK, JNK, phospho-IκB, IκB, phospho-p38, and p38 (images of one representative experiment of six is shown). B, quantitative analysis of relative levels of phosphorylated JNK, IκB, and p38 using ImageJ software. Results are expressed as relative protein levels compared to untreated control cells. Data are presented as mean ± SEM ($n = 6$ independent determinations). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* TNF-α alone using one-way ANOVA with Dunnett's post test.

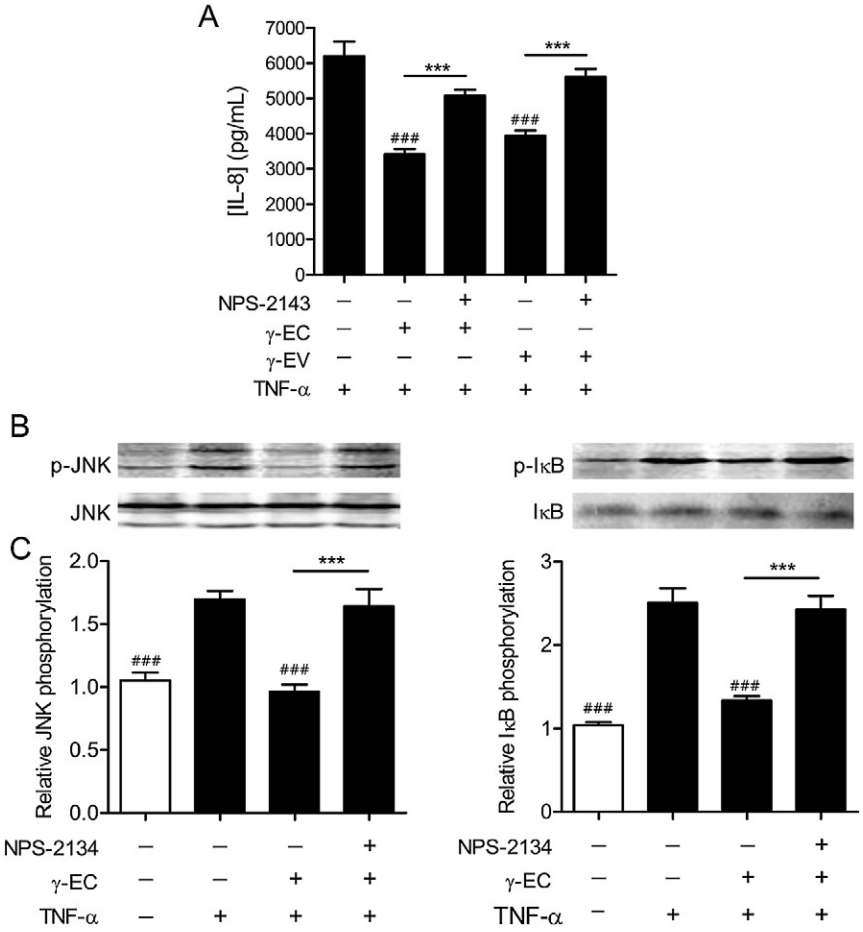

Fig. 5. NPS-2143 blocks anti-inflammatory effects of γ-EC and γ-EV in Caco-2 cells. Caco-2 cells were pre-treated with 1 μM NPS-2143 for 45 min, followed by incubation with 0.5 mM γ-EC or γ-EV for 2 h. Cells were then stimulated with 2 ng/mL TNF-α for 30 min (Western blotting) or 4 h (ELISA). A, IL-8 concentration in supernatants was measured by ELISA. Data are presented as mean ± SEM ($n = 4$ independent determinations). $^{***}p < 0.001$ *versus* peptide treatment without inhibitor using unpaired Student's *t* test. $^{###}p < 0.001$ *versus* TNF-α alone using one-way ANOVA with Dunnett's post test. B, Western blot analysis of cell lysates was performed using antibodies against phospho-JNK, JNK, phospho-IκB, and IκB (images of one representative experiment of six is shown). C, quantitative analysis of relative levels of phosphorylated JNK and IκB using ImageJ software. Results are expressed as relative protein levels compared to untreated control cells. Data are presented as mean ± SEM ($n = 6$ independent determinations). $^{***}p < 0.001$ *versus* peptide treatment without inhibitor using unpaired Student's *t* test. $^{###}p < 0.001$ *versus* TNF-α alone using one-way ANOVA with Dunnett's post test.

and demonstrate that γ-EC or γ-EV can exert potent anti-inflammatory effects *in vivo*.

3.5. γ-EC and γ-EV reduce DSS-induced pro-inflammatory cytokine expression via CaSR activation

Based on the previous *in vitro* and *in vivo* results, we next examined if the ability of γ-EC and γ-EV to reduce the severity of DSS-induced inflammation in mice was similarly mediated by CaSR. The use of NPS-2143 has been reported to block CaSR activation in rat and mouse models, and was found to inhibit CaSR-mediated gastrin secretion in the stomach of mice following i.v. administration [32], and so was used here to block CaSR activation by the γ-glutamyl peptides in a mouse model of DSS-induced colitis. NPS-2143 or vehicle was given daily before oral administration of γ-EC and γ-EV for 14 days (Fig. 8A). After 7 days of DSS administration, relative mRNA expression in the colon was measured. Treatment with NPS-2143 restored DSS-induced mRNA expression of TNF-α, INF-γ, IL-6, IL-1β, and IL-17A when compared to mice treated without inhibitor (Fig. 8B–F), suggesting that NPS-2143 also interfered with the anti-inflammatory effects of the peptides *in vivo*. NPS-2143 also prevented the γ-EC- and γ-EV-induced increase in IL-10 expression (Fig. 8G), although this effect was not as pronounced as with the other cytokines. These results confirm that γ-EC and γ-EV can reduce DSS-induced inflammation *via* CaSR-mediated signaling pathways.

3.6. Role of β-arrestin2 in the CaSR-mediated anti-inflammatory effects of γ-glutamyl peptides

β-Arrestin2 has been shown to be involved in the regulation of CaSR function [33] and participate in downstream anti-inflammatory signaling mechanisms in immune cells [11]. Thus, we hypothesized that β-arrestin2 may similarly be involved in the CaSR-mediated signaling events observed here. To determine whether β-arrestin2 was required for the anti-inflammatory effects of γ-EC, Caco-2 cells with reduced β-arrestin2 expression were generated. Caco-2 cells were transiently transfected with specific β-arrestin2 siRNA, to produce β-arrestin2 knockdown cells (β-arr2 KD) (Fig. 9A) and the effect of γ-EC on TNF-α-stimulated cytokine production and cell signaling was compared to control cells transfected with control siRNA (Scramble). In β-arrestin2 knockdown cells, the γ-EC-mediated anti-inflammatory activity was reduced when compared to control cells (Scramble), resulting in an increase in IL-8 levels in β-arr2 KD cells treated with γ-EC and TNF-α (Fig. 9B). Although a decrease in TNF-α-stimulated IL-8 secretion was still observed in response to γ-EC in the β-arr2 KD cells, this was to a lesser extent than that observed in control

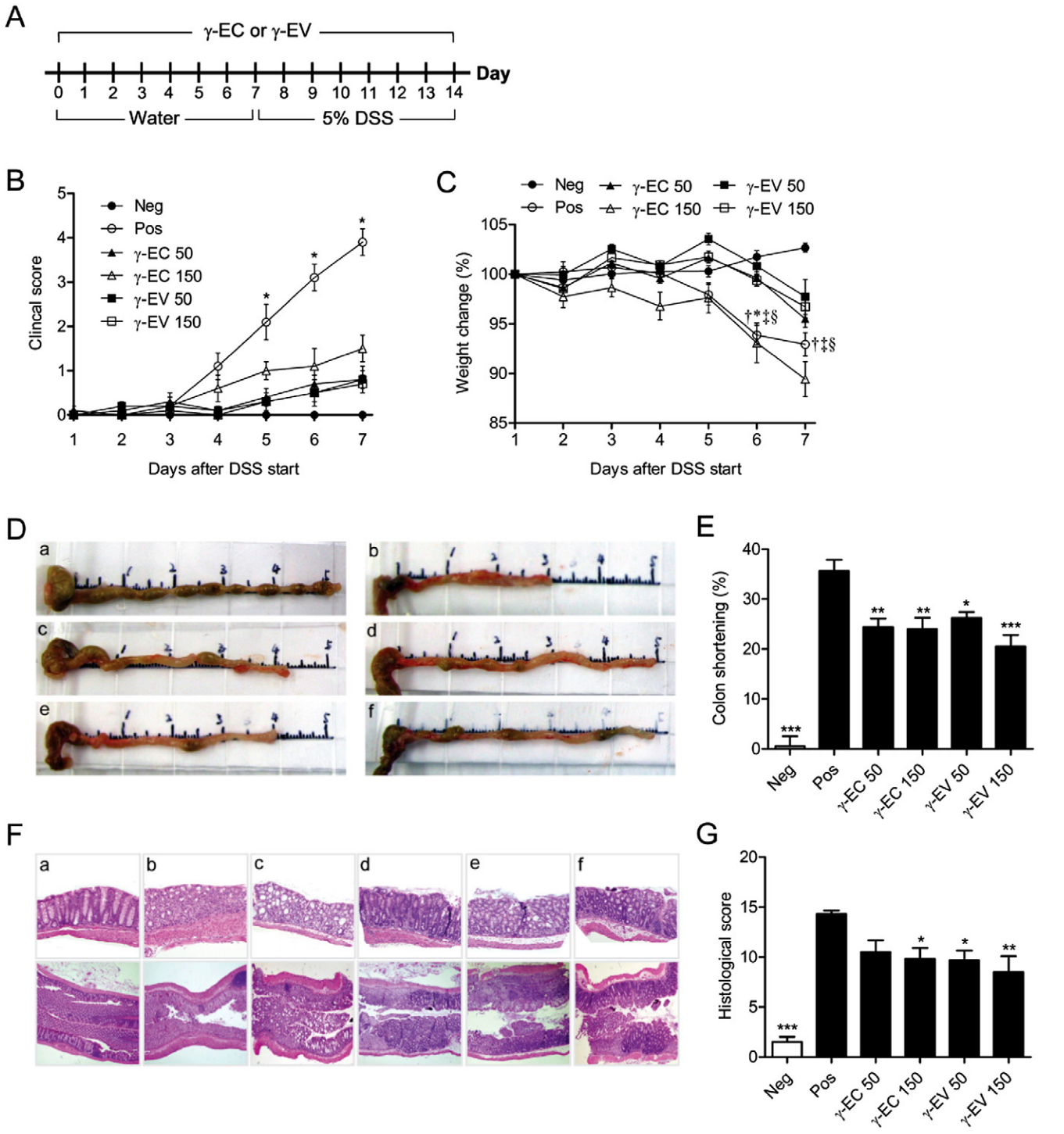

Fig. 6. γ-EC and γ-EV reduce severity of histopathological and clinical parameters of DSS-induced colitis in mice. A, experimental design. Mice were given 50 or 150 mg/kg γ-EC or γ-EV orally for 14 d. To induce acute colitis, 5% DSS was added to drinking water on day 7. B, clinical score was determined by assessing stool consistency, presence of blood in stool or rectal bleeding, and general appearance. Clinical scores ranged from 0 to 7. Data is presented as mean score ± SEM ($n = 12$ per group). $^{*}p < 0.05$ *versus* all other groups using one-way ANOVA with Dunnett's post test. C, body weight loss after induction of colitis was calculated as mean percent of initial weight ± SEM ($n = 12$ per group). $^{\dagger}p < 0.05$ for Pos *versus* Neg; $^{*}p < 0.05$ for Pos *versus* γ-EC 50; $^{\ddagger}p < 0.05$ for Pos *versus* γ-EV 50; $^{\S}p < 0.05$ for Pos *versus* γ-EV 150 using unpaired Student's *t* test. D, length of colons from a) Neg, b) Pos, c) γ-EC 50, d) γ-EC 150, e) γ-EV 50, and f) γ-EV 150 groups. Images are representative examples of each group. E, colon shortening is presented as mean change in percent colon length compared to Neg ± SEM ($n = 12$ per group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* Pos. F, degree of ulceration, tissue damage and cellular infiltration in colons of a) Neg, b) Pos, c) γ-EC 50, d) γ-EC 150, e) γ-EV 50, and f) γ-EV 150 groups. Images are representative examples of each group at 50× (upper panel) and 20× (lower panel) magnification. G, histological scoring was carried out to assess colonic tissue injury and inflammation. Data is presented as mean score ± SEM ($n = 6$ per group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* Pos using one-way ANOVA with Dunnett's post test.

cells, and was likely due to partial expression of β-arrestin2. The ability of γ-EC to reduce TNF-α-stimulated phosphorylation of JNK and IκB was also abolished in β-arrestin2-deficient cells (Fig. 9C and D), suggesting that β-arrestin2 expression is required for the anti-inflammatory activity of γ-EC.

We further investigated the role of β-arrestin2 with respect to potential cross-talk between CaSR and TNFR-mediated signaling pathways. TGF-β-activated kinase 1 (TAK1) is an intermediate regulator in the TNFR signaling pathway, and is activated by TNF-α, resulting in the association of TAK1 with TAK1 binding protein (TAB1) and the

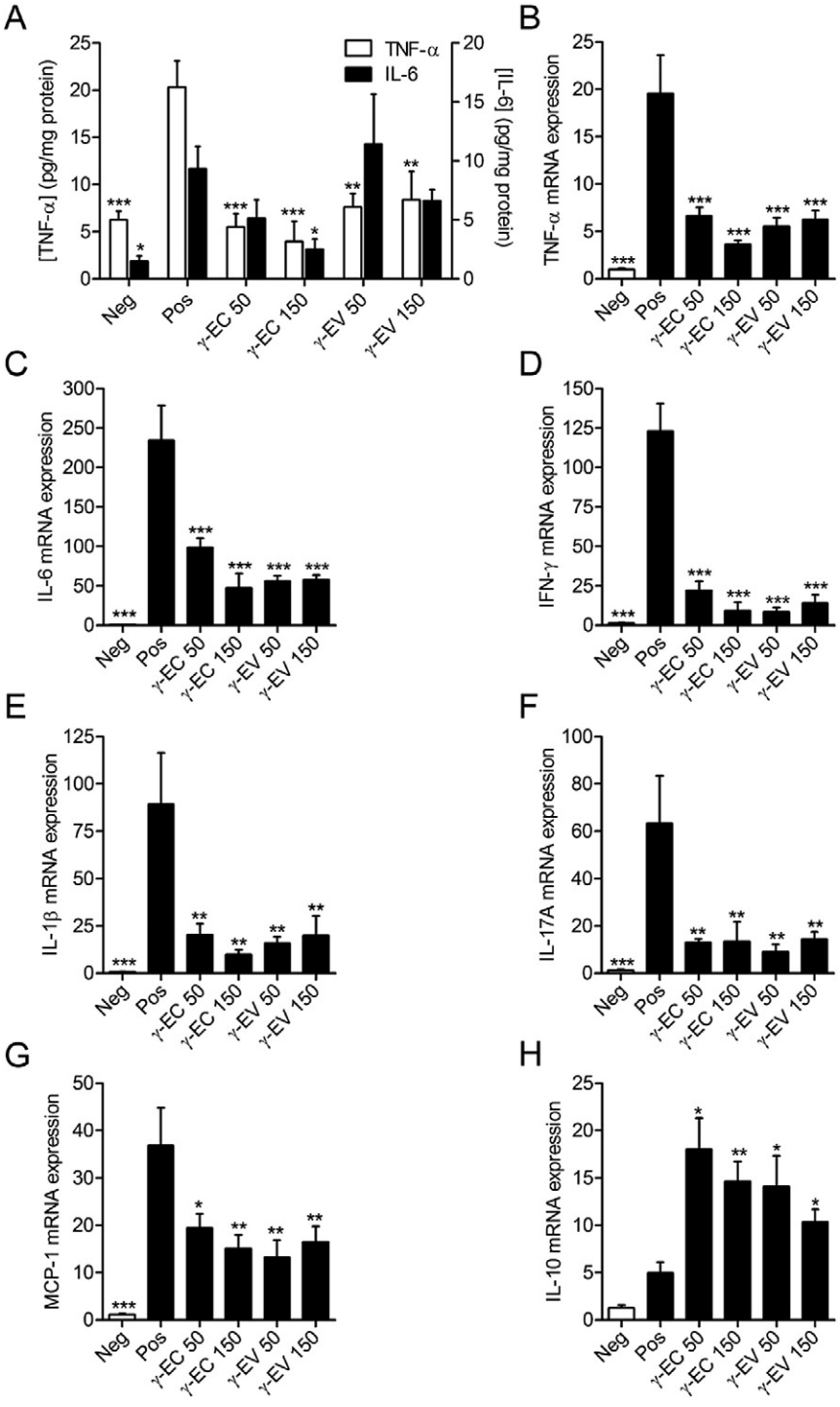

Fig. 7. γ-EC and γ-EV reduce expression of DSS-induced pro-inflammatory mediators in mice. A, TNF-α and IL-6 concentrations in colon homogenates were measured by ELISA. Results are expressed as cytokine amount per total protein concentration. Data are presented as mean ± SEM ($n = 6$ per group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* Pos. B, cytokine and chemokine mRNA expression in the colon was measured by quantitative real-time RT-PCR using GAPDH as the reference gene. Results are expressed as mRNA level relative to untreated (Neg) mice. Data are presented as mean ± SEM ($n = 6$ per group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* Pos using one-way ANOVA with Dunnett's post test.

activation of downstream IKK and JNK inflammatory pathways [34,35]. Lysates from Caco-2 cells treated with γ-EC and/or TNF-α were subjected to immunoprecipitation using antibodies against β-arrestin2 or TAK1, and co-immunoprecipitated proteins were detected by Western blotting analysis using TAB1- or CaSR-specific antibodies. γ-EC treatment resulted in association between β-arrestin2 and CaSR (Fig. 10A), both in the presence or absence of TNF-α (Fig. 10A). γ-EC also caused the interaction of β-arrestin2 with TAB1 (Fig. 10A), and reduced TNF-α-stimulated TAK1–TAB1 complex formation (Fig. 10B). In similar co-immunoprecipitation experiments using β-arr2 KD cells, γ-EC did not reduce TNF-α-stimulated TAB1–TAK1 association in β-arrestin2-deficient cells, in contrast to the corresponding control cells (Scramble) treated with both TNF-α and γ-EC (Fig. 10C). These results indicate that the activation of CaSR by γ-EC leads to the association of β-arrestin2

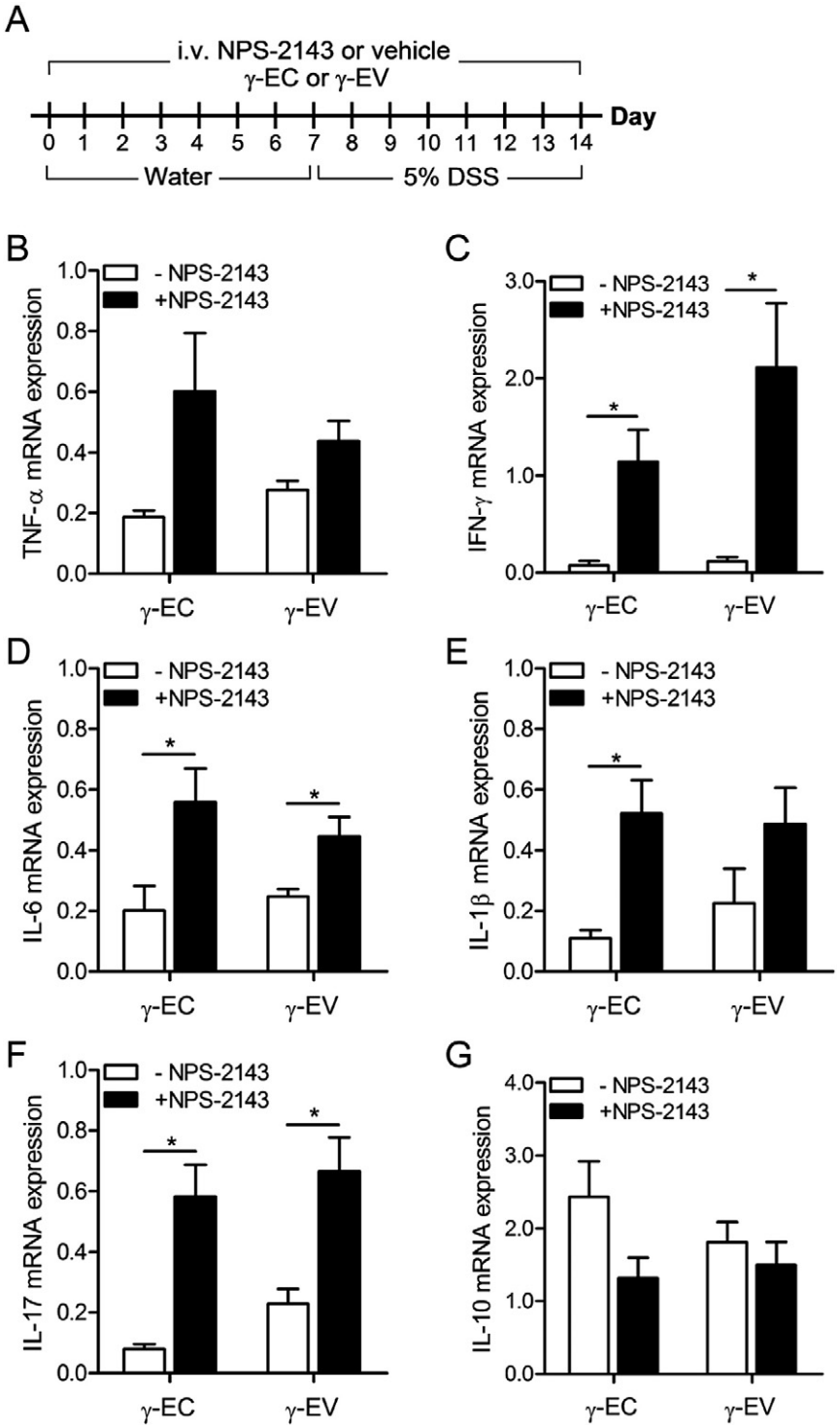

Fig. 8. NPS-2143 reduces anti-inflammatory effects of γ-EC and γ-EV in DSS-induced colitis. A, experimental design. Mice were injected i.v. with 1 mg/kg NPS-2143 or vehicle, followed by treatment with γ-EC or γ-EV (150 mg/kg) by oral gavage for 14 d. To induce acute colitis, 5% DSS was added to drinking water on day 7. B, cytokine mRNA expression in the colon was measured by quantitative real-time RT-PCR using GAPDH as the reference gene. Results are expressed as mRNA level relative to untreated (Neg) mice. Data are presented as mean ± SEM ($n = 6$ per group). *$p < 0.05$ *versus* corresponding group receiving no NPS-2143 antagonist using unpaired Student's *t* test.

with CaSR, and the subsequent binding of this complex to TAB1, reducing TAB1–TAK1 binding and thereby preventing TNF-α-induced stimulation of inflammatory pathways through TNFR, as illustrated in Fig. 11.

4. Discussion

We have demonstrated here that γ-EC and γ-EV exert CaSR-mediated anti-inflammatory activity *in vitro* in IECs and in a mouse model of intestinal inflammation. *In vitro*, the intracellular signaling triggered by γ-EC binding with CaSR interfered with TNF-α-stimulated pro-inflammatory signaling events, inhibiting activation of both IκBα and JNK pathways. We further demonstrated that γ-EC-induced activation of CaSR led to the recruitment of β-arrestin2 and subsequent association with TAB1, thereby interfering with TAK1-dependent inflammatory signaling activated by extracellular TNF-α. This is also the first such report of interaction, or cross-talk, between CaSR and TNFR in IECs.

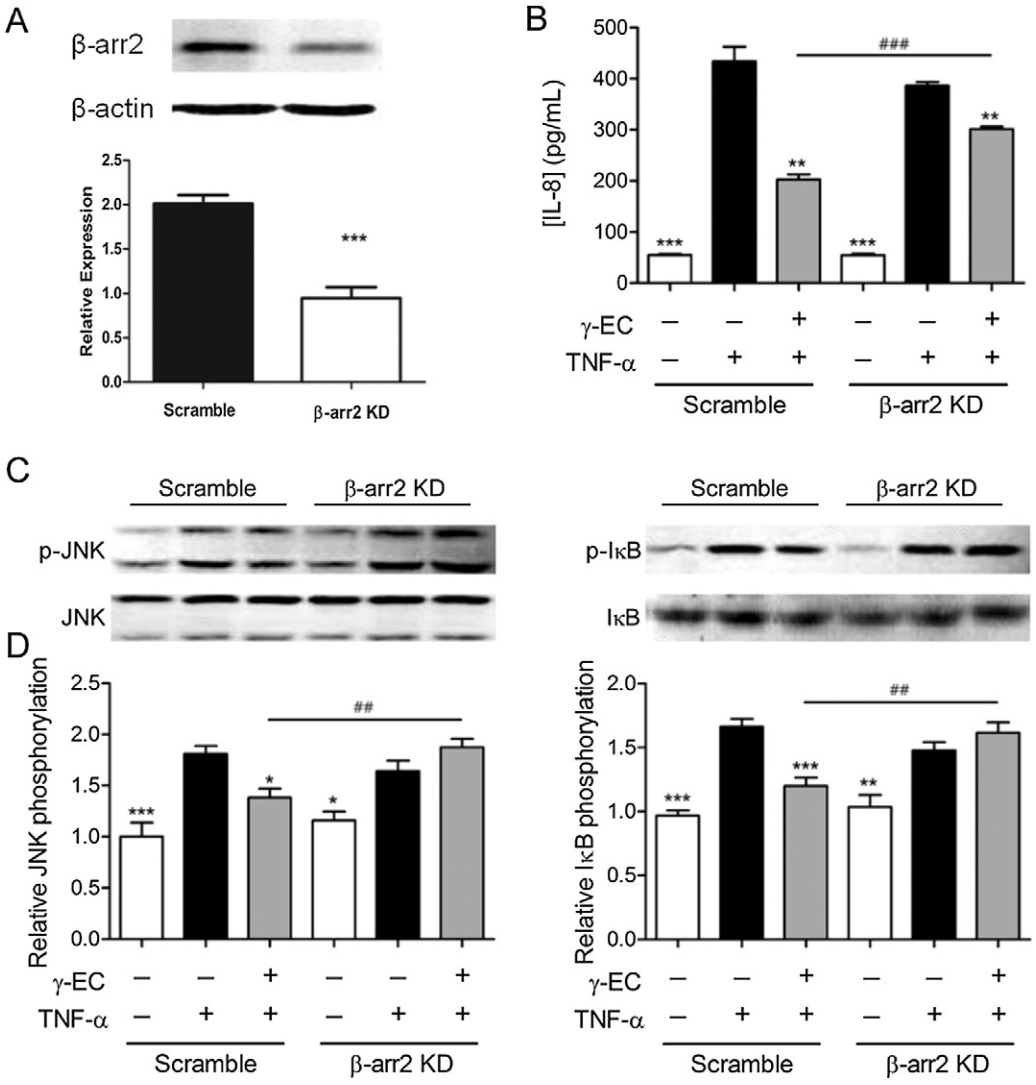

Fig. 9. Expression of β-arrestin2 is required for anti-inflammatory activity of γ-EC in Caco-2 cells. Caco-2 cells were transiently transfected with control siRNA (Scramble) or specific β-arrestin2 siRNA to produce β-arrestin2 knockdown cells (β-arr2 KD). β-arr2 KD or control (Scramble) Caco-2 cells were pre-treated for 2 h with 0.5 mM of γ-EC followed by stimulation with 2 ng/mL TNF-α for 30 min (Western blotting) or 4 h (ELISA). A, β-arrestin2 knock-down was confirmed by Western blotting, using β-actin as loading control. B, IL-8 concentration in supernatants was measured by ELISA. Data are presented as mean ± SEM ($n = 4$ independent determinations). $p < 0.001$ for interaction, and $p < 0.001$ for treatment effects using two-way ANOVA. $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* TNF-α alone. $^{\#\#}p < 0.01$ *versus* control (Scramble) cells using Tukey's post test. C, Western blot analysis of cell lysates was performed using antibodies against phospho-JNK, JNK, phospho-IκB, and IκB (images of one representative experiment of six is shown). D, quantitative analysis of relative levels of phosphorylated JNK and IκB using ImageJ software. Results are expressed as relative protein levels compared to untreated control cells. Data are presented as mean ± SEM ($n = 6$ independent determinations). $p < 0.01$ for interaction, $p < 0.001$ for treatment effects, and $p = 0.053$ (JNK) or $p = 0.1$ (IκB) for genotype effect (Scramble *versus* β-arr2 KD) using two-way ANOVA. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *versus* TNF-α alone. $^{\#\#}p < 0.01$ *versus* control (Scramble) cells using Tukey's post test.

During IBD, dysregulated mucosal immune responses to normal microflora result in damage to the intestinal epithelial barrier and activation of immune cells in the lamina propria, leading to the overproduction of pro-inflammatory cytokines [7,36]. The increased expression of pro-inflammatory mediators including TNF-α and IFN-γ induces the production of additional inflammatory cytokines such as IL-1β, IL-6 and IL-17. Current evidence suggests that TNF-α plays a key role in IBD pathogenesis, disrupting intestinal homeostasis and preventing activation of regulatory cells [9,37]. As such, TNF-α and its associated pathways have become an important therapeutic target, and biological therapies, including anti-TNF-α monoclonal antibodies, have been shown to aid in successfully managing IBD symptoms in humans [10]. *In vitro*, reduction of TNF-α-associated expression of the chemokine IL-8 by Caco-2 cells has been linked to inhibition of NF-κB activation, and correlated with *in vivo* anti-inflammatory activity in mice [38]. Here, we observed that both γ-EC and γ-EV reduced TNF-α-induced IL-8 secretion, as well as the expression of TNF-α-induced pro-inflammatory mediators *in vitro* in Caco-2 cells. Moreover, γ-EC and γ-EV increased IL-10 expression, an anti-inflammatory or regulatory cytokine important in maintaining intestinal homeostasis [39].

JNK, ERK 1/2, and p38 MAPK and their related signaling components play an important role in the perpetuation of the inflammatory response in IBD, and MAPK inhibitors, including those against JNK and p38 have been examined as possible IBD therapeutics in animal models and human studies [40]. In Caco-2 cells, TNF-α similarly activates MAPK and NF-κB pathways, leading to the production of pro-inflammatory mediators [41]. Treatment of Caco-2 cells with γ-EC reduced phosphorylation of JNK and IκBα, which is necessary for NF-κB transcriptional activation [42], in response to TNF-α. However, p38 was not affected by either TNF-α or γ-EC treatment. This is consistent with reports that dietary anti-inflammatory compounds can exert their effects by blocking MAPK signaling and NF-κB activation [43], and a recent study demonstrated that anti-inflammatory polyphenols could inhibit TNF-α-stimulated JNK phosphorylation and NF-κB translocation in Caco-2 cells, but did not prevent p38 or ERK activation [44]. This would suggest that the *in vitro* anti-inflammatory activity of γ-glutamyl

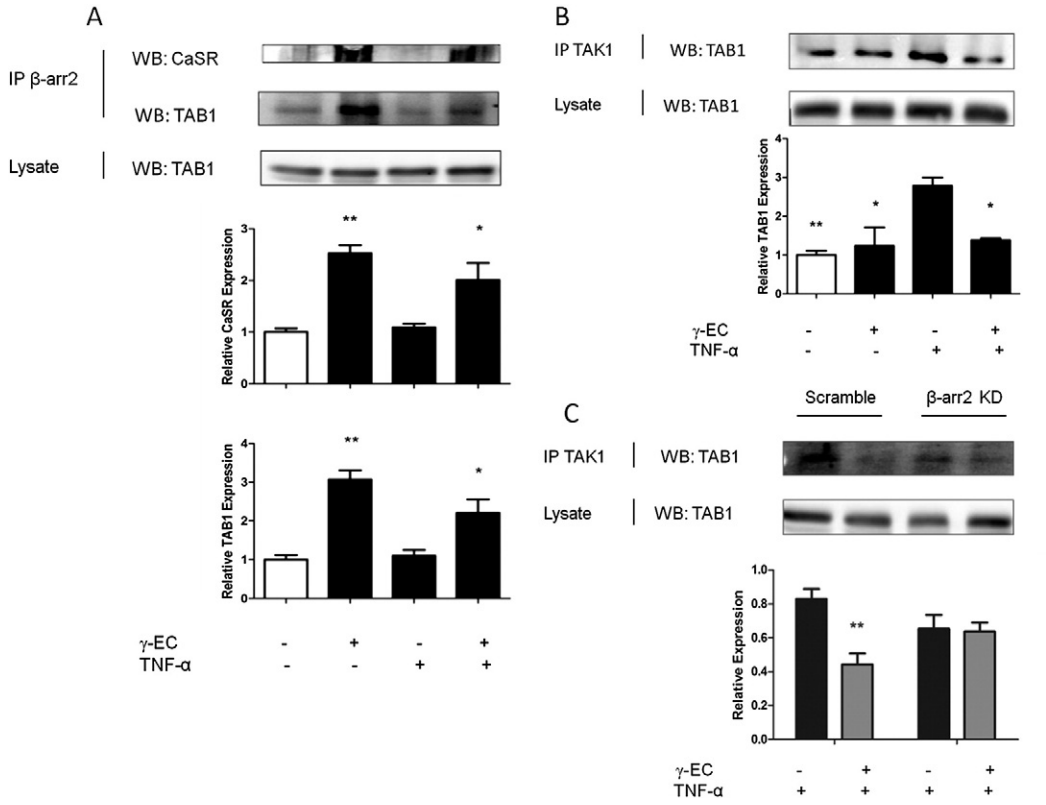

Fig. 10. Effect of γ-EC on TNF-α-stimulated TAB1–TAK1 association in Caco-2 cells. A, Caco-2 cells were treated for 2 h in the presence or absence of 0.5 mM of γ-EC followed by stimulation with or without 2 ng/mL TNF-α for 30 min. Cell lysates were subjected to immunoprecipitation (*IP*) using anti-β-arrestin2 (β-arr2) antibodies, and Western blotting (*WB*) was performed using anti-CaSR or anti-TAB1 antibodies. B, Caco-2 cells were treated for 2 h in the presence or absence of 0.5 mM of γ-EC followed by stimulation with or without 2 ng/mL TNF-α for 30 min. Cell lysates were subjected to immunoprecipitation (*IP*) using anti-TAK1 antibodies, and Western blotting (*WB*) was performed using anti-TAB1 antibodies. C, β-arrestin2 knock-down (β-arr2 KD) or control (Scramble) Caco-2 cells were pre-treated for 2 h in the presence or absence of 0.5 mM of γ-EC followed by stimulation with 2 ng/mL TNF-α for 30 min. Cell lysates were subjected to immunoprecipitation (*IP*) using anti-TAK1 antibodies, and Western blotting (*WB*) was performed using anti-TAB1 antibodies. An aliquot of the cell lysate was also analyzed by Western blotting using antibodies against TAB1. Data are presented as mean ± SEM (n = 3), *p<0.05 and ** p<0.01 versus TNF-α alone using one-way ANOVA with Dunnett's post test.

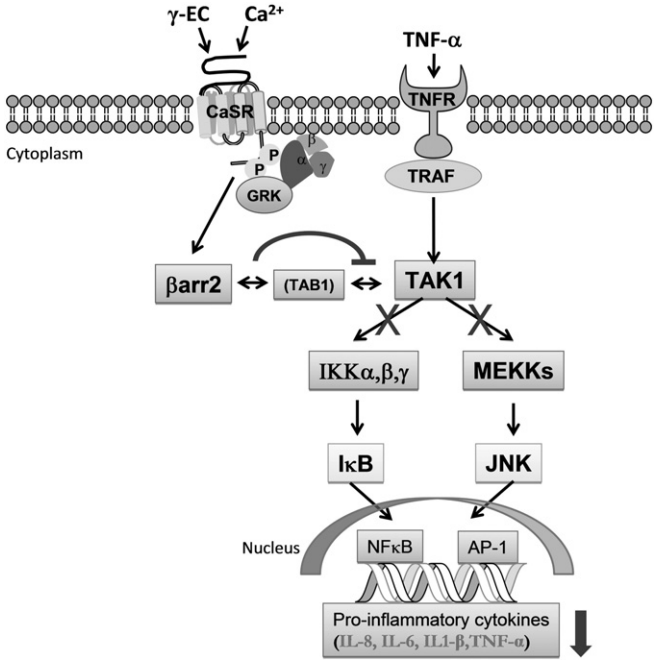

Fig. 11. Putative mechanism for CaSR-mediated anti-inflammatory effect of γ-EC in Caco-2 cells. Ligand activation of CaSR by γ-EC leads to phosphorylation of CaSR by GRK, resulting in the interaction of β-arrestin2 with CaSR and TAB1, reducing TNF-α-induced TAB1–TAK1 complex formation and activation of JNK and IκB inflammatory pathways.

peptides may be due in part to their ability to inhibit JNK phosphorylation and reduce NF-κB activation. CaSR is known to be expressed by intestinal epithelial cell lines, including Caco-2 cells [45,46]. The CaSR-specific antagonist NPS-2143 was employed here to confirm that the anti-inflammatory effects of γ-EC and γ-EV were mediated *via* activation of CaSR. As expected, NPS-2143 abrogated the *in vitro* anti-inflammatory effects of γ-EC and γ-EV, restoring IL-8 secretion, JNK and IκB phosphorylation in Caco-2 cells treated with TNF-α. Similarly, activation of the GPCR GPR120 in mouse macrophage cells was found to inhibit TNF-α-mediated inflammatory responses, preventing phosphorylation of JNK and IKKβ, IκB degradation, and inflammatory cytokine expression [11].

A mouse model of DSS-induced colitis was used to evaluate the anti-inflammatory effects of γ-EC and γ-EV *in vivo*. DSS causes damage to the intestinal epithelium and impairs gut barrier function by activating the NF-κB pathway, leading to mucosal inflammation and ulceration, neutrophil infiltration, colon shortening and diarrhea [47,48]. We have previously shown that dietary di- and tri-peptides could reduce the severity of inflammation and down-regulate the expression of pro-inflammatory mediators in animal models of colitis [26,49]. Here, treatment with γ-EC and γ-EV reduced DSS-induced clinical colitis signs, colon shortening, and histological damage. Surprisingly, the high dose of γ-EC did not reduce DSS-induced weight loss, despite an apparent reduction in all other colitis parameters, suggesting that there might be other potential effects of a high concentration of γ-EC on growth rate which will need to be further examined. In line with our *in vitro* results in Caco-2 cells, γ-EC and γ-EV also reduced the expression of pro-inflammatory cytokines and chemokines in the colon, and up-regulated the expression of IL-10. The *in vivo* anti-inflammatory effects of γ-EC and γ-EV were likewise shown to be mediated by CaSR

activation, providing the first evidence that the CaSR agonist γ-glutamyl peptides may be effective for the reduction of intestinal inflammation.

To elucidate the mechanism by which γ-glutamyl dipeptides could reduce TNF-α-stimulated inflammation, we examined the role of β-arrestin-mediated signaling and cross-talk between CaSR and TNFR. β-Arrestins can regulate immune responses by coordinating NF-κB-dependent signaling pathways in cells, and association of β-arrestins with IκBα has been found to inhibit NF-κB transcriptional activation in various cell types [22,50]. In the present study, the expression of β-arrestin2 in Caco-2 cells was required for the anti-inflammatory activity of γ-EC, and in particular JNK and IκB phosphorylation. Because TNF-α-induced phosphorylation of both JNK and IκB was inhibited in γ-EC-treated Caco-2 cells, it suggests that the underlying mechanism may involve the interaction of β-arrestin2 with upstream signal proteins in the TNF-α pathway. The importance of TNF-α and its receptor, TNFR, in inflammation and IBD is well known [51]. Upon TNF-α binding to TNFR, TNFR-associated factors (TRAFs) detach from the receptor and form a trimeric complex with TAK1, TAB1 and TAB2, which mediates activation of MAPKs and NF-κB signaling [52–54]. We therefore undertook to examine the potential role of TAK1 involvement in cross-talk between CaSR- and TNFR-mediated signaling pathways. TAB1 is an adaptor protein and plays a critical role to activate TAK1 [34], and recently it was demonstrated that activation of the GPCR GPR120 could mediate association between β-arrestin2 and TAB1, thereby inhibiting TAK1-activated inflammatory responses in mouse macrophage cells [11]. Here we have shown that cross-talk between CaSR and TAK1-mediated inflammatory pathways was bridged by β-arrestin2 associated with TAB1 in Caco-2 cells. These results suggest that interaction between β-arrestin2 and TAB1 inhibited TNF-α-induced TAK1 activation and subsequent downstream signal transduction in IECs treated with γ-EC. The non-canonical activation of CaSR by allosteric modulation may play the key role in cross-talk with TNF-α-activated signaling event.

In conclusion, we have shown that the γ-glutamyl dipeptides γ-EC and γ-EV can act *via* allosteric ligand activation of CaSR to exert intestinal anti-inflammatory effects, and may be a novel nutraceutical or pharmaceutical-based strategy for the prevention of inflammation in IBD. The results of this study also revealed that the activation of CaSR by γ-glutamyl dipeptides leads to the association of β-arrestin2 with TAB1 to block activation of the TNF-α-dependent pro-inflammatory signaling cascade through TNFR, a previously unknown function of CaSR.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.12.023.

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