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Basic Study

Impact of intrarectal chromofungin treatment on dendritic cells-related markers in different immune compartments in colonic inflammatory conditions

Kunal Kapoor, Nour Eissa, Diane Tshikudi, Charles N Bernstein, Jean-Eric Ghia

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Abstract

BACKGROUND

Chromofungin (CHR: chromogranin-A 47-66) is a chromogranin-A derived peptide with anti-inflammatory and anti-microbial properties. Ulcerative colitis (UC) is characterized by a colonic decrease of CHR and a dysregulation of dendritic CD11c⁺ cells.

AIM

To investigate the association between CHR treatment and dendritic cells (DCs)-related markers in different immune compartments in colitis.

METHODS

A model of acute UC-like colitis using dextran sulphate sodium (DSS) was used in addition to biopsies collected from UC patients.

RESULTS

Intrarectal CHR treatment reduced the severity of DSS-induced colitis and was associated with a significant decrease in the expression of CD11c, CD40, CD80, CD86 and interleukin (IL)-12p40 in the inflamed colonic mucosa and CD11c,

Bernstein CN has been on the advisory boards for Abbvie Canada, Amgen Canada, Bristol Myers Squibb Canada, Janssen Canada, Roche Canada, Sandoz Canada, Takeda Canada, Pfizer Canada and consulted to Takeda and Mylan Pharmaceuticals. He has received educational grants from Abbvie Canada, Pfizer Canada, Takeda Canada, Janssen Canada. He has been on speaker's panel for Abbvie Canada, Medtronic Canada and Janssen Canada. The other authors declare that they have no conflicts of interest.

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CD80, CD86 IL-6 and IL-12p40 within the mesenteric lymph nodes and the spleen. Furthermore, CHR treatment decreased CD80 and CD86 expression markers of splenic CD11c⁺ cells and decreased NF- κ B expression in the colon and of splenic CD11c⁺ cells. In vitro, CHR decreased CD40, CD80, CD86 IL-6 and IL-12p40 expression in naïve bone marrow-derived CD11c⁺ DCs stimulated with lipopolysaccharide. Pharmacological studies demonstrated an impact of CHR on the NF- κ B pathway. In patients with active UC, CHR level was reduced and showed a negative linear relationship with CD11c and CD86.

CONCLUSION

CHR has protective properties against intestinal inflammation *via* the regulation of DC-related markers and CD11c⁺ cells. CHR could be a potential therapy of UC.

Key Words: Chromofungin; Chromogranin-A; Colitis; Cytokines; Dendritic cells; Gut hormones

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Core Tip: Ulcerative colitis (UC) is characterized by a colonic decrease of chromofungin (CHR: chromogranin-A 47-66) and a dysregulation of CD11c⁺ dendritic cells (DC). Using a UC-like model (dextran sulphate sodium) and biopsies collected from UC patients, we demonstrated a protective effect of CHR *via* the regulation of DC-related markers and CD11c⁺ cells at the colonic, mesenteric lymph node and spleen levels, through a potential effect *via* the NF- κ B pathway. In patients with active UC, CHR level showed a negative linear relationship with CD11c⁺ marker. CHR could be a potential therapy of UC, but larger samples and additional experiments are needed.

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INTRODUCTION

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD), disorders in which the gastrointestinal (GI) tract becomes ulcerated and inflamed[1,2]. To date, the exact etiopathology and mechanism behind them are unknown. It is believed that genetic predisposition and environmental factors lead to an abnormal mucosal immune response in the intestinal lining[3-5]. Over the last decade, the diseases have increased worldwide, but the greatest prevalence is evident in western countries[6,7]. Although treatments and therapeutic strategies are evolving quickly, treatments are still inadequate for a substantial percent of those with active IBD, and some therapies may have serious adverse side effects. Therefore, IBD needs new therapeutic approaches associated with higher efficacy and limited side effects[8].

IBD is characterized by an ongoing inflammatory process manifested by an increase of immune cell infiltration and accompanied by an up-regulation of pro-inflammatory cytokines, including interleukin (IL)-6 and IL-12p40[9]. The continuing release of mediators damages the intestinal epithelium, leading to abnormal activities of enterochromaffin cells (EC), goblet cells and Paneth cells, and impaired tissue repair[10,11]. UC is represented by an overactivation of the adaptive immune system represented by T cells which is considered a consequence of abnormal activation of innate immune cells[12] such as dendritic cells (DCs)[10,11,13-17].

DCs are produced by the bone marrow and can be located in the mucosal tissues or circulate in the lymph tissues and the blood[18]. DCs interaction and antigen presentation to T cells and their subsequent polarization can be induced within mesenteric lymph nodes (MLNs) and the spleen[19]. In UC, as in other chronic inflammatory diseases, DC activation is represented by an up-regulation of the CD80, CD86, and CD11c markers and is associated with an increased release of IL-6 and IL-12p40 to

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promote a Th1 cell polarization[20,21]. In UC, the IL-12p40 pathway's importance has been confirmed by genome-wide association studies, identifying the IL-12 genes as a major contributor[22,23]. Intracellularly, the production of IL-12p40 is regulated by different pathways, including NF- κ B. In IBD, a significant increase in NF- κ B p65 has been observed in the gut epithelial and immune cells isolated from colonic biopsies [24]. More specifically related to DCs, the NF- κ B pathway plays a crucial role in maintaining their intracellular activation responsible for T-cell polarization[25]. Therefore, the excessive pro-inflammatory cytokine production can in part be related to the overactivation of DCs in the gut[26]. Nevertheless, in certain conditions, the level and expression of DCs activation markers are not quite equivalent when comparing the different immune compartments (*i.e.* colon, MLN, spleen)[19].

Chromogranin-A (CHGA) is the second most prominent protein found in the secretory granules of ECs in the GI tract. For decades, CHGA has been known to be associated with IBD, but few data have demonstrated a plausible mechanism of association due to the lack of a specific CHGA receptor[27]. An increase in the total number of endocrine cells defined as CHGA-immunoreactivity has been described in several cohorts of patients with active IBD[28]. In parallel, the serum CHGA concentration has been reported to be significantly increased compared to controls[29-32]. CHGA is considered a prohormone and can be the precursor of several bioactive peptides having a wide variety of functions in the human body affecting the cardiovascular, endocrine, neuroendocrine and immune systems[33]. One of the peptides generated, CHR (CHGA₄₇₋₆₆), encoded by Exon-IV located at the N-terminal section of CHGA, has been demonstrated to depict anti-fungal and anti-microbial activities[34]. Recently, in the context of UC and experimental colitis, new data highlighted an essential anti-inflammatory role through epithelial cells and peritoneal macrophage regulation[25], but no data exist regarding the broader effect within the immune system and its different compartments.

Taken together, we hypothesized that CHR regulates DCs activation in the context of UC experimental colitis, and we evaluated CHR's effects on different DCs-related markers at various immune compartments and defined a potential intracellular pathway implicated.

MATERIALS AND METHODS

Human participants

Biopsies were collected from participants undergoing colonoscopy with known UC or with no IBD. A total of four biopsies were taken from inflamed sites identified endoscopically in persons with active UC ($n = 10$) and from normal tissue from healthy participants ($n = 10$). Biopsy collection was approved by the University of Manitoba Research Ethics Board. Participants were ≥ 18 years. All concomitant IBD therapies, including 5-amino salicylates, corticosteroids, and methotrexate, were permitted in active UC patients. Healthy control individuals had no history of abdominal afflictions, especially IBD. For more details, see Kermarrec *et al*[20]. The individuals in the control group were not on any regular medication at the time of the study. The University of Manitoba Research Ethics Board [HS14878 (E)] approved the study.

Animal and ethics statement

All the experiments were conducted under protocols # 15-010 and #19-014, approved by the University of Manitoba Ethics Committee under Canadian animal research guidelines. In-house groups of 6 male C57BL/6 mice (six to eight weeks old) with bodyweight between 20 g to 25 g were used. All animals were kept in the specific pathogen-free barrier facility maintained at the University of Manitoba animal care facility. Animals were kept in a 12-h dark/light cycle and fed ad libitum.

Peptide

CHGA₄₇₋₆₆: RILSILRHQNLKELQDLAL[35-39] were synthesized by a solid-phase method and purified by reverse-phase high-performance liquid chromatography to reach $< 98\%$ purity (Pepmic Co., Suzhou, China). As previous studies from our laboratory demonstrated an absence of effect of the scramble version[25], this group was not added to our experimental plan.

Dextran sulphate sodium-induced experimental colitis and experimental plan

Based on previously published data, CHR's dose was adjusted to 2.5 mg/kg per day

[40]. The 1% of phosphate buffer saline (PBS, 1%) (Life Technology, Grand Island, NY, United States) was given intra-rectally to the controls[40], all the injections of both CHR and PBS started one day before the induction of experimental colitis and lasted for 5 d. Dextran sulphate sodium (DSS) (molecular weight, 40 kDa; MP Biomedicals, Soho, OH, United States) was added to regular drinking water and freshly replaced every two days at a concentration of 5% (wt/vol) for 5 d till the mice are sacrificed [41]. The average consumption of DSS was noted per cage each day. Animals were randomized, 6 mice per group were assigned for each experiment. Time matching of the controls was done with mice receiving normal drinking water only.

Assessment of DSS-induced colitis

Disease activity index (DAI) included a combined score of weight loss, stool consistency, and bleeding was recorded daily[42] from day zero to day five during DSS treatment. Blood in the stool was evaluated using the Hemocult II test (Beckman Coulter, Oakville, ON, Canada). Colon length and macroscopic scores[42] were assessed on sacrifice day.

Spleen and MLN isolation

After the induction of DSS colitis on sacrifice day, the spleen and MLN were collected and digested in 2 mg/mL collagenase D (Roche Diagnostics, Meylan, France) dissolved in RPMI 1640 (Life Technologies) at 37 °C for 30 min associated with a 10 min intermittent shaking. To stop the reaction and disrupt the DC-T cell complexes, the cell suspension was supplemented with 5 mmol/L EDTA (Sigma, Mississauga, ON, Canada) during the last 5 min. The homogenate was then passed through a 70-mm cell strainer (VWR, Mississauga, ON, Canada) and then washed with RPMI-1640 at 1200 rpm for 5 min. Red blood cells (RBC)'s lysis was done using ACK lysis buffer (150 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA; Life Technologies).

MACS CD11c sorting

EasySep™ Mouse CD11c Positive selection kit (Stemcell Technologies) was used according manufacturer's instructions. The cells were then collected, counted and used for stimulatory experiments.

Isolation and stimulation of bone marrow-derived DCs

Six to eight weeks naïve mice were sacrificed by cervical dislocation. In sterile conditions, the femur and tibia were removed and soaked in RPMI-1640 medium [containing 10% heat-inactivated fetal bovine serum (FBS), 25 mg/mL gentamicin, 2 mmol/L L-glutamine) (Life Technologies)]. Bone marrow cell extraction was performed by cutting both ends of the bones and flushing them with 1 mL of sterile RPMI-1640 into a sterile Petri dish. The cell suspension was collected and centrifuged for 5 min at 1000 rpm. The supernatant was discarded, and pellets were resuspended in RBC ACK lysis buffer (150 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA; Life Technologies) for the lysis of RBC. After the second centrifugation, the pellet was resuspended in RPMI-1640 and counted[43]. The purity of the cells was then verified by flow cytometry. Granulocyte-macrophage induced colony-stimulating factor (GM-CSF, Cedarlane, Burlington, ON, Canada) induced culture of bone marrow-derived DCs (BMDCs) were used for the cell culture. Cells were suspended at a concentration of 1 × 10⁶ cells/mL in culture plates containing complete RPMI 1640 medium and stimulated with 20 ng/mL of GM-CSF. The cell culture was then put in an incubator at 37 °C with 5% CO₂. GM-CSF cultured media was replaced on the 3rd and 6th day. On the 8th day, the semi-suspended cells were collected with gentle pipetting, and the loosely attached cells were scraped using a cell scraper generating mature cells[43,44]. The CD11c⁺ cells (1 × 10⁶ per well) isolated *via* MACS were cultured in complete RPMI 1640 medium (Life Technologies) containing 10% heat-inactivated FBS, 25 mg/mL, gentamicin and 2 mmol/L L-glutamine (Life Technologies) in 12-well plates in the presence or absence of 10⁻⁶ M of CHR for 12 h. After 12 h, they were stimulated with 100 ng/mL of lipopolysaccharide (LPS; Sigma) for 24 h. The cell culture was collected for quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis, and ELISA assessed the supernatant. To determine the intracellular pathway, pharmaceutical blocker/activator, betulinic acid (NF-κB activator, 10 μmol/L; Sigma) or BAY 11-7082 (NF-κB inhibitor, 10 μmol/L; Sigma) were added to the culture medium for 24 h at the same time of LPS.

qRT-PCR

Total RNA from colonic tissue, MLN, splenocytes, CD11c⁺ splenic and CD11c⁺ BMDC

isolated cells was extracted using TRIzol™ Plus RNA Purification Kit (Life Technologies) and reverse-transcribed using SuperScript VILO cDNA Synthesis Master Mix (Life Technologies). Gene expression was measured by a qRT-PCR using a Roche Light Cycler 96 Real-Time System and Power SYBR green master mix (Life Technologies). Differences in the threshold cycle (Δ Ct) number between the target genes and the optimal reference gene Eukaryotic elongation factor 2 (*Eef2*)[45] were used to calculate differences in expression. Human and mouse primer sequences used are provided in Tables 1 and 2.

RT2-profiler qPCR array

RT2 profiler™ PCR array mouse dendritic and antigen-presenting cell (Qiagen Inc, Toronto, ON Ref #330231) was performed for the quantitative PCR according to the manufacture instructions to profile the expression of 84 essential genes. Changes in gene expression between different experimental groups and the heat maps (colour-coded graphs with groups in columns and genes in rows) were generated using the web-based program of RT2 profiler PCR array data analysis.

Protein quantification

Enzyme-linked immunosorbent assays (ELISAs) were used for protein quantification from full-thickness tissue homogenates and/or supernatants from the cell cultures. Mouse commercial ELISA kits were used to detect IL-6 and IL-12p40 (R & D Systems, Inc., Minneapolis, United States).

Statistical analysis

Data are expressed as the mean \pm SE. Unpaired Mann-Whitney *U* test was applied to compare between two groups. To compare between more than two groups, One-Way ANOVA followed by a post-hoc test was used. The analysis DAI repeated measure Two-Way ANOVA followed by a post-hoc test was used. To analyze the association between different markers studied, Spearman's correlation test was used. The statistical two-tailed significance level was determined at $P = 0.05$. GraphPad Prism software (version 6; GraphPad Software, Inc, La Jolla, CA, United States) was used to compute the statistics.

RESULTS

In colitic mice, in vivo CHR treatment decreased colonic inflammatory macro- and micro-scopic scores

As reported previously[40], we confirmed CHR's anti-inflammatory effect on experimental colitis induction. CHR treatment (2.5 mg/kg/day, *i.r.*) resulted in a marked reduction in the external DAI (Figure 1A). No difference was seen within the first two days, but starting day 3, a significant difference was detected, culminating at a 3-fold reduction on day 5 when compared with the colitic group. We also confirmed the beneficial effect of the CHR treatment on the macroscopic score ($P < 0.0002$) when distal colonic sections were examined (Figure 1B).

In colitic mice, in vivo CHR treatment decreases whole colonic section DCs-related surface and functional markers

RT²-PCR array analysis of DCs-related membrane and functional markers was conducted on whole colonic sections. As presented in Figure 1C, whole colitic samples isolated from mice treated with CHR demonstrated a significant downregulation of surface and intracellular markers, notably: CD86 4.43-fold ($P < 0.04$, $n = 6$ per group), CD80 4.21-fold ($P < 0.04$, $n = 6$ per group) and NF- κ B1 11.01-fold ($P < 0.02$, $n = 6$ per group). To confirm the data, gene expression level using a single target qRT-PCR technique was used. Development of colitis induced a significant colonic increase of mRNA expression of surface markers 86 ($P = 0.0089$, $n = 6$ per group) and CD80 ($P = 0.0005$) when compared with the non-colitic PBS group (Figure 2A and B). Treatment with CHR significantly decreased the two markers ($P = 0.0001$ and $P < 0.0001$ respectively, $n = 6$ per group). Besides, two others DC-related markers not present in our initial array were quantified. Induction of colitis induced a significant colonic increase of CD40 ($P < 0.0001$, $n = 6$ per group) and CD11c ($P < 0.0001$, $n = 6$ per group) when compared with the non-colitic PBS group. The same pattern of significant decrease was found for CD40 ($P = 0.0002$, $n = 6$ per group) and CD11c ($P < 0.0001$, $n = 6$ per group) (Figure 2C and D) in colon treated with CHR when compared with the

Table 1 Human primers sequences

Gene name	Forward (5'-3')	Reverse (3'-5')
<i>INFg</i>	GCCTATCTIGTCACCATCTCATC	CCCTCGGCTGCTGGTATTTAT
<i>CCR7</i>	GTTCAAATCTCAGGTCCCTCTC	TACCCCTGGTGATCCTCATCTTA
<i>CD74</i>	CCCAAGGAAGAGCCAATGT	CATGGCCCTGAAAAGCTGATA
<i>Il12p40</i>	ACCAGAGCAGTGAGGTCTTA	CTCCTTTGTGACAGGTGTACTG
<i>CD86</i>	CAGACCACATTCCTTGGATCA	CCGCTTCTTCTTCTCCATTTC
<i>TBP</i>	CCCGAAACGCCGAATATAATCC	AATCAGTGCCGTGGTTCGTG
<i>CD11C</i>	ACTCAGATCGGCTCCTACTT	TCGGGTCTGCTCGTAGTAAT
<i>IL12A</i>	ATTCCAGAGAGACCTCTTTCATAAC	CCACCTGGTACATCTTCAAGTC
<i>IL23A</i>	CAGGTCACTATTCAATGGGATGC	GCAGTCTTAATTGCTGCTTGG

Table 2 Mouse primers sequences

Gene	Forward (5'-3')	Reverse (3'-5')
<i>IL6</i>	CCTGAACCTTCCAAAGATGGC	TTCACCAGGCAAGTCTCCTCA
<i>CD86</i>	TTACGGAAGCACCCACGATG	ACTACCAGCTCACTCAGGCT
<i>CD11c</i>	CCAAGACATCGTGTCTCTGATT	ACAGCTTTAACAAAGTCCAGCA
<i>Eef2</i>	TGTCAGTCATCGCCCATGTG	CATCCTTGGCAGTGTGTCAGTGA
<i>CD80</i>	TCGGCCGAGTAATAACAGTC	GTTTCTCTGCTTGCCTCATTTTC
<i>CD40</i>	GTCACACAGGAGGATGGTAAAG	AAAGCAGTTCACAGGTTTCAG
<i>IL12p40</i>	TGGTTTGCCATCGTTTTGCTG	ACAGGTGAGGTTCACTGTTTCT
<i>CCR7</i>	TGGCTCTCTTGTCAATTTCCA	CTTGAAGCACACCGACTCGTA
<i>IFNγ</i>	CTCTTCTCATGGCTGTTTCT	TTCTTCCACATCTATGCCACTT

untreated group. Besides, a significant increase in colonic mRNA expression and protein quantification of IL-12p40 ($P < 0.0001$, $n = 6$ per group) were detected in colitic conditions when compared with the untreated group, and CHR treatment decreased those two markers ($P < 0.0001$ and $P < 0.0043$ respectively, $n = 6$ per group) (Figures 2E and F) when compared with the untreated group.

In colitic mice, in vivo CHR treatment decreases MLN DCs-related surface and functional markers

Next, we assessed the regulation of the different markers within the MLN. Induction of colitis was associated with a significant increase of CD86 ($P < 0.0001$, $n = 6$ per group), CD80 ($P < 0.0001$, $n = 6$ per group) and CD11c ($P < 0.0001$, $n = 6$ per group) mRNA expression (Figures 3A-C) when compared with non-colitic PBS group. In colitic mice, when compared with PBS group, CHR treatment decreased significantly CD86 ($P < 0.0001$, $n = 6$ per group) (Figure 3A), CD80 ($P < 0.0001$, $n = 6$ per group) (Figure 3B) and CD11c ($P < 0.0001$, $n = 6$ per group) (Figure 3C) mRNA expression. Also, induction of colitis induced a significant increase of IL-12p40 ($P < 0.0001$, $n = 6$ per group) and IL-6 ($P < 0.0001$, $n = 6$ per group) within the MLN when compared with the PBS group, and CHR treatment significantly decreased those two markers ($P < 0.0001$, $n = 6$ per group) (Figure 3D and E) when compared with untreated group. No significant changes were observed for CD40 (data not shown).

In colitic mice, in vivo CHR treatment decreases splenocytes DCs-related surface and functional markers

We assessed the expression of the different markers within the spleen cells. Induction of colitis was associated with a significant increase of splenocytes CD86 ($P < 0.0001$, $n = 6$ per group), CD80 ($P < 0.0001$, $n = 6$ per group) and CD11c ($P < 0.0001$, $n = 6$ per group) mRNA expression (Figures 4A-C) when compared with PBS group. In colitic

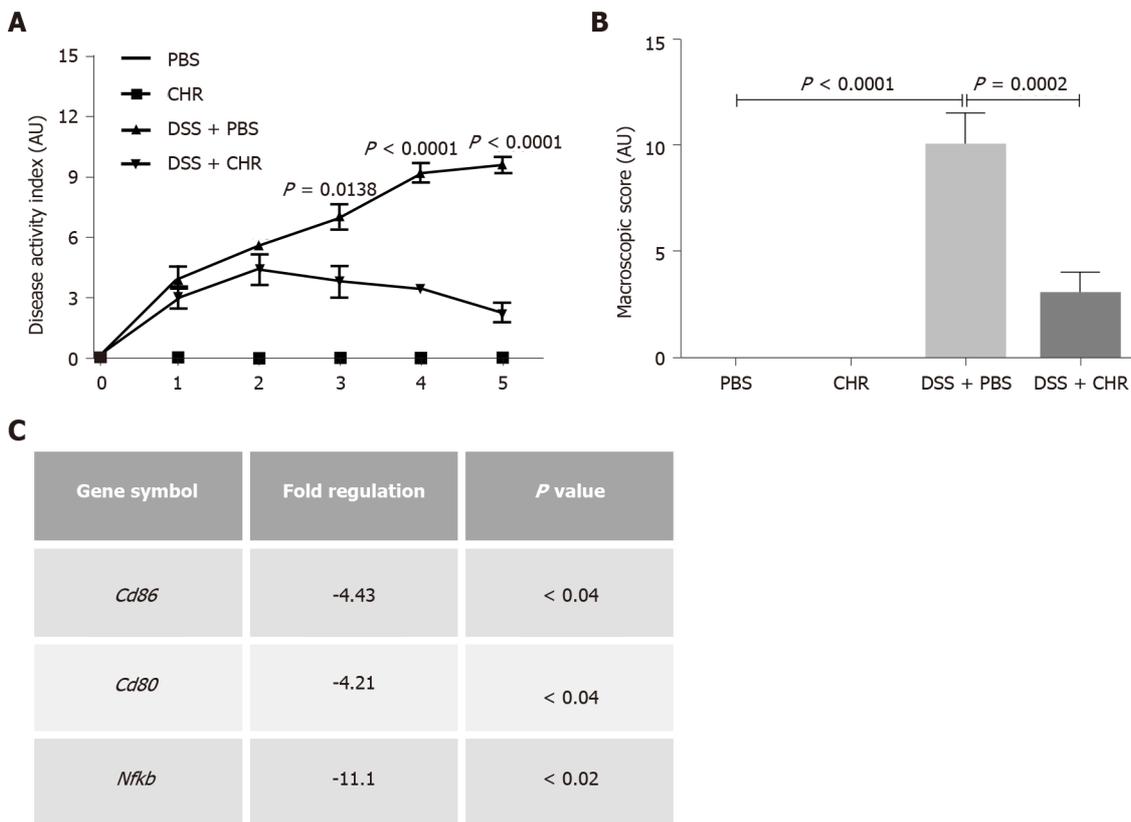


Figure 1 Chromofungin (chromogranin-A 47-66) treatment decreases dextran sulphate sodium-induced experimental colitis and colonic colonic cluster of differentiation markers and NF-κB gene expression in dextran sulphate sodium-induced experimental colitis. Colitis was induced with 5% dextran sulphate sodium in drinking water to C57BL/6 male mice, and the control group of mice received regular drinking water. Chromofungin (2.5 mg/kg per day, *i.r.*) was given as a preventive treatment or phosphate buffer saline (1%) starting from one day before the induction of colitis till the fifth day when the mice were sacrificed. A-C: External disease activity index recorded over the period of 5 d (A), macroscopic scores at sacrifice day (B), CD86 and 80 and NF-κB gene expression profile by RT² profiler PCR array at sacrifice day (C). Unpaired two-tailed Mann-Whitney *U* and Two-way repeated measures or One-way ANOVA followed by multiple comparison tests were used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean ± SE, *n* = 6 mice/group. PBS: Phosphate buffer saline; CHR: Chromofungin; DSS: Dextran sulphate sodium.

mice, when compared with untreated group, CHR treatment significantly decreased splenocytes' CD86 ($P < 0.0001$, $n = 6$ per group), CD80 ($P < 0.0001$, $n = 6$ per group) and CD11c ($P < 0.0001$, $n = 6$ per group) (Figures 4A-C) mRNA expression. Induction of colitis induced a significant increase of splenocytes mRNA expression of IL-12p40 ($P < 0.0001$, $n = 6$ per group) and IL-6 ($P < 0.0001$, $n = 6$ per group) when compared with the PBS group and CHR treatment was associated with a significant decrease of IL-12p40 ($P < 0.0001$, $n = 6$ per group) and IL-6 ($P < 0.0001$, $n = 6$ per group) (Figures 4D and E) when compared with the colitic PBS group. No significant changes were observed for CD40 (data not shown).

In colitic mice, in vitro CHR treatment decreases splenic CD11c⁺ cells DCs-related surface and functional markers

Next splenic CD11c⁺ cells were isolated from the colitic group treated or not with CHR *via* the MACS technique. As presented in Figure 5, RT²-PCR array analysis demonstrated a significant downregulation of DC-related surface and intracellular markers after CHR treatment: CD86 (4.0-fold) ($P < 0.04$, $n = 6$ per group), CD80 (3.8-fold) ($P < 0.04$, $n = 6$ per group) and NF-κB (20-fold) ($P < 0.04$, $n = 6$ per group) marker.

CHR decreases DC-related markers in BMDCs treated with LPS or NF-κB activator

Finally, we determined the intracellular pathway implicated. In BMDCs CD11c⁺ cells, treatment with NF-κB stimulator significantly increased the mRNA expression of CD86 ($P = 0.0088$, $n = 6$ per group), CD80 ($P = 0.0026$, $n = 6$ per group), CD40 ($P = 0.0119$, $n = 6$ per group) and CHR treatment abolished the increase (Figures 6A-C). LPS treatment increased the mRNA expression of the same three markers, and treatment with NF-κB inhibitor or CHR abolished the increase. CHR treatment decreased the harmful effect of LPS stimulation even in the presence of the NF-κB activator.

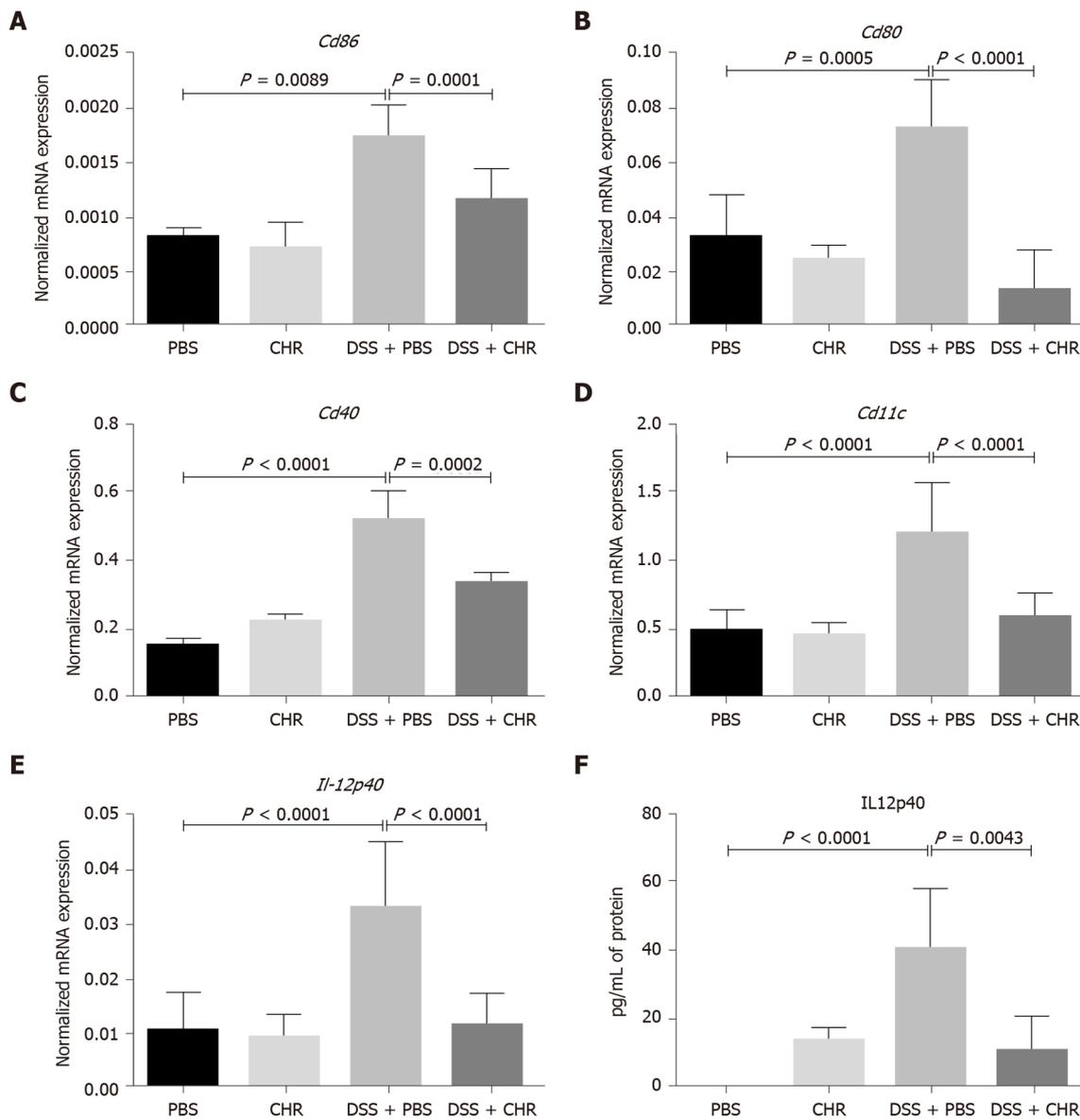


Figure 2 Chromofungin (chromogranin-A 47-66) treatment decreases colonic cluster of differentiation markers and interleukin-12 level in dextran sulphate sodium-induced experimental colitis. Colitis was induced with 5% dextran sulphate sodium in drinking water to C57BL/6 male mice, and the control group of mice received regular drinking water. Chromofungin (2.5 mg/kg per day, *i.r.*) was given as a preventive treatment or phosphate buffer saline (1%) starting from one day before the induction of colitis till the fifth day when the mice were sacrificed. A-F: CD86 (A), CD80 (B), CD40 (C), CD11c (D), interleukin (IL)-12p40 mRNA expression (E), IL-12p40 protein level (F). mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction and protein level by ELISA. One-way ANOVA followed by multiple comparison tests was used to analyze the data, and adjusted P equal to or smaller than 0.05 is believed to be significant. Each value represents the mean \pm SE, $n = 6$ mice/group. PBS: Phosphate buffer saline; CHR: Chromofungin; DSS: Dextran sulphate sodium.

In parallel, the NF- κ B stimulator increased significantly the protein concentration of IL-6 ($P < 0.0001$, $n = 6$ per group) and IL-12p40 ($P < 0.0001$, $n = 6$ per group) and CHR treatment significantly decreased the level of the two inflammatory proteins (Figure 6D and E). LPS treatment increased significantly the level of IL-6 ($p < 0.0001$, $n = 6$ per group) and IL-12p40 ($P < 0.0001$, $n = 6$ per group), and NF- κ B inhibitor or CHR treatments decreased the status of the two markers. CHR treatment decreased the deleterious effect of LPS stimulation even in the presence of an NF- κ B activator.

Relationship between CHGA-Exon-IV and DCs-related markers in colonic biopsies of active UC patients

Colon biopsies from UC patients were analyzed using qRT-PCR to assess the relationship between CHGA-Exon-IV and CD11c⁺ cells-related markers. We used our previously published data on Exon-IV[25] to perform a new correlation analysis. Our data demonstrated an upregulation of surface markers related to DCs (Figure 7A and

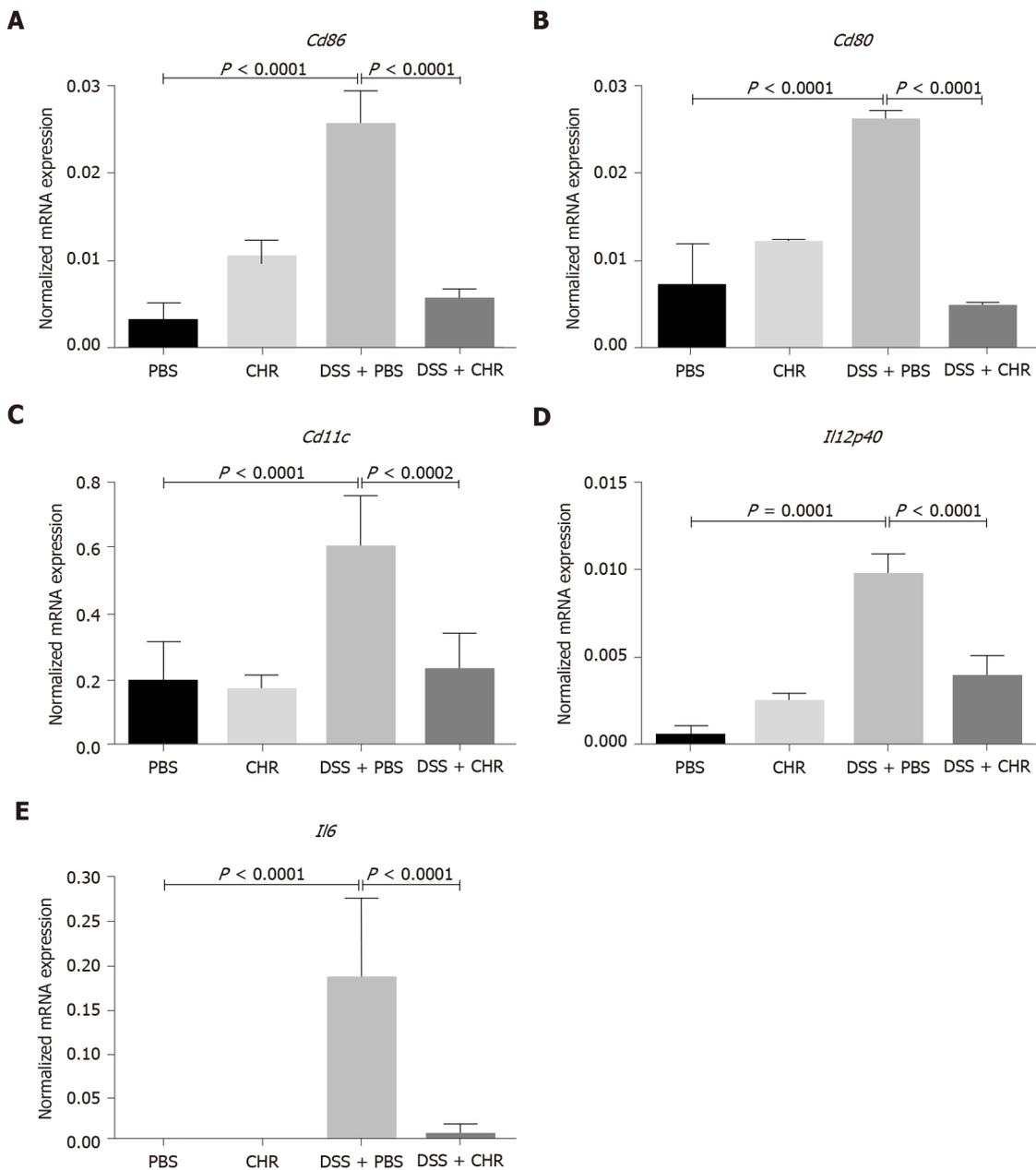


Figure 3 Chromofungin (chromogranin-A 47-66) treatment decreases mesenteric lymph node cluster of differentiation markers and cytokine levels in dextran sulphate sodium-induced experimental colitis. Colitis was induced with 5% dextran sulphate sodium in drinking water to C57BL/6 male mice, and the control group of mice received regular drinking water. Chromofungin (2.5 mg/kg per day, *i.r.*) was given as a preventive treatment or phosphate buffer saline (1%) starting from one day before the induction of colitis till the fifth day when the mice were sacrificed. A-E: CD86 (A), CD80 (B), CD11c (C), interleukin (IL)-12p40 (D), IL-6 mRNA expression (E). mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction. One-way ANOVA followed by multiple comparison tests was used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean \pm SE, *n* = 6 mice/group. PBS: Phosphate buffer saline; CHR: Chromofungin; DSS: Dextran sulphate sodium.

B): CD86 (*P* = 0.0002, *n* = 10 per group), CD11c (*P* = 0.0002, *n* = 10 per group). *Exon-IV* showed a strong to moderate negative correlation with DCs-related surface markers (Figure 7C and D) such as CD86 (*r* = -0.6765, *P* = 0.0051, *n* = 10 per group), CD11c (*r* = -0.4647, *P* = 0.0710, *n* = 10 per group).

DISCUSSION

Overactivation of the immune system through the dysregulation of DCs and its subsequent T-cell polarization is one of the cytokine storm features seen in inflamed mucosa[46]. Over the last five years, a lot of attention has been given to CHGA and its derived peptides in regulating the immune components. However, little is known as to

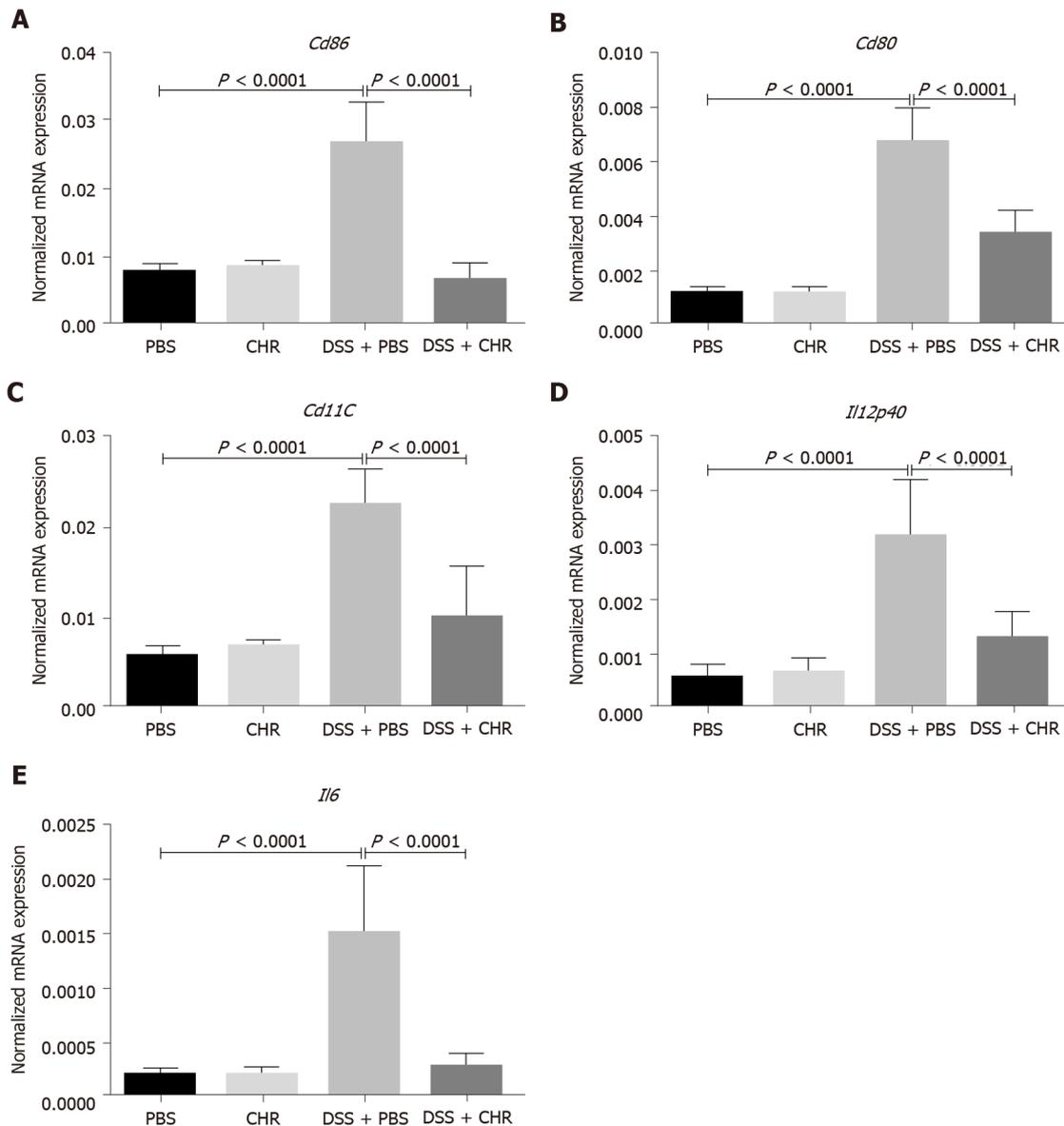


Figure 4 Chromofungin (chromogranin-A 47-66) treatment decreases splenocyte cluster of differentiation markers and interleukin-12 level in dextran sulphate sodium-induced experimental colitis. Colitis was induced with 5% dextran sulphate sodium in drinking water to C57BL/6 male mice, and the control group of mice received regular drinking water. Chromofungin (2.5 mg/kg per day, *i.r.*) was given as a preventive treatment or phosphate buffer saline (1%) starting from one day before the induction of colitis till the fifth day when the mice were sacrificed. A-E: CD86 (A), CD80 (B), CD11c (C), interleukin (IL)-12p40 (D), IL-6 mRNA expression (E) from splenic cells. mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction. One-way ANOVA followed by multiple comparison tests was used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean \pm SE, *n* = 6 mice/group. PBS: Phosphate buffer saline; CHR: Chromofungin; DSS: Dextran sulphate sodium.

how those peptides can modulate innate immune cells in different immune compartments. Using an experimental colitis model mimicking UC and colonic biopsies obtained from participants with active UC compared with biopsies from healthy controls, we assessed CHR's impact in regulating DCs-related markers. Our findings suggest a novel effect by which CHR promotes a down-regulation of DCs-related functional markers *via* the NF- κ B pathway within different immune compartments.

In our UC-like experimental colitis model, we first confirmed the beneficial effect of CHR on qualitative and quantitative inflammatory markers described in our previous publications[25,27,40]. Because of the peptide's specific action demonstrated in our previous study on different cytokines like TNF- α [25,40], we did not consider using a scramble version; this can be regarded as a limitation. However, in a few experimental plans (not shown), the scramble version was used, and no significant effect was seen. With CHR treatment, DAI and macroscopic scores were significantly downregulated, and in addition to the decrease previously seen in IL-6[25], there was a significant decrease in the concentration of IL-12p40 at the mucosal level. This decrease was also

Gene symbol	Fold regulation	<i>P</i> value
<i>Cd86</i>	-4.40	< 0.04
<i>Cd80</i>	-3.80	< 0.04
<i>Nfkb</i>	-20.01	< 0.04

Figure 5 Chromofungin (chromogranin-A 47-66) treatment decreases dextran sulphate sodium-induced experimental colitis and colonic cluster of differentiation markers and NF- κ B gene expression in dextran sulphate sodium-induced experimental colitis. CD86 and 80 and NF- κ B gene expression profile by RT² profiler polymerase chain reaction array at sacrifice day. One-way ANOVA followed by multiple comparison tests were used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean \pm SE, *n* = 6 mice/group.

seen at the mRNA level, suggesting the peptide's ability to down-regulate pro-inflammatory markers at the protein and gene levels. These data confirm GWAS studies, which have shown the importance of several APC-related cytokines, most prominently, the ones related to DCs: IL-6, IL-12 which have a critical role during the development of UC[47].

In our study, experimental colitis induced a significant colonic increase of DCs-associated markers CD86, CD80, CD40 and CD11c mRNA expression, and CHR treatment decreased all of them. DCs play a critical role during inflammation[48] as they activate and differentiate T cells and play an essential role in regulating adaptive immunity by releasing IL12p40[20]. At the colonic level, CD11c⁺ cells show a crucial role in IBD progression, colitis being associated with an increased level of CD11c^{hi} cells [46]. In parallel, in inflammatory conditions like asthma, arthritis, other markers like CD86, 80 and 40 are highly expressed in CD11c⁺ cells[49,50]. T-cell activation can be blocked by suppressing CD86 and CD80[51,52] and the release of IL-12p40 or 6[53,54]. Therefore, our data support the hypothesis that disruption of intestinal DCs-related markers can contribute to the perpetuation of the inflammatory process seen in IBD and that attenuation of DC's activation could lead to a better outcome.

DCs are essential in detecting antigens at the mucosal level and presenting them within other immune compartments, where, within the MLN, their roles in the regulation of immune activation are pivotal[55]. Human DCs, specifically myeloid (mDCs) CD11c⁺ DC-SIGN⁺, are characterized by producing many cytokines and further activating the adaptive immune cells[56-58]. In the context of gut inflammation, increased CD11c^{hi} cells' level is found in the draining MLN and associated with a marked enhancement of CD11c^{hi}HLADR^{int} DC[46]. In IBD patients, plasmacytoid DCs isolated from MNL exhibit overexpression of CD86, CD80 and an increase of IL-12p40 and IL-6 is seen[55]. In our study, in the context of experimental colitis, our data confirmed an up-regulation of CD86, 80 and 11c markers and IL-12p40 and IL-6. We demonstrated a significant decrease in CD86, CD80 and CD11c in the group treated with CHR. This highlights CHR's critical role in regulating CD markers and depicts the treatment's effectiveness in controlling IL-12p40 and IL-6 within the MNL. Surprisingly no effects were seen on CD40.

In addition to the MLN, the spleen plays a significant role in the crosstalk between the innate and adaptive immune systems. Within the spleen, CD11c⁺ DCs act as first responder cells during the immune response development[59]. Studies have shown that surface costimulatory molecules CD86, 80, and 40 present on isolated splenic CD11c⁺ cells are modified during the inflammatory process[60]. Using splenocytes isolated cells from colitic mice, our study confirmed these elements by depicting a decreased expression of CD86, CD80 and CD11c, and IL-12p40 and IL-6 levels when treated with CHR. We were also able to confirm the importance of the CD11c⁺ population, as our RT²-PCR array demonstrated a significant decrease of CD86 and 80 in that population. This was consistent with our data presented in our next experimental plan when we determined the direct impact of CHR on the CD11c⁺ BDMC cell population. Again here, like within the MLN, no effect was visible on the expression of CD40.

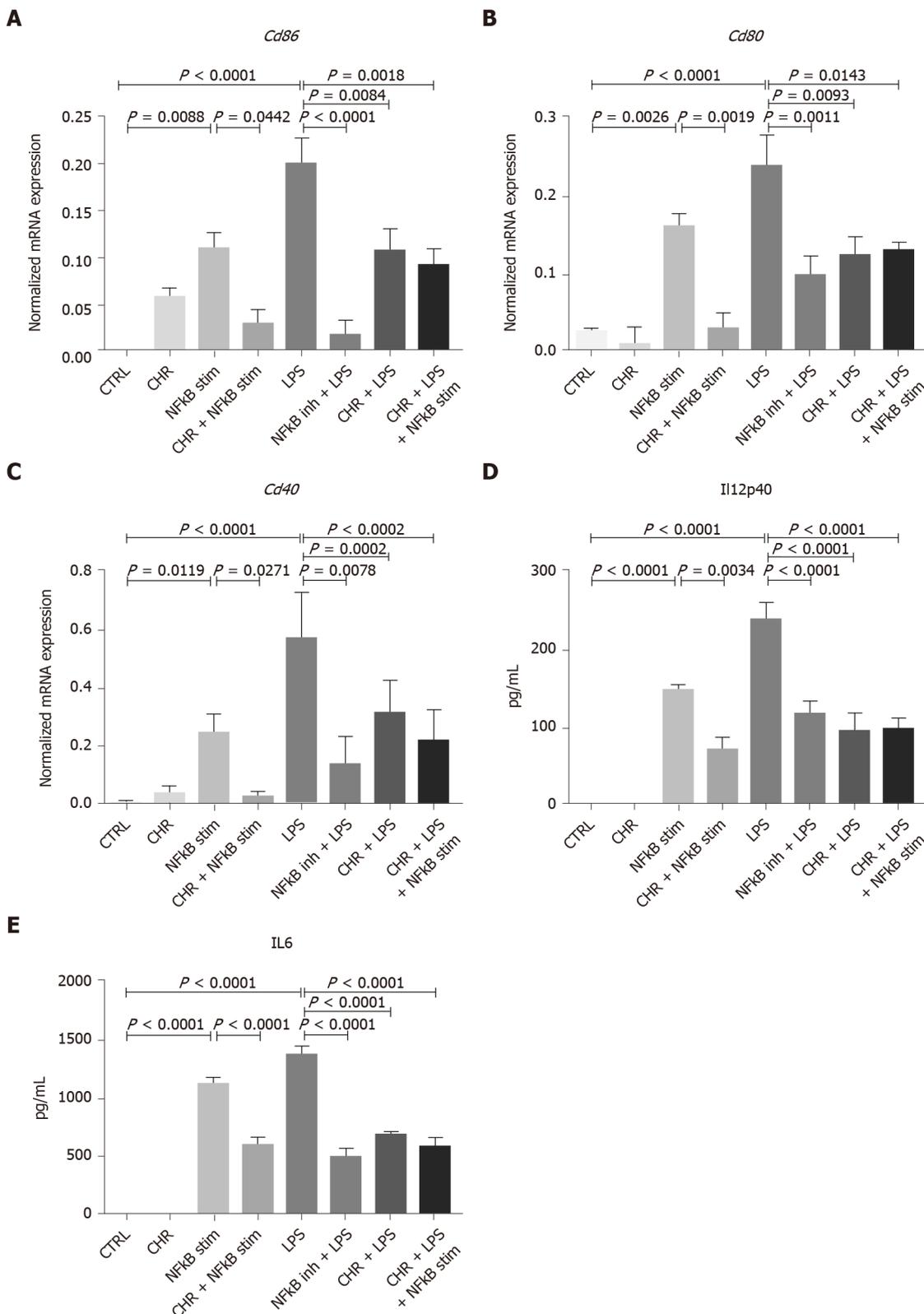


Figure 6 Chromofungin (chromogranin-A 47-66) treatment decreases lipopolysaccharide-stimulated bone marrow-derived CD11c⁺ cells' cluster of differentiation markers and cytokine-associated level via the NF- κ B pathways. Bone marrow-derived CD11c⁺ cells using MACS technique were isolated and cultured with granulocyte-macrophage induced colony-stimulating factor for 8 d until full maturation. Cells were treated with CHR (10⁻⁶ M/mL) for 12 h and then stimulated with lipopolysaccharide (100 ng/mL) in the presence or absence of NF- κ B activator/stimulator (10 μ M) for 24 h. A-E: CD86 (A), CD80 (B), CD40 (C), mRNA expression and IL-12p40 (D), IL-6 (E) medium protein level. mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction and protein levels were quantified by ELISA. One-way ANOVA followed by multiple comparison tests was used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean \pm SE, *n* = 6 mice/group. LPS: Lipopolysaccharide; CHR: Chromofungin.

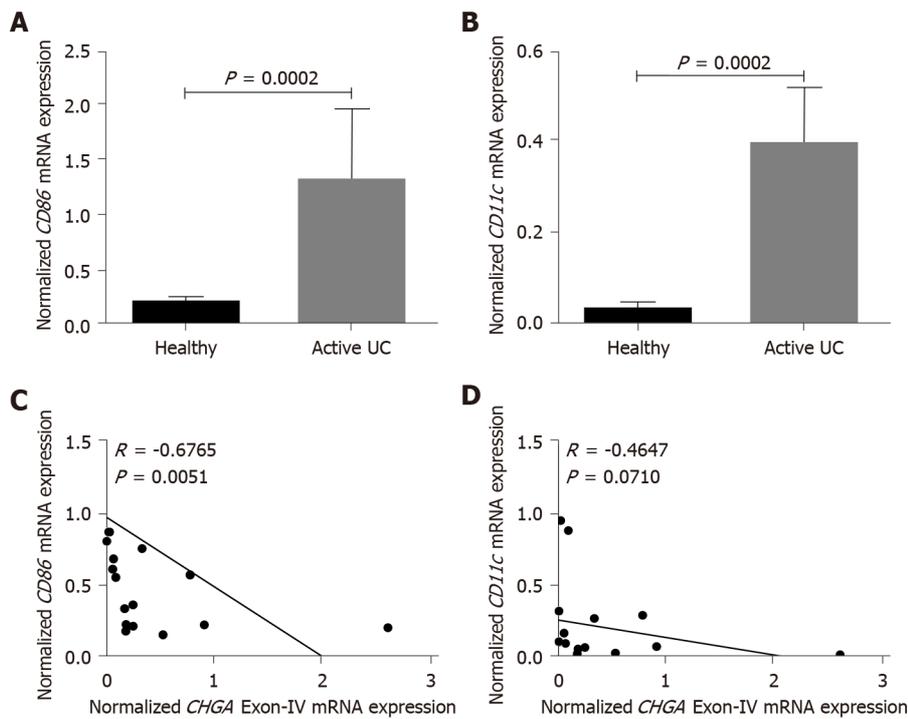


Figure 7 Chromofungin (CHGA Exon-IV) correlates negatively with cluster of differentiation 86 and 80 mRNA expression. A: mRNA expression of CD86 in the colonic tissue of healthy individuals ($n = 10$) and participants with active ulcerative colitis (UC) ($n = 10$); B: Correlation analysis between biopsy CHGA Exon IV and CD86 mRNA expression; C: mRNA expression of CD80 in the colonic tissue of healthy individuals ($n = 10$) and participants with active UC ($n = 10$); D: Correlation analysis between biopsy CHGA Exon IV and CD80 mRNA expression. mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction. Student *t*-test and Mann-Whitney test and Spearman's correlation were used to analyze, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. UC: Ulcerative colitis.

In the context of acute and chronic inflammatory conditions, several inflammatory cytokines are regulated by NF- κ B, and previous studies have reported that blocking NF- κ B in mice can be considered as a potential treatment for preventing gut inflammation[61-65]. NF- κ B plays an essential role in regulating the expression of CD86, CD80, CD40 and CD11c[66-68]. In our study, the RT²-PCR array performed on colonic colitic samples, and splenic CD11c⁺ cells demonstrated a down-regulation of NF κ B1 after CHR treatment. Consistent with our *in vivo* data, mimicking an inflammatory activation state, mouse LPS-stimulated CD11c⁺ BMDCs showed an increase of CD86, 80, 40 mRNA expression. The difference in CD40 expression between the colon, the MLN, spleen, and the CD11c⁺ BMDCs can be explained by a potential weak stimulus of CHR in that marker in an *in vivo* condition in contrast to a strong effect when the peptide had a direct action on the CD11c⁺ BMDCs, as presented by other when deciphering the impact of commensal bacteria on DC cells in different immune compartments[69].

In addition, the presence of CHR abolished colitis in the same way as the NF- κ B inhibitor. To confirm NF- κ B as a potential target, using an NF- κ B stimulator, we demonstrated the impact of CHR's inhibitory effect on the detrimental impact induced by the stimulator. Our *in vitro* evaluation also showed a significant decrease in IL-6 and IL-12p40 in LPS-stimulated CD11c⁺ BMDCs. As CHR can decrease the effect caused by the LPS and NF- κ B stimulator, we can postulate that the targeted protein resides at one of the NF- κ B targets. These data agree with human data, showing that anti-IL-12p40 monoclonal antibodies have demonstrated excellent clinical efficacy in a group of UC patients[70]. In the clinical and experimental models of colitis, NF- κ B has been demonstrated to be a major immune regulator[61,62,65] when inflammation is developed. However, at that stage, we cannot confirm if the effect of CHR is a direct or an indirect effect on NF κ B. Many upstream pathways can be modulated, as for example, TLR4[71], ER stress[71]. However, there are some early indications that the phosphoinositide-3-kinase-protein kinase B/Akt (PI3K-PKB/Akt) pathway maybe be of interest[72], as CHR can produce a cardioprotective action by regulating the PI3K pathways[37]. Thus, considering the previous literature related to NF- κ B and IBD, our results support the concept that CHR can potentially decrease intestinal inflammation by regulating the NF- κ B pathway in CD11c⁺ cells.

We previously demonstrated that *CHGA* (*Exon-IV*) was highly down-regulated in UC patients showing that the disease is affecting the levels of *CHGA* (*Exon-IV*)[27,73]. As a novel finding, in colonic biopsies from UC participants, we demonstrated that CD11c and its related costimulatory surface markers CD86 were significantly upregulated, leading to a potential enhancement of colitis. Furthermore, in active UC participants, we observed a negative correlation between the *CHGA* (*Exon-IV*) with CD86 markers. These alterations can be explained by previous data, which demonstrated changes at the level of the ECs during the inflammatory process[32]. Our small sample size likely led to our inability to show a significant difference in CD11c bearing cells.

Some limitations exist in our study. Regarding the overall impact of CD11c⁺ cell deactivation, we did not demonstrate a direct impact on T-cell activation, a regulation that remains elucidated *via* the use of a co-culture model. Further studies are also required to investigate the functional consequences of CHR on other immune cells or epithelial cells and angiogenesis, all implicated in the pathogenesis of colitis. Moreover, we acknowledge that CD11c⁺ cells isolated from the lamina propria need to be studied, and new studies should define if DC expressing CD11c markers are the main target of CHR. Additional biopsies need to be added to determine a compelling correlation between various markers, and a proteomics approach should be used for protein quantification in the colon, and all correlation analyses should be repeated accordingly.

Several other factors might lead to CHR's protective effect, which we have observed in our study. For example, previous studies demonstrated a link between gut microbiota and experimental colitis and human IBD, and there is evidence showing that CHR can have anti-microbial function[34,35]. Therefore, in our *in vivo* model, *i.r.* injection could have induced a microbial change in favour of beneficial bacteria; further metagenomic analyses are needed to study this aspect, but that would not account for the beneficial effect seen on CD11c⁺ BMDCs.

CONCLUSION

In summary, our study shows that treatment with CHR led to lower colonic inflammation, which was associated with decreased levels of CD11c⁺-associated cytokines through an NF- κ B-dependent mechanism. Hence, CHR seems to act as an NF- κ B blocker, reducing primary APCs' activation and ultimately decreasing the intestinal inflammatory process. CHR alone had no side effect on the mice or the cells, indicating CHR's relative safety at the studied dose. This previously unknown spatial impact of CHR in colonic inflammation may help broaden research done by other groups on the overall effect of *CHGA* and its derived peptides on immune regulation in the context of IBD. This might lead to future novel CHR-based therapies in IBD.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is a disorder in which the gastrointestinal tract becomes ulcerated and inflamed. Although treatments and therapeutic strategies are evolving quickly, treatments are still inadequate for a substantial percentage of those with active UC, and some therapies may have serious adverse side effects. Therefore, UC needs new therapeutic approaches associated with higher efficacy and limited side effects. In UC, there is an overactivation of innate immune cells such as macrophages and dendritic cells (DCs). Recently, in the context of UC and experimental colitis, new data highlighted an essential anti-inflammatory role of chromofungin (CHR), a chromogranin-A derived peptide, on peritoneal macrophages.

Research motivation

To evaluate CHR's effects on different DCs at various immune compartments and to define a potential intracellular pathway implicated.

Research objectives

To investigate the association between CHR treatment and DCs-related markers in colitis.

Research methods

Participants with active UC and a model of acute UC-like colitis using dextran sulphate sodium were used. We used cell culture and quantitative reverse transcription-polymerase chain reaction to analyze the relative expression levels of CD11c, CD80, CD86, interleukin (IL)-6 and IL-12p40 within the colonic samples, mesenteric lymph nodes and the spleen.

Research results

In a preclinical setting, CHR treatment the expression of CD11c, CD40, CD80, CD86 and IL-12p40 in the inflamed colonic mucosa and CD11c, CD80, CD86, IL-6 and IL-12p40 within the mesenteric lymph nodes and the spleen. In addition, CHR treatment decreased CD80 and CD86 expression markers of splenic CD11c⁺ cells and decreased NF- κ B expression in the colon and splenic CD11c⁺ cells. *In vitro*, CHR decreased CD40, CD80, CD86, IL-6, and IL-12p40 expression in naïve bone marrow-derived CD11c⁺ DC stimulated lipopolysaccharide. Using a pharmacological approach, we demonstrated the impact of CHR on the NF- κ B pathway. In a clinical setting, in patients with active UC, CHR level was reduced and showed a negative linear relationship with CD11c and CD86.

Research conclusions

CHR has protective properties against intestinal inflammation, potentially through the regulation of DC-related markers and CD11c⁺ cells.

Research perspectives

Although we have demonstrated that CHR may have a potential therapeutic interest, additional markers and detailed mechanisms of action need to be determined in a large sample.

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