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Anti-inflammatory Effects of Ganoderma *Lucidum* Triterpenoid in Human Crohn's Disease Associated with Down-Regulation of NF-κB Signaling

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Abstract

Background—Crohn's disease (CD) is a chronic inflammatory disease of the gastrointestinal tract. Current medications have potentially serious side effects. Hence there is increasing interest in alternative therapies. We previously demonstrated the anti-inflammatory effects of FAHF-2 in-vitro on PBMCs and mucosa from CD subjects. Here we investigated the anti-inflammatory effects of a bioactive compound isolated from Ganoderma *lucidum* (G. *lucidum*), a key herbal constituent of FAHF-2, in CD *in vitro*.

Methods—Triterpene ganoderic acid C1 (GAC1) was isolated from G. *lucidum*. Stimulated RAW 264.7 macrophages were treated with GAC1. Human peripheral blood mononuclear cells (PBMCs) and colonic biopsies were obtained from children with CD and cultured with or without GAC1. TNF-α and other pro-inflammatory cytokine levels were measured in the culture supernatant. NF-κB signaling was investigated in PBMCs and colonic mucosa treated with GAC1 by In-Cell Western and western blot analysis.

Results—GAC1 decreased TNF-α production by macrophages and PBMCs from CD subjects. GAC1 significantly decreased TNF-α, IFN-γ, and IL-17A production by inflamed colonic biopsies from CD subjects. These effects were due to down-regulation of the NF-κB signaling pathway.

Conclusions—GAC1 inhibited production of TNF-α and other pro-inflammatory cytokines by PBMCs and inflamed CD colonic mucosa due to blockage of NF-κB activation. GAC1 is a key beneficial constituent in G. *lucidum* and the FAHF-2 formula in suppressing the inflammatory cytokines found in CD and warrants clinical investigation for the treatment of CD.

Keywords

Crohn's disease; Triterpene; TNF-α; NF-κB; FAHF-2

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INTRODUCTION

Crohn's disease (CD), an inflammatory bowel disease (IBD), is an immune-mediated disorder characterized by relapsing and remitting inflammation of the gastrointestinal tract. The exact etiology of Crohn's disease is unknown, but both genetic and environmental causes have been implicated.[1] Crohn's disease affects 1.4 million Americans, of which 140,000 are under the age of 18. Approximately 25% of all new cases in the population are under 20 years of age, and roughly 30,000 new patients are diagnosed annually.[2;3]

CD is considered to be a result of a multifactorial interplay that is driven by innate and adaptive immune responses. Studies in mice and humans implicate dysregulation of intestinal $CD4^+$ T cell subgroups leading to an abnormal immune response to bacterial antigens in a genetically predisposed individual. Th1/Th17 cells lead to increased production of effector T cell responses and an imbalance with regulatory T cells. CD pathogenesis includes upregulation of multiple cytokines including: TNF-α, IFN-γ, IL-1, IL-2, IL-6, IL-12, and IL-17.[4;5]

Current therapies work by either broadly suppressing the immune system or by suppressing specific aspects of the inflammatory pathway. Despite the predominance of numerous inflammatory cytokines (TNF-α, IFN-γ, IL-17) involved in the pathogenesis of CD, the only cytokine-directed monoclonal antibodies that have shown efficacy in the treatment of CD are those targeted against TNF-α.[6] Treatments for CD, including corticosteroids, immunomodulators and biologics, are each associated with significant toxicities. Corticosteroids may cause suppression of the hypothalamic-pituitary adrenal axis, bone demineralization and growth suppression. Immunomodulators also have potential adverse effects including myelosuppression, hepatitis and increased risk of malignancies. Patients treated with anti-TNF-α therapy may develop antigenicity,[7] loss of response, and toxicities such as infection, lupus-like syndrome and malignancy. Treatment with biologic agents is also expensive.[6] Additionally, treatment failure is common, with up to 18% of children requiring surgery within 5 years of disease onset.[8] Therefore, alternative less toxic, orally administered interventions are being sought.

Food Allergy Herbal Formula-2's (FAHF-2) effects on CD were examined in a recent study which demonstrated that FAHF-2 reduced production of TNF-α, IFN-γ and other inflammatory cytokines by PBMCs and inflamed colonic biopsies from CD subjects. FAHF-2 was also effective in treating colitis in a murine model. This established the potential of FAHF-2 as a novel treatment for CD.[9] Clinical trials of FAHF-2 in patients with food allergies showed that it was safe, well tolerated and had multiple immunomodulatory effects.[10;11] However, therapeutic doses in humans for the treatment of inflammation are impractically large. Current trials of FAHF-2 for food allergy require subjects to take 36 tablets daily.[10] Despite its safety and tolerability, this large dosage makes adherence difficult.[18] Thus, identification of the specific bio-active compounds of FAHF-2 would improve compliance by decreasing the pill burden. Isolation of the active compounds would also facilitate investigation of underlying mechanisms. Therefore, the 9 herbal components of FAHF-2 were screened for anti-inflammatory activity.[12]

Ganoderma *lucidum* (G. *lucidum*) is a major constituent in FAHF-2. It is also a major constituent in other traditional Chinese medicine formulas such as anti-asthma herbal medicines intervention (ASHMI or ASHMI™). ASHMI was recently shown to be effective in a neutrophil predominant, steroid resistant asthma model.[13] The therapeutic effect was associated with significant suppression of pro-allergic Th2 cytokines and pro-inflammatory cytokines TNF-α and IL-17.[14;15] Ganoderic acid C1 (GAC1) is a triterpenoid isolated from G. *lucidum*. A number of previous studies reported that polysaccharides from *G. lucidum* modulated cytokines.[16;17] In unpublished work, we found that the triterpenoid fraction of *G. lucidum* was more potent than the polysaccharide fractions in suppression of TNF-α. GAC1 was the most potent triterpenoid. GAC1 inhibited TNF-α production by a murine macrophage cell line via down-regulation of the NF-κB signaling pathway, a key feature of both neutrophil predominant asthma and CD.[18] We therefore hypothesize that GAC1 may be effective in suppression of inflammatory responses in CD.

The aim of this study was to investigate the potential of GAC1 to inhibit production of TNF-α and other pro-inflammatory cytokines by peripheral blood mononuclear cells (PBMCs) and inflamed colonic mucosa from pediatric CD patients, and to determine the underlying mechanism of action.

MATERIALS AND METHODS

Extraction and Isolation of Compound from G. *lucidum*

G. *lucidum* aqueous extract was manufactured by the Sino-Lion Pharmaceutical Company (a GMP certified facility in Weifang, China) as described previously.[19;20] A dried aqueous extract of G. *lucidum* was dissolved in water and extracted with methylene chloride (MC); the water layer was further extracted with n-butanol. The MC, butanol and water layers were concentrated and dried under pressure to produce a powder. The MC fraction was further fractionated and purified by using repeated silica gel, preparative HPLC, and sephadex LH-20 column chromatography methods to obtain GAC1.

Identification of GAC1 Isolated from G. *lucidum*

^{1}H (300 MHz) and ^{13}C (75 MHz) NMR were collected on a JEOL ECX-300 instrument. Samples were dissolved in DMSO-d_6, containing TMS as an internal standard, and MS spectra were recorded on an Agilent 6130 single quadrupole LC–MS. The ^{1}H-NMR (DMSO-d_6) spectrum of GAC1 ($C_{30}H_{42}O_7$) showed a proton signal at δ 4.80 (1H, m, H-7). ^{13}C-NMR (DMSO-d_6) spectra showed signals at δ 216.0 (C-15), 215.1 (C-3), 208.6 (C-23), 198.0 (C-11), 176.7 (C-26), 159.4 (C-9), 139.8 (C-8), and 64.8 (C-7).

RAW 264.7 Cell Culture

The murine macrophage cell line RAW 264.7 was purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/L streptomycin. Linearly growing cells were detached using a cell scraper and 5×10^5 cells per well were seeded onto 24-well culture plates. The cells were pre-incubated with or without GAC1 treatment in various concentrations (2.5, 5, 10, 20, 40 μg/mL) for 24 hours followed by an

additional 24-hour incubation with LPS (Sigma, *Escherichia coli* 0111:B4) (1 μg/mL). GAC1 was dissolved initially in DMSO and the final concentration of DMSO in all culture conditions, including medium alone and LPS alone groups, was 0.1%. Cell viability was determined by trypan blue dye exclusion. The ratio of viable cells to total cells was calculated.

Subjects

Human studies were approved by the Institutional Review Board of the Icahn School of Medicine at Mount Sinai. Blood samples (n=12) and inflamed colonic biopsy specimens (n=15) were collected from newly diagnosed pediatric CD patients seen by faculty of the Division of Pediatric Gastroenterology (age: 8–19 yrs). Patients were diagnosed with CD based upon standard clinical criteria. Subjects were not on immunomodulating medications including steroids, thiopurines or biologics at the time specimens were obtained. Additionally, colonic biopsy specimens were obtained from non-IBD pediatric patients undergoing colonoscopy due to gastrointestinal symptoms (controls) (n=5) (11–18 yrs). Controls had macroscopically normal colons and normal colonic histology.

PBMC Isolation and Cell Culture

PBMCs were isolated by Ficoll Hypaque (ThermoFisher Scientific, Piscataway, NJ) with density-gradient centrifugation at 1800 RPM for 30 minutes, and washed 3 times in PBS. Purified PBMCs (2×10^6/well) were incubated in RPMI 1640 supplemented with 25 mM Hepes 10% (v/v) heat-inactivated FBS, 60 mg/L (100U/mL) penicillin, 100 mg/L streptomycin and 0.29 g/L L-glutamine in 24-well plates with or without GAC1 (20 μg/mL) for 24 hours. LPS (2 μg/mL) was added and culture conditions maintained for another 24 hours. At the end of the incubation, supernatants were harvested for TNF-α measurement. In parallel culture experiments, PBMCs (2×10^5) were cultured in a 96-well plate overnight in serum free medium with or without GAC1 (20 μg/mL). Cells were stimulated with LPS (2μg/mL) or TNF-α (10 μg/mL) for 10 minutes. In-Cell Western Assay (Li-Cor, Lincoln, Nebraska) was performed according to the manufacturer's instructions. Cell viability was determined by trypan blue dye exclusion. The ratio of viable cells to total cells was calculated.

Biopsy Preparation and Culture

Inflamed colonic biopsies from CD subjects and non-inflamed colonic biopsies from controls were placed into culture with proportionate volumes (14.5μl/mg) of complete RPMI, based upon the weight of the specimen, with or without GAC1 (20 μg/mL) for 24 hours. Supernatants were filtered and kept for measurement of cytokines.

Cytokine Measurement: ELISA and Cytometric Bead Array

TNF-α levels in the Raw 264.7 cell and PBMC supernatants were measured by ELISA according to the manufacturer's protocol (BD Biosciences, San Diego, CA). Briefly, 96-well plates were coated with capture antibody to anti-TNF-α at the recommended dilution. The following day, plates were washed and blocked with 10% FBS for 2 hours. Samples and standards were added in various dilutions and incubated for 2 hours. Plates were then

washed and detection antibody, biotinylated anti-TNF-α, and streptavidin-horseradish peroxidase conjugate were added. Plates were washed, developed with TMB substrate reagent and the reaction halted with 2N H_2SO_4. Cytokines (IL-1, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ, and IL-17A) from supernatant of biopsy cultures were assessed in duplicate by cytometric bead array (BD Biosciences) according to the manufacturer's instructions. Briefly, undiluted supernatants and standards were incubated with appropriate capture beads and PE detection reagent for 3 hours at room temperature. Samples were then washed and resuspended in wash buffer. Samples were acquired from a 96 well plate using a BD LSR II flow cytometer. Data were analyzed using FlowJo (Tree Star, Ashland, OR) and Graphpad Prism 4 (La Jolla, CA).

Lamina Propria Mononuclear Cell Isolation and Cell Culture

Lamina Propria Mononuclear Cells (LPMCs) were isolated from inflamed colonic surgical specimens from adult subjects with CD who underwent bowel resection at the Mount Sinai Medical Center. LPMCs were isolated according to an established protocol using Dispase II (Roche Diagnostics, Indianapolis, IN) and collagenase (Sigma, St. Louis, MO) treatment.[21;22] LPMCs were cultured overnight in serum-free medium. GAC1 (40μg/mL) was then added for 1 hour, and cells were stimulated with TNF-α (10 μg/mL) for 10 min. In-Cell Western Assay (Li-Cor, Lincoln, Nebraska) was performed according to the manufacturer's instructions.

In-Cell Western Assay

In-Cell Western Assay (Li-Cor, Lincoln, Nebraska) was performed according to the manufacturer's instructions and as previously described.[9] Cells were incubated with antibodies against p-IκBα (1/1000) (Cell Signaling Technology, Danvers MA) and β-actin (1/1000) (Santa Cruz Biotechnology, Birmingham AL) as a loading control for 2 hours. This was followed by incubation with secondary antibodies IRDye800CW donkey anti-goat (1/1000) and IRDye680RD donkey anti-rabbit (1/1000) (Li-Cor) for 1 hour with protection from light. Plates were scanned, florescence detected at 700 and 800 nm, and data normalized using an Odyssey CLx Infared Imaging System.

Western Blot Analysis

Colonic biopsies from pediatric CD subjects were cultured with or without GAC1 (20 μg/mL) in complete RPMI (500μL/15mg) for 24 hours. The whole cell protein was extracted using the manufacturer's protocol (Active Motif, nuclear extract kit). The protein concentration was determined according to the manufacturer's protocol (Bio-Rad laboratories, Hercules, CA), and stored at −80 °C. Total cell proteins (20–40 μg) were separated by SDS-PAGE and were transferred to nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin in TBS-Tween 20 solution and was further incubated with the corresponding antibody: p-p65 (rabbit, 1:1000) and β-actin (rabbit, 1:5000) at 4°C overnight. Membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin diluted 1:3000 in TBS-Tween 20 for 1 hour at room temperature with continuous shaking. Reactive bands were visualized with HRP-coupled respective secondary antibodies via an enhanced chemiluminescence detection system according to the manufacturer's procedures (Denville Scientific, Metuchen, NJ).

Statistical Analysis

All statistical analyses were performed using Graphpad Prism4 software. Differences between multiple groups were analyzed by one-way ANOVA followed by the Dunnett test, and the differences between 2 groups with paired samples were analyzed by paired t test. A p-value <0.05 was considered to be statistically significant.

RESULTS

GAC1 Inhibited TNF-α Production by RAW 264.7 Macrophages

GAC1 was isolated from G. *lucidum* and identified by Nuclear Magnetic Resonance and Liquid chromatography–mass spectrometry with the chemical structure shown in Figure 1A. We first established the inhibitory effects of GAC1 on TNF-α production by LPS stimulated-RAW 264.7 macrophages. LPS (1μg/mL) enhanced TNF-α production, and pretreatment with GAC1 decreased this TNF-α production in a dose-dependent manner with a maximal suppressive effect at 20 and 40μg/mL. There was no significant difference in the suppressive effect at 20 or 40μg/mL. GAC1 showed a significant inhibitory effect at concentrations as low as 10μg/mL (Figure 1B). GAC1 showed no significant cytotoxicity at any dose tested (data not shown).

GAC1 Suppressed TNF-α Production by PBMCs from CD Subjects

Given the importance that TNF-α plays in the pathogenesis of CD and the inhibitory effects of GAC on TNF-α production *in vitro*, we next tested the effect of GAC1 on TNF-α secretion by PBMCs from CD subjects. LPS significantly enhanced TNF-α production by PBMCs from CD subjects. Simultaneous treatment with GAC1 (20μg/mL) reduced TNF-α secretion from 2089 ±1240 pg/mL to 992 ± 1292 pg/mL (Mean ± SD, n=12, $p<0.01$) (Figure 2A). GAC1 caused no cytotoxicity (Figure 2B).

GAC1 Suppressed Inflammatory Cytokine Production by Mucosa from CD Subjects

We next sought to assess the effects of GAC1 on colonic mucosa, the site of inflammation in CD where medications are likely to have the largest effect. Inflamed colonic biopsies from CD subjects (n=10) and non-inflamed colonic biopsies from controls (n=5) were incubated with and without GAC1. Cytokine levels were quantified in the supernatant. Inflamed mucosa from CD subjects had significantly more production of TNF-α, and IFN-γ production compared with non-CD controls. GAC1 significantly suppressed the production of TNF-α (n=12, $p<0.05$), IFN-γ (n=10, $p<0.05$), and IL-17A (n=10, $p<0.05$) but not IL-6 production (n=10, $p=0.09$) from the inflamed CD biopsies (Figure 3). CD biopsies showed negligible production of other tested cytokines. GAC1 had no effect on cytokine production by biopsies from control subjects.

GAC1 Inhibited the NF-κB Signaling Pathway in PBMCs

We next sought to understand the mechanism by which GAC1 inhibits the production of inflammatory cytokines. LPS and TNF-α stimulation of cells induces the secretion of pro-inflammatory cytokines through activation of the NF-κB signaling pathway. We therefore examined phosphorylation of IkB-α in human PBMCs from CD subjects by In-Cell

Western. Phosphorylation of IkB-α was enhanced by both LPS (2μg/mL) and TNF-α (10μg/mL) stimulation. Pre-treatment with GAC1 (20μg/mL) significantly inhibited this phosphorylation ($p<0.05$) (Figure 4), thus indicating disruption of the NF-κB signaling pathway.

GAC1 Inhibited the NF-κB Signaling Pathway in the Mucosa

Given the results in PBMCs and to elucidate the mechanism of GAC1 on the mucosa, we examined phosphorylation of IkB-α in human LPMCs from CD subjects by In-Cell Western. Phosphorylation of IκB-α occurred by 10 minutes after cells were stimulated with TNF-α. GAC1 significantly inhibited this phosphorylation ($p<0.05$) (Figure 5A and B). We also examined phosphorylation of IκBα in three inflamed colonic biopsies from CD subjects by western blot analysis. Compared with the medium alone group, GAC1 treatment significantly inhibited IκBα activation (p-IκBα expression) (Figure 5B). These results demonstrate that GAC1 disrupts the NF-κB pathway in PBMCs, LPMCs, and colonic biopsies, leading to decreased production of TNF-α and other pro-inflammatory cytokines.

DISCUSSION

In this study, we demonstrated for the first time that GAC1 exhibited inhibitory effects on TNF-α and other inflammatory cytokines including IFN-γ and IL-17 by PBMCs and inflamed intestinal biopsy tissue from CD subjects. GAC1 did not show signs of cytotoxicity *in vitro*.

Monoclonal antibodies against TNF-α have revolutionized the treatment of CD and therefore our initial focus of investigation was on suppressing TNF-α production. $CD14^+$ macrophages, adipocytes, fibroblast and T cells produce increased amounts of TNF-α, which perpetuates inflammation in CD. TNF-α levels are elevated in the serum and inflamed mucosa in patients with CD. Serum levels of TNF-α have been shown to correlate with clinical and laboratory indices of intestinal disease activity.[23;24] TNF-α is also secreted by LPMCs from CD patients. TNF-α effects include hypervasculization, angiogenesis, and increased pro-inflammatory cytokine production by macrophages and T-cells. Moreover it causes mucosal barrier alterations, cell death of intestinal epithelial cells, tissue destruction, suppression of regulatory macrophages and activation of NF-κB.[4;25] Since GAC1 significantly inhibited TNF-α production by both PBMCs and inflamed mucosa from CD, it is likely to be beneficial in combating inflammation in CD.

Unlike monoclonal antibodies against TNF-α, GAC1 is able to abrogate secretion of multiple inflammatory cytokines implicated in the pathogenesis of CD. CD is driven by a Th-1/Th-17 profile leading to increased secretion of IFN-γ and IL-17.[25;26] IFN-γ is a pro-inflammatory cytokine that activates macrophages, augments antigen processing, alters tight junction activity and induces the death of epithelial cells. IL-17, part of the Th-17 response, mediates pro-inflammatory functions such as the recruitment of neutrophils and the secretion of matrix metalloproteinases.[4] It has been linked to degradation of tissue during inflammatory responses.[27] IL-17 may also have a protective role within the intestine that has yet to be fully elucidated.[28;29] Increased IL-1, IL-17A/F, IFN-γ and IL-6 are found in inflamed mucosa of CD. Interestingly, monoclonal antibodies targeted against IFN-γ

(fontolizumab) and IL-17A (secukinumab) were not effective.[30;31] This suggests that a multi-pronged approach that suppresses multiple cytokines may be necessary. GAC1 inhibits IFN-γ and IL17A production in addition to TNF-α, and therefore may be efficacious in attenuating the inflammation that occurs in CD.[4]

NF-κB is known to play a central role in immune and inflammatory responses and is involved in transcriptional regulation of many cytokine genes, including TNF-α. Inflammatory cytokines and intestinal microorganisms activate the NF-κB transcription factor process by inducing the phosphorylation and consequent degradation of IκB by its kinase. This allows NF-κB translocation into the cell nucleus to activate gene expression for relevant inflammatory proteins.[32] The activated form of this transcription factor has been detected in mononuclear and epithelial cells of inflamed colon.[33] Recent studies have shown that NF-κB may be a good target for CD therapy.[6;34;35] GAC1 inhibited IκB activation both in PBMCs and mucosa, suggesting that it may work to decrease mucosal inflammation by down-regulation of the NF-κB signaling pathway.

GAC1, alone or perhaps together with other compounds in FAHF-2 or ASHMI, has potential to be developed as a therapy for CD and other inflammatory conditions. We have previously demonstrated that FAHF-2, the formulation containing GAC1, has anti-inflammatory effects both in human samples from CD and in a murine model of colitis. Although allergic and non-allergic immune disorders are different clinical entities, neutrophil-predominant steroid-resistant asthma, food allergy and CD share some similar innate and adaptive immunological pathways. The common abnormalities include: increased TNF-α and activation of the NF-κB signaling pathway.[36–38] We have shown that *G. lucidum*, a key herbal constituent in FAHF-2 and ASHMI, has anti-inflammatory effects on cytokines involved in both allergic and non-allergic disorders.[39–41]

In conclusion, this study establishes a framework for refining an herbal therapy into its active components to ease use in clinical studies in inflammatory bowel disease patients. GAC1 has beneficial anti-inflammatory effects on cytokine production by PBMCs and colonic mucosa from CD subjects, showing promise for further development as a treatment for CD.

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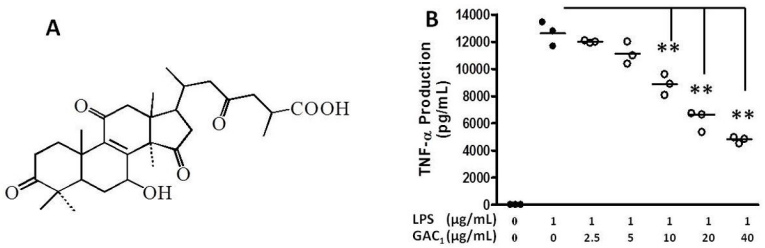

Figure 1. GAC1 inhibited TNF-α production by RAW 264.7 cells

A. Chemical structure of GAC1. **B.** Concentration dependent inhibitory effect of GAC1 on TNF-α production by LPS stimulated RAW 264.7 cells. Bars represent means, **p<0.01 vs. LPS group, n=3.

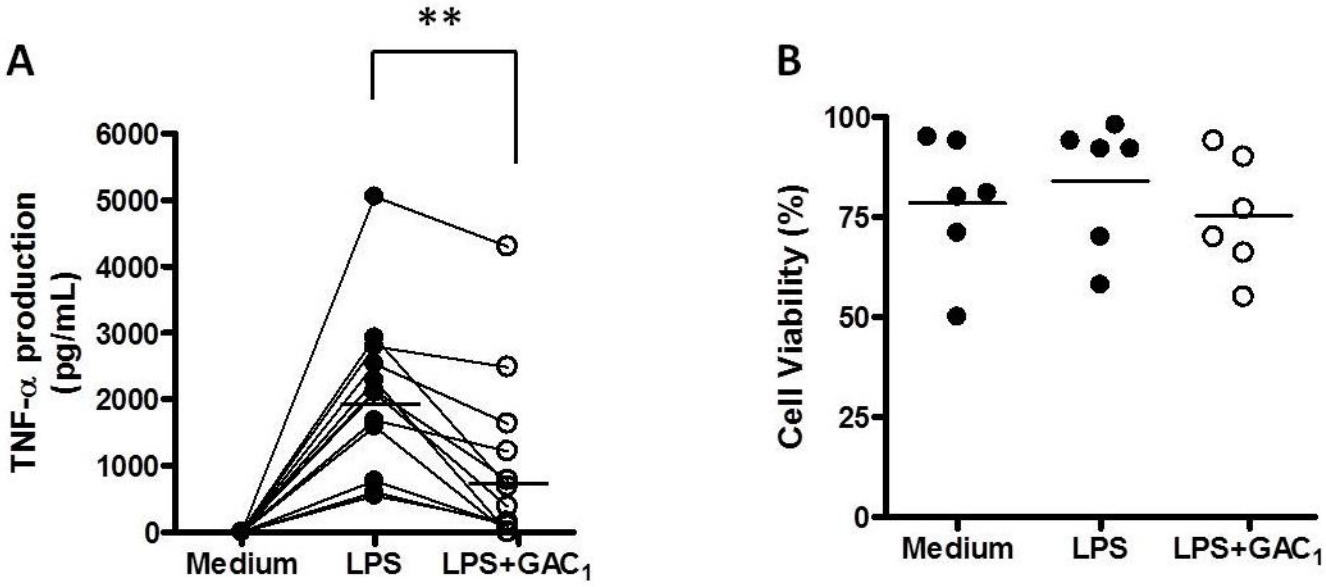

Figure 2. GAC1 inhibited TNF-α production by PBMCs from CD subjects

A. Inhibitory effect of GAC1 (20 μg/mL) on TNF-α production by PBMCs from CD subjects stimulated with LPS (Bars represent means, n=12). **B**. Cell viability after treatment with GAC1 (20 μg/mL) on CD subjects PBMCs stimulated with LPS. Bars represent means, ** $p<0.01$ vs. LPS alone group, n=12.

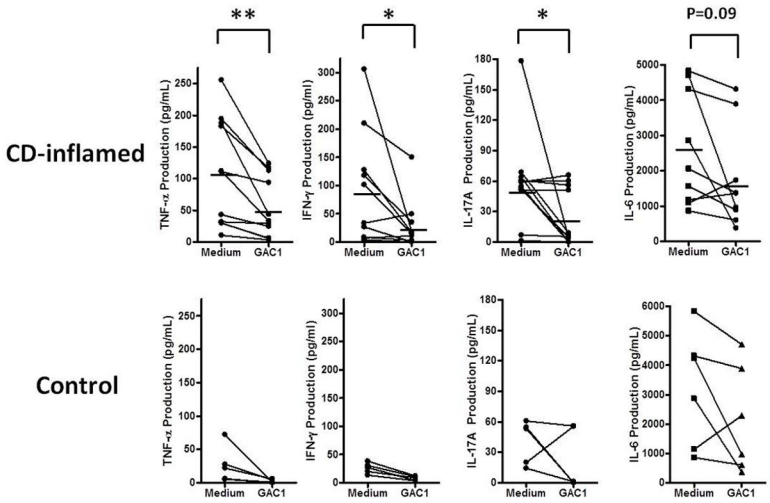

Figure 3. GAC1 inhibited cytokine production from colonic biopsies from CD subjects and controls

Inflamed biopsies from CD and non-inflamed biopsies from control subjects were cultured with or without GAC1 (20 μg/mL) for 24 hours. Cytokine levels were measured from the supernatants by cytometric bead array. Cytokine levels (TNF-α, IFN-γ, IL-17A, and IL-6) without (medium group) and with treatment with GAC1. CD inflamed: n =15; non-CD control (control): n = 5. Bars represent means, *$p<0.05$.

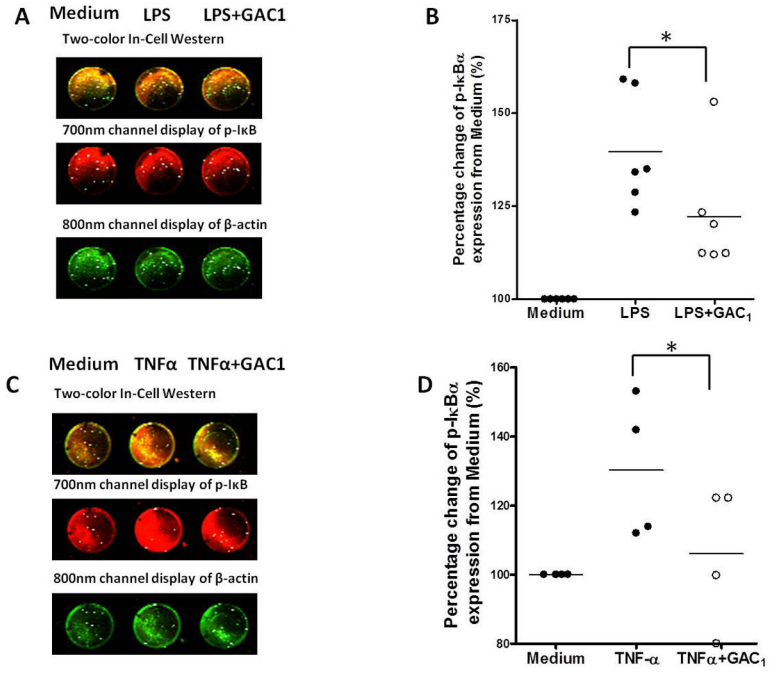

Figure 4. GAC1 inhibited p-IκBα expression by PBMCs from CD subjects

A. Representative In-Cell Western Assay with 700 and 800 nm channels for detecting p-IκBα and β-actin in PBMCs cultured in medium, stimulated with LPS (2μg/mL), or stimulated and treated with GAC1. **B.** Quantitative In-Cell Western Assay of p-IκBα expression in PBMCs cultured in medium, stimulated with LPS, or stimulated with LPS and treated with GAC1 ($p<0.05$ vs. LPS alone, n=6). **C.** Representative In-Cell Western Assay with 700 and 800 nm channels for detecting p-IκBα and β-actin in PBMCs cultured in medium, stimulated with TNF-α (10 μg/mL), or stimulated and treated with GAC1. **D.** Quantitative In-Cell Western Assay of p-IκBα expression in PBMCs cultured in medium, stimulated with TNF-α, or stimulated and treated with GAC1 ($p<0.05$ vs. LPS alone, n=4). (Bars represent means)

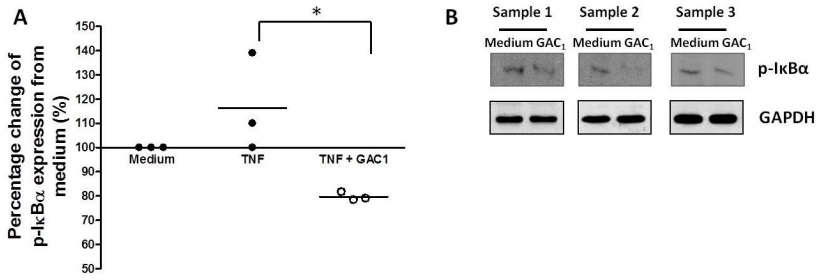

Figure 5. GAC1 inhibited p-IκBα expression by mucosa from CD subjects

A. Percentage of phosphorylated IκB from LPMCs measured by In-Cell Western after TNF-α (10 μg/mL) stimulation and with GAC1 (40μg/mL) treatment as compared with medium alone (n =3). All values normalized to β-actin. Bars represent means, *p< 0.05. **B.** Representative protein expression of phospho-IκB and β-actin from inflamed colonic biopsies from CD subjects (n=3) treated with or without GAC1 (20μg/mL).