Effects of Complement 3 deficiency on its receptor downstream signaling pathway-mediated inflammatory response in knockout mice

Yun Ju Choi
Pusan National University

Ji Eun Kim
Pusan National University

Su Jin Lee
Pusan National University

Jeong Eun Gong
Pusan National University

Ho Lee
National Cancer Center

Dae Youn Hwang (dyhwang@pusan.ac.kr)
Pusan National University

Research Article

Keywords: Complement C3, inflammation, iNOS, COX-2, Cytokines, Transverse colon

DOI: https://doi.org/10.21203/rs.3.rs-402686/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

The current study measured alterations in the inducible nitric oxide synthase (iNOS)-mediated cyclooxygenase-2 (COX-2) induction pathway, inflammasome pathway, NF-kB activation, and inflammatory cytokine expressions in the transverse colon of C3 knockout (KO) mice, to determine whether complement component 3 (C3) deficiency affects its receptor downstream-mediated inflammatory response. Compared to wild type (WT) mice, the expression level of C3 protein was successfully suppressed in the transverse colon of C3 KO mice. Significant enhancement was observed in expression levels of important members of the iNOS-mediated COX-2 induction pathway, and in the phosphorylation of mitogen-activated protein (MAP) kinase members. Also, a similar pattern of increase was observed in the expression levels of inflammasome proteins in C3 KO mice. Moreover, compared to WT mice, C3 KO mice showed remarkably enhanced phosphorylation of NF-kB and IkB-α, which was reflected in entirety as increased expressions of TNF-α, IL-6 and IL-1α. Taken together, results of the current study indicate that C3 deficiency induces activation of the iNOS-mediated COX-2 induction pathway, ASC-inflammasome pathway, and NF-kB signaling pathway, resulting in the enhancement of inflammatory cytokine expressions in the transverse colon of C3 KO mice.

Introduction

The complement system plays a key role in the opsonization of pathogens and injured cells, induction of inflammation, and destruction of microorganisms, in the innate immune system of various organs including the heart, lung, liver, kidney and gut [1, 2]. These responses of the complement are mediated via three routes, viz., the classical, lectin and alternative pathways, subsequently resulting in elimination of the antigenic agent and activation of the inflammatory response [2]. During activation of the classical and alternative pathways, C3 is important for regulating various innate immune responses including promotion of opsonic phagocytosis, regulation of humoral immune response, and some T-cell biology [3]. C3 convertase is formed with fragments produced in the classical and lectin pathways (C4bC2b, formerly C4b2a) or the alternative pathway (C3bBb), which subsequently splits the C3 protein into C3a and C3b via proteolytic activity [4]. Subsequently, C3a acts as an anaphylatoxin, while C3b participates to form C5 convertase, following which it contributes to formation of the membrane attack complex (MAC) comprising C5b, C6, C7, C8 and polymeric C9 [5]. Based on the key role of C3 within the complement cascade, this protein has received great attention as a target of complement-directed therapeutic intervention [6].

The correlation between C3 concentration and inflammatory response is known to play a significant role in several gastrointestinal inflammatory diseases. Patients with inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), show enhanced catabolism and deposition of C3 as well as circulating C3 conversion products at the site of inflammation [7–9]. Stimulation of C3 and IL-6 production has also been detected in basolateral and apical membranes of Caco-2 cells treated with IL-1β [10]. Moreover, expressions of IL-17 and C3 mRNA were remarkably increased in UC and CD, whereas levels of these two cytokines in the same afflictions were suppressed by p42/44, Mitogen-activated
protein kinase (MAPK) inhibitor, and p38 MAPK inhibitor [11]. Furthermore, complement deficiency significantly suppresses tumor development through activation of the intestinal IL-1β/IL-17A axis in the azoxymethane and dextran sulfate sodium (DDS)-induced colitis-associated colorectal cancer (CAC) model [12]. Furthermore, activation of the C3 receptors (C3R), including C3a and C3b receptor (C3aR and C3bR), on the cell membrane is linked to regulation of the inflammatory response within various cells. C3aR activation amplifies the MAPK signaling pathway, leading to enhanced expression of proinflammatory cytokines. However, the involvement of iNOS and COX-2 proteins are yet to be examined [13, 14]. This activation signal induces NLRP inflammasome activation via an increase of ERK1/2 and interaction with the NF-κB activation [14, 15]. Furthermore, addition of C3 stimulates increased expressions of iNOS, IL-6 and IL-1β, and decreases the TGF-β and TNF-α expressions [16]. However, alterations in the C3R downstream-mediated inflammatory response in transverse colon of mice during C3 deficiency is poorly understood, although the complement cascade has been considered a novel therapeutic target for IBD.

In the current study, we investigated alternative regulation of the C3R downstream-mediated inflammatory response during C3 deficiency. Our study especially focuses on regulation of the iNOS-mediated COX-2 induction pathway, ASC-inflammasome pathway, NF-κB phosphorylation, and inflammatory cytokine expression in the transverse colon of C3 KO mice.

Results

Suppression of C3 protein in the kidney, spleen and thymus of C3 KO mice

To verify the suppression of C3 protein, expression levels of the C3 protein were measured in the kidney, spleen and thymus of C3 KO mice. Compared to levels obtained in WT mice, we determined successful decrease in expression levels of the protein in the kidney, spleen and thymus of C3 KO mice. (Fig. 1C). These results indicate that the expression of C3 protein is successfully inhibited in the kidney, spleen and thymus of C3 KO mice, generated using the CRISPR/Cas9-mediated technique.

Suppression of C3 and C3R protein in the transverse colon of C3 KO mice

To verify the suppression of C3 protein, expression levels of the C3 protein were measured in the transverse colon of C3 KO mice. Compared to levels obtained in WT mice, we determined successful decrease in expression levels of the protein and mRNA, whereas C3aR and C3bR protein levels were higher in the transverse colon of C3 KO mice (Fig. 2A,B). Moreover, tissue distribution of the C3 protein was detected in the intestinal villus of transverse colon of WT mice, whereas no significant color change was observed for C3 proteins in sections obtained from C3 KO mice (Fig. 2C). These results indicate that
the expression of C3 protein is successfully inhibited in the transverse colon of C3 KO mice, generated using the CRISPR/Cas9-mediated technique.

**Regulatory effects of C3 deficiency on MAPK signaling pathway**

Since the MAPK signaling pathway mediates the transfer of signals derived from C3R [13, 14], we first investigated whether C3 deficiency affects regulation of the MAPK signaling of the C3R downstream pathways in transverse colon. To achieve this, alterations in the phosphorylation of ERK, JNK and p38 were measured in the colon of C3 KO mice. As shown in Fig. 3, phosphorylation levels of three members in the MAPK signaling pathway are remarkably enhanced in KO mice, although the increase rates differed for each protein. The highest protein phosphorylation level was observed in JNK and p38. These results indicate that the MAPK signaling pathway of C3R downstream pathways may be activated in the transverse colon during C3 deficiency.

To investigate whether activation of the MAPK signaling pathway in C3R downstream pathways is accompanied with changes in the iNOS-mediated COX-2 induction pathway during C3 deficiency, alterations in the expression levels of iNOS, COX-2 were measured in the transverse colon of C3 KO mice. A similar pattern of regulation was observed in all three mediators of the iNOS-mediated COX-2 induction pathway. The expression levels of COX-2, iNOS proteins were significantly increased in the transverse colon of C3 KO mice as compared with WT mice, although the rate of increase was varied (Fig. 4). These results indicate that activation of the MAPK signaling pathway and iNOS-mediated COX-2 induction pathway in C3R downstream pathways is tightly linked to C3 deficiency in the transverse colon of C3 KO mice.

**Regulatory effects of C3 deficiency on the ASC-inflammasome pathway**

To investigate whether C3 deficiency can affect regulation of the ASC-inflammasome pathway of the C3R downstream pathways in transverse colon, altered expressions of NLRP3, Cleaved cas1/Cas1 and ASC were measured in the transverse colon of C3 KO mice. The expression level of ASC was remarkably increased in KO mice compared with WT mice. A similar pattern was observed for the expressions of the three inflammasomal proteins (Fig. 5). These results indicate that C3 deficiency is associated with upregulation of the ASC-inflammasome pathway of the C3R downstream pathway.

**Regulatory effects of C3 deficiency on the NF-κB signaling pathway**

NF-κB pathway is important for the transcriptional regulation of numerous genes involved in the host immune and inflammatory response, and also for the proliferation and survival of cells [20]. We examined
whether upregulation of the ASC-inflammasome pathway and activation of iNOS-mediated COX-2 induction pathway is accompanied by changes in the NF-κB signaling pathway during C3 deficiency. To achieve this, we measured for altered phosphorylation levels of NF-κB and IκB in the colon of C3 KO mice. As shown in Fig. 6, phosphorylation levels of two members in the NF-κB signaling pathway were remarkably enhanced in KO mice, although the increase rates differed for both proteins. Especially, the highest phosphorylation level of protein was observed in IκB. These results suggest that activation of the NF-κB signaling pathway regulated by upregulation of the ASC-inflammasome pathway and iNOS-mediated COX-2 induction pathway is associated with C3 deficiency in the transverse colon of C3 KO mice.

**Regulatory effects of C3 deficiency on inflammatory cytokines**

Finally, we examined whether activation of the NF-κB signaling pathway during C3 deficiency induces regulation of inflammatory cytokine expressions in the transverse colon. To achieve this, the transcript levels of NF-κB, TNF-α, IL-6 and IL-1α were evaluated by RT-qPCR of the transverse colon in C3 KO mice. A similar pattern of regulation was observed for all four inflammatory cytokines. The mRNA levels of NF-κB, TNF-α, IL-6 and IL-1α cytokines were remarkably increased in C3 KO mice, as compared with WT mice. TNF-α exhibited the highest increase, while NF-κB showed the lowest rate of increase (Fig. 7A). A similar increase was observed for levels of the IL-6 protein, in transverse colon obtained from C3 KO mice (Fig. 7B). Moreover, the ELISA assay determined that protein levels of TNF-α and IL-6 were constantly maintained in the serum of WT and C3 KO mice (Fig. 7C). These results indicate that upregulation of the inflammatory cytokines regulated by activation of the NF-κB signaling pathway is associated with C3 deficiency in the transverse colon of C3 KO mice.

**Discussion**

Chronic gastrointestinal inflammation is the most common inflammatory response linked to the development of various IBDs, including CD and UC [21]. Complications of long-term inflammation are proposed to be a major contributor to the development of colorectal cancer (CRC). During the inflammatory response, diverse physiological events occur in the gastrointestinal mucosa and lumen, including cell activation, cytokine production, complement activation and tissue damage [22, 23]. The regulatory factors assessed in this study are considered important for the balance between pro- and anti-inflammatory responses, which is the driving force behind inflammation and immune response to treat these diseases [21]. We therefore undertook to investigate the effects of C3 deficiency on the C3R downstream-mediated inflammatory response of the gastrointestinal tract, by analyzing alterations in the iNOS-mediated COX-2 induction pathway, ASC-inflammasome pathway, NF-κB signaling pathway, and inflammatory cytokine expression, in the transverse colon of C3 KO mice. Results of the present study provide first evidence that C3 deficiency may be tightly linked with upregulation of the inflammatory response of the C3R downstream signaling pathway in transverse colon of C3 KO mice. Furthermore,
these results indicate that activation of C3 can be considered as one of the important factors during IBD. However, more studies are required to verify the molecular mechanism of C3 and C3 receptors in the transverse colon of C3 KO mice.

Several split products of complement, including C3a and C5a, play a major role in regulating the immune system activity including degranulation, extravasation and chemotaxis, as well as activation of the immune cells and non-myeloid cells [24, 25]. Considering all factors of the complement, C3a is known to have conflicting functions on the inflammatory response. Briefly, this product contributes to the pro-inflammatory response, including enhancement of the G-protein coupled C3aR expression in immune cells and non-myeloid cells, induction of oxidative burst in macrophages, and stimulation of histamine release from basophils and mast cells [25–28]. However, the anti-inflammatory role was also investigated in ischemia-reperfusion injury and in the sepsis model showing acute phase of inflammation [29, 30]. Especially, C3a prevents the migration and degranulation of neutrophils, although other granulocytes are activated [31]. Furthermore, some significant alterations on the expression level of inflammatory cytokines were observed in C3 KO mice. The cytokine ratio between IL-10 and interferon (IFN)-γ or IL-17 levels shows a shift in the jejunum of ovalbumin challenged C3 KO mice [32]. The increase of TNF-α and IL-12 levels was greater in the colon of DSS-treated C3 KO mice than DSS-treated WT mice [33]. In the current study, the iNOS-mediated COX-2 induction pathway and inflammatory cytokine expressions were upregulated in the transverse colon of C3 KO mice. These results provide additional evidence that C3 deficiency plays an alternative role in the inflammatory response of transverse colon, although further studies are required to determine the molecular mechanism.

In the current study, we measured the mRNA level of some cytokines in the transverse colon of C3 KO mice. Enhanced levels of TNF-α, IL-6 and IL-1α transcripts were observed in the transverse colon during C3 deficiency, although the rate of increase varies widely. It is impossible to directly compare our findings with previous results because the disease model used in each study was different. However, few studies revealed a correlation between C3 and inflammation in the gastrointestinal tract. Previous studies also reported significant alterations in the levels of cytokines and C3 protein in IBD. C3 metabolism (including cleavage, circulation and deposition) was significantly increased in IBD, while levels of IL-17 and C3 mRNA were enhanced in UC and CD [7–9, 11]. Furthermore, IL-1β-induced C3 and IL-6 production was observed at the apical or basolateral membrane or chamber of CaCo-2 cells [10]. A similar response was also observed in macrophages after treatment with C3 peptides, where increased iNOS, IL-6 and IL-1β expressions and decreased TGF-β and TNF-α expressions were determined [16].

Oxidative stress is a condition in which the balance of oxidative stimulators and inhibitors in the body is disturbed by events such as inflammation. This ultimately causes oxidative damage to cells and the human body [34]. During this balancing, the iNOS-mediated COX-2 induction pathway is considered a key regulatory mechanism, since expressions of iNOS and COX-2 proteins are induced by a variety of pro-inflammatory stimuli (such as LPS and TNF-α) in various diseases [35, 36]. The overexpression and activation of iNOS promotes the production of NO, which stimulates the activation of COX-2 [37]. This process, mediated by the NF-κB and MAPK signaling pathways, has a critical role in the regulation of cell
growth and differentiation, as well as in the control of cellular responses to cytokines and stresses [38–40]. Also, the above inflammatory pathway regulated by the iNOS-mediated COX-2 induction pathway was observed to improve with diet components, including fibers, polyphenols, and poly-unsaturated fatty acids, as well as lifestyle changes including fasting and physical exercise [41]. In the current study, we measured whether the iNOS-mediated COX-2 induction pathway of the C3R downstream pathway can be activated by C3 deficiency in the transverse colon. We observed significantly enhanced expression levels of COX-2 and iNOS in the transverse colon of C3 KO mice, as compared to WT mice. These results provide additional evidence that the role of C3 in transverse colon is associated with the iNOS-mediated COX-2 induction pathway, although we were unable to directly compare our findings to previous studies.

Taken together, our study undertook to investigate the effects of C3 deficiency on the C3R downstream signaling pathway-mediated inflammatory response in the transverse colon. Our results indicate that C3 deficiency induces the upregulation of inflammatory cytokines through activation of the iNOS-mediated COX-2 induction pathway, ASC-inflammasome pathway, and NF-κB signaling pathway, in the transverse colon of C3 KO mice (Fig. 8). Furthermore, we provide additional evidence for the role of C3 in inflammatory responses and mucosal damage to the colon. However, this study provides limited information on the correlation between C3 deficiency and C3R downstream-mediated inflammatory response, since the functional validation for C3 deficiency was not analyzed in the transverse colon of C3 KO mice. Therefore, more experimental evidence is required to clarify the functional mechanism of the inflammatory response during C3 deficiency.

Materials And Methods

Animal care and use

All experiments and methods were performed in accordance with relevant guidelines and regulations. The experiments complied with the ARRIVE guidelines. Protocol for the C3 KO mice study was approved by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC; Approval Number PNU-2020-2657). The C3 KO and WT mice were handled at the Pusan National University-Laboratory Animal Resources Center, which is accredited by the Korea Food and Drug Administration (FDA) (Accredited Unit Number: 000231), and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International (Accredited Unit Number: 001525). Seven-week-old C3 KO (n = 7) and WT (n = 7) mice having the Friend Virus B Type NIH (FVB) genetic background were kindly provided by the Department of Laboratory Animal Resources at the National Institute of Food and Drug Safety Evaluation (NIFDS, Chungju, Korea). The C3 KO mice have an 11-nt deletion mutation in exon 2 of the C3 gene, which is achieved using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) design tool (crispor.tefor.net) (Fig. 1A) [17]. Throughout the experimental period, all animals were provided ad libitum access to water, and a standard irradiated chow diet (Samtako BioKorea Co., Osan, Korea) consisting of moisture (12.5%), crude protein (25.43%), crude fat (6.06%), crude fiber (3.9%), crude ash (5.31%), calcium (1.14%), and phosphorus (0.99%). All animals were maintained in a specific pathogen free (SPF) state at 23 ± 2°C and 50 ± 10% relative humidity under a strict light cycle (lights on at
08:00 h and off at 20:00 h). After adaptation for 1 week, the mice were euthanized using a chamber filled with CO₂ gas, and transverse colons were subsequently harvested for further analysis.

Wild type (WT) and C3 KO mice were identified using tail genomic DNA PCR analysis. After preparation of the reaction mixture, two sets of primers were added to detect the C3 gene: set 1, forward primer (5'-CAR CTG CTC CAG TGA GAA C-3') and reverse primer (5'-CTT CTC AGA TGT CCA CTG GCT C-3'), and set 2, forward primer (5'-CAT CTG CTC CAG YGA GAA C-3') and reverse primer (5'-TGC CTC TTT AGG AAG TCT TG-3'). All reactions were performed at 38 cycles of amplification on a Perkin-Elmer Thermal Cycler, using the following conditions: 30 sec, 94°C; 30 sec, 60°C; 1 min, 72°C. After amplification, the 291 bp and 280 bp final PCR products obtained were electrophoresed on 1% agarose gels (Fig. 1B).

**Quantitative Real-Time PCR Analysis (RT-qPCR)**

RT-qPCR was applied to assess the relative quantities of inflammatory cytokine mRNAs. Briefly, total RNA molecules were isolated from frozen transverse colon tissues using RNA Bee solution (Tet-Test Inc., Friendswood, TX, USA). After quantification of RNA, complement DNA (cDNA) was synthesized using a mixture of oligo-dT primer (Thermo Fisher Scientific Inc., Waltham, MA, USA), dNTP and reverse transcriptase (Superscript II, 18064-014, Thermo Fisher Scientific Inc.). RT-qPCR was then achieved using a cDNA template and 2: Power SYBR Green (TOYOBO Co., Osaka, Japan), as described in a previous study [18]. The primer sequences used to evaluate the mRNA levels were as follows: C3, sense primer 5'-CATAT GCTCC AGCAC TGAGA AC-3', antisense primer 5'-TGCCCT CTTTA GGAAG TCTTG-3'; C3aR, sense primer 5'-GTGAA CACAG TCTGG TTCCT CCATC TC-3', antisense primer 5'-GGTTG TTGGT TTGCT GAATC TAGAG AG-3'; C3bR, sense primer 5'-CACCA TTCAG ACATG GTGAT TCTGT GAC-3', antisense primer 5'-CTTCA TTACA GGAGA AGTAC ACAGT TGTG-3'; NF-κB, sense primer 5'-GTAAC AGCAG GACCC AAGGA-3', antisense primer 5'-AGCCC CTAAT ACACG CCTCT-3'; Tumor necrosis factor (TNF)-α, sense primer 5'-CCTGT AGCCC ACGTC GTAGC-3', antisense primer 5'-TTGAC CTCAG CGCTG ACTTG-3'; IL-6, sense primer 5'-TTGGG ACTGA TGTTG TTGAC A-3', antisense primer 5'-TCATC GCTGT TGATA CAATC AGA-3'; IL-1α, sense primer 5'-CAGTTCTGGCATTGACCAT-3', antisense primer 5'-TCTCAGAACTCAGGCT-3'; β-actin, sense and antisense primers 5'-TGGAA TCCTG TGGCA TCCAT GAAC-3' and 5'-TAAAA CGCAG CTCAG TAACA GTCCG-3', respectively. The reaction cycle at which PCR products exceeded the fluorescence intensity threshold during the exponential phase of PCR amplification was considered as the threshold cycle (CT).

**Histopathological analysis**

Transverse colons collected from mice of the subset group were fixed in 10% formalin for 48 h, embedded in paraffin wax, and then sectioned into 4 µm thick slices. The colon sections were collected on glass slides and stained with Hematoxylin & Eosin (H&E) (Sigma-Aldrich Co., St. Louis, MO, USA), after which they were examined by light microscopy for histopathology, at 400× magnification.

**Immunohistochemical (IHC) Staining Analysis**
Tissue distribution of the C3 protein was detected by IHC staining using light microscopy, as previously described [19]. Briefly, the transverse colon tissue samples were fixed in 10% formalin for 12 h, embedded in paraffin, and sliced into 4 µm thick sections. These sections were subsequently deparaffinized with xylene, rehydrated, and pretreated for 30 min at room temperature with 1× PBS blocking buffer containing 10% goat serum (Vector Laboratories, Burlingame, CA, USA). The sections were then incubated with primary anti-C3 antibody (Abcam Com., Cambridge, UK), diluted 1:300 in 1× PBS blocking buffer. The antigen-antibody complexes were visualized with biotinylated secondary antibody (goat anti rabbit)-conjugated horseradish peroxidase (HRP) streptavidin (Histostain-Plus Kit, Zymed, South San Francisco, CA, USA), at a dilution of 1:300 in PBS blocking buffer. Finally, C3 proteins were detected using stable diaminobenzidine (DAB) (Invitrogen Co., Carlsbad, CA, USA) and the Leica Application Suite (Leica Microsystems, Wetzlar, Germany).

Western blot

Total homogenate proteins were extracted from the transverse colon of C3 KO and WT mice using the Pro-Prep Protein Extraction Solution (Intron Biotechnology Inc., Seongnam, Korea). Following centrifugation of tissue homogenates at 13,000 rpm for 5 min, protein concentrations were determined using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). Proteins (30 µg) were then separated by 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 3 h, following which the resolved proteins were transferred to nitrocellulose membranes for 2 h at 40 V. Each membrane was then incubated separately with the following primary antibodies, overnight at 4°C: anti-C3 (Abcam Com.), anti-C3aR (Bioskis Inc.), anti-C3bR (LSBio Inc.), anti-COX-2 (Cell Signaling Technology Inc., Danvers, MA, USA), anti-NLR family pyrin domain containing 3 (NLRP3) (Cell Signaling Technology Inc.), anti-Caspase 1 (Cell Signaling Technology Inc.), anti-Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (Cell Signaling Technology Inc.), anti-iNOS (Thermo Fisher Scientific Inc.), anti-Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (Cell Signaling Technology Inc.), anti-p-ERK (E-4) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), anti-c-Jun N-terminal kinase (JNK) (Cell Signaling Technology Inc.), anti-p-JNK (Cell Signaling Technology Inc.), anti-p38 (Cell Signaling Technology Inc.), anti-p-p38 (Cell Signaling Technology Inc.), anti-p-Nuclear factor-κB (NF-κB) (Boster Bio Inc., Pleasanton, CA, USA), anti-Inhibitor of κB (IκB) (Cell Signaling Technology Inc.), anti-p-IκB (Cell Signaling Technology Inc.), anti-IL-6 (Santa Cruz Biotechnology Inc.) or anti-β-actin (Sigma-Aldrich Co.). The probed membranes were subsequently washed with washing buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, and 0.05% Tween 20), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000 dilution) (Zymed Laboratories, South San Francisco, CA, USA) at room temperature for 2 h. Finally, the blots were developed using a Chemiluminescence Reagent Plus kit (Pfizer Inc., Gladstone, NJ, USA). The signal band image for each protein was acquired using a digital camera (1.92 MP resolution) of the FluorChem® FC2 Imaging system (Alpha Innotech Corporation, San Leandro, CA, USA). Protein densities were semi-quantified using the AlphaView Program version 3.2.2 (Cell Biosciences Inc., Santa Clara, CA, USA).
Enzyme-linked immunosorbent assay (ELISA) for evaluating TNF-α and IL-6 cytokines

Serum concentrations of TNF-α and IL-6 cytokines were measured using a mouse TNF-α enzyme-linked immunosorbent assay kit (Biolegend, San Diego, CA, USA) and IL-6 ELISA kit (Biolegend), according to the manufacturer's protocols. Briefly, serum was isolated from whole blood of each mouse, diluted 1:75,000, and pipetted into designated wells of the kit. The plate was incubated for 20 min at room temperature, followed by washing and addition of 1× enzyme-antibody conjugate. After incubation for 20 min at room temperature and additional washing, tetramethylbenzidine (TMB) substrate was added to each well, and the color alteration was determined using a Vmax plate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

Statistical Analysis

Statistical significance was evaluated using the one-way analysis of variance (ANOVA) (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL, USA) followed by Tukey’s post hoc t-test for multiple comparisons. Data are presented as mean ± standard deviation (SD). p < 0.05 is considered to indicate a statistically significant difference.

Abbreviations

C3: Complement component C3; KO: knockout; WT: wild type; HT: heterogenous type; iNOS: nitric oxide synthase; COX-2: cyclooxygenase-2; MAPK: mitogen-activated protein kinase; IBD: Inflammatory bowel diseases; UC: ulcerative colitis; CD: Crohn's disease; CAC: Colitis-associated colorectal cancer; DDS: dextran sulfate sodium; MAC: membrane attack complex; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; PNU-IACUC: Pusan National University-Institutional Animal Care and Use Committee; FDA: Food and Drug Administration; CT: threshold cycle; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ASC: the adaptor molecule apoptosis-associated speck-like protein containing a CARD; FVB/N: Friend Virus B Type NIH

Declarations

Availability of data and material

Available.

Competing interests

The authors declare that they have no competing interests.

Funding
This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (2019R1A2C1084140). This study was supported by the BK21 FOUR Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Korea (F20YY8109033 and F21YY8109033).

Authors’ contributions

Y.J.C. were involved in experimental design, performing experiments and interpretation of data. J.E.K. performed experiments, interpreted the data. S.J.L., J.E.G. participated in data collection and analysis. D.Y.H. contributed to conception, experimental design and writing the manuscript. H.L. participated in reviewing and editing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Miss Jin Hyang Hwang, the animal technician, for directing the animal care and use at the Laboratory Animal Resources Center at Pusan National University.

References

1. Venkatesha, R. T., Berla, T. E., Zaidi, A. K. & Ali, H. Distinct regulation of C3a-induced MCP-1/CCL2 and RANTES/CCL5 production in human mast cells by extracellular signal regulated kinase and PI3 kinase. *Mol Immunol.* **42**, 581–587 (2005).

2. Ehrnthaller, C., Ignatius, A., Gebhard, F. & Huber, L. M. New insights of an old defense system: structure, function, and clinical relevance of the complement system. *Mol Med.* **17**, 317–329 (2011).

3. Ricklin, D., Hajishengallis, G., Yang, K. & Lambris, J. D. Complement: A key system for immune surveillance and homeostasis. *Nat Immunol.* **11**, 785–797 (2010).

4. Ke, L. *et al.* Functional modulation of human monocytes derived DCs by anaphylatoxins C3a and C5a. *Immunobiology.* **217**, 65–73 (2012).

5. Dalmasso, A. P., Falk, R. J. & Raij, L. The pathobiology of the terminal Complement complexes. *Complement Inflamm.* **6**, 36–48 (1989).

6. Ricklin, D. & Lambris, J. D. Complement in immune and inflammatory disorders: therapeutic interventions. *J Immunol.* **190**, 3839–3847 (2013).

7. Goska, D. T. Deficiencies and excessive human complement system activation in disorders of multifarious etiology. *Adv Clin Exp Med.* **21**, 105–114 (2012).

8. Teiberg, P. & Gjone, E. Humoral immune system activity in inflammatory bowel disease. *Stand J Gastroenterol.* **10**, 545–549 (1975).

9. Barberio, B., Gubbiotti, A., Albertoni, L., Ghisa, M. & Savarino, E. Gastrointestinal: An unusual rectal finding in a patient with ulcerative colitis. *J Gastroenterol Hepatol.* **35**, 179 (2019).

10. Moon, M. R. *et al.* Interleukin-1beta induces complement component C3 and IL-6 production at the basolateral and apical membranes in a human intestinal epithelial cell line. *Shock.* **13**, 374–378.
11. Sugihara, T. et al. The increased mucosal mRNA expressions of complement C3 and interleukin-17 in inflammatory bowel disease. *Clin Exp Immunol.* **160**, 386–393 (2010).

12. Ning, C. et al. Complement activation promotes colitis-associated carcinogenesis through activating intestinal IL-1β/IL-17A axis. *Mucosal Immunol.* **8**, 1275–1284 (2015).

13. Napier, B. A. et al. Complement pathway amplifies caspase-11-dependent cell death and endotoxin-induced sepsis severity. *J Exp Med.* **213**, 2365–2382 (2016).

14. Zhang, X. et al. Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. *Blood.* **110**, 228–236 (2007).

15. Asgari, E. et al. C3a modulates IL-1beta secretion in human monocytes by regulating ATP efflux and subsequent NLRP3 inflammasome activation. *Blood.* **122**, 3473–3481 (2013).

16. Liu, Y. et al. Complement C3 produced by macrophages promotes renal fibrosis via IL-17A secretion. *Front Immunol.* **9**, 2385 (2018).

17. Park, J. W. et al. Deficiency of complement component 3 may be linked to the development of constipation in FVB/N-C3em1Hlee/Korl mice. *FASEB J.* **35**, e21221 (2021).

18. Park, J. W. et al. Anti-oxidant activity of gallotannin-enriched extract of galla rhois can associate with the protection of the cognitive impairment through the regulation of BDNF signaling pathway and neuronal cell function in the scopolamine-treated ICR mice. *Antioxidants (Basel).* **8**, 450 (2019).

19. Park, J. J. et al. Deletion of NNX3.1 via CRISPR/Cas9 induces prostatic intraepithelial neoplasia in C57BL/6 mice. *Technol Cancer Res Treat.* **19**, 1533033820964425 (2020).

20. Yamamoto, Y. & Gaynor, R. B. IkB kinases: key regulators of the NF-kB pathway. *Trends Biochem Sci.* **29**, 72–79 (2004).

21. Bamford, K. B. Chronic gastrointestinal inflammation. *FEMS Immunol Med Microbiol.* **24**, 161–168 (1999).

22. Nakayama, Y. et al. Proinflammatory cytokines induce amelotin transcription in human gingival fibroblasts. *J. Oral Sci.* **56** (4), 261–268 (2014).

23. Camilleri, M. The Leaky Gut: Mechanisms, measurement and clinical implications in humans. *HHS Public Access.* **68**, 1516–1526 (2019).

24. Klos, A. et al. The role of the anaphylatoxins in health and disease. *Mol Immunol.* **46**, 2753–2766 (2009).

25. Klos, A., Wende, E., Wareham, K. J. & Monk, P. N. International union of basic and clinical pharmacology. LXXXVII. ComplementpeptideC5a, C4a, and C3a receptors. *Pharmacol Rev.* **65**, 500–543 (2013).

26. Murakami, Y., Imamichi, T. & Nagasawa, S. Characterization of C3a anaphylatoxin receptor on guinea-pig macrophages. *Immunology.* **79**, 633–638 (1993).

27. Kretzschmar, T. et al. Chronic myelogenous leukemia-derived basophilic granulocytes express a functional active receptor for the anaphylatoxin C3a. *Eur J Immunol.* **23**, 558–561 (1993).
28. Schäfer, B. et al. Mast cell anaphylatoxin receptor expression can enhance IgE dependent skin inflammation in mice. *J Allergy Clin Immunol.* **131**, 541–581 (2012).

29. Hollmann, T. J., Mueller-Ortiz, S. L., Braun, M. C. & Wetsel, R. A. Disruption of the C5a receptor gene increases resistance to acute Gram-negative bacteremia and endotoxic shock: opposing roles of C3a and C5a. *Mol Immunol.* **45**, 1907–1915 (2008).

30. Wu, M. C. L. et al. The receptor for complement component C3a mediates protection from intestinal ischemia-reperfusion in juries by inhibiting neutrophil mobilization. *Proc Natl Acad Sci USA.* **110**, 9439–9444 (2013).

31. Elsner, J., Oppermann, M., Czech, W. & Kapp, A. C3a activates the respiratory burst in human polymer phonuclear leukocytes via pertussis toxin-sensitive G-proteins. *Blood.* **83**, 3324–3331 (1994).

32. Pekkarinen, P. T. et al. Impaired intestinal tolerance in the absence of a functional complement system. *J Allergy Clin Immunol.* **131**, 1167–1175 (2013).

33. Elvington, M., Schepp-Berglind, J. & Tomlinson, S. Regulation of the alternative pathway of complement modulates injury and immunity in a chronic model of dextran sulphate sodium-induced colitis. *Clin Exp Immunol.* **179**, 500–508 (2015).

34. John, J. H. Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. *Cell Signal.* **14**, 879–897 (2003).

35. Endo, K., Yoon, B. I., Pairojkul, C., Demetris, A. J. & Sirica, A. E. ERBB–2 overexpression and cyclooxygenase–2 up–regulation in human cholangiocarcinoma and risk conditions. *Hepatology.* **36**, 439–450(2002).

36. Jaiswal, M., LaRusso, N. F., Burgart, L. J. & Gores, G. J. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide–dependent mechanism. *Cancer Res.* **60**, 184–190 (2000).

37. Huang, F. Y. et al. Characterization of interleukin-1β in Helicobacter pylori-induced gastric inflammation and DNA methylation in interleukin-1 receptor type 1 knockout (IL-1R1(-/-)) mice. *Eur J Cancer.* **49**, 2760–2770 (2013).

38. Hemish, J., Nakaya, N., Mittal, V. & Enikolopov, G. Nitric oxide activates diverse signaling pathways to regulate gene expression. *J Biol Chem.* **278**, 42321–42329 (2003).

39. Vanden, B. W. et al. p38 and extracellular signal–regulated kinase mitogen–activated protein kinase pathways are required for nuclear factor–kappa B p65 transactivation mediated by tumor necrosis factor. *J Biol Chem.* **273**, 3285–3290 (1998).

40. Margină, D. et al. Chronic inflammation in the context of everyday life: Dietary changes as mitigating factors. *Int J Environ Res Public Health.* **17**, 4135 (2020).

41. Margina, D. et al. Analysis of the intricate effects of polyunsaturated fatty acids and polyphenols on inflammatory pathways in health and disease. *Food Chem Toxicol.* **143**, 111558 (2020).
Figure 1

Targeting scheme for the C3 gene, identification of C3 KO mice, and expression of C3 protein. (A) 11 nucleotides in exon 2 of the C3 gene was deleted with a mixture of Cas9 protein and 2 sgRNA, as presented in Materials and Methods. (B) Deletion of C3 gene was identified by DNA analysis using genomic DNA isolated from tails of founder mice. M and HT indicate the maker and heterogenous type. (C) The expressions of C3 protein in the kidney, spleen and thymus tissues were measured with Western blot analysis using anti-C3 antibody and HRP-labeled anti-rabbit IgG antibody. Band intensities were determined using an imaging densitometer, and expressions of the proteins were calculated relative to the intensity of β-actin. Three to five mice per group were used for the preparation of tissue lysates, and Western blots were assayed in duplicate for each sample. Data are reported as the mean ± SD. * indicates p < 0.05 compared to the WT mice.
Figure 2

Expression Levels of C3 protein and mRNA in the transverse colon of C3 KO mice. (A) The expressions of C3 protein and mRNA in the transverse colon were measured with Western blot and RT-PCR analysis using anti-C3 antibody and C3 specific primers. After determining the intensity of each band using an imaging densitometer, relative levels of the C3 protein were calculated, based on the intensity of β-actin. The mRNA level of the C3 gene was calculated based on the intensity of β-actin as an endogenous.
control. Three to five mice per group were used for preparing total RNA, and RT-PCR analysis was assayed in duplicate for each sample. (B) The expressions of C3aR, C3bR protein in the transverse colon were measured with Western blot analysis using anti- C3aR, C3bR antibody. After determining the intensity of each band using an imaging densitometer, relative levels of the C3aR, C3bR protein were calculated, based on the intensity of β-actin. Three to five mice per group were used for preparation of the tissue lysates, and Western blots were assayed in duplicate for each sample. Data are reported as the mean ± SD. * indicates p < 0.05 compared to the WT mice. (C) Tissue distribution of C3 protein was analyzed in the transverse colon of WT and C3 KO mice. The C3 protein-specific antibody-stained sections of the transverse colon from the WT and KO mice were observed at 400× magnification using a light microscope. The large image in the right column is a magnified image of the rectangle in the left column. H&E-stained sections (low rectangle in left corner) were observed at 400× magnification using a light microscope.
Figure 3

Expression Levels of members in the MAPK signaling pathway. (A) Expression levels of ERK, p-ERK, JNK, p-JNK, p38 and p-p38 proteins were determined by Western blot analysis using specific primary antibody and HRP-labeled anti-rabbit IgG antibody. (B) Band intensities were determined using an imaging densitometer, and expressions of the proteins were calculated relative to the intensity of β-actin. Three to five mice per group were used for the preparation of tissue lysates, and Western blots were assayed in
duplicate for each sample. Data are reported as the mean ± SD. * indicates p < 0.05 compared to the WT mice.

Figure 4

Expression levels of members in the iNOS-mediated COX-2 induction pathway. (A) Expression levels of COX-2 and iNOS proteins were determined by Western blot analysis using specific primary antibody and HRP-labeled anti-rabbit IgG antibody. (B) Band intensities were determined using an imaging
densitometer, and expressions of the proteins were calculated relative to the intensity of β-actin. Three to five mice per group were used for the preparation of the tissue lysates, and Western blots were assayed in duplicate for each sample. Data are reported as the mean ± SD. * indicates p < 0.05 compared to the WT mice.

Figure 5
Expression levels of members in the ASC-inflammasome pathway. (A) Expression levels of NLRP3, Cleaved cas1/Cas1 and ASC proteins were determined by Western blot analysis using the specific primary antibody and HRP-labeled anti-rabbit IgG antibody. (B) Band intensities were determined using an imaging densitometer, and protein expressions were calculated relative to the intensity of β-actin. Three to five mice per group were used for the preparation of tissue lysates, and Western blots were assayed in duplicate for each sample. Data are reported as the mean ± SD. * indicates p < 0.05 compared to the WT mice.
Figure 6

Expression levels of members in the NF-κB signaling pathway. (A) Expression levels of NF-κB and IκB proteins were determined by Western blot analysis using specific primary antibody and HRP-labeled anti-rabbit IgG antibody. (B) Band intensities were determined using an imaging densitometer, and protein expressions were calculated relative to the intensity of β-actin. Three to five mice per group were used for the preparation of the tissue lysates, and Western blots were assayed in duplicate for each sample. Data are reported as the mean ± SD. * indicates p < 0.05 compared to the WT mice.

Figure 8

Suggested mechanism of inflammatory response in the C3R downstream pathway during C3 deficiency. In this scheme, the downregulation of C3 concentration is thought to activate the iNOS-mediated COX-2 induction pathway, MAPK signaling pathway, ASC-inflammasome pathway, and NF-κB signaling pathway. Finally, activated NF-κB translocate into nucleus, and promotes the expression of inflammatory cytokines in the transverse colon.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformationChoietal.pdf