Effectiveness of C5a aptamers in a TNBS-induced colitis mouse model

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Abstract. The complement-activated product, complement component 5a (C5a), is a potent inflammatory peptide with a broad spectrum of functions. *In vivo* and *in vitro* studies have demonstrated that C5a serves an important role in inflammation; however, the role of C5a in the pathogenesis of inflammatory bowel disease (IBD) is not known. The purpose of the current study was to investigate the role of C5a in IBD using an experimental mouse model of colitis. Colitis was induced in mice using 2,4,6-trinitrobenzene sulfonic acid (TNBS), and C5a aptamers were subsequently administered via intraperitoneal injection. Clinical symptoms of the disease, histopathological analysis of the colon and the level of inflammatory components were examined. The symptoms of colitis, including changes in behavior, weight loss, colon damage and an increase in inflammatory cytokines, were attenuated following the treatment of mice with TNBS-induced colitis with C5a aptamers. The aptamer-treated mice exhibited a marked attenuation of colitis when compared with untreated mice, as demonstrated by the phenotypic observations, histological examinations and inflammatory cytokine levels. Colitis is characterized by an imbalance between pro-inflammatory and anti-inflammatory mediators. The results of the current study suggest that C5a may serve a critical role in inflammation in IBD.

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Introduction

Crohn disease (CD) and ulcerative colitis (UC) are chronic relapsing gastrointestinal disorders, two main components of inflammatory bowel disease (IBD) (1). To date, the etiology of IBD is unknown; however, various inflammatory reactions constantly stimulate the mucosal and systemic immune systems and are involved in the inflammatory cascade (2). Approximately 25% of IBD cases occur before the age of 20 years, and 4% occur in persons younger than 5 years of age (3). In the treatment of IBD, a variety of pharmacological agents that target the inflammatory process are effective in controlling disease in patients and in sustaining symptomatic remission for prolonged periods, including tumor necrosis factor blockers, probiotics and corticosteroids (4).

The complement system is widely considered to function to protect the host against invading microorganisms (5). However, previous studies investigating complement system activation have demonstrated that it may serve an injurious role in the pathogenesis of a number of inflammatory and immunological diseases (6,7). Complement activation products include complement component (C) 3a, C4a, C5a and C5b-9 and the membrane attack complex. C5a is an anaphylatoxin that exhibits chemotactic activities and promotes oxidative bursts, phagocytosis and the release of granule enzymes from neutrophils, monocytes and macrophages (7).

A study involving C5a knockout mice has demonstrated that C5a serves a critical role in inflammatory diseases (8). Several inflammatory diseases have been attributed to the ability of C5a to bind with high-affinity C5a receptors (9,10), including the C5a anaphylatoxin chemotactic receptor and C5L2, which is expressed on numerous myeloid and non-myeloid cells (11). Inhibition of C5a using a C5a antagonist has been considered as an intervention that may protect against excessive C5a production and the associated systemic inflammatory response impairment (12). Antibodies are the primary antagonists that have been used to inhibit the biological activity of C5a (12). However, the disadvantages of using protein-based C5a inhibitors include an increased risk of immunogenicity due to their chronic use, as well as the cost of production, resulting in expensive therapies for patients (13). Similar to antibodies, aptamers can be designed to bind to multiple

targets. Aptamers exhibit a high affinity for their targets, as their dissociation constants, which are comparable to those of certain monoclonal antibodies, are typically between the μM and low pM range (14,15). Therefore, the present study aimed to investigate the effect of C5a in a mouse model of acute 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced experimental colitis. In addition, macroscopic and histological parameters, and the diversification of inflammatory cytokine expression were analyzed. The results suggest that C5a serves a critical role in inflammation.

Materials and methods

Animals and grouping. A total of 24 adult female, 10-week-old BALB/c mice (weight, 20-24 g; Laboratory Animal Center of Jilin University, Jilin, China) were employed in the present study. All animals were maintained in a temperature-controlled environment (temperature, 23˚C, humidity, 50-55%), were group-housed (2 mice per cage) in a pathogen-free facility with a 12 h light/dark cycles and were provided mouse chow and water *ad libitum*. The animals were handled in accordance with the Welfare and Ethics of Laboratory Animals of China guidelines, and protocols were approved by Military Veterinary Institute Animal Investigational Committee (Changchun, China), and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health of China.

The mice were randomly and equally allocated to the following three groups (8 mice/group): TNBS-induced colitis with no treatment, TNBS-induced colitis with C5a aptamer treatment (TNBS+C5a aptamer), and a negative control group. In the experimental colitis groups, colitis was induced with a single dose of intrarectal injections of 2.75 mg TNBS. A total of 2 days later, 8 mice from the TNBS-induced colitis group were intraperitoneally injected with 10 mg/kg C5a aptamers. The C5a aptamers coupled with 40 kDa polyethylene glycol were obtained from Ms Man Chen (The Institute of Military Veterinary, Academy of Military Medical Sciences, Changchun, China). In the negative control group, 50% ethanol was intrarectally instilled into the colon. The weight and behavior changes were observed for 4 days after treatment.

TNBS-induced colitis. Following fasting for 24 h, the mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg, cat no. 76744; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequent to anesthesia, the mice were intrarectally administered with 70 μl TNBS (2.75 mg; Sigma-Aldrich; Merck KGaA) dissolved in 50% ethanol. Mice in the negative control group received 70 μl ethanol (50%) under the same conditions. A total of 8 mice of the TNBS-induced colitis group were intraperitoneally injected with C5a aptamers 48 h after induction of colitis. The weight of all the mice was recorded every morning for 6 days by electronic balance (0.01 accuracy). On the 7th morning, blood samples from mice in each group were collected from the retro-orbital plexus. Serum samples obtained from three groups of mice blood were centrifuged at 1,000 x g for 3 min at 4˚C.

Determination of C5a serum concentration. The quantitative sandwich enzyme immunoassay technique was performed using the Mouse Complement fragment 5a, ELISA kit (cat no. CSB-E08514m; Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturer's protocol. The antibodies specific to C5a were used to pre-coat wells of a microplate. A total of 200 μl mouse serum samples and standards from the kit were pipetted into the wells, and C5a bound to the immobilized antibodies. Following the removal of all unbound substances, a biotin-conjugated antibody directed against C5a was added to the wells. Subsequent to washing the wells with wash buffer (25X) from the Mouse Complement fragment 5a ELISA kit, avidin-conjugated horseradish peroxidase was added to the wells. The plate was washed to remove any unbound avidin-enzyme reagent, and a substrate solution was added to the wells. The subsequent extent of color change was proportional to the quantity of C5a present in the sample. Color development was then inhibited and the microplate was read at 450 nm within 5 min using a microplate reader.

Quantitative analysis of mouse cytokine levels. To quantify the concentration of cytokines in serum, the Quantibody® Mouse Inflammation Array Q1 kit (cat no. QAM-INF-1-1; Raybiotech, Inc., Norcross, GA, USA) was used according to the manufacturer's protocol. Similar to a traditional sandwich-based ELISA, pairs of cytokine-specific antibodies were used for detection. A variety of cytokine antibodies were coated on glass slides, and following incubation with the sample, any target cytokines present within the sample bound to antibodies in the solid surface. A second biotin-labeled detection antibody was subsequently added, which recognized a different isotope on the target cytokine. The antibody-cytokine-antibody-biotin complex was visualized through the addition of the streptavidin-labeled cyanine 3 equivalent dye using a laser scanner (535 nm). By comparing the signals produced by unknown samples with the signal curve produced by the standards, the cytokine concentration in the samples was determined.

Histopathology. Colon specimens from mice in the three groups were collected 7 days after the establishment of the model and fixed in 10% buffered formalin for 2 days at 23˚C, embedded in paraffin and then cut into 3-μm sections. The sections were stained with hematoxylin for 5 min and eosin for 1 min at 23˚C and observed at a magnification of x132 using a light microscope.

Immunohistochemistry. Inflammatory cells in colon tissue samples were detected by assessing the level of the inflammatory marker, myeloperoxidase (MPO), by immunohistochemical analysis. The method of fixing and slicing tissue samples was the same as previously described. The sections were then deparaffinized and rehydrated via standard protocols according to the manufacturer's guidelines of UltraSensitive™ SP IHC kit (MXR® Corporation, Fuzhou, China) and 3% H_2O_2 was subsequently added to the sections until they were covered. Bovine serum albumin (3%) was then added to the sections to inhibit non-specific binding. Tissue sections were incubated with a 500-fold dilution of primary anti-MPO rabbit monoclonal antibody (cat no. RB-373-R7; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C for

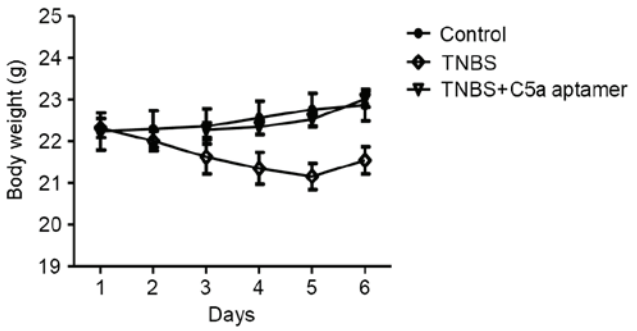


Figure 1. C5a aptamer treatment attenuates weight loss in mice with TNBS-induced colitis. TNBS, 2,4,6-trinitrobenzene sulfonic acid; C5a, complement component 5a.

24 h. This was followed by incubation with streptavidin peroxidase-conjugated biotinylated goat anti-rabbit IgG secondary antibody (UltraSensitive™ SP IHC kit). Tissue sections were subsequently stained with a 3,3'-diaminobenzidine for 15 min at room temperature (Sigma-Aldrich; Merck KGaA) and counterstained with Mayer's hematoxylin for 5 min at 23˚C. The result from stained tissue sections observed at a magnification of x132 using a light microscope.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). The results were expressed as the mean ± standard deviation, and were analyzed by a one-way analysis of variance followed by Tukey's post hoc. $P<0.0001$ was considered to indicate a statistically significant difference (Student's t-test). All experiments were repeated three times.

Results

Aptamer treatment attenuates TNBS-induced weight loss, lethargy and hoarding. Mice in the TNBS group exhibited a marked reduction in weight and appetite decline (weight was reduced from 22.32±0.23 to 21.55±0.32 g) when compared with the control (weight changed from 22.24±0.45 to 22.87±0.37 g) and TNBS+C5a aptamer group (weight changed from 22.28±0.18 to 23.02±0.19 g; Fig. 1). Mice in the TNBS group compared with the control exhibited greater lethargy, were more crowded together and half-closed their eyes or were more immobile (data not shown).

C5a aptamer treatment significantly attenuates the TNBS-induced increase in serum C5a levels. In order to determine the concentration of C5a in mouse serum samples, a standard curve of C5a concentration vs. absorbance was generated ($r^2=0.97$; Fig. 2A). The concentration of C5a in the serum of mice from the TNBS group (82.93±3.31 mg/ml) was significantly higher when compared with those from the TNBS+C5a aptamer (24.07±4.10 mg/ml) and control (2.94±0.90 mg/ml) groups ($P<0.0001$; Fig. 2B).

Treatment with C5a aptamers reverses the TNBS-induced damage to epithelial, submucosal and lamina propria layers. Colon tissue samples from mice in all groups were histopathologically analyzed by hematoxylin and eosin staining. In the TNBS group, the epithelial layer was damaged and the villi were absent; there was also evidence of edema in the submucosa and lamina propria, with congested and dilated blood vessels (Fig. 3A). By contrast, no evidence of congestion and vasodilation in the lamina propria was observed in the C5a aptamer-treated group (Fig. 3B). The histological appearance of the colonic walls from the mice in the control group was normal (Fig. 3C).

Table I. Level of inflammatory cytokines.

Cytokine (pg/ml)	Control	TNBS	TNBS+C5a aptamers
BLC	0.0	86,452.2	1,936.7
Eotaxin	20.3	2,070.4	327.1
Eotaxin-2	0.0	2,597.2	507.2
IFNγ	611.7	4,771.8	170.8
IL-6	0.0	189.6	17.3
IL-7	0.0	9.4	824.0
IL-10	0.0	22.1	168.1
IL-12p70	128.3	14,934.7	1,269.5
IL-21	0.0	393.7	6,659.0
KC	12.5	358.0	696.5
MCP-1	0.0	107.0	30.7
MCP-5	20.6	575.0	131.8
MCSF	0.0	12.1	0.0
MIG	1,204.0	8,552.2	2,931.8
MIP-1α	0.0	54.8	1,831.9
MIP-1γ	969.3	37.4	1,672.2
TARC	0.0	24.2	10.3
TCA-3	0.3	3.3	11.0

BLC, B lymphocyte chemoattractant; IFN, interferon; IL, interleukin; KC, platelet-derived growth factor-inducible protein KC; MCP, monocyte chemoattractant protein; MCSF, macrophage colony-stimulating factor; MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; TARC, thymus and activation-regulated chemokine; TCA, T-cell activation protein.

Treatment with C5a aptamers attenuates the TNBS-induced increase in MPO expression. MPO expression was observed in lymphocytes within the colonic mucosal lamina propria in mice from the TNBS group (Fig. 4A). However, reduced MPO expression was observed in the colons of mice from the TNBS+C5a aptamer group (Fig. 4B), whereas no MPO expression was observed in colon tissue samples from mice in the control group (Fig. 4C). These results indicate that C5a aptamers exhibit therapeutic effects in IBD.

C5a treatment altered the level of cytokines in the serum. The level of the majority of cytokines in mice with TNBS-induced colitis was observed to increase when compared with mice in the control group (Table I). The level of macrophage inflammatory protein (MIP)-1γ was decreased in the TNBS group compared with the control and TNBS+C5a aptamer groups, whereas the serum concentration of B lymphocyte

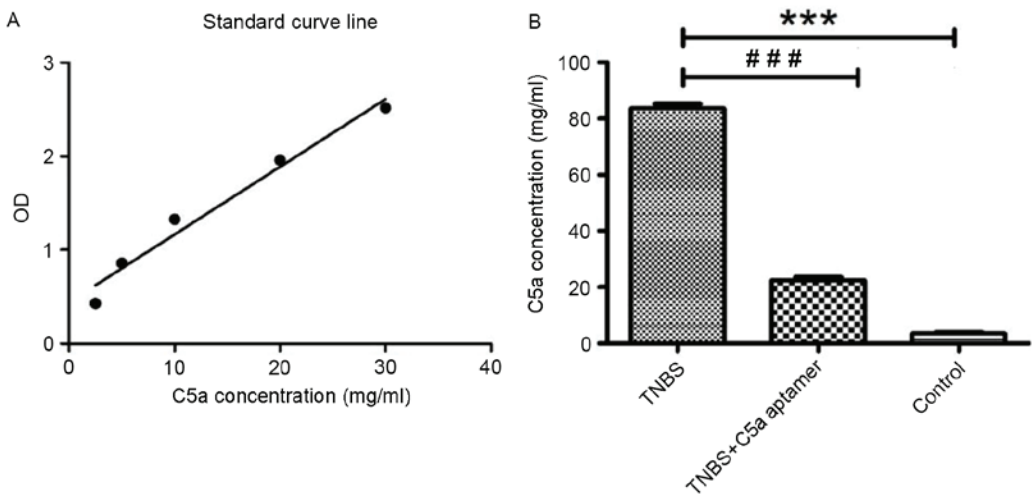


Figure 2. C5a aptamer treatment significantly attenuates the TNBS-induced increase in serum C5a levels. (A) The standard curve of mouse serum C5a standards. (B) Serum concentration of C5a in the TNBS, TNBS+C5a aptamer treatment and control groups. ***P<0.0001 and ###P<0.0001 as indicated. TNBS, 2,4,6-trinitrobenzene sulfonic acid; C5a, complement component 5a; OD, optical density.

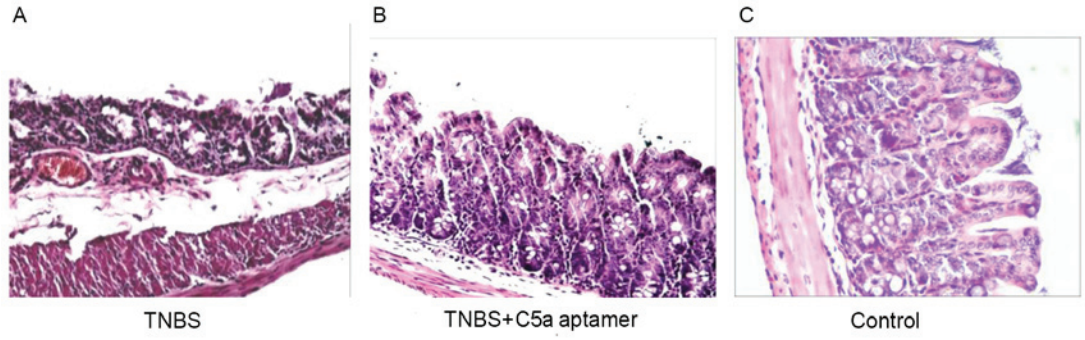


Figure 3. Colon wall layers are damaged by colitis in the TNBS group compared with the control and C5a aptamer-treated groups. (A) The epithelial, submucosal and lamina propria layers were damaged and the villi were absent in the TNBS group. There were also signs of edema in the submucosa and lamina propria, with congested and dilated blood vessels. (B) There was no evidence of congestion and vasodilation in the lamina propria of the TNBS+C5a aptamer group. (C) The wall of the colon was normal in the control group. Magnification, x132. TNBS, 2,4,6-trinitrobenzene sulfonic acid; C5a, complement component 5a.

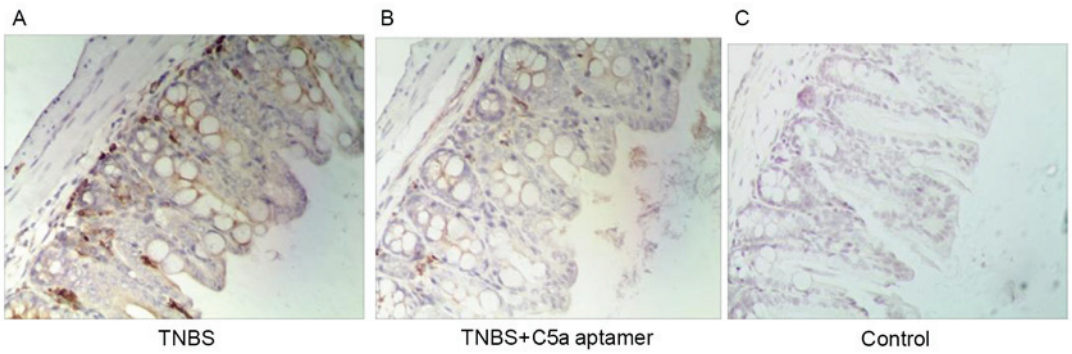


Figure 4. Treatment with C5a aptamers attenuates the TNBS-induced increase in MPO expression. (A) The level of MPO expression was high in the colonic mucosal lamina propria in mice from the TNBS group. (B) Partial MPO expression was observed in mice from the TNBS+C5a aptamer group, and (C) no MPO expression was observed in mice from the control group. Magnification, x132. TNBS, 2,4,6-trinitrobenzene sulfonic acid; C5a, complement component 5a; MPO, myeloperoxidase.

chemoattractant (BLC) and interferon-γ (IFN-γ) in the TNBS group was >25-fold higher when compared with the TNBS+C5a aptamer group (Table I). Following treatment with C5a aptamer, the concentration of ~50% of the inflammatory cytokines decreased to a level similar to that observed in the control group. By contrast, interleukin (IL)-7, IL-10 and IL-21, platelet-derived growth factor-inducible protein KC (KC), MIP-1α, MIP-1γ and T-cell activation protein-3 (TCA-3)

concentrations in mice from the TNBS+C5a aptamer group were higher compared with the TNBS and control groups (Table I).

Discussion

IBD is used to describe two chronic diseases that lead to inflammation of the intestines, including Crohn's disease and ulcerative colitis (4). Although the diseases share common features, there are important differences. The results of previous studies suggest that specific C3 and mannose-binding lectin genotypes, as well as associated multi-gene regions that encode complement molecules, are associated with IBD (4,16). Despite these observations, complement molecules have not emerged as a cause of monogenic IBD (16,17). Instead, hyperactivation of the complement system or the lack of regulators, may underlie chronic inflammatory diseases such as IBD (18,19).

The complement system is considered to serve a central role in the innate immune system, which functions as a host defense mechanism against invading pathogens and in the clearance of potentially damaging cell debris (20-23). However, it has been confirmed that complement activation is also involved in the pathogenesis of several inflammatory and immunological diseases (24). A previous study has indicated that the establishment of a TNBS-induced experimental colitis in the intestinal mucosa was associated with the increased production of the pro-inflammatory cytokine, IL-6, and decreased production of the anti-inflammatory cytokine, IL-10 (25). The present study demonstrated that the C5a aptamers reduced the level of specific cytokines in the serum of mice with TNBS-induced colitis, including BLC, eotaxin, eotaxin-2, IL-6, IFNγ, IL-12p70, monocyte chemoattractant protein 1 and 5, macrophage colony-stimulating factor, monokine induced by IFN-γ, and thymus and activation-regulated chemokine, when compared with the TNBS group and control group. By contrast, treatment with a C5a aptamer increased the production of IL-7, IL-10, KC, IL-21, MIP-1α, MIP-1γ and TCA-3 in mice with TNBS-induced colitis. Colitis is characterized by an imbalance in pro-inflammatory and anti-inflammatory mediators, which may result in an exaggerated pro-inflammatory response, anti-inflammatory response syndrome, immunosuppression, apoptosis, organ dysfunction or clinical manifestations such as weight loss (26). The effect of C5a aptamer treatment in a mouse model of colitis was examined in the present study. Following C5a aptamer treatment, the body weight and levels of inflammatory cytokines in mice with colitis decreased and were closer to the values observed in mice from the control group.

Currently, immunomodulators are commonly used to treat IBD. The side effects of these agents include nephrotoxicity, nausea, tremor, headaches and gingival hyperplasia (27). Oligonucleotides have emerged as a promising class of biopharmaceuticals. Aptamers constitute a novel class of oligonucleotides that have gained therapeutic importance (28). In the present study, the importance of C5a in IBD was addressed by treating mice with TNBS-induced colitis using C5a aptamers. Aptamers are selected from random-sequence oligonucleotide pools to bind a wide range of molecules, ranging from small inorganic molecules to biomacromolecules with affinities and specificities that are comparable to antibodies (29). Some aptamers have entered the clinical trials process (30). The greatest success in the therapeutic application of an aptamer, which was approved by the US Food and Drug Administration, was an anti-vascular endothelial growth factor aptamer for the treatment of age-associated macular degeneration (31). A number of aptamers are currently being evaluated in clinical trials (32). In addition, numerous aptamers have exhibited efficacy in tissue culture experiments and animal models, including those targeting thrombin, factor IXa, IFN-γ and epidermal growth factor receptor (33-36). In the current study, the mouse C5a aptamers were selected to bind to the C5a protein, and were revealed to markedly reduce inflammation in mice with TNBS-induced colitis. Therefore, C5a aptamers may present a potential therapeutic candidate to treat patients with IBD.

Acknowledgements

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