

ARTICLE

Enolase-specific cross antibodies induce neutrophilic inflammation in the intestine

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Abstract

The pathogenesis of ulcerative colitis (UC) is to be further investigated. House dust mites (HDM) are highly associated with the pathogenesis of immune inflammation in the body. This study aims to investigate the role of enolase (one of the HDM-derived proteins)-specific cross Abs in the induction of UC-like inflammation. The enolase specific IgG (EslgG) was identified in UC patients by mass spectrometry. Mice were treated with EslgG to induce inflammation in the colon mucosa. EslgG was detected in the serum and the colon tissues of UC patients, which was positively correlated with the polymorphonuclear neutrophil (PMN) counts in the blood and colon tissues of UC patients. EslgG formed immune complexes with the constitutive enolase in the UC colon epithelium that activated complement, induced epithelial cell apoptosis, compromised epithelial barrier functions, and resulted in UC-like inflammation in the mouse colon. In summary, UC patients have high serum levels of Abs against HDM-derived enolase and intestinal epithelial cell-derived enolase. These Abs attack the colonic epithelium to induce UC-like inflammation.

KEYWORDS

ulcerative colitis, intestine, inflammation, house dust mites, neutrophil

1 | INTRODUCTION

The prevalence of ulcerative colitis (UC) is about 0.1–0.3% in the world and kept rising in the last few decades.¹ Although research about UC advanced rapidly in the recent years, the causative factors of UC remain elusive and need to be further investigated. It is the consensus that aberrant immune responses play a central role in the pathogenesis of UC.¹ However, the initiation factors of the aberrant immune responses are largely unknown.

Published data indicate that house dust mite (HDM)-derived factors can be detected in the digestive tract, affect intestinal barrier function, and possibly involved in the pathogenesis of intestinal inflammation.²

The association between HDM and human diseases has been extensively studied, and mostly focuses on allergic diseases, including allergic rhinitis, allergic asthma, and allergic dermatitis.³ The HDM-specific IgE binds the high-affinity IgE receptors on the surface of mast cells to make mast cells sensitized. Sensitized mast cells can be activated by re-exposure to HDM allergens and release pro-inflammatory mediators, such as histamine, leukotrienes, serotonin, and TNF- α , to induce inflammation in the local tissues.⁴ However, HDM still induces inflammation in the absence of mast cells or IgE receptor-deficient.⁵ This may be because besides IgE, HDM also induces IgG production.⁶ Whether HDM-specific IgG contributes to the pathogenesis of intestinal inflammation, such as UC, is to be investigated.

IgG is a fraction of the 5 immunoglobulins in the body. The canonical function of IgG is to eliminate invading Ags. It forms complexes with the Ags; the complexes can be engulfed by phagocytes to be eliminated.⁷

Abbreviations: EslgG, enolase specific IgG; HDM, house dust mite; PMN, polymorphonuclear neutrophil; slgE, specific IgE; UC, Ulcerative colitis.

On the other hand, the complexes may activate complement.⁸ Activated complement chemoattracts and activates polymorphonuclear neutrophils (PMN),⁹ and induce inflammation in the local tissues. HDM-specific IgG was detected in asthma patients,⁶ whether UC patients also respond to HDM-specific IgG has not been investigated. Since HDM distribute extensively in the human living environment, it is logical that HDM gets into the digestive tract following dietary activities.²

Enolase is one of the constitutive proteins of HDM that highly homologous to human enolase.¹⁰ Previous studies found that enolase-specific Abs could be found in the serum of UC patients.^{11,12} Enolase is a lyases that can cleave the carbon-oxygen bonds. Enolase is associated with some disease activities, such as astrocytoma,¹³ UC, primary sclerosing cholangitis, autoimmune hepatitis, and vasculitis.^{11,12} Whether enolase involves in the UC pathogenesis remains to be further investigated.

Therefore, we hypothesize that HDM-derived factors, such as enolase, may induce specific IgG Ab production in the human body; the Abs recognize HDM-derived factors as well as the constitutive molecules in the intestinal tissues; it triggers the immune complex-directed proinflammatory cell activation and induces inflammation in the local tissues. Therefore, we obtained blood and colon biopsies from UC patients. The interaction between HDM and the cross Abs from UC patients was observed. The results showed that high enolase-specific cross-Ab levels in UC patients in the serum. The findings were expanded in a murine model. Exposure to the enolase-specific Ab resulted in intestinal epithelial barrier dysfunction and induced neutrophilic inflammation in the intestine. The data suggest that extrinsic enolase-specific cross Ab may play an important role in the pathogenesis of colitis.

2 | MATERIALS AND METHODS

2.1 | Reagents

Abs of enolase (with or without labeling with FITC), C3a, C5a, C5b-9, caspase (csp)-3, and csp9 (with or without labeling with Alexa Fluor® 680) were purchased from Santa Cruz Biotech (Santa Cruz, CA). ELISA kits of specific IgG (mIgG), mIgG4, sIgE, TNF- α , IFN- γ , and IL-1 β were purchased from Dakewe Biomart (Beijing, China). ELISA kits of C3a, C5a, and C5b-9 were purchased from Biocompare (South San Francisco, CA). FITC-dextran, Z-VAD-FMK, protein G, reagent kits of TUNEL, and Annexin V were purchased from Sigma-Aldrich (St. Louis, MO). RNAi kits of enolase and csp9 were provided by GenScript (Nanjing, China). Abs of CD11b (BUV563) and Ly-6G (APC-cy7) for flow cytometry were purchased from BD Biosciences (Franklin Lakes, NJ). Reagents and materials for RT-qPCR, Western blotting, and immunoprecipitation were purchased from Invitrogen (Carlsbad, CA).

2.2 | Human subjects

UC patients were recruited into this study. The diagnosis and management of UC were carried out by our physicians following our established procedures. The demographic data of human subjects are

presented in Table 1. Biopsy specimens per endoscopy were collected in the colon in the vicinity of lesions or ulcerations. The samples were processed and stained with H&E and observed under a light microscope. The histological disease activity was semi-quantitatively with a published scoring system.¹⁴ Inflammatory items observed include PMN infiltration, crypt abscesses, loss of glandular parallelism, crypt shortening, and/or ramification, mucus epithelial depletion, involvement of muscularis mucosae. The score was ranged from 0 (normal) to 3 (1 mild, 2 moderate, and 3 severe). In addition, the total number of PMNs, eosinophil, and mononuclear cells in the lamina propria was counted in 10 high-power fields (400 $\times$), the selection was based on high cellular density. The experimental procedures using human tissue in the present study were approved by the Human Ethic Committee at Zhengzhou University (ZZUH#2017003). A written informed consent was obtained from each human subject. Patients or the public were not involved in the design, or conduct, or reporting, or dissemination of our research.

2.3 | Enzyme-linked immunosorbent assay

Cytokine levels in the serum and culture supernatant were determined by ELISA with commercial reagent kits following the manufacturer's instructions.

2.4 | Assessment of serum HDM-specific IgE (sIgE)

The sIgE for HDM, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, was measured by immunoCAP (Pharmacia AB Diagnostic, Uppsala, Sweden) following the manufacturer's instructions. According to the WHO Standard, IgE with a range of 0.35–100 kUA/L was considered positive sIgE and <0.35 kUA/L was considered nonspecific.

2.5 | Assessment of serum HDM-specific IgG

Serum mIgG levels were measured by using ELISA. In brief, 96-well microtiter plates (Corning, Corning, NY, USA) were coated with HDM extracts (10 μ g/ml; 100 μ l/well, made by our laboratory)¹⁵ and incubated at 4°C overnight. After washing with PBS 3 times, the plates were incubated with 3% BSA in PBS for 30 min. Diluted (1:10) serum was added to the plates (100 μ l/well) and incubated for 2 h. Then, biotin-labeled goat anti-human IgG Ab (Santa Cruz Biotech) at 1:1000 dilution was added and incubated for 1 h to detect mIgG. After washing, the plates were incubated with streptavidin-peroxidase (Sigma Co.) at 1:1000 dilution for 30 min at room temperature. After washing, the O-phenylene diamine was added as a substrate solute. Optical densities were recorded by using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 405 nm.

2.6 | Generation of polyclonal EslgG

BALB/c mice were subcutaneously injected with recombinant HDM-specific enolase (0.1 mg/mouse, provided by Sangon Biotech, Shanghai, China) mixed in complete Freund adjuvant on the back

TABLE 1 Demographic data of human subjects

	UC	HC	Colon cancer
Age (year), mean $\pm$ SD	44 $\pm$ 11	41 $\pm$ 9	55 $\pm$ 15
Sex (M/F)	22/18	20/20	5/5
Duration of disease (year), mean $\pm$ SD	5.8 $\pm$ 1.9	—	0.8 $\pm$ 0.5
Mayo score, median (range)	8.5 (7.2–10)	—	—
Colonic area involved			
Left-side	15	—	5
Right-side	13	—	5
Both left and right-side	12	—	—
Histologic score, median (IQR)	2.1 (2–2.5)	—	—
Active UC	0	—	—
Remission UC	40 (100%)	—	—
Under medication	0	—	—
C-reactive protein mg/L, median (IQR)	3.06 (1.6–10.4)	—	—
Hemoglobin g/dl, median (IQR)	12.68 (10.6–13.5)	—	—
Albumin g/L, median (IQR)	3.6 (3.2–4.3)	—	—
Fecal calprotectin mg/kg, median (IQR)	1210 (300–1800)	—	—

skin at multiple spots on day 1, day 7, and day 14, respectively. The mice were boosted with enolase mixed with incomplete Freud adjuvant every 2 weeks 4 times. One week after the last boost, blood was collected from mice by the eyeball-pulling method.

2.7 | Purification of EslgG

Blood samples were collected from 10 UC patients. The samples were placed at 4°C overnight. The serum was collected. The total IgG in the serum (the serum from 10 patients was pooled) was precipitated by protein A. Following published procedures,¹⁶ EslgG was purified from the total IgG by affinity chromatography in which the sepharose beads were saturated with recombinant enolase (provided by the Shanghai Sangon Biotech, Shanghai, China).

2.8 | Mass spectrometry

Following established procedures,¹⁵ whole HDM proteins were fractionated by the 2-dimensional (2D) PAGE, immunoblotted with serum from UC patients. Positive spot gel was cut; proteins were extracted from the gel and analyzed by matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry (MALDI-TOF-MS) performed by professional staff. The data were also analyzed by professional staff in MS facility.

2.9 | EslgG forms complexes with intestinal epithelial cell-derived enolase

Protein extracts were prepared from the colon epithelial cell line, T84 cells. EslgG was mixed with the protein extracts at a ratio of 1:1. The samples were analyzed by immunoprecipitation (IP) with anti-mouse IgG as the precipitation Abs (mouse was the host produced the EslgG).

After desolation, the proteins were analyzed by MS to determine if the protein was enolase.

2.10 | Western blotting

Total proteins were extracted from cells collected from relevant experiments, fractionated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking by incubating with 5% skim milk for 30 min, the membrane was incubated with the primary Abs of interest overnight at 4°C, washed with TBST (Tris-buffered saline containing 0.1% Tween20) 3 times, incubated with peroxidase-labeled secondary Abs for 2 h at room temperature, washed with TBST 3 times. Immunoblots on the membrane were developed by the enhanced chemiluminescence and photographed in an imaging station.

2.11 | Immunoprecipitation

Cells were collected from relevant experiments. Proteins were extracted from the cells and precleared by incubating with protein G agarose beads for 2 h to remove the pre-existing immune complexes. The beads were removed by centrifugation at 5000 $\times$ g for 10 min. Supernatant was collected and incubated with the primary Abs of interest or isotype IgG overnight. Immune complexes in the samples were precipitated by incubating with protein G agarose beads for 2 h. The beads were collected by centrifugation at 5000 $\times$ g for 10 min. Proteins on the beads were eluted with an eluting buffer and analyzed by Western blotting. All the procedures were performed at 4°C.

2.12 | Flow cytometry

T84 cells were fixed with 1% paraformaldehyde (containing 0.05% Triton X-100) for 1 h, washed with PBS 3 times, and incubated with

FITC-enolase Ab (1:100 dilution) or Alexa Fluor® 680-csp9 Ab (1:100 dilution) on ice for 30 min. Control samples were stained with isotype IgG. The cells were washed with PBS 3 times and analyzed with a flow cytometer (FACSCanto II, BD Bioscience). The data were analyzed by Flowjo (Tree Star Inc., Oakland, OR) with the data obtained from the isotype IgG staining as gating reference.

2.13 | Apoptosis cell assessment by flow cytometry

Cells were harvested from relevant experiments and washed with PBS, and then stained Annexin-V-FITC and PI according to manufacturer's instructions. The cells analyzed with a flow cytometer.

2.14 | Assessment of apoptotic epithelial cells in situ by TdT mediated-dUTP nick-end labeling

Mouse intestinal segments were excised upon the sacrifice. Cryosections were prepared with the intestinal samples, fixed with 4% paraformaldehyde for 2 h at room temperature, washed with PBS, and then stained with a TUNEL kit following manufacturer's instructions.

2.15 | Immunohistochemistry

Intestinal segments were excised and snap-frozen by liquid nitrogen. Cryosections were prepared, fixed with cold acetone for 20 min. After blocking by incubating with 1% BSA for 30 min, sections were incubated with the first Abs of interest at 4°C overnight, washed with PBS 3 times, incubated with fluorochrome-labeled secondary Abs for 2 h at room temperature, washed with PBS for 3 times, contrast staining with propidium iodide (PI), mounted with cover slips, and observed under a fluorescent microscope.

2.16 | Assessment of the effects of EslgG on impairing T84 monolayer barrier functions

T84 cells were cultured into monolayers in Transwells. The monolayers were regarded as confluence when the transepithelial electric resistance (TEER; recorded with an Ohmmeter) reached or over 1000 ohm·cm². EslgG was added to the apical chambers at 5 µg/ml. TEER was recorded before the addition of EslgG and 12 h later. FITC-dextran (MW = 40 kDa; used as a tracer of the barrier permeability) was added to the apical chambers at 20 µg/ml 12 h after the addition of EslgG. Samples were collected from the basal chambers 1 h later. FITC levels in the samples were determined by fluorescent spectrometry and used as an indicator of the barrier permeability.

2.17 | Activation of complement in T84 cells by EslgG

T84 cells were exposed to EslgG in the culture at 5 µg/ml. Cells and supernatant were collected 30 min later. Levels of C3a, C5a, and C5b-9 in cell extracts and supernatant were determined by ELISA.

2.18 | Myeloperoxidase assessment

Colon segments were excised upon the sacrifice. The tissues were cut into small pieces and homogenized in a tube containing protein extracting buffer containing proteinase inhibitor cocktail in an ice bath. The lysates were centrifuged at 13,000 × g for 10 min at 4°C. The supernatant was collected. MPO levels in the supernatant were determined by ELISA with a commercial reagent kit following the manufacturer's instructions.

2.19 | Mice

BALB/c mice and C57BL/6 mice (6–8-week-old) were purchased from the Guangdong Experimental Animal Center (Guangzhou, China). The enolase-1 KO mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained in a specific pathogen-free facility with accessing food and water freely. The experimental procedures were approved by the Animal Ethnic Committee at Shenzhen University.

2.20 | Inflammatory scores of mouse colitis

Following published procedures,¹⁷ the histopathological score of lesions was scored (0–4 increasing severity) with following parameters: (1) presence of tissue loss/necrosis, (2) severity of mucosal epithelial lesion, (3) inflammation, (4) grade 1 – the percentage of colon tissues was mildly affected and (5) grade 2 – the percentage of colon was severely affected. The colitis severity was calculated by summing the individual scores (max score = 20).

2.21 | Depletion of csp9 or enolase expression in T84 cells

Expression of csp9 or enolase in T84 cells was depleted by RNA interference (RNAi) with commercial reagent kits following manufacturer's instruction. The effects of RNAi in T84 cells were assessed by Western blotting and flow cytometry 48 h later.

2.22 | Induction of colon inflammation in mice by EslgG

EslgG was generated in mice by immunizing mice with enolase and completed Freund adjuvant as described above. EslgG was introduced into the colon (0.1 mg/mouse; the same as the TNBS-colitis procedures¹⁸) every other day for 3 times. Body weight was recorded daily. Mice were sacrificed 1 day after the last treatment. Blood samples were collected and colon segments were excised and processed for histology and determining inflammation in the colon following our established procedures that can be found elsewhere.^{18,19}

2.23 | Statistical analysis

Data were expressed as mean ± SEM. The Student's *t*-test was performed to determine the difference between the 2 groups. ANOVA followed by the Dunnett's test or the Bonferroni test were used for

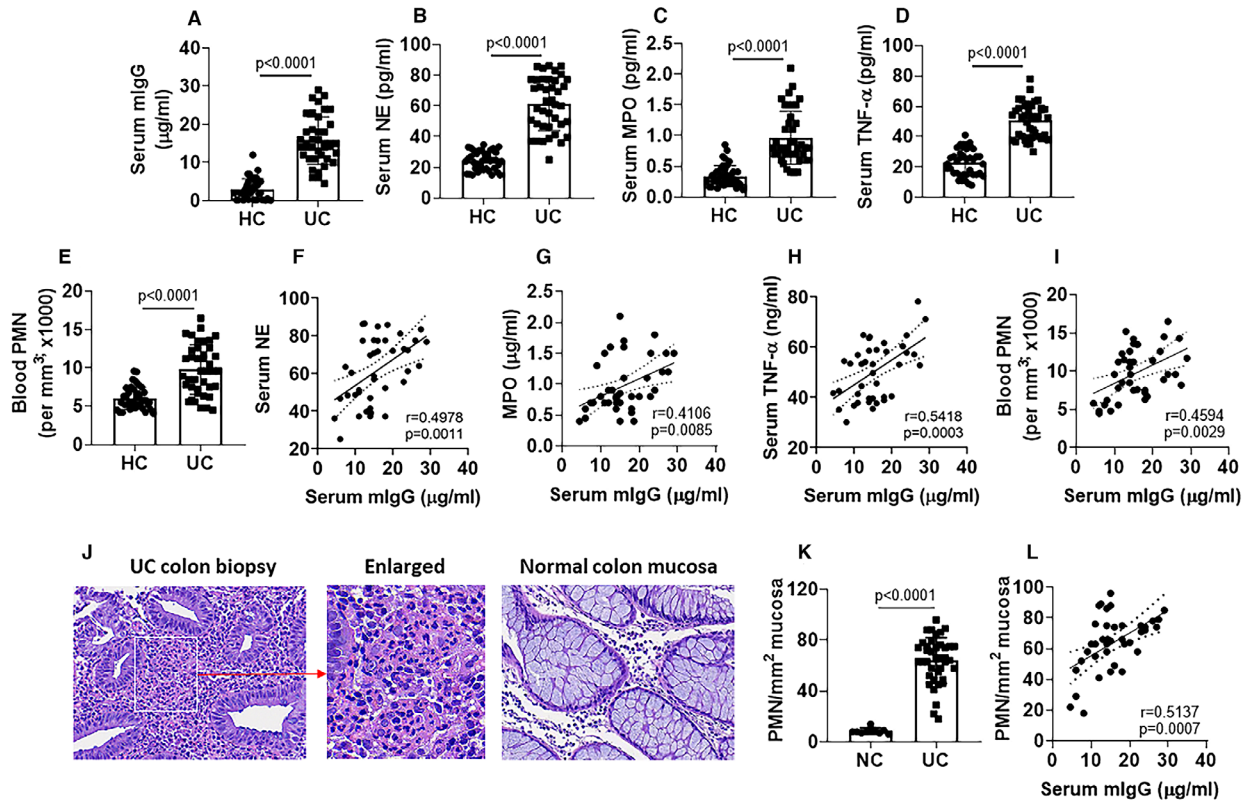


FIGURE 1 HDM-specific IgG (mIgG) is associated with PMN activities in UC patients. Blood samples were obtained from 40 UC patients and 40 healthy control (HC) subjects. (A–D) Serum levels of mIgG (mite-specific IgG), NE (neutrophil elastase), MPO (myeloperoxidase), and TNF- α . (E) Blood PMN counts. (F–I) Positive correlation between serum mIgG levels and NE, MPO, TNF- α , or blood PMN counts. (J and K) Colon biopsies were taken from the 40 UC patient; marginal normal control (NC) colon tissues were obtained from 10 surgically removed colon cancer tissues. (H) Representative colon histology images (original magnification: $\times 200$) of UC and NC samples show PMN infiltration. (K) Summarized PMN counts in colon tissue sections. (L) Positive correlation between serum mIgG and colon PMN counts. Data of bars are presented as median (IRQ). Each dot presents data obtained from 1 human subject. Statistics: The Mann-Whitney *U*-test (A–D, K) or Pearson correlation assay (F–I, L)

multiple comparisons. The Mann-Whitney *U* test was used to compare differences in 2 groups when the data distribution was not normal. The Pearson test was performed for correlation analyses. A *P*-value of less than 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Serum HDM specific IgG is positively correlated with PMN activities of UC patients

Published data indicate that, apart from inducing airway inflammation, HDM is also involved in the intestinal barrier dysfunction.² As epithelial barrier dysfunction is one of the major features of IBD,²⁰ HDM-derived factors may involve in the pathogenesis of UC. To test this, blood samples were obtained from UC patients. HDM-specific Abs in the serum were assessed by ImmunoCAP and ELISA. The results showed that both HDM-specific IgE (mIgE) and mIgG were detected in the serum of UC patients. The levels of mIgG in UC patients were significantly higher than that in healthy control (HC) subjects (Fig. 1A), while the mIgG4 and mIgE levels were comparable between UC patients and HC subjects (Supplementary Fig. S1A and B). PMN-derived cytokines,

neutrophil elastase (NE), myeloperoxidase (MPO), and TNF- α , were higher in the UC serum than that of HC serum (Fig. 1B–D). Other UC-related cytokines, IFN- γ and IL-17, in the serum of UC patients were comparable to HC subjects (Supplementary Fig. S1C and D). In addition, peripheral PMN counts were also higher in the UC group than that of the HC group (Fig. 1E). Importantly, a positive correlation was detected between serum mIgG levels and serum NE, MPO, TNF- α , and blood PMN counts, respectively (Fig. 1F–I). No correlation was found between serum mIgG and serum IFN- γ or IL-17 (Supplementary Fig. S1E and F). Furthermore, profound PMN infiltration was observed in UC colon biopsies (Fig. 1J and K) that were also positively correlated with serum mIgG levels (Fig. 1L). The results demonstrate that higher PMN activities were detected in UC patients; the mIgG may be associated with the high PMN activities.

3.2 | HDM-derived enolase specific IgG (EslgG) recognizes colon epithelial cell-derived enolase

Next, we purified mIgG from UC patients by affinity chromatography (Fig. 2A). The mIgG was incubated with UC colon biopsy protein extracts. The immune complexes of mIgG and colon tissue extracts were formed as detected by immunoprecipitation (IP) (Fig. 2B). The

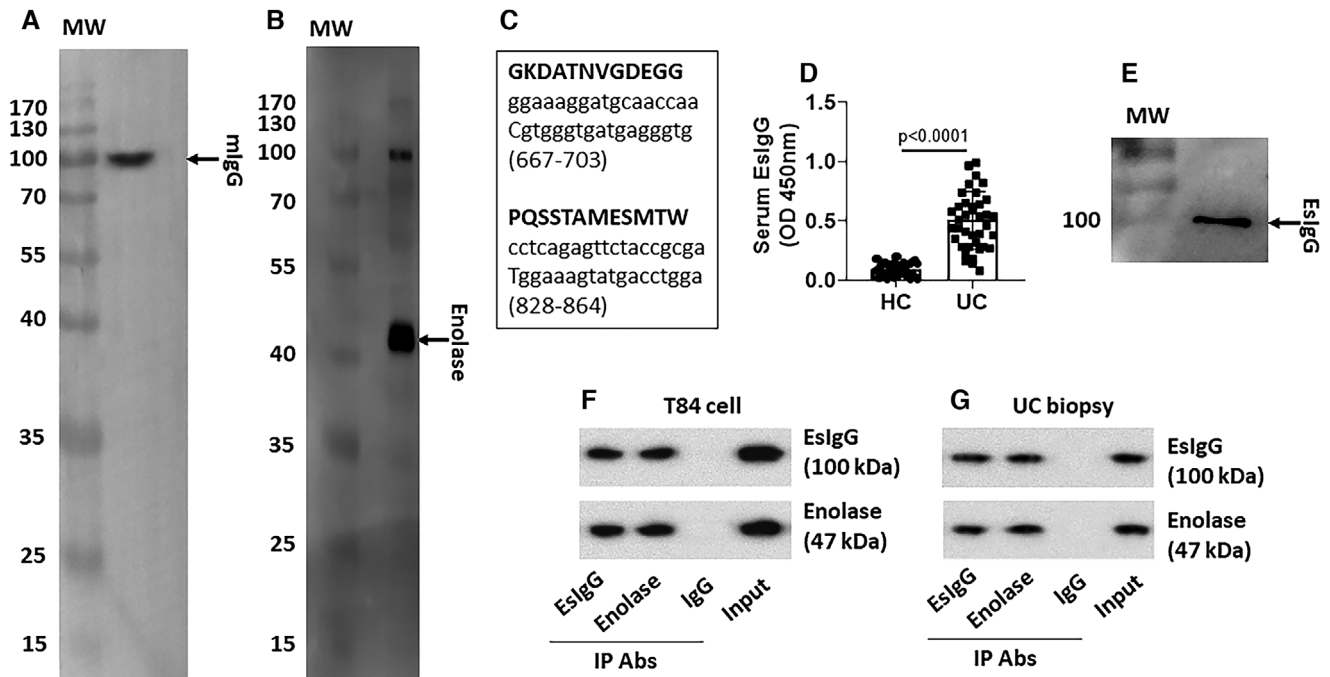


FIGURE 2 EslgG recognizes colon epithelial cell-derived enolase. Blood samples were collected from 40 UC patients and 40 healthy control (HC) subjects. (A) mlgG (HDM-specific IgG) was purified from the blood samples (by affinity chromatography). (B) The immunoblot indicates the protein precipitated by purified mlgG (it was identified latterly as enolase by MS). (C) The precipitated protein in panel (B) was analyzed by mass spectrometry (MS). The amino acid sequences (highlighted) are 2 representative peptides of enolase- α identified by MS (the corresponding gene sequences and locations in the enolase- α gene are presented below). (D) Bar graphs show the serum enolase-specific IgG (EslgG) of HC subjects and UC subjects. The data of bars are presented as median (IQR). Each dot presents data obtained from 1 patient. Statistics: The Mann-Whitney *U*-test. (E) The immunoblots show the affinity chromatography purified EslgG from the UC patient serum. (F and G) the purified EslgG was incubated with T84 cell extracts or UC biopsy extracts, and then precipitated by co-IP. The immunoblots show EslgG forms a complex with enolase extracted from T84 cells (F) or UC colon epithelium (G). The data represent at least 6 independent experiments

precipitated proteins were analyzed by MS. The results showed that enolase was the protein to form complexes with mlgG (Fig. 2C). The results were verified by ELISA that showed enolase-specific IgG (EslgG) was detected in the serum that was significantly higher in UC samples than HC samples (Fig. 2D). We then purified EslgG from the UC serum by affinity chromatography (Fig. 2E). The EslgG was incubated with protein extracts from T84 cells or UC colon biopsies and analyzed by IP. The results showed that EslgG formed complexes with enolase of T84 cell protein extracts or colon biopsies (Fig. 2F and G). The results indicate that the UC patient serum contains high levels of EslgG that can recognize the constitutive enolase of colon epithelial cells.

3.3 | EslgG/enolase complexes activate complement in intestinal epithelial cells

It is known that Ag/Ab complexes can activate complement.⁸ Intestinal epithelial cells express complement.²¹ Thus, we inferred that the EslgG/enolase complexes (as shown in Fig. 2) could activate complement in colon epithelial cells. To test this, T84 cells were exposed to EslgG in the culture. This markedly induced complement activation in an EslgG dose-dependent manner; the levels of C3a, C5a, and C5b-9 were detected in culture supernatant as well as in cell extracts, which

was abolished by knocking down the expression of enolase gene in T84 cells (Fig. 3A–F; Supplementary Fig. S2). This phenomenon was reproduced in vivo. After treating mice through introducing EslgG into the colon every other day for 3 times, the colon segments were excised. Significantly more activated complement in colon epithelial cells as well as in the surrounding tissues were observed, which was abolished by knocking out the enolase gene (Fig. 3G). High levels of activated complement were also observed in UC colon epithelial cells (Fig. 3H–K). The results demonstrate that EslgG can form complexes with the constitutive enolase in intestinal epithelial cells to activate complement in epithelial cells.

3.4 | EslgG activates complement to induce intestinal epithelial cell apoptosis

Complement activation can induce apoptosis.²² We found that after exposing to EslgG in the culture for 48 h, T84 cell apoptosis was detected; blocking complement with CVF (a complement inhibitor) or depletion of enolase expression abolished this phenomenon (Fig. 4A and B). Prompted by the previous finding that C5b-9 was colocalized with apoptotic cells,²³ we inferred that C5b-9 might physically interact with the apoptosis-initiating molecules. Indeed, we found that the EslgG-activated C5b-9 formed a complex with caspase-9 (csp9) in T84

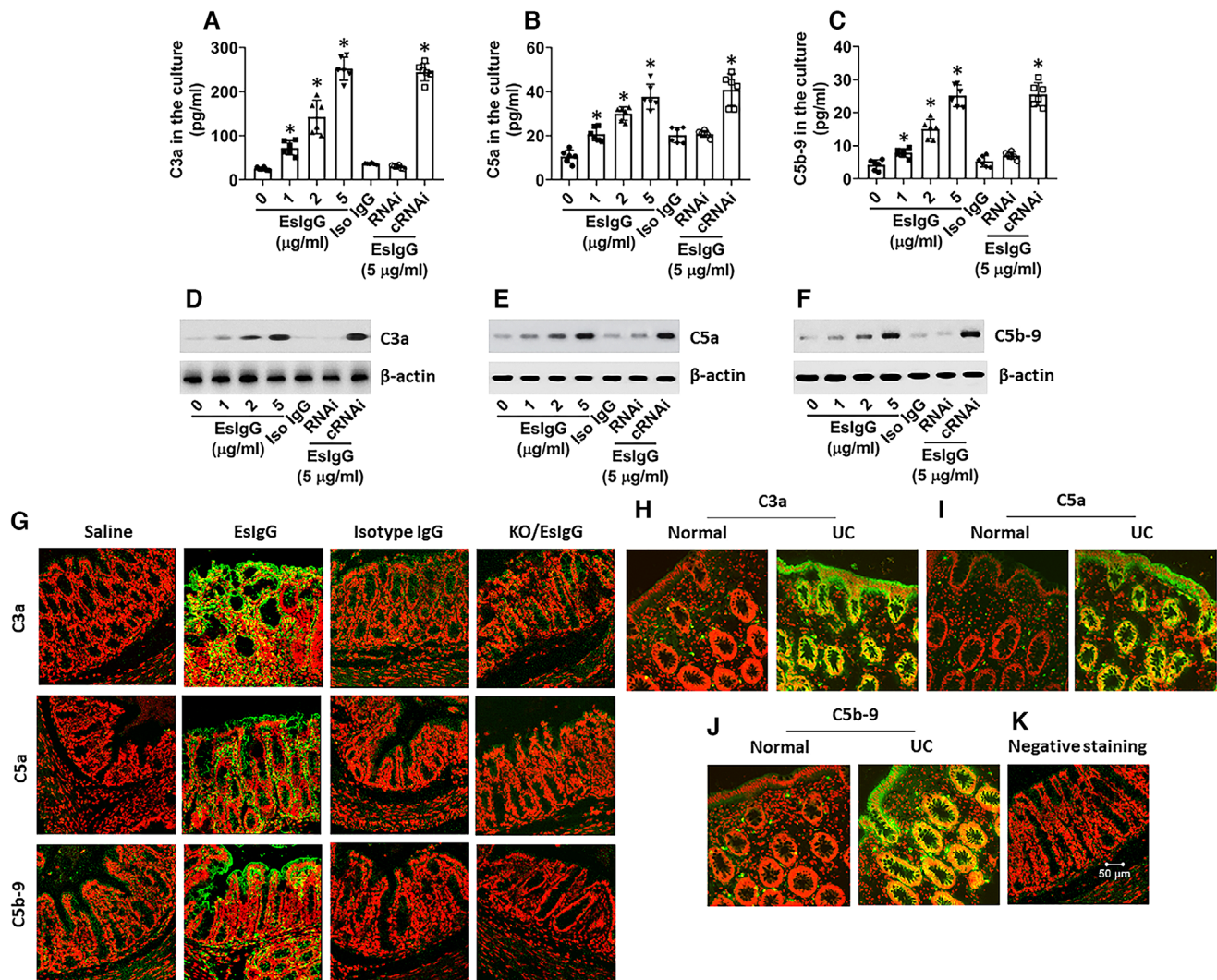


FIGURE 3 EslgG activates complements in intestinal epithelial cells. (A–F) T84 cells were cultured in the presence of EslgG (5 μg/ml) overnight. (A–C) Bars show activated complements in the culture supernatants. (D–F) Immunoblots show activated complements in the cytosolic protein extracts of the T84 cells. RNAi: enolase-deficient T84 cells prepared by RNAi. cRNAi: T84 cells were treated with control RNAi. Data of (A–F) represent 6 independent experiments. (G–K) Colon segments were obtained from mice (G) and human (H–K). Representative immunohistochemistry images show activated compliments (in green) in colon epithelial cells and the surrounding tissues, in which naive mice were introduced with EslgG (saline and isotype IgG were used as controls) into the colon (0.1 mg/mouse/time) every other day for 3 times. KO: enolase deficient mice. Human colon biopsies were obtained from normal colon mucosa or UC mucosa. (K) Isotype IgG negative staining. Statistics: ANOVA followed by the Bonferroni test. Each group consists of 6 mice

cell extracts (Fig. 4C) that cleaved csp9 to its active forms; such an effect of EslgG was abolished by depletion of enolase expression, or the presence of cobra venom factor (CVF) (Figure 4D). The active csp9 in turn cleaved csp3 to active csp3 that could be abrogated by depletion of enolase expression, or the presence of CVF, or depletion of csp9 expression (Supplementary Fig. S3) in T84 cells (Fig. 4E). Blocking csp3 inhibited the EslgG-induced T84 cell apoptosis (Fig. 4A(e)). The results indicate that EslgG forms complexes with enolase on T84 cells; the complexes activate complement to induce T84 cell apoptosis. Such a phenomenon was reproduced in vivo. After treating mice with EslgG daily for 3 times, apoptotic epithelial cells were detected in the colon that was abolished by depletion of enolase expression or administration of CVF (Fig. 4F).

3.5 | EslgG impairs intestinal epithelial barrier function

Since complement activation can induce epithelial barrier dysfunction,²⁴ we next assessed the effects of EslgG on regulating T84 monolayer barrier functions in a Transwell system. In response to EslgG stimulus, the transepithelial electric resistance (TEER) of T84 monolayers dropped markedly and the permeability was significantly elevated (Fig. 5A and B). This EslgG-altered T84 monolayer barrier function was abolished by the presence of CVF.²⁵ On the other hand, EslgG was introduced into mice via intraperitoneal injection daily for 7 times. The epithelial barrier permeability was significantly increased by EslgG, which was abrogated by

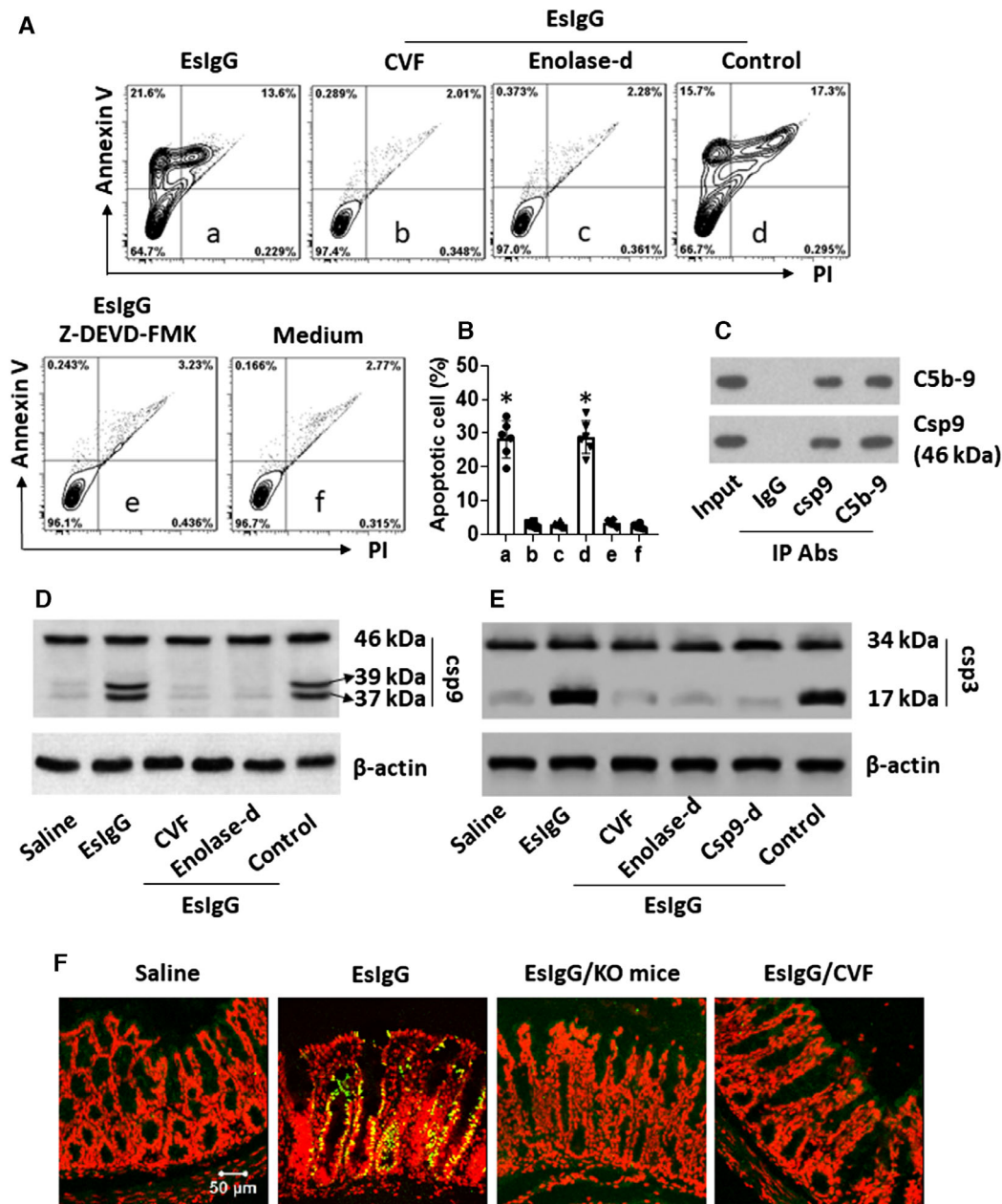


FIGURE 4 EslgG induces intestinal epithelial cell apoptosis. (A) T84 cells were treated with the procedures denoted above each subpanel for 48 h, stained with propidium iodide (PI) and Annexin V reagent, and analyzed by flow cytometry. Gated dot plots show apoptotic cells. EslgG (5 μ g/ml); cobra venom factor (CVF) (0.2 U/ml). Enolase-d: Enolase-deficient T84 cells (by CRISPR). control: cells were treated with control CRISPR reagents. (B) Summarized data of panel (A) (mean $\pm$ SEM; each dot presents data obtained from 1 experiment). * $P < 0.01$, compared with group b (ANOVA followed by the Dunnett's test). (C) EslgG induces a complex of C5b-9 and pro-csp9 (by IP) in T84 cells. (D) EslgG activates complements to cleave csp9 in T84 cells. (E), EslgG induces active csp9 to cleave csp3. csp9-d: csp9-deficient T84 cells (by CRISPR). (F) EslgG induces colon epithelial cell apoptosis (in green, by TUNEL staining) after introducing EslgG the mouse colon daily for 3 times. KO mice: Enolase KO mice. CVF: Mice were treated with CVF (200 U/mice; i.p.)

administration of CVF or depletion of enolase expression (Fig. 5C). The results indicate that EslgG can impair intestinal epithelial barrier functions by activating complement.

3.6 | EslgG induces inflammation in the colon

We next treated mice with EslgG (0.1 mg/mouse; i.p.) daily for 7 days. The mice showed UC-like symptoms and immunopathological

changes, including loss of the body weight (Fig. 6A), increases in MPO levels in colon protein extracts (Fig. 6B), colon tissue inflammation and tissue structure destruction (Fig. 6C and D), and the high PMN frequency in the colon tissues (Fig. 6E and F), which were blocked by CVF, or enolase KO, or blocking caspases (Fig. 6A–F). The results indicate that EslgG can induce UC-like inflammation in the colon by activating complement, inducing epithelial barrier dysfunction, and chemoattracting PMNs to the colon tissues.

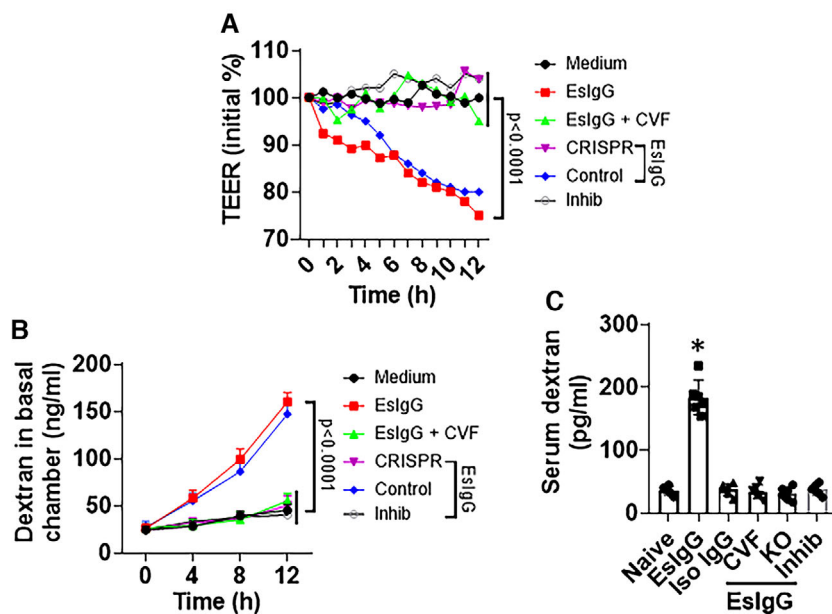


FIGURE 5 EslgG compromises intestinal epithelial barrier functions. (A and B) T84 monolayers were treated with the indicated procedures, including EslgG: 5 μ g/ml; CVF (cobra venom factor): 0.2 U/ml; CRISPR (or control): T84 cells were treated with enolase CRISPR (or control reagents) to deplete the enolase expression. inhib: Z-VAD-FMK (20 μ M; a caspase inhibitor). (A) Dynamic changes of T84 monolayer TEER. (B) Dynamic changes of T84 monolayer permeability. (C) Naive wild type (WT) or enolase KO mice (enolase-KO mice; 6 per group) were peritoneally injected with EslgG (0.1 mg/mouse) daily for 7 days. The mice were introduced with FITC-dextran (0.5 g/kg) by intra-colon instillation. One hour later, the blood samples were collected from mice and analyzed by fluorospectrometry. Bars (mean $\pm$ SEM) show serum dextran levels. * $P < 0.01$, compared with group naive (ANOVA followed by the Dunnett's test). CVF: 200 U/kg. Each dot present data obtained from 1 mouse. Data of (A) and (B) represent 6 independent experiments

4 | DISCUSSION

The present data show that enolase can induce cross Abs (the EslgG) against the constitutive enolase in intestinal epithelial cells. EslgG forms complexes with enolase in epithelial cells. The complexes activate complement in the epithelial cells to impair the epithelial barrier function and induce UC-like inflammation in the intestine. The data demonstrate that the EslgG is a cross Ab and can induce neutrophilic inflammation in the intestinal mucosa. This can be regarded as one of the causative factors in the pathogenesis of UC.

Enolase, also known as phosphopyruvate hydratase, is a metalloenzyme responsible for the catalysis of the conversion of 2-phosphoglycerate to phosphoenolpyruvate, one of the steps of glycolysis. Enolase has 3 isozymes; enolase-1 is expressed almost by all tissues; enolase-2 is mainly expressed by nerve tissues; and enolase-3 is expressed by muscle cells.²⁶ Previous studies indicate the enolase-specific autoantibodies can be found in patients with IBD, primary sclerosing cholangitis, autoimmune hepatitis, vasculitis, non-IBD gastrointestinal controls, and some of the healthy controls.^{11,12} In line with these pioneer studies, the present study also found the enolase-specific Abs in UC patients. Our study expanded the findings by showing that enolase-specific Ab could recognize the constitutive enolase in intestinal epithelial cells that induced intestinal epithelial barrier dysfunction and resulted in neutrophilic inflammation in the intestine.

HDM distributes extensively in the human living environment.²⁷ HDM's body components and products have competent antigenicity to induce allergic responses in the body.²⁷ It is well known that HDM-induced IgE triggers rhinitis, asthma, and dermatitis.³ The present data demonstrate that HDM also can induce a cross Ab, an IgG against enolase, production to induce an immune response and induce inflammation in the colon. Previous studies also found the HDM-specific IgG (mIgG) in allergic patients. Okazaki et al. reported sIgE, mIgG, and mIgG4 in the serum of asthma patients. The levels of mIgE and mIgG

could not be changed by specific immunotherapy, although the mIgG4 levels were increased.²⁸ Our data show that the serum mIgG4 levels could be detected in UC patients, but was not significantly different from HC subjects. Whether up-regulation of mIgG4 in UC patients can have therapeutic effects on UC is an interesting topic and is worth to be further investigated.

The data show that EslgG recognizes human intestinal epithelial cell-derived enolase and forms a complex with it. Ab/Ag complexes can activate complement.⁸ As shown by the present data, activated complement, including C3a, C5a, and C5b-9, were found in UC patient colon biopsies, as well as in T84 cells, and in the culture supernatants of T84 cells after exposure to EslgG. The data indicate that EslgG forms complexes with the constitutive enolase in T84 cells that can be released into the surrounding tissues. Activated complements are associated with the pathogenesis of inflammation. C3a and C5a can induce anaphylactic mediators, such as histamine release and attract phagocytes to the sites^{29,30}; C5b-9 induces target cell apoptosis.³¹ These factors from complement activation are closely associated with the pathogenesis of inflammation.³²

We found that EslgG induces intestinal epithelial cell apoptosis, in which complement activation plays a critical role. By exposing intestinal epithelial cells to EslgG in the culture or administration of EslgG in mice, colon epithelial cell apoptosis was observed that could be abolished by inhibiting complement by the presence of CVF, an inhibitor of complement, or depletion of enolase in T84 cells or in mice. The data demonstrate that EslgG recognizes the constitutive enolase in intestinal epithelial cells, activates complement in the cells, and induces epithelial cell apoptosis. Others also observed complement induced cell apoptosis. For example, Farkas et al. found that C5a could induce neuroblastoma cell apoptosis.³³ Shi et al. reported that C5b-9 induced photoreceptor cell apoptosis and necroptosis.³⁴ Apoptosis is associated with the pathogenesis of UC.³⁵ The present data expand our knowledge in this area by showing that a cross Ab, the EslgG, is one

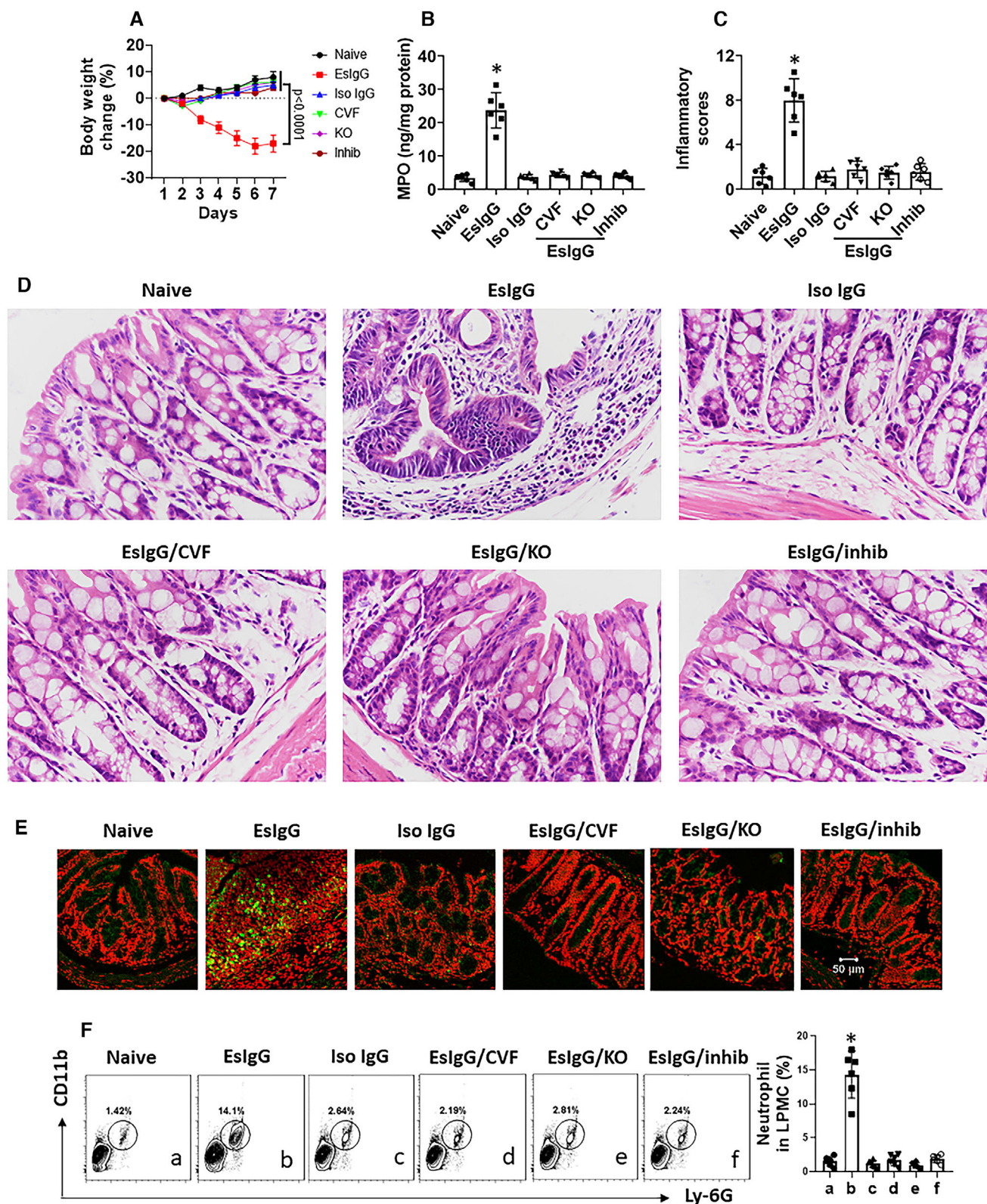


FIGURE 6 EslgG induces colitis-like inflammation. C57BL/c (wild type) mice and enolase-knockout (KO) mice were treated with indicated procedures daily for 7 days. EslgG: 0.1 mg/mouse (ip). Iso IgG: Isotype IgG (0.1 mg/mouse; ip). CVF: 200 U/mouse; i.p.; inhib: Z-VAD-FMK (1.5 mg/kg; ip; a caspase inhibitor). (A) Mouse body weight changes. (B) MPO levels in colon tissue protein extracts. (C) Colon tissue inflammatory scores. (D) Representative colon tissue histology images. Original magnification: $\times 400$. (E) Representative colon tissue PMN (NIMP-R14; in green) immunohistochemistry images. Original magnification: $\times 200$. (F) Gated dot plots show PMN frequency in colon tissue LPMCs; bars show summarized PMN frequency. Each group consists of 6 mice. $^*P < 0.01$, compared with the naive group (ANOVA followed by the Dunnett's test)

of the causative factors inducing intestinal epithelial cell apoptosis; this may contribute to the development of intestinal inflammation such as UC.

The data show that C5b-9 forms a complex with csp9 in colon epithelial cells, both in vitro and in vivo. This cleaves csp9 to its active forms. Previous studies also found the colocalization of C5b-9, csp9, and csp3 in the human arterial wall with atherosclerosis.²³ Such an anatomical relation may imply a functional association between these molecules. Our data support this previous finding by showing that C5b-9 physically contacts and activates csp9; the latter then activates csp3 and induces epithelial cell apoptosis. Other supporting evidence is that depletion of csp9 can inhibit EslgG-induced epithelial cell apoptosis.

Cumulative reports show the epithelial barrier dysfunction in the colon of UC patients,³⁶ of which the pathogenesis is to be further investigated. Although some inflammatory factors, such as TNF- α , IFN- γ , and IL-1 β , associating with the epithelial barrier dysfunction,³⁷ can be found in the colon tissues of UC subjects,³⁸ it is not certain whether these factors trigger the epithelial barrier dysfunction or are only the results of epithelial barrier dysfunction-induced inflammation in the local tissues. The present data reveal a novel causative factor associating with epithelial barrier dysfunction. The HDM-derived enolase specific cross Abs (EslgG) are associated with intestinal epithelial barrier dysfunction in UC patients. The cross Abs form complexes with the constitutive enolase in epithelial cells; the complexes activate complement, and induce epithelial barrier dysfunction. Since homologous gene sequences are common between humans and other species, such as HDM, plenty of cross Abs may be produced. Whether those cross Abs are also associated with epithelial barrier dysfunction of other organs needs to be further investigated.

The data show that EslgG induces neutrophilic inflammation in the colon. Neutrophilia has been observed in the colon mucosa of UC patients.³⁹ Neutrophils can release reactive oxygen species, serine proteases, matrix metalloproteinases, and myeloperoxidase (MPO), and subsequently, induces inflammation in the local tissues.³⁹ The most common factor to attract PMNs to the local tissues is bacterial infection. Besides, activated complement, such as C5a, are also the common factors to cause PMN infiltration in the tissue.⁹ Our data show that serum EslgG levels are positively correlated with neutrophilia in the UC patient colon tissues, suggesting that EslgG may be an important factor in the pathogenesis of UC. Although most studies about HDM focus on the role of HDM in the induction of airway allergy, recent studies indicate that HDM also compromises the epithelial barrier function in the gastrointestinal tract.² Since HDM distributes extensively in the human living environment, it is possible that HDM contaminates food that can be ingested into the gastrointestinal tract to induce EslgG production, and therefore, induces inflammation in the intestine.

In summary, the present data show that UC patients have high serum EslgG levels. EslgG forms complexes with the constitutive enolase in intestinal epithelial cells, activates complement, induces epithelial cell apoptosis, compromises epithelial barrier functions, and induces neutrophilic inflammation in the intestine.

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DISCLOSURE

The authors declare no conflict of interest.

AUTHORSHIP

J.L., B.S.F., N.H., N.M., Z.Q.L., and Q.S. performed experiments, analyzed data, and reviewed the manuscript. P.C.Y., Z.G.L., and N.Z. organized the study and supervised experiments. P.C.Y. and J.L. designed the project. P.C.Y. prepared the manuscript.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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