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Effects of dietary exposure to chlorpyrifos on immune cell populations and inflammatory responses in mice with dextran sulfate sodium-induced colitis

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ABSTRACT

This study investigated the effects of chlorpyrifos (CPF) on immune-cell populations and intestinal inflammation using a mouse model of inflammatory bowel disease induced by dextran sulfate sodium (DSS). C57BL/6 mice were randomly assigned to five groups with one normal control (NC) and four DSS-treated groups. Mice in the NC group were given distilled water, whereas the DSS-treated groups received distilled water containing 3% DSS for 6 days to induce colitis. The NC and disease control (DC) groups were fed a control semipurified diet, while the remaining groups were exposed to CPF in the AIN-93 diet at doses of 1, 2.5, or 5 mg/kg/day throughout the study. Results showed that dietary exposure to CPF in colitic mice significantly increased circulating classical monocytes and upregulated gene expressions of chemokines in the colon compared to the NC group. Meanwhile, CPF exposure groups had lower plasma cholinesterase activities and higher percentages of circulating neutrophils than those of the DC group. A shorten length, tissue edema, and lipid peroxidation of the colon were also observed in all CPF-exposed mice. These findings suggest that dietary exposure to CPF affected immune-cell populations and inflammatory responses, which led to more severe tissue injury in mice with DSS-induced colitis.

1. Introduction

Inflammatory bowel disease (IBD), a chronic and recurrent gastrointestinal disorder, is a global disease with increasing incidence (Ng et al., 2018). IBD is caused by a complex interplay of environmental factors (such as diet and antibiotics), susceptibility genes, gut microbiota, and dysregulation of immune responses (Kaser et al., 2010). Hyper-reactive inflammatory responses against commensal microflora leading to disruption of the epithelial barrier underlies the pathogenesis of IBD (Kaser et al., 2010; de Souza and Fiocchi, 2016). The adaptive immune system, especially T cells, has traditionally been considered to play a major role in driving dysregulated immune responses against gut microbiota in IBD (Zenewicz et al., 2009). However, recent studies suggested that the innate immune system is equally as important in inducing gut inflammation (Geremia et al., 2014; Huang and Chen, 2016). Phagocytes of the innate immune system not only provide a first line of defense against microorganisms but also play a crucial part in the initiation and subsequent direction of adaptive immune responses via cytokine production and antigen presentation (Geremia et al., 2014).

Neutrophils and macrophages are key myeloid phagocytes which collaboratively work as complementary and cooperative partners in innate immunity (Silva, 2010). Neutrophils are absent from the healthy intestine (Kolaczkowska and Kubes, 2013), while macrophages are one of the most abundant leucocytes in the gut mucosa (Bain and Mowat, 2014a). Macrophages are essential for maintaining intestinal homeostasis, which requires continual renewal from circulating blood monocytes (Bain and Mowat, 2014b). During perturbations of homeostasis, intestinal resident macrophages contribute to the recruitment of neutrophils through the production of chemokines (Steinbach and Plevy, 2014). Neutrophil infiltration contributes to oxidative stress and inflammation, which was correlated with the severity of IBD (Fournier and Parkos, 2012; Bressenot et al., 2015).

Chlorpyrifos (CPF), an organophosphate pesticide, is widely used to control insects in agriculture and household environments worldwide (Eaton et al., 2008). Although residential use of CPF was banned in the

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United States (USEPA, 2002) and the European Union (EU, 2007) over a decade ago, it remains one of the most commonly used insecticides in agriculture worldwide. Previous studies of CPF mainly emphasized its neurotoxicity due its ability to inhibit cholinesterase (ChE) (Pereira et al., 2014). Considering the oral route of CPF exposure, several studies in rodent models began to focus on impacts of CPF on the intestinal barrier and gut microbiome. It was demonstrated that oral exposure to CPF increased the intestinal permeability by disturbing the expression and localization of tight junction proteins (Joly Condette et al., 2014). The gut microbiota was perturbed by oral CPF exposure (Joly et al., 2013; Fang et al., 2018), and changes in the microbial composition were similar to those seen in IBD patients and which result in intestinal injury (Zhao et al., 2016). However, it is still unclear whether dietary exposure to CPF affects the immune system and inflammatory responses in IBD.

Dextran sulfate sodium (DSS)-induced colitis in mice is a useful model of IBD for investigating the innate response (Chassaing et al., 2014). C57BL/6 mice with acute colitis induced by DSS exhibit similar expression profiles of inflammatory mediators and histological changes to those observed in human IBD (Melgar et al., 2005). In this study, we used DSS-induced acute colitis in C57BL/6 mice to investigate the effects of CPF on immune cells and inflammatory mediators. Also, biochemical and histopathological markers of colon injuries were measured to evaluate the impacts of oral exposure to CPF during colitis.

2. Materials and methods

2.1. Animals

Eight-week-old male C57BL/6 mice were used in this study. Conventional mice were maintained in a temperature- and humidity-controlled room and were fed a standard chow diet ad libitum before the study. Care of laboratory animals was in full compliance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), and protocols were approved by the institutional Animal Care and Use Committee of Taipei Medical University (LAC-2017-0145).

2.2. Experimental protocols

After 1 week of acclimation, 50 mice were randomly assigned to one normal control (NC) group and four colitis groups ($n = 10$ in each group). The NC group was given distilled water, while the colitis groups received 3% (wt/vol) DSS (MW 40 kDa; MP Biomedicals, Solon, OH, USA) in the drinking water for 6 days to induce colitis. During the period of colitis induction, mice in the NC and disease control (DC) groups were fed a control semipurified diet comparable to AIN-93G, while other colitis groups received a semipurified diet containing different concentration of CPF (purity ≥98.0%; Sigma-Aldrich, St. Louis, MO, USA). The CPF levels in the diets were 7, 17.5, and 35 ppm, which provided dietary CPF at estimated respective doses of 1, 2.5, and 5 mg/kg/day (WHO, 2000). The lowest dose (1 mg CPF/kg) used in this study corresponds to the ingestible daily dose per person (Cochran et al., 1995; Joly et al., 2013) and the oral "no observed effect level" (NOEL) for inhibition of brain ChE activity in animals, which is used to establish the acceptable daily intake for human (Cochran et al., 1995). The highest dose was set to 5 mg/kg based on the effects of immune alterations observed in rodents (Blakley et al., 1999). There were five groups in total in this study: control diet with distilled water (NC group); control diet with DSS water (DC group); low-dose CPF diet (7 ppm) with DSS water (DL group); medium-dose CPF diet (17.5 ppm) with DSS water (DM group); and high-dose CPF diet (35 ppm) with DSS water (DH group). A flow diagram of the study design and the diet formulations are shown in Supplementary Fig. S1 and Table 1, respectively. Actual CPF concentrations in the diets were determined by gas chromatography-tandem mass spectrometry using a QuEChERS-based method at an accredited laboratory (Food Safety Research Center in Taipei Medical University).

Table 1
Diet compositions (g/kg).

Component	Control diet	Chlorpyrifos diet
Corn starch	529.5	529.5
Casein	200	200
Sucrose	100	100
Soybean oil	70	70
Fiber	50	50
Mineral mixture[a]	35	35
Vitamin mixture[b]	10	10
L-cystine	3	3
Choline	2.5	2.5
Chlorpyrifos	–	0.007/0.0175/0.035
tert-Butylhydroquinone	0.014	0.014

[a] The salt mixture contains the following (mg/g): calcium phosphate dibasic, 500; sodium chloride, 74; potassium sulfate, 52; potassium citrate monohydrate, 20; magnesium oxide, 24; manganese carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; curpric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; and chromium potassium sulfate, 0.55.

[b] The vitamin mixture contains the following (mg/g): thiamin hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride, 0.7; nicotinic acid, 3; calcium pantothenate, 1.6; D-biotin, 0.05; cyanocobalamin, 0.001; retinyl palmitate, 1.6; DL-α-tocopherol acetate, 20; cholecalciferol, 0.25; and menaquinone, 0.005.

All mice had free access to food and water throughout the study. Food consumption and body weight (BW) were recorded every 3 days. At the end of the experiment, mice were anesthetized and sacrificed by cardiac puncture. Fresh blood samples were collected in heparin-containing tubes. Whole blood was split into two vials with 100 μL in each vial for analysis by flow cytometry, and the remaining blood was centrifuged at 3000 × *g* for 10 min at 4 °C to obtain plasma. The colon was cut close to the ileocecal valve, and its length and weight were measured. Sections (1 cm) of the distal colon were collected and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Plasma and the remaining colon tissues were kept at −80 °C until being processed for further analysis.

2.3. Measurements of haptoglobin and ChE activity in plasma

Plasma haptoglobin concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) kit (ICL, Newberg, OR, USA). Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were determined with commercial fluorometric kits (Invitrogen, Carlsbad, CA, USA). All procedures followed the manufacturer's instructions.

2.4. Distribution of circulating leukocytes

A five-color flow cytometric analysis was performed to determine the phagocyte population and lymphocyte subsets in blood. Blood samples were incubated with antibodies against mouse leukocyte surface antigens for 30 min at 4 °C in the dark. After lysis of red blood cells, stained cells were suspended in staining buffer and then analyzed with a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA).

To measure the phagocyte population, whole blood was incubated with PerCP-conjugated anti-CD45 (Biolegend, San Diego, CA, USA), Pacific blue-conjugated anti-CD11b (Biolegend), FITC-conjugated anti-Ly6G (Biolegend), PE-conjugated anti-Ly6C (Biolegend) and APC-conjugated anti- C–C chemokine receptor type 2 (CCR2) antibodies (R&D, Minneapolis, MN, USA). CD45-positive cells were gated to select the leukocyte population. $CD45^+CD11b^+Ly6G^+$ cells were identified as neutrophils, whereas $CD45^+CD11b^+Ly6G^-$ cells were considered to be macrophages/monocytes. Classical monocytes ($Ly6C^{high}CCR2^+$ monocytes) and patrolling monocytes ($Ly6C^{low}CCR2^-$ monocytes) were

identified based on surface marker expression profiles.

PerCP-conjugated anti-CD45 (Biolegend), FITC-conjugated anti-CD3ε (eBioscience, San Diego, CA, USA), Pacific blue-conjugated anti-CD19 (Biolegend), APC-conjugated anti-CD4 (eBioscience) and PE-conjugated anti-CD8 (eBioscience) antibodies were used to identify lymphocyte subsets. $CD45^+CD3\varepsilon^+$ and $CD45^+CD19^+$ cells were respectively considered to be T and B cells. T cells were further subdivided into CD4-positive (T helper cells) and CD8-positive (cytotoxic T cells) subpopulations.

2.5. RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was isolated from colon tissues using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), followed by chloroform phase separation and 2-propanol precipitation. RNA was purified by lithium chloride precipitation to remove DSS as previously described (Viennois et al., 2013). RNA was reverse-transcribed using oligo (dT) 18 primers with a complementary (c)DNA synthesis kit (Fermentas, Vilnius, Lithuania) according to standard protocols. For the real-time PCR, 50 ng of cDNA was amplified in a 25-μL PCR volume containing 100–400 nM of each primer and 1× SYBR green master mix reagent (Thermo Scientific, Waltham, MA, USA). The reaction was performed with a Roche LightCycler® 480 Real-Time PCR System (Pleasanton, CA, USA). Amplification was performed under the following conditions: 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 30 s, with a final dissociation curve analysis. Primer sequences were as follows: mouse C-X-C motif chemokine ligand 1 (CXCL1) (5′-TGGG AGGCTGTGTTTGTATG-3′ and 5′-GAGACGAGACCAGGAGAAAC-3′), mouse C–C motif chemokine ligand 2 (CCL2) (5′-ACTGAAGCCAGCTC TCTCTTCCTC-3′ and 5′-TTCCTTCTTGGGTCAGCACAGAC-3′) and mouse β-actin (5′- ACCCACACTGTGCCCATCTAC-3′ and 5′-TCGGTGA GGATCTTCATGAGGTA-3′). All samples were analyzed in triplicate, and multiple changes of gene expression were calculated by equation $(2)^{-\triangle\triangle Ct}$ ($\triangle$Ct indicates the difference in threshold cycles between the test gene and β-actin, and $\triangle\triangle$Ct indicates the difference of $\triangle$Ct between the colitis and the NC groups).

2.6. Thiobarbituric acid-reactive substance (TBARS) assay

The amount of lipid peroxidation end products in colon tissue was measured using a previously described method (Hou et al., 2009). Colon homogenates at 20% were prepared in ice-cold phosphate-buffered saline (PBS) using a homogenizer. Trichloroacetic acid (10%) was added to homogenates to precipitate proteins. After centrifugation at 14,000 × *g* for 15 min, supernatants were collected for the analysis of TBARSs. The production of TBARSs consists of the thiobarbituric acid-heated lipid peroxidation end product, malondialdehyde (MDA). 1,1,3,3-Tetramethoxypropane, an MDA precursor, was used to prepare standard curves. TBARSs were determined with a spectrofluorometer with excitation and emission wavelengths set to 515 and 555 nm, respectively. TBARS concentrations in colon tissues were expressed as micromolar.

2.7. Histopathology

Specimens of the distal colon fixed with paraformaldehyde were embedded in paraffin. Series of 5-μm-thick sections stained with hematoxylin and eosin (H&E) were examined to determine the colon morphology. Digital images at 100× magnification per section were captured with an Olympus BX43 Upright microscope (Waltham, MA, USA) and a Canon EOS 700D digital camera (Tokyo, Japan). Five fields per section were examined to determine morphological lesions and changes in the colon mucosa. The degree of colon injury was assessed using a modified scoring system based on Hou et al. (2013), which is shown in Supplementary Table S1. The total histological score ranged 0–12, which represented the summed scores of loss of epithelium, length of crypts, depletion of goblet cells, and infiltration of leukocytes, with a higher score indicating more severe injuries.

2.8. Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Differences among groups were analyzed by a one-way analysis of variance (ANOVA) with Tukey's post-hoc test. A two-way ANOVA with the Bonferroni post-test was used to analyze differences in BW. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. BW changes and actual CPF exposure doses

There were no differences in initial BWs among the five groups. BW loss was observed at day 7 in all DSS-treated groups compared to the NC group. BWs did not differ between the DC group and CPF-exposed groups during the experimental period (Supplementary Fig. S2). Reduction of food intake was also observed at day 7 in all DSS-treated groups. Comparing food consumption between days 4 and 7, food intake on day 7 had decreased 10.8% in the DC group, whereas CPF-exposed groups showed lower food intake levels by 35.9% (DL group), 32.0% (DM group), and 33.4% (DH group). Actual CPF concentrations of diets in the CPF groups were 5.84 ppm (DL group), 15.4 ppm (DM group), and 36.1 ppm (DH group). Average CPF exposure doses during the study were as follows: 0.80 mg/kg/day in the DL group; 2.00 mg/kg/day in the DM group; and 6.66 mg/kg/day in the DH group. The DL and DM groups had lower, whereas the DH group had a higher exposure dose to CPF than expected.

3.2. Plasma ChE activity

Reductions in plasma AChE and BChE activities were observed in all DSS-induced colitic mice. Compared to the DC group, CPF exposure suppressed plasma AChE and BChE activities in dose-dependent manners (Fig. 1).

3.3. Blood leukocyte populations

All DSS-treated colitis groups had a higher neutrophil percentage, and lower populations of patrolling monocytes and lymphocytes in blood compared to the NC group. There were no differences in percentages of blood macrophages/monocytes and T-cell subsets between normal mice and those with DSS-induced colitis (Fig. 2 and Fig. 3). Compared to the DC group, CPF exposure significantly elevated the neutrophil percentage in blood. CPF-exposed groups also had higher populations of circulating classical monocytes than the NC group (Fig. 2). Myeloid leukocytes in blood were affected by CPF exposure; however, no dose-dependent response was observed.

3.4. Colon inflammation and tissue injury

A shortened colon length and elevated plasma haptoglobin concentration, which respectively indicate intestinal and systemic inflammation, were observed in all DSS-treated colitis groups compared to the NC group (Table 2). CPF exposure resulted in a shorter colon length than the DC group. In addition, the DM and DH groups exhibited significantly upregulated plasma haptoglobin levels compared to the DC group (Table 2). The colon weight/length ratio, an indicator of colonic edema, and the MDA content in colon tissues, a marker of lipid oxidation, were significantly higher in all CPF-exposed groups than in the NC group (Table 2). Also, mice exposed to CPF had significantly higher gene expression levels of the neutrophil-attracting chemokine, CXCL1, and the classical monocyte-attracting chemokine, CCL2, in colons compared to the NC group (Fig. 4). CPF exposure exacerbated

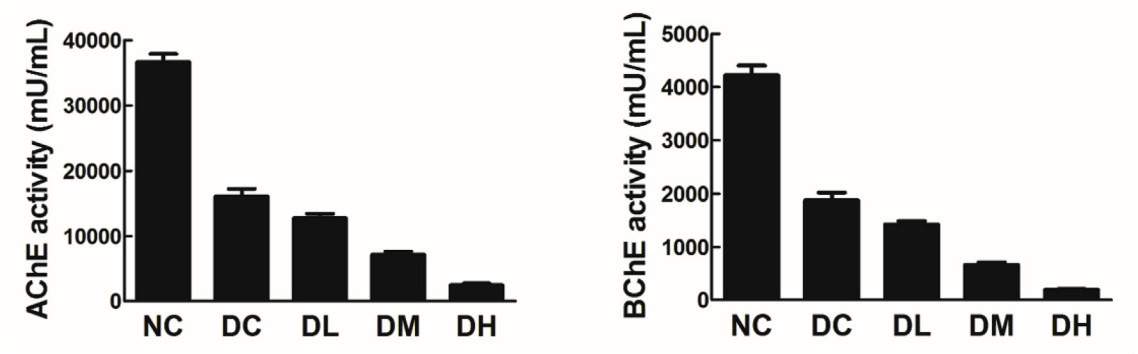

Fig. 1. Plasma cholinesterase activity. AChE, acetylcholinesterase; BChE, butyrylcholinesterase. Data are expressed as the mean ± SEM. Differences among groups were analyzed by a one-way ANOVA with Tukey's post-hoc test. All groups significantly differed from each other ($p < 0.05$).

colon inflammation and tissue injury in mice with DSS-induced colitis.

3.5. Histopathological aspects of the colon

Colon tissue sections from the NC group showed intact epithelium, a well-defined gland length, and no leukocyte infiltration in the mucosa (Fig. 5). In contrast, DSS-induced colitis resulted in mucosal ulceration, infiltration of leukocytes, crypt distortion, and hyperplastic epithelium. Colon injury scores were significantly higher in all DSS-treated groups than in the NC group; however, there were no difference between the DC group and CPF exposure groups (Fig. 5).

4. Discussion

Intestinal homeostasis depends on the interplay between microbiota, intestinal epithelium, and host immune system. Disruption of intestinal homeostasis leads to gut inflammation, which is responsible for the pathogenesis of IBD (Maloy and Powrie, 2011). Previous studies using normal rodents indicated that chronic oral exposure to CPF has unfavorable effects on intestinal homeostasis by increasing the intestinal permeability (Joly Condette et al., 2014) and perturbing the gut microbiota (Joly et al., 2013; Zhao et al., 2016; Fang et al., 2018). In this study, dietary exposure to CPF for 6 days altered the myeloid leukocyte population and subsequently exacerbated colon inflammation and injury in mice with DSS-induced colitis, even at the exposure dose close to NOEL. Our results indicated that dietary CPF exposure

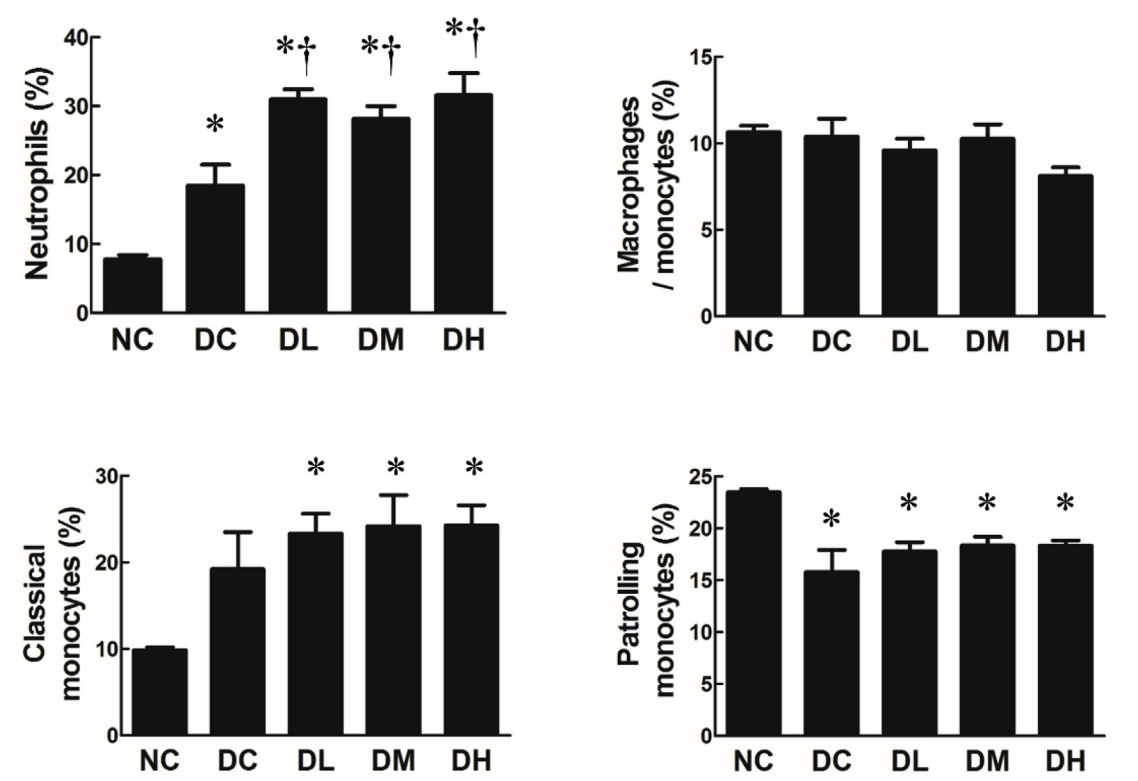

Fig. 2. Myeloid leukocyte populations in the blood. Neutrophils and macrophages/monocytes were respectively defined as $CD45^+CD11b^+Ly6G^+$ and $CD45^+CD11b^+Ly6G^-$ cells and are shown as percentages of the leukocyte ($CD45^+$ cells) population. Monocyte subpopulations are presented as percentages of classical monocytes ($Ly6C^{high}CCR2^+$) and patrolling monocytes ($Ly6C^{low}CCR2^-$) among macrophages/monocytes. Data are expressed as the mean ± SEM. Differences among groups were analyzed by a one-way ANOVA with Tukey's test. * Significant difference from the NC group. † Significant difference between the DC group and the chlorpyrifos-exposed groups ($p < 0.05$).

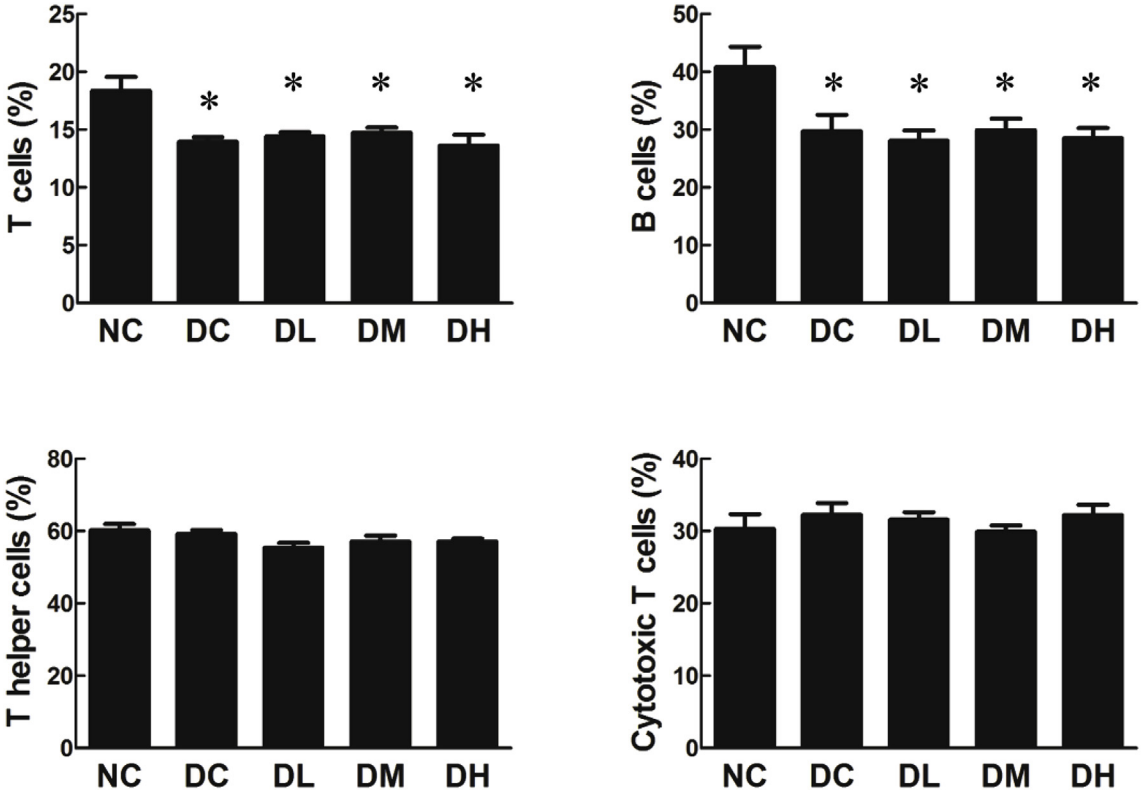

Fig. 3. Blood lymphocyte populations. T cells ($CD45^+CD3^+$) and B cells ($CD45^+CD19^+$) are shown as percentages of the leukocyte ($CD45^+$ cells) population. T-cell subpopulations are presented as percentages of T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) among T cells. Data are expressed as the mean ± SEM. Differences among groups were analyzed by a one-way ANOVA with Tukey's test. * Significant difference from the NC group ($p < 0.05$).

aggravated colonic and systemic inflammation in colitic mice. To best of our knowledge, this is the first study to investigate the effects of short-term exposure to CPF on the immune system using a murine model of IBD.

Drinking water administration of DSS, a heparin-like polysaccharide, results in disruption of the intestinal epithelium barrier and activation of mucosal macrophages, which in turn induce gut inflammation (Kawada et al., 2007). DSS-induced colitis is characterized by extensive damage to the intestinal epithelium with ulceration, tissue edema, and leukocyte infiltration predominantly in the distal colon which resembles histological features of ulcerative colitis (UC), a principal type of IBD (Okayasu et al., 1990). Although DSS-induced colitis does not accurately mimic the T-cell responses of human IBD, acute DSS colitis is a suitable model to investigate the contribution of the innate immune system to intestinal inflammation because it can be induced without the help of T cells (Kiesler et al., 2015). Regarding the high susceptibility to DSS of the C57BL/6 strain (Melgar et al., 2005), DSS-induced acute colitis in C57BL/6 mice were used in this study to

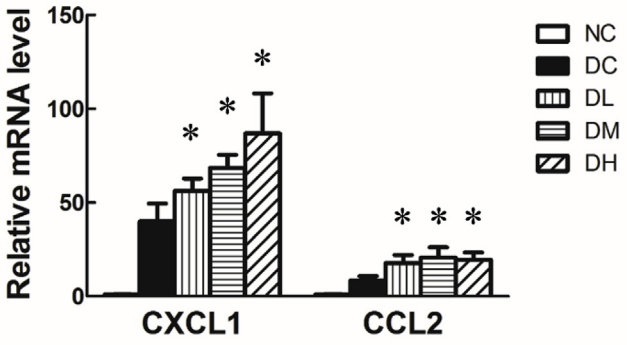

Fig. 4. Expressions of chemokine genes in the colon. CXCL1, C-X-C motif chemokine ligand 1; CCL2, C–C motif chemokine ligand 2. Data are expressed as the mean ± SEM. Differences among groups were analyzed by a one-way ANOVA with Tukey's test. * Significant difference from the NC group ($p < 0.05$).

Table 2
Inflammatory and tissue injury-related markers in the colon.

	NC	DC	DL	DM	DH
Colon					
Weight (g)	0.31 ± 0.01	0.31 ± 0.03	0.29 ± 0.01	0.30 ± 0.01	0.33 ± 0.02
Length (cm)	7.08 ± 0.32	6.20 ± 0.11*	5.39 ± 0.07*†	5.50 ± 0.15*†	5.45 ± 0.07*†
Weight/Length (g/cm)	0.043 ± 0.002	0.051 ± 0.003	0.058 ± 0.002*	0.060 ± 0.002*	0.059 ± 0.003*
Colon homogenates					
MDA (μM)	1.81 ± 0.20	2.28 ± 0.09	2.95 ± 0.09*	3.10 ± 0.26*	3.13 ± 0.16*
Plasma					
Haptoglobin (μg/mL)	0.10 ± 0.01	20.04 ± 1.58*	31.23 ± 3.0*	34.45 ± 3.33*†	35.41 ± 5.41*†

MDA, malondialdehyde. Data are expressed as the mean ± SEM. Differences among groups were analyzed by a one-way ANOVA with Tukey's test. *Significant difference from the NC group. †Significant difference between the DC group and the chlorpyrifos-exposed groups ($p < 0.05$).

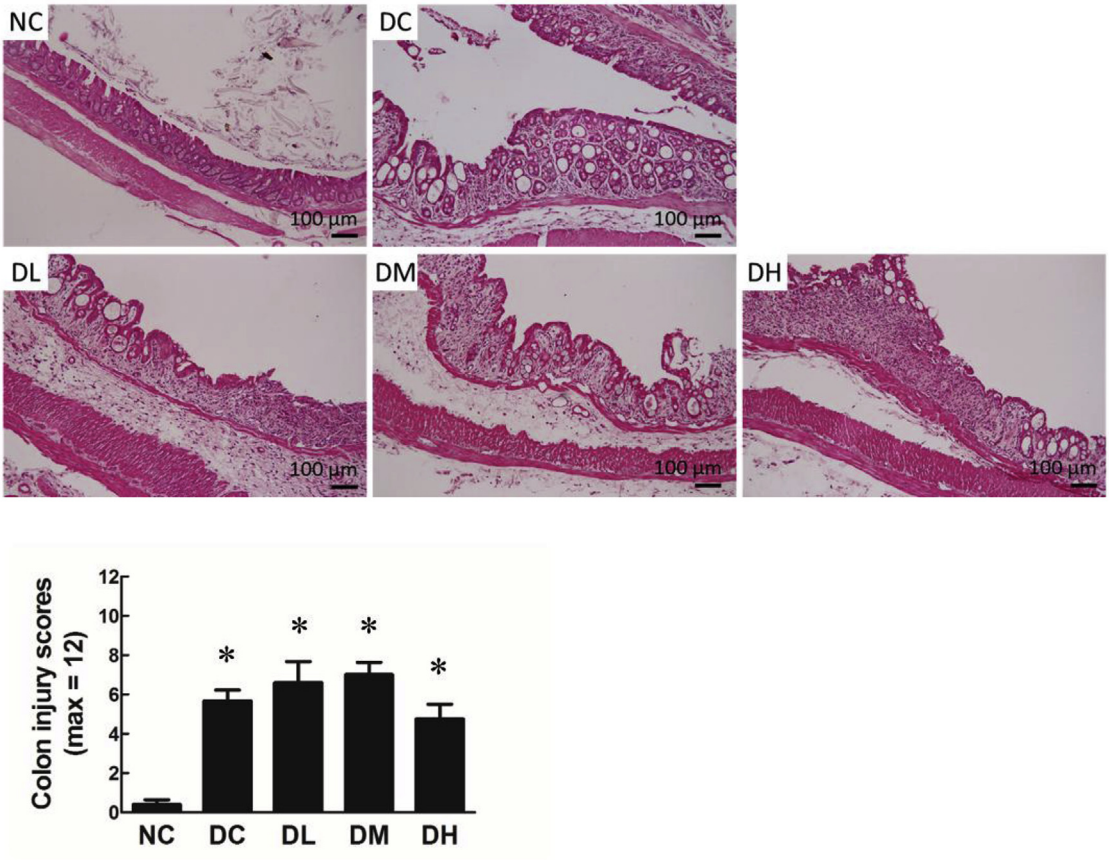

Fig. 5. Histopathology of colon tissues. Representative histological images are shown at 100 × magnification. Histological scores of colon injury are presented as the mean ± SEM, which were determined as described in "Section 2". Differences among groups were analyzed by a one-way ANOVA with Tukey's test. * Significant difference from the NC group ($p < 0.05$).

investigate the consequences of dietary CPF exposure on immune cell populations and colon inflammation.

Both neutrophils and macrophages contribute to the pathogenesis of IBD. Neutrophils are released from the bone marrow, age in the blood, and are absent from the intestinal mucosa under physiological condition (Furze and Rankin, 2008). Macrophages are resident sentinel cells which contribute to the first-line defense in the gut. When sensing pathogens or foreign antigens, these cells release pro-inflammatory mediators and CXC chemokines to promote neutrophil activation and recruitment (Kuhl et al., 2015). Activated neutrophils secrete reactive oxygen species, and prolonged excessive oxidative stress leads to significant tissue damage. Massive neutrophil infiltration into the colon mucosa is a hallmark of IBD, especially UC (Muthas et al., 2017). The extent of neutrophil infiltration correlates with the severity of UC (Bressenot et al., 2015), and the neutrophil-to-lymphocyte ratio in the blood is elevated in patients with active UC (Demir et al., 2015). Neutrophil accumulation was also observed in DSS-treated colitis (Natsui et al., 1997). Lipid peroxidation is significantly increased in the colon during DSS treatment (Naito et al., 2007).

Intestinal macrophages require continuous replenishment by blood monocytes which are derived from bone marrow (Bain and Mowat, 2014b). In mice, two main subsets of blood monocytes can be identified on the basis of expression levels of monocyte markers and chemokine receptors (Geissmann et al., 2003). Classical monocytes express high levels of Ly6C in conjunction with CCR2 ($Ly6C^{high}CCR2^{+}$), whereas patrolling monocytes have low/negative expression levels of Ly6C and CCR2 ($Ly6C^{low}CCR2^{-}$). CCL2/CCR2-dependent recruitment of classical monocytes is the major source of intestinal macrophages (Takada et al., 2010; Bain et al., 2013). Patrolling monocytes are mainly involved in maintaining of the vasculature (Carlin et al., 2013). Previous studies indicated that classical monocytes are pro-inflammatory and directly pathogenic in DSS-induced colitis (Platt et al., 2010; Zigmond et al., 2012).

In the present study, DSS colitic mice exposed to CPF for 6 days had elevated levels of blood neutrophils and classical monocytes which were concomitant with higher expressions of neutrophil- and monocyte-attracting chemokines in colon tissues. Lipid peroxidation in the colon was enhanced in all CPF groups, whereas plasma haptoglobin levels were significantly increased in the DM and DH groups. Haptoglobin, an acute-phase protein, is mainly produced by hepatocytes under inflammatory conditions (Vanuytsel et al., 2013). It is used as a systemic marker to monitor disease activity in murine models of colitis (Melgar et al., 2005). Mucosal injury results in shortening the colon length, which is a useful indicator of DSS-induced colitis (Park et al., 2015). The colon weight/length ratio, a measurement indicative of colonic edema, was found to be highly correlated with colon inflammation and injury (Ostanin et al., 2006). Our results indicated that CPF exposure shortened the colon length and worsened colon edema in colitic mice although there was no difference in histologic findings between the DC and CPF-exposed groups. There being no change in histology can be explained by DSS being administrated throughout the study which continually caused epithelial injury in the colon. These findings suggest that dietary CPF exposure facilitates recruitment of myeloid leukocytes to the colon by increasing circulating leukocytes and promoting colonic chemokine expression. The inference was supported by more severe colonic and systemic inflammation being noted in colitic mice with CPF exposure.

There is little information on the immune toxicity of CPF, which mainly focused on adaptive immunity. In rodent studies, oral CPF exposure (5 mg/kg) twice weekly for 28 days suppressed T-lymphocyte

blastogenesis (Blakley et al., 1999), whereas exposure to CPF (1 mg/kg) during the developmental period significantly impaired T-cell responses (Navarro et al., 2001). In this study, dietary CPF exposure exacerbated inflammation by innate immune cells during colitis, which had unfavorable effects on intestinal homeostasis. Our study provides new insights into the immune toxicity of CPF in immune toxicity under disease conditions. The unfavorable effects of CPF exposure observed in this study may be explained by interactions between acetylcholine (ACh) and ACh receptors (AChRs). ACh binds to both nicotinic and muscarinic AChRs (n- and mAChRs), which regulate neuronal excitability in the nervous system and help maintain and optimize cell functions in non-neuronal cells (Wessler and Kirkpatrick, 2008). ACh is hydrolyzed by ChE which is divided into AChE and BChE. AChE, mainly found in nerves, muscles and erythrocytes, has high substrate preferences for ACh, whereas tissue-abundant BChE has a lower affinity for ACh (Davis et al., 1997; Li et al., 2000). Inhibition of ChE was thought to be neurotoxic due to the accumulation of ACh (Pereira et al., 2014). In recent years, studies began to investigate the effects of cholinergic signaling on immune regulation because many non-neuronal cells were found to synthesize ACh and express AChRs, including immune cells and the intestinal epithelium (Klapproth et al., 1997; Fujii et al., 2017). Both pro- and anti-inflammation mediated by ACh were reported in previous studies, depending on the specific ACh receptor subtype activated. Macrophage activity was suppressed by ACh through the α7 nAChR pathway (Pohanka, 2014; Fujii et al., 2017), whereas activation of M3 mAChR promotes release of pro-inflammatory mediators (Koarai et al., 2012; Kistemaker et al., 2013). A previous study indicated that ChE synthesis was suppressed by endotoxins and cytokines, leading to ChE reduction in severe IBD patients (Tromm et al., 1992). Consistent with the previous study, our results indicated that both plasma AChE and BChE activities were reduced in all colitic mice. Since the CPF doses used in this study were higher than the NOEL for inhibition of blood ChE activity in rodents with repeated oral exposures to CPF (0.1 mg/kg/day) (Marty et al., 2012), we also observed that ChE inhibition was more prominent in CPF exposed groups. Although ChE inhibition contributes to ACh accumulation, we speculated that the cholinergic anti-inflammatory effects through α7 nAChR activation might be diminished by CPF exposure, because CPF and its oxon metabolite bind to and desensitize the nAChR (Katz et al., 1997). Future studies are required to clarify which ACh receptor subtype is involved in colonic and systemic inflammation induced by CPF exposure during DSS-induced colitis.

The limitation of this study was that the effects of CPF on the immune system and inflammatory response were not evaluated in healthy mice. Healthy mice exposed to dietary CPF were not included in the present study because CPF was only administrated for 6 days. Previous studies suggested that it would require a longer exposure time at the dose ranging from 1 to 5 mg/kg of CPF to investigate its effects on the immune function in normal rodents (Blakley et al., 1999; Navarro et al., 2001). In addition, the relevance of animal research to human response should be taken into consideration. A previous study indicated that the generation velocity of the toxic metabolite of CPF, CPF oxon was higher in the rodent liver than in the human liver (Tang et al., 2001). DSS-induced colitis does not completely mimic the immune responses of human IBD and the variables such as medication and diet adjustment for IBD patients were not considered in the present study. Whether dietary exposure to CPF affects immune function in IBD patients is unknown.

5. Conclusions

In summary, this study showed that dietary exposure to CPF in colitic mice promoted percentages of circulating neutrophils and classical monocytes. Upregulated gene expressions of neutrophil- and monocyte-attracting chemokines were also observed in the colon. Damage to colon tissues was more severe in colitic mice exposed to CPF. Our study proposes another potential adverse mechanism of dietary exposure to CPF.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110596.

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