Endogenous Interleukin-17 as a Factor Determining Severity of *Clostridium difficile* Infection in Mice

Running title: Role of Interleukin-17 in *C. difficile* infection

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Abbreviations: BHI, brain-heart-infusion; TY, tryptone-yeast extract; qPCR, quantitative polymerase-chain-reaction; G-CSF, granulocyte-colony stimulating factor; CXCL2, CXC chemokine ligand 2; HBSS, Hank's balanced salt solution; FBS, fetal bovine serum; RPMI, Roswell Park Memorial Institute

ABSTRACT

Clostridium difficile infection (CDI) is a toxin-mediated intestinal disease. Toxin A, toxin B and binary toxin are believed to be responsible for the pathogenesis of CDI, which is characterized by massive infiltration of neutrophils at the infected intestinal mucosa. Interleukin-17 (IL-17) is one of the cytokines that play critical roles in several inflammatory and immunological diseases through several actions, including promoting neutrophil recruitment. The aim of this study is to examine the role of this cytokine in CDI by applying IL-17 A and F double knockout (IL-17 KO) mice for CDI model. We demonstrated that IL-17 KO mice were more resistant to CDI than wild type (WT) mice, evaluated in several factors, such as diarrhea score, weight change and survival rate. Although the bacterial numbers of *C. difficile* in feces were not different, the inflammatory mediator levels at the large intestine on day 3 post infection were attenuated in IL-17 KO mice. Finally, we proved that infiltration of neutrophil, but not macrophage, in the large intestine was significantly decreased in IL-17 KO mice than that of WT mice. In conclusion, the present data demonstrated that endogenous IL-17 may be a factor determining severity of CDI in mice. Although the mechanism was fully unknown, IL-17-mediated inflammatory responses, such as cytokine/chemokine productions and neutrophil accumulation, may be a plausible target for future's investigation.

INTRODUCTION

Clostridium difficile infection (CDI) is becoming a serious problem in the world, especially in elderly and debilitating individuals as a hospital and healthcare facility-associated infection (Miller et al., 2011). It is reported that one of the reasons of CDI issues may be associated with emergence and spreading of hypervirulent strain BI/NAP1/027 (O'Connor et al., 2009). The primary virulence factors of *C. difficile* were believed to be toxins, like toxin A, B and binary toxin, which trigger dysregulation of intestinal mucosal cells (ex. Rho GTPases inactivation) leading to cellular damage and/or apoptosis. In addition to the direct toxin-mediated pathogenic effects on the target cells, these toxins were shown to induce profound immunological and inflammatory responses (Hirota et al., 2012).

Neutrophils are a key player in the pathogenesis of CDI, because a peripheral leukocytosis and massive infiltration of neutrophils into the sites of infection are demonstrated to be one of characteristics and a predictor of severity of this disease (Fujitani et al., 2011). Although several lines of evidence demonstrated that excessive accumulation and activation of neutrophils may be involved in the pathogenesis of CDI, whether neutrophil influx is beneficial or detrimental are still controversial (Kelly et al., 1994; Qiu et al., 1999; Jarchum et al., 2012; Hasegawa et al., 2011).

Interleukin (IL)-17 is a proinflammatory cytokine, and plays a crucial role in bridging between innate and adaptive immunity. Although there are at least 6 molecules identified in IL-17 family, IL-17 A and IL-17 F are the most studied subtypes, which share high homology in structure and use the same receptor on host cells. Contribution of IL-17 in host defense systems and pathogenesis of diseases were well characterized in neutrophil

recruitment and antimicrobial peptides production, especially in skin, lungs and intestinal tracts (Lochner et al., 2008). In chronic diseases, persistent uncontrolled production of IL-17 may be associated with autoimmune/inflammatory diseases, such as psoriasis and inflammatory bowel disease (IBD) (Pappu et al., 2010). On the other hand, there are growing evidences of the roles of IL-17 in several infectious diseases (Ishigame et al., 2009). The recent data demonstrated up-regulation of IL-17 and IL-23, a positive regulator for IL-17 production, at the site of infected mucosa (Buonomo et al., 2013; Hirota et al., 2012). Interestingly, a link between CDI and IBD was reported by the fact that IBD patients are more susceptible to CDI (Issa et al., 2007).

From these backgrounds, we investigated roles of IL-17 in a mice model of CDI. Particularly, the survival data obtained from IL-17 A and F double knockout (IL-17 KO) mice experiments, along with diarrhea score, macroscopic/microscopic findings and cytokine/chemokine responses, strongly suggested a role of endogenous IL-17 in a determining factor for inflammatory responses and severity of CDI in mice.

METHODS

C. difficile strain and cultivation methods

A clinical isolate of *C. difficile* BI/NAP1/027 was kindly provided by Thomas V Riley, division of Microbiology and Infectious Diseases, Path West Laboratory Medicine, Perth, Australia. Freeze stock of the bacteria was cultured onto brain-heart-infusion (BHI) agar (Becton Dickinson) supplemented with 0.1 % w/v L-cysteine (Sigma-Aldrich) and 0.1 % w/v taurocholic acid (Sigma-Aldrich) at 37 °C in anaerobic chamber (Mitsubishi gas chemical). After 48 hours, a single colony grown on BHI agar was suspended in pre-deoxidized TY (tryptone-yeast extract) medium (Becton Dickinson) containing 0.1 % w/v thioglycollate (Wako pure chemical) and incubated for 48 hours anaerobically (Sorg & Dineen, 2009; Fang et al., 2009). The suspension was centrifuged for 10 min at 10,000 g and the pellets containing vegetative cells were re-suspended in the used TY media to adjust the concentration for 1.0×10^6 CFU/ml.

Animal models of CDI

Specific-pathogen-free BALB/c mice (6 to 9 weeks of age) (WT) were purchased from Charles River Laboratories Japan, Inc. IL-17 KO mice on a BALB/c genetic background, were previously established at the Institute of Medical Science, University of Tokyo (Nakae et al., 2002). All mice were maintained under specific-pathogen-free conditions within the animal care facility in the Laboratory Animal Research Center of Toho University School of Medicine. Animal protocols were approved by the institutional animal care and use committee (approval number 13-51-220). The intestinal flora of WT and IL-17 KO mice were checked by the absence of specific pathogens in the feces, and

compared by composition and number of major culturable bacteria. Murine CDI model was prepared according to the protocol, as described previously (Chen et al., 2008). Briefly, mice were administered antibiotics cocktail (kanamycin 0.4 mg/ml, gentamycin 0.035 mg/ml, colistin 850 U/ml, metronidazole 0.215 mg/ml, and vancomycin 0.045 mg/ml) in the drinking water from 6 days to 3 days before the infection. On one day before the infection, mice were injected with clindamycin 300 μg in 0.25 ml PBS intraperitoneally. Mice were intragastrically challenged with 5.0×10^5- 1.0×10^6 CFU of *C. difficile*. After the infection, the survival rate, the diarrhea scoring and the weight change of the mice was monitored daily until 8 days after the infection. The stool condition was scored as follows: 0, normal stool; 1, loose stool; 2, mushy stool; 3, watery stool. The highest stool score after the infection was used for the analysis. On day 3 post-infection, the mice were sacrificed by CO_2 asphyxia, and then each colon was collected to measure weight and length for evaluation of fluid accumulation and inflammation, as previously described (Inui et al., 2011). For bacterial count of *C. difficile* in feces, stool pellets were suspended in PBS. The suspension was inoculated onto a selective medium containing cysteine hydrochloride, moxalactam, and norfloxacin after 1:10 serial dilutions, and then cultivated anaerobically, as mentioned above (Aspinall & Hutchinson, 1992). For histological analysis, cecum was harvested on day 3 post-infection. The tissues were fixed and stained with hematoxylin-eosin.

Gene expressions and protein levels of inflammatory mediators

Expressions of mRNA for Inflammatory mediators in the colon were evaluated by real-time quantitative PCR (qPCR). Total RNA was extracted by using RiboPure kit

(Ambion), according to the manufacturer's instructions. TURBO DNA-free kit (Ambion) was used to treat RNA with DNase, then reverse transcribed by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Data analysis using Fast SYBR Green Mater Mix (Applied Biosystems) was performed on 7500 Fast Real-time PCR system (Applied Biosystems). PCR primers used were shown in Table 1.

Scraped mucosa of the cecum were suspended in 8-fold amount of PBS containing 1 % Triton X-100 (Sigma-Aldrich) and complete protease inhibitor (Roche) and incubated for 20 min on ice. The suspension was centrifuged for 15 min at 15,000 g to collect supernatants. The concentrations of IL-1 beta, IL-6, granulocyte-colony stimulating factor (G-CSF) and CXC chemokine ligand 2 (CXCL 2) in the cecum was measured by ELISA according to the manufacturer's instructions (R&D systems).

Isolation of intestinal immune cells and flow cytometric analysis

Small pieces of PBS-washed cecum and colon were incubated in HBSS (Gibco) containing 1mM ethylenediaminetetraacetic acid (Wako Pure Chemical), 2 % FBS (Gibco) and 1 mM dithiothreitol (Wako Pure Chemical) for 20 min at 37 °C with 160 rpm shaking. The tissue pieces were washed with HBSS and minced, then incubated in RPMI 1640 medium (Gibco) containing 2 % FBS, 0.5 mg/ml collagenase D (Roche) and 150 μg/ml DNase (Roche) for 50 min at 37 °C with 160 rpm shaking. Suspensions were passed through a strainer and centrifuged 1,500 rpm for 5 min. Pellets were washed with PBS and centrifuged. The samples were re-suspended in 10 ml 40 % Percoll (GE Healthcare) diluted with RPMI 1640 and layered on 2 ml 80 % Percoll diluted with RPMI 1640 before centrifugation at 2,300 rpm for 20 min. Viable immune cells were treated

with CD 16/32 (eBioscience), then stained with PerCP/Cy 5.5-conjugated Ab CD11b (M1/70; Biolegend), FITC-conjugated Ab Ly6G (1A8; Biolegend) and PE-conjugated Ab F4/80 (BM8; Biolegend). After staining, samples were washed with PBS and treated with 4 % paraformaldehyde PBS (Wako Pure Chemical). Fluorescence was assessed with FACSCanto 2 (Becton Dickinson) and analyzed with FlowJo 7.6.5 (TreeStar).

Statistical analysis

Statistical analysis was performed in Kaplan-Meier plots by log-rank (Mantel-Cox) test (GraphPad Prism 5: GraphPad software). All values are expressed as mean ± standard error of the mean (SEM). Mann-Whitney U test was used throughout the study. A threshold for statistical significance was set at $P< 0.05$.

RESULTS

Susceptibility of IL-17 KO mice to CDI

To investigate the lethal susceptibility of IL-17 KO mice to CDI, we compared survival rate, diarrhea score and weight changes between WT and IL-17 KO mice. As shown in Figure 1a, IL-17 KO mice demonstrated a significantly higher survival rate compared with that of WT mice. Although all WT mice showed watery diarrhea within 3 days after the infection, only about 40% of IL-17 KO mice did. The average diarrhea score of IL-17 KO mice group was significantly lower than that of WT mice group (Fig. 1b). In addition, milder weight loss was recorded in IL-17 KO mice than that of WT mice group during the course of infection (Fig. 1c). These data demonstrated that IL-17 KO mice were more resistant to *C. difficile* challenge, compared with WT mice.

C. difficile number in feces of IL-17 KO and WT mice

To compare the bacterial burden of *C. difficile* in the intestinal tracts of WT and IL-17 KO mice, we examined the number of *C. difficile* in the feces 3 days after the challenge of 10^5- 10^6 CFU of *C. difficile*. Although more than 1,000 times of increase of *C. difficile* was observed in all feces of mice, there were no significant difference at this time point between WT mice and IL-17 KO mice (Fig. 2). These results suggested that the resistant features of IL-17 KO mice to *C. difficile*, as shown in Figure 1, are not associated with the bacterial burden of *C. difficile* in the feces at the early stage of the infection. Before these experiments, we compared the composition and number of major enteric bacteria in feces, such as *Escherichia coli* and *Bacteroides* spp. and *Clostridium* spp., and it

confirmed that there were no differences, at least in the organisms examined, between WT and IL-17 KO mice.

Evaluation of inflammatory responses in the large intestine of IL-17 KO mice

In our mice model of CDI, the predominant inflammation was observed in the large intestine, as reported previously (Sun et al., 2011). To compare the degree of inflammation, we evaluated the weights and lengths of the large intestines and the macroscopic/microscopic examination of the gut on day 3 after the infection of *C. difficile*. As shown in Figure 3a, an increase of the weight/length ratio in large intestines of WT mice was observed after the *C. difficile* infection. Significantly a lower response in the weight/length ratio was observed in IL-17 KO, comparing to that of WT mice. The macroscopic observations clearly showed that the shortening of the large intestines was observed in both infected WT and IL-17 KO mice, comparing to that of the uninfected WT mice (Fig. 3b). Interestingly, more reddish color in the large intestine was observed in WT mice infected with *C. difficile*, which suggested congestion, increase of vascular permeability and/or micro-bleeding. Associated with the macroscopic findings, the microscopic observation demonstrated remarkable inflammatory changes in the large intestines of *C. difficile*-infected WT mice, characterized with epithelial cell damage, submucosal edema, fibrin synthesis and inflammatory cell infiltration (Fig. 3c). These results showed weaker inflammatory responses in the large intestines of IL-17 KO mice after the *C. difficile* infection.

Inflammatory mediators in the large intestines of IL-17 KO mice

Next, we evaluated inflammatory mediator expressions and productions in the large intestine of the infected mice by qPCR and ELISA. The *C. difficile* infection induced up-regulation of all the genes examined in WT mice, as shown in Figure 4a. In IL-17 KO mice, clearly lower levels of expressions of these genes were demonstrated, which were substantially comparable to those of the uninfected WT mice. Well correlated with the gene expression data, substantially similar results were obtained at the protein level, which mean lower productions of inflammatory mediators in IL-17 KO mice (Fig. 4b). Particularly, the prominent amount of production was observed in IL-1 beta, followed by G-CSF, CXCL 2 and IL-6.

Inflammatory cell infiltrations into the large intestines of IL-17 KO mice

Since accumulation of inflammatory cells, especially neutrophils, was well known to be a crucial factor for determining CDI pathogenesis and severity, we evaluated the infiltration of neutrophils and macrophages in the large intestine on day 2 after the infection. As shown in Figure 5a, we found the significant numbers of $CD11b^{high}Ly6G^{high}$ cells, which were defined to be neutrophils, in the *C. difficile* infected mice. Figure 5a data clearly showed a lower number of neutrophils in IL-17 KO mice, comparing to that of WT mice. This difference was calculated to be statistically significant in neutrophils, but not in macrophages, as shown in Figure 5b. These results demonstrated a lower neutrophil accumulation into the site of infection in IL-17 KO mice at the early stage of *C.difficile* infection.

DISCUSSION

The present data clearly demonstrated that IL-17 KO mice were more resistant to the challenge of *C. difficile*, as evidenced by diarrhea score, weight change, macroscopic/microscopic findings and survival rates. These data suggested that endogenous IL-17 may be a responsible factor for the pathogenesis of CDI. Since there were no changes in the burdens of *C. difficile* in the gut of IL-17 KO mice, the host responses may be important for resistant phenotype in IL-17 deficient situation. In this point, our data showed significantly lower cytokine and chemokine responses and less neutrophil accumulation in the infected site of IL-17 KO mice.

IL-17 is a potent inducer of inflammatory cytokines, such as IL-1 beta, IL-6, IL-8, TNF alpha, G-CSF, and chemokines, and recruits neutrophils and monocytes to the site of inflammation(Jovanovic et al., 1998; Happel et al., 2005). Thus, deficiency and disturbance in IL-17-axis were reported to be strongly associated with high susceptibility to bacterial and fungal infections. This fact is true, not only in mice, but also in several human diseases, including Job's syndrome (or hyper-IgE syndrome) and chronic mucocutaneous candidiasis (McDonald, 2012). In our CDI model, we observed significantly less responses of IL-1 beta, IL-6, G-CSF and CXCL 2 in the gut of IL-17 KO mice. The reduced productions of inflammatory mediators were well correlated with lower numbers of neutrophils and less severe courses of CDI in IL-17 deficient situations. Although IL-17 is a responsible cytokine for bridging of innate and adaptive immunity, uncontrolled or dysregulated production of IL-17 was reported to be associated with several disorders, such as psoriasis, IBD and infectious diseases (Pappu et al., 2010). Zelante et al. (2007) have reported dual potential of IL-17-associated host responses in

296 fungal infections. Especially, the previous data demonstrated induction of IL-17 and IL-
297 23 (a crucial driver for IL-17 axis) may be associated with activation of neutrophils (ex.
298 matrix metalloprotease 9, myeloperoxidase), which likely accounts for the high
299 inflammatory pathology and tissue destruction in a mice model of mucosal candidiasis.
300 These data pointed a detrimental effect for IL-17 on neutrophil function, and also
301 suggested a possibility that IL-17 play a different role in different types/sites of infection.
302 In this respect, the decreased influx of neutrophils accounted for the high susceptibility of
303 IL-17 receptor deficient mice to systemic candidiasis (Huang et al., 2004), but not to
304 mucosal candidiasis (Zelante et al., 2007). In CDI, Buonomo et al. (2013) have reported
305 the increased IL-23 in human colon biopsy specimens from CDI patients, and mice
306 lacking IL-23 signaling had a significant increase of survival in CDI.
307 The source of IL-17 is believed to be restricted to hematopoietic cells, while the
308 heterodimeric IL-17 receptor is widely expressed by hematopoietic and non-
309 hematopoietic cells, such as endothelial and epithelial cells. Skin, lungs and intestines
310 are most investigated organs (Lochner et al., 2008), and high levels of IL-17 are induced
311 in response to a range of infections, as well as during the development of several
312 autoimmune diseases. IBD is one of the most investigated entities, and up-regulation of
313 IL-17 was reported in active Crohn's disease and ulcerative colitis (Pappu et al., 2010). In
314 gut, it seems likely that IL-17 has distinctive roles, because Paneth cells of the crypts
315 constitutively stored IL-17 inside-cells and then rapid release of this cytokine upon
316 certain stimuli (Takahashi et al., 2008). A rapid local amplification mechanism needed
317 for defense may turn out detrimental in certain inflammatory/immunological and
318 infectious diseases, such as IBD and CDI.

The discovery of IL-17 axis has contributed to elucidate new pathways of pathogenesis, host responses and tissue damage in a variety of diseases, as well as open new avenues for development of therapeutic strategies. In respect of blocking of IL-17, secukinumab (an IL-17A antibody), brodalumab (an IL-17 receptor antibody) were recently introduced for treatment of IBD (Fitzpatrick, 2013). There are a limited information investigating a relation between IL-17 blocking and CDI (Reichert, 2013).

There are several limitations in the present study. Firstly, although the bacterial numbers in the feces were compared between WT and IL-17 KO mice, there were no data of microbiota in IL-17 KO mice. Since IL-17 was reported to play a crucial role in mucosal immunity (Atarashi et al., 2011), deficiency of IL-17 may induce changes of commensal bacterial flora, which may affect toxin productions of *C. difficile*. In this point, microbiota analysis is ongoing in our laboratory (Fukui et al., 2015). Secondly, we have only examined infiltration of neutrophils and macrophages, but not other cell types, such as gamma-delta T cells and natural killer T cells, which are also important IL-17 producers (Cua & Tato, 2010). Sequential changes of these cell populations in the site of infection, in addition to analysis of activation and secretions of related mediators, are necessary for better understanding of pathogenesis of CDI. Finally, there were no direct explanations of the mechanisms of death in mice with CDI. Since these mice were not bacteremic, and toxin-deficient strains were less virulent (data not shown), the toxin-mediated cellular and organ damages may be associated with lethality of mice with CDI.

In summary, the present study demonstrated a role of endogenous IL-17 in mice model of CDI. These data suggested that targeting regulation of IL-17 axis may be a potential therapeutic approach for CDI.

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TABLES

Table. 1 Sequence of the primers used for qPCR

Gene	Sequence
IL-1 beta	F) 5'-CAACCAACAAGTGATATTCTCCATG-3' R) 5'-GATCCACACTCTCCAGCTGCA-3'
G-CSF	F) 5'-AGATATTCGAGCAGGGTCTAC-3' R) 5'-GGGATATCAGTCAGAAAGGTT-3'
CXCL 2	F) 5'-TCCAGAGCTTGAGTGTGACG-3' R) 5'-TCCAGGTCAGTTAGCCTTGC-3'
IL-6	F) 5'-ACACATGTTCTCTGGGAAATCGT-3' R) 5'-AAGTGCATCATCGTTGTTCATACA-3'
IL-17 A	F) 5'-TTTAACTCCCTTGGCGCAAAA-3' R) 5'-CTTTCCCTCCGCATTGACAC-3'
IL-17 F	F) 5'-TGCTACTGTTGATGTTGGGAC-3' R) 5'-AATGCCCTGGTTTTGGTTGAA-3'
beta-actin	F) 5'-AGAGGGAAATCGTGCGTGAC-3' R) 5'-CAATAGTGATGACCTGGCCGT-3'

FIGURES

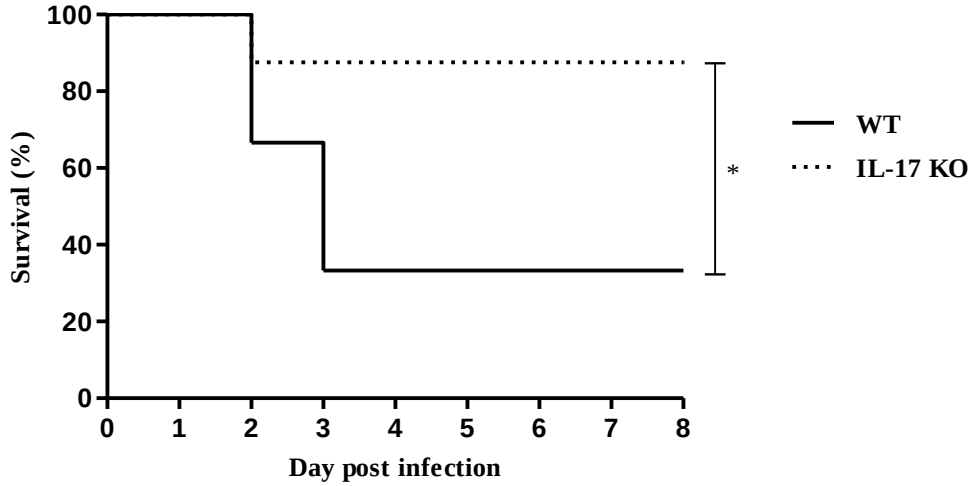


Fig. 1(a)

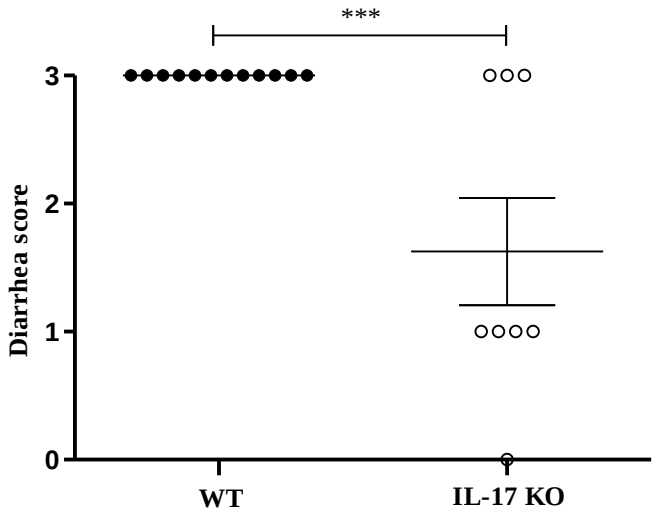


Fig. 1(b)

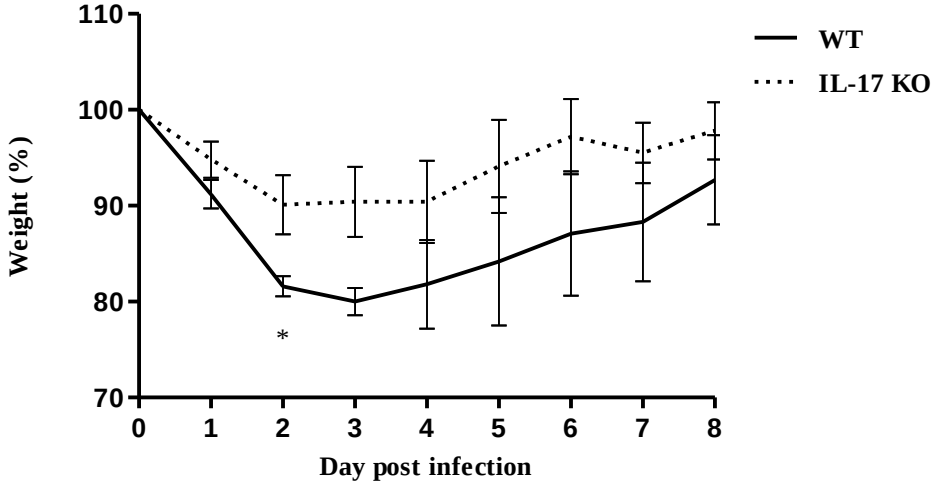


Fig. 1(c)

Fig 1(a), (b), (c). Susceptibility of IL-17 KO mice to CDI

Susceptibility of IL-17 KO mice (n=8) to CDI were compared to those of WT mice (n=12) in several points, such as survival rate (a), diarrhea score (b) and weight change

(c). Solid and dotted lines in (a) and (c) show WT and IL-17 KO mice, respectively. Values in Fig.1(b) and (c) were presented in mean ± SEM. The asterisks denote significant differences (*, $P< 0.05$; ***, $P< 0.005$). At least three independent experiments were performed and the representative data were demonstrated.

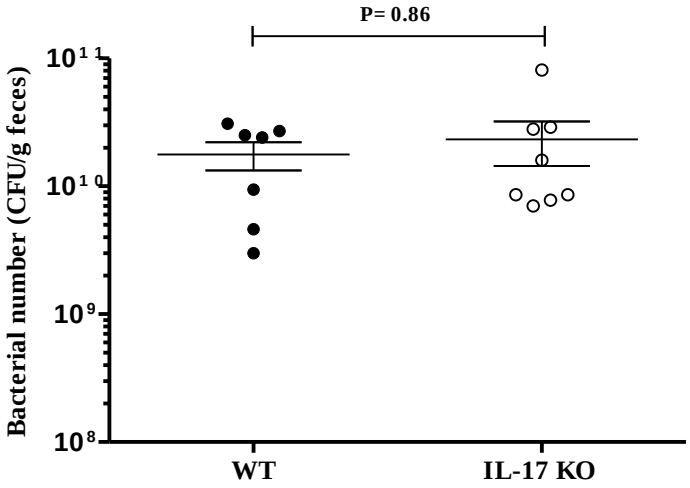


Fig. 2. The number of *C. difficile* in feces in WT and IL-17 KO mice

The number of *C. difficile* in feces of WT mice (n=7) and IL-17 KO mice (n=8) were compared on day 3 after the infection. The data were demonstrated in mean ± SEM. Four independent experiments were performed and the representative data were demonstrated.

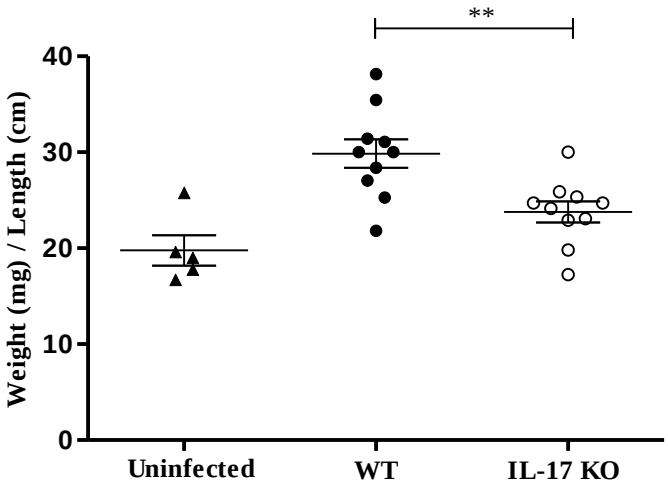

**
40
30
20
10
0
Weight (mg) / Length (cm)
Uninfected
WT
IL-17 KO

Fig. 3(a)


WT IL-17 KO
Uninfected

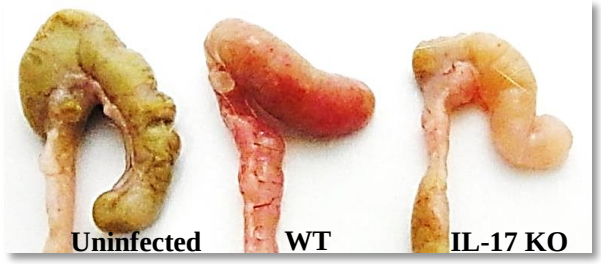
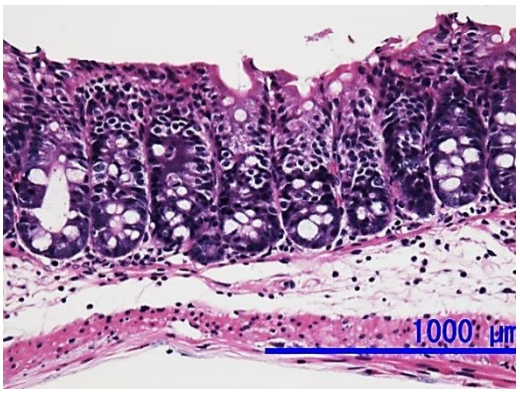
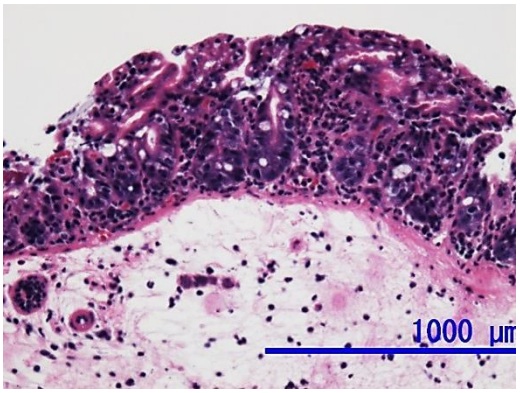

Uninfected
WT
IL-17 KO

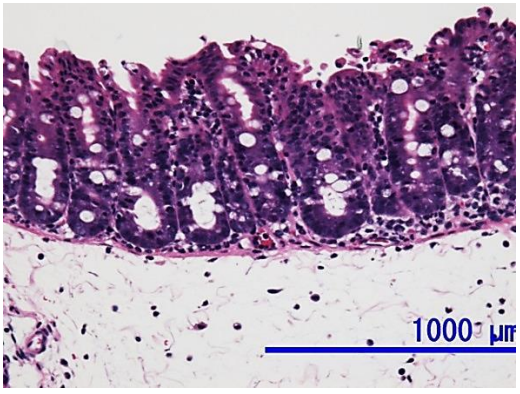
Fig. 3(b)



Uninfected



WT



IL-17 KO

Fig. 3(c)

Fig. 3(a), (b), (c). Inflammatory responses in the large intestine of IL-17 KO mice

Inflammatory responses were compared in WT mice without infection (uninfected; n=5), WT mice with infection (WT; n=10) and IL-17 KO mice with infection (IL-17 KO; n=10). (a) shows weight/length ratio of colon on day 3 after the infection, respectively. The data were shown in mean ± SEM. The asterisks denote significant differences (*, $P < 0.05$; **, $P < 0.01$). Five independent experiments were performed and the representative data were demonstrated. (b) and (c) show macroscopic and microscopic findings in the large intestines, respectively.

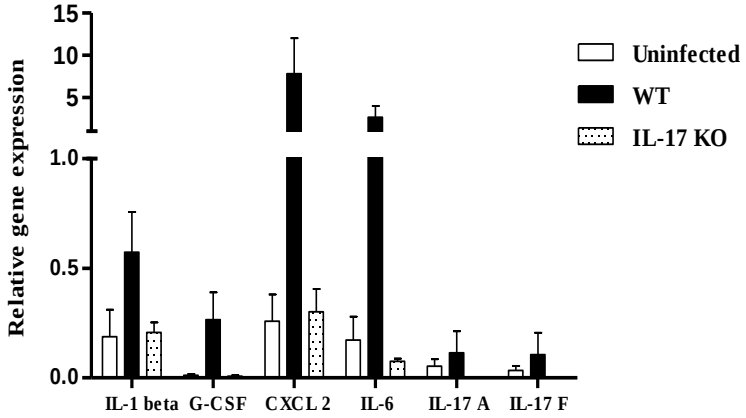


Fig. 4(a)

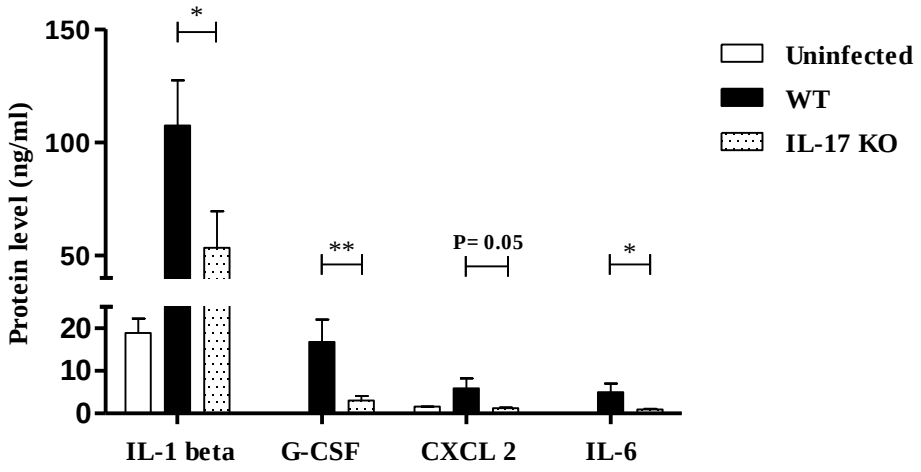
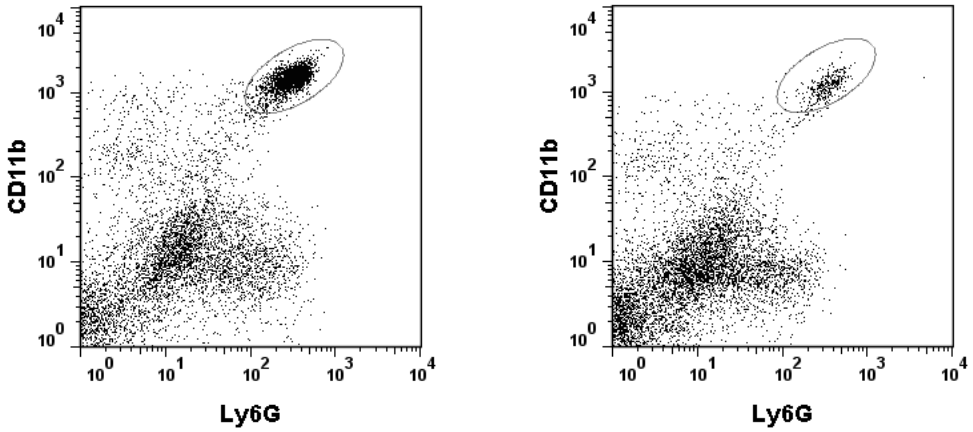


Fig. 4(b)

Fig. 4(a), (b). Gene and Protein levels of inflammatory mediators in the large intestine of IL-17 KO mice

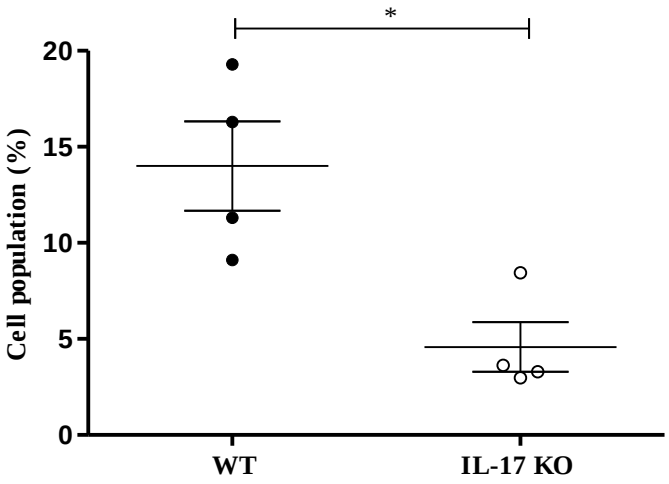
We analyzed gene and protein levels of inflammatory mediators in the large intestine of WT mice (n=8) and IL-17 KO mice (n=8) by qPCR (a) and ELISA (b) on day 3 after the infection. The data were expressed in mean ± SEM. The asterisks denote significant differences (*, $P < 0.05$; **, $P< 0.01$). Three independent experiments were performed and the representative data were demonstrated.



529

530 **WT** **IL-17KO**

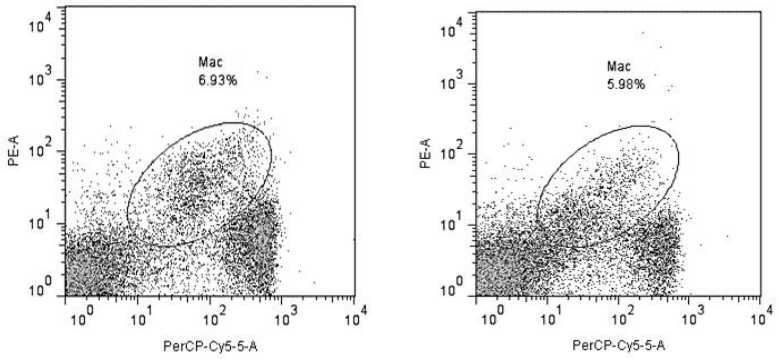
531



532

533 **Fig. 5(a)**

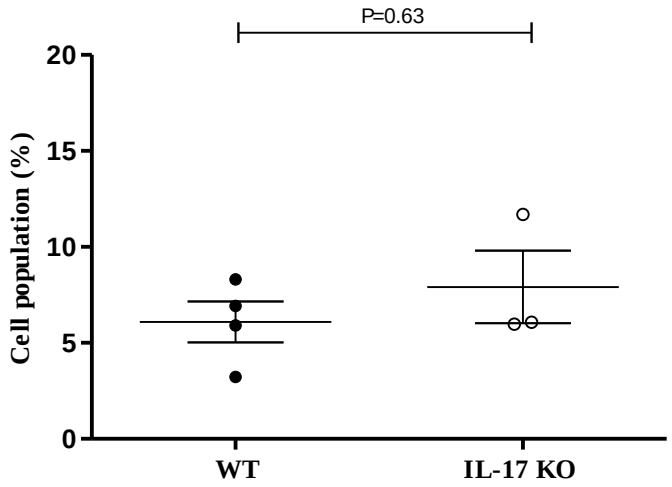
534



535

536 **WT** **IL-17 KO**

537



538

539 **Fig. 5(a), (b). Analysis of accumulation of neutrophils and macrophages into the**

540 **large intestines in IL-17 KO mice**

541 We analyzed accumulation of neutrophils and macrophages into the large intestines of

542 WT mice and IL-17 KO mice by using flowcytometry on day 2 after the infection. The

543 circle shows cell population of neutrophils (a) and macrophages (b), respectively. The

544 data were expressed in mean ± SEM. The asterisks denote significant differences (*, $P <$

545 0.05). Three independent experiments were performed and the representative data were
546 demonstrated.