

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

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

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29 chemokine ligand 2; HBSS, Hank's balanced salt solution; FBS, fetal bovine serum;

30 RPMI, Roswell Park Memorial Institute

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48 **ABSTRACT**

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

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71 **INTRODUCTION**

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

82 Neutrophils are a key player in the pathogenesis of CDI, because a peripheral
83 leukocytosis and massive infiltration of neutrophils into the sites of infection are
84 demonstrated to be one of characteristics and a predictor of severity of this disease
85 (Fujitani et al., 2011). Although several lines of evidence demonstrated that excessive
86 accumulation and activation of neutrophils may be involved in the pathogenesis of CDI,
87 whether neutrophil influx is beneficial or detrimental are still controversial (Kelly et al.,
88 1994; Qiu et al., 1999; Jarchum et al., 2012; Hasegawa et al., 2011).

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

94 recruitment and antimicrobial peptides production, especially in skin, lungs and intestinal
95 tracts (Lochner et al., 2008). In chronic diseases, persistent uncontrolled production of
96 IL-17 may be associated with autoimmune/inflammatory diseases, such as psoriasis and
97 inflammatory bowel disease (IBD) (Pappu et al., 2010). On the other hand, there are
98 growing evidences of the roles of IL-17 in several infectious diseases (Ishigame et al.,
99 2009). The recent data demonstrated up-regulation of IL-17 and IL-23, a positive
100 regulator for IL-17 production, at the site of infected mucosa (Buonomo et al., 2013;
101 Hirota et al., 2012). Interestingly, a link between CDI and IBD was reported by the fact
102 that IBD patients are more susceptible to CDI (Issa et al., 2007).

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

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116 **METHODS**

117 ***C. difficile* strain and cultivation methods**

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

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130 **Animal models of CDI**

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

139 compared by composition and number of major culturable bacteria. Murine CDI model
140 was prepared according to the protocol, as described previously (Chen et al., 2008).
141 Briefly, mice were administered antibiotics cocktail (kanamycin 0.4 mg/ml, gentamycin
142 0.035 mg/ml, colistin 850 U/ml, metronidazole 0.215 mg/ml, and vancomycin 0.045
143 mg/ml) in the drinking water from 6 days to 3 days before the infection. On one day
144 before the infection, mice were injected with clindamycin 300 µg in 0.25 ml PBS
145 intraperitoneally. Mice were intragastrically challenged with 5.0×10^5 - 1.0×10^6 CFU of *C.*
146 *difficile*. After the infection, the survival rate, the diarrhea scoring and the weight change
147 of the mice was monitored daily until 8 days after the infection. The stool condition was
148 scored as follows: 0, normal stool; 1, loose stool; 2, mushy stool; 3, watery stool. The
149 highest stool score after the infection was used for the analysis. On day 3 post-infection,
150 the mice were sacrificed by CO₂ asphyxia, and then each colon was collected to measure
151 weight and length for evaluation of fluid accumulation and inflammation, as previously
152 described (Inui et al., 2011). For bacterial count of *C. difficile* in feces, stool pellets were
153 suspended in PBS. The suspension was inoculated onto a selective medium containing
154 cysteine hydrochloride, moxalactam, and norfloxacin after 1:10 serial dilutions, and then
155 cultivated anaerobically, as mentioned above (Aspinall & Hutchinson, 1992). For
156 histological analysis, cecum was harvested on day 3 post-infection. The tissues were
157 fixed and stained with hematoxylin-eosin.

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159 **Gene expressions and protein levels of inflammatory mediators**

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

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

167 Scraped mucosa of the cecum were suspended in 8-fold amount of PBS containing 1 %
168 Triton X-100 (Sigma-Aldrich) and complete protease inhibitor (Roche) and incubated for
169 20 min on ice. The suspension was centrifuged for 15 min at 15,000 g to collect
170 supernatants. The concentrations of IL-1 beta, IL-6, granulocyte-colony stimulating
171 factor (G-CSF) and CXC chemokine ligand 2 (CXCL 2) in the cecum was measured by
172 ELISA according to the manufacturer's instructions (R&D systems).

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174 **Isolation of intestinal immune cells and flow cytometric analysis**

175 Small pieces of PBS-washed cecum and colon were incubated in HBSS (Gibco)
176 containing 1mM ethylenediaminetetraacetic acid (Wako Pure Chemical), 2 % FBS
177 (Gibco) and 1 mM dithiothreitol (Wako Pure Chemical) for 20 min at 37 °C with 160
178 rpm shaking. The tissue pieces were washed with HBSS and minced, then incubated in
179 RPMI 1640 medium (Gibco) containing 2 % FBS, 0.5 mg/ml collagenase D (Roche) and
180 150 µg/ml DNase (Roche) for 50 min at 37 °C with 160 rpm shaking. Suspensions were
181 passed through a strainer and centrifuged 1,500 rpm for 5 min. Pellets were washed with
182 PBS and centrifuged. The samples were re-suspended in 10 ml 40 % Percoll (GE
183 Healthcare) diluted with RPMI 1640 and layered on 2 ml 80 % Percoll diluted with RPMI
184 1640 before centrifugation at 2,300 rpm for 20 min. Viable immune cells were treated

185 with CD 16/32 (eBioscience), then stained with PerCP/Cy 5.5-conjugated Ab CD11b
186 (M1/70; Biolegend), FITC-conjugated Ab Ly6G (1A8; Biolegend) and PE-conjugated Ab
187 F4/80 (BM8; Biolegend). After staining, samples were washed with PBS and treated with
188 4 % paraformaldehyde PBS (Wako Pure Chemical). Fluorescence was assessed with
189 FACSCanto 2 (Becton Dickinson) and analyzed with FlowJo 7.6.5 (TreeStar).

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191 **Statistical analysis**

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

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205 **RESULTS**

206 **Susceptibility of IL-17 KO mice to CDI**

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

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

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

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

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

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

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

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

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

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

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273 **DISCUSSION**

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

282 IL-17 is a potent inducer of inflammatory cytokines, such as IL-1 beta, IL-6, IL-8, TNF
283 alpha, G-CSF, and chemokines, and recruits neutrophils and monocytes to the site of
284 inflammation(Jovanovic et al., 1998; Happel et al., 2005). Thus, deficiency and
285 disturbance in IL-17-axis were reported to be strongly associated with high susceptibility
286 to bacterial and fungal infections. This fact is true, not only in mice, but also in several
287 human diseases, including Job's syndrome (or hyper-IgE syndrome) and chronic
288 mucocutaneous candidiasis (McDonald, 2012). In our CDI model, we observed
289 significantly less responses of IL-1 beta, IL-6, G-CSF and CXCL 2 in the gut of IL-17
290 KO mice. The reduced productions of inflammatory mediators were well correlated with
291 lower numbers of neutrophils and less severe courses of CDI in IL-17 deficient situations.

292 Although IL-17 is a responsible cytokine for bridging of innate and adaptive immunity,
293 uncontrolled or dysregulated production of IL-17 was reported to be associated with
294 several disorders, such as psoriasis, IBD and infectious diseases (Pappu et al., 2010).
295 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.

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

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

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

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457 **TABLES**

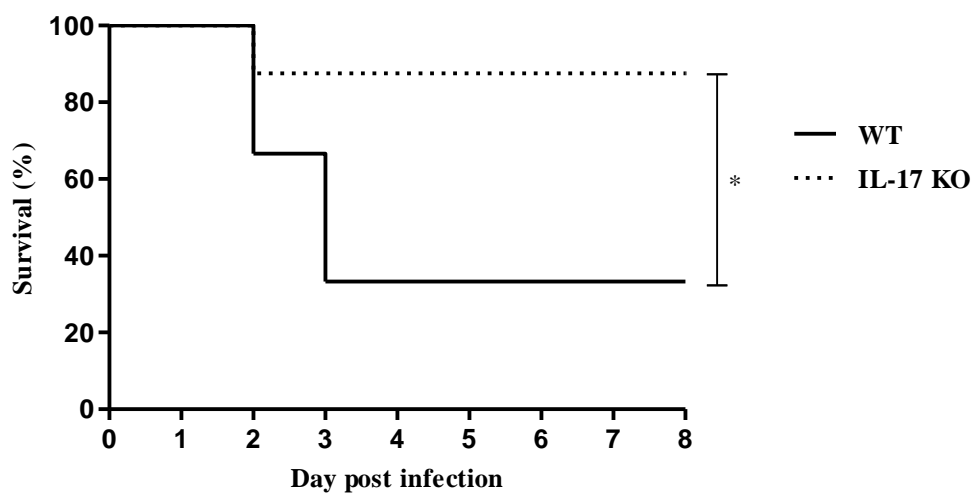
458 **Table. 1** Sequence of the primers used for qPCR

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'-AAGTGCATCATCGTTGTTTCATACA-3'
IL-17 A	F) 5'-TTTAACTCCCTTGGCGCAAAA-3' R) 5'-CTTCCCTCCGCATTGACAC-3'
IL-17 F	F) 5'-TGCTACTGTTGATGTTGGGAC-3' R) 5'-AATGCCCTGGTTTTGGTTGAA-3'
beta-actin	F) 5'-AGAGGGAAATCGTGCGTGAC-3' R) 5'-CAATAGTGATGACCTGGCCGT-3'

459

460 **FIGURES**

461

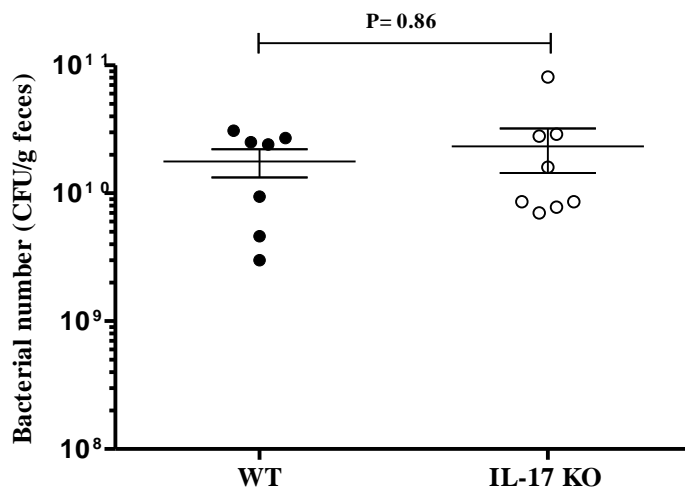


462

463 **Fig. 1(a)**

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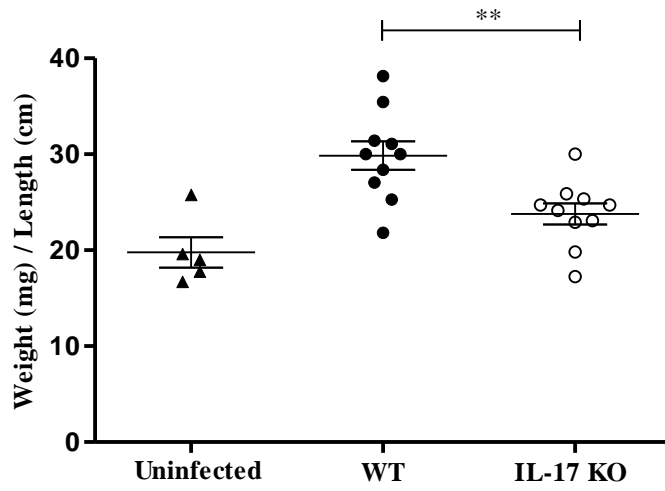
474 (c). Solid and dotted lines in (a) and (c) show WT and IL-17 KO mice, respectively.
475 Values in Fig.1(b) and (c) were presented in mean \pm SEM. The asterisks denote
476 significant differences (*, $P < 0.05$; ***, $P < 0.005$). At least three independent
477 experiments were performed and the representative data were demonstrated.
478



479

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

481 The number of *C. difficile* in feces of WT mice (n=7) and IL-17 KO mice (n=8) were
482 compared on day 3 after the infection. The data were demonstrated in mean \pm SEM. Four
483 independent experiments were performed and the representative data were demonstrated.
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485

486 **Fig. 3(a)**

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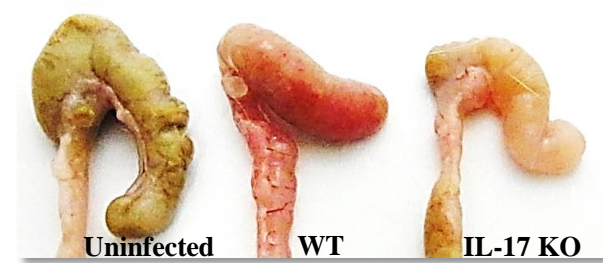
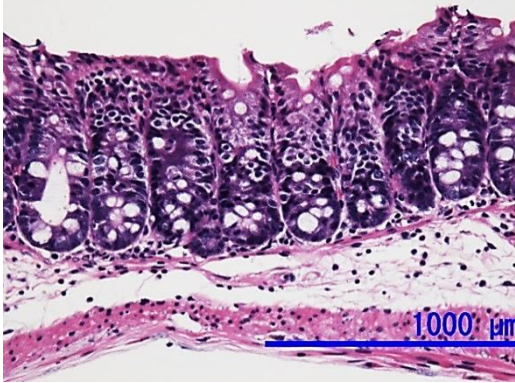
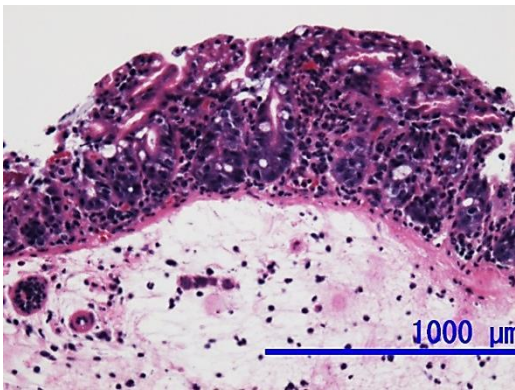


Fig. 3(b)



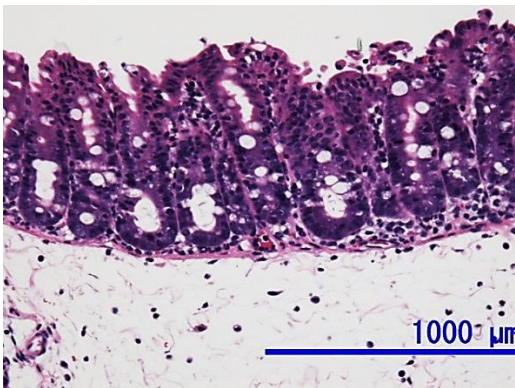
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499 **Uninfected**



500

501 **WT**



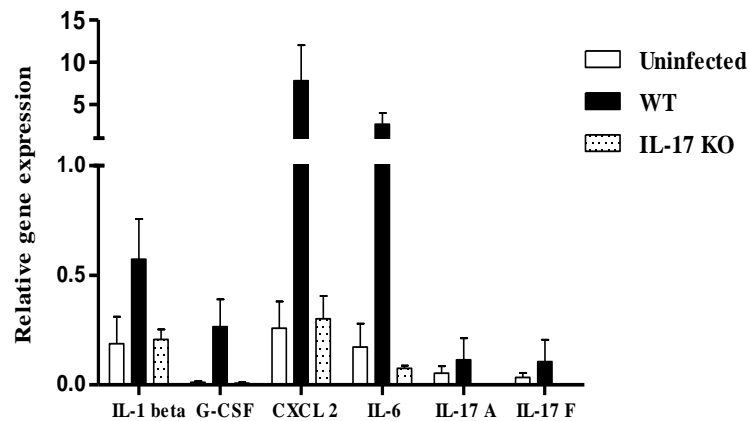
502

503 **IL-17 KO**

504 **Fig. 3(c)**

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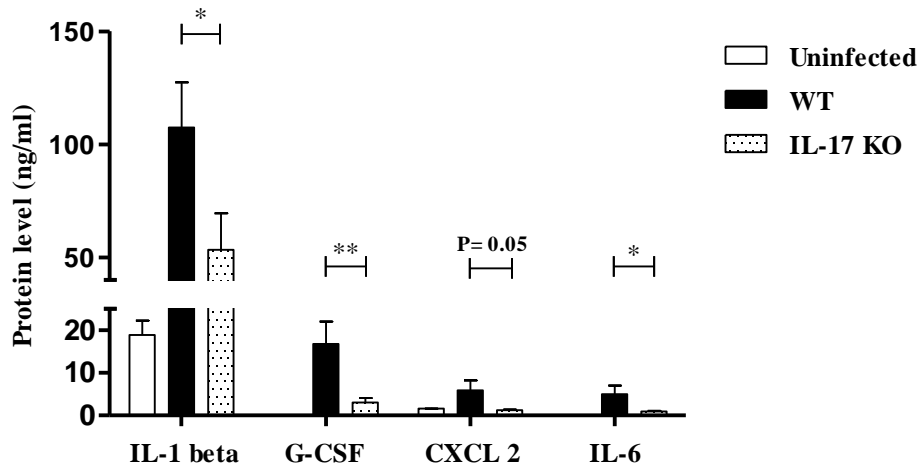
506 **Fig. 3(a), (b), (c). Inflammatory responses in the large intestine of IL-17 KO mice**
507 Inflammatory responses were compared in WT mice without infection (uninfected; n=5),
508 WT mice with infection (WT; n=10) and IL-17 KO mice with infection (IL-17 KO;
509 n=10). (a) shows weight/length ratio of colon on day 3 after the infection, respectively.
510 The data were shown in mean \pm SEM. The asterisks denote significant differences (*, $P <$
511 0.05; **, $P <$ 0.01). Five independent experiments were performed and the representative
512 data were demonstrated. (b) and (c) show macroscopic and microscopic findings in the
513 large intestines, respectively.
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516 **Fig. 4(a)**

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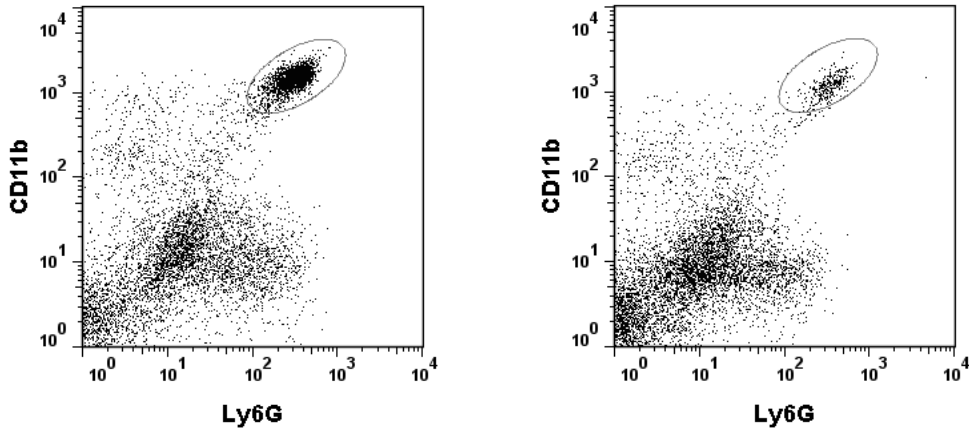
519 **Fig. 4(b)**

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

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

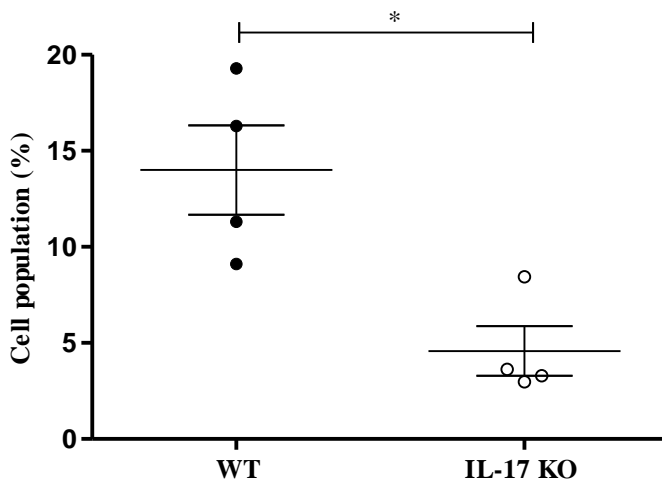
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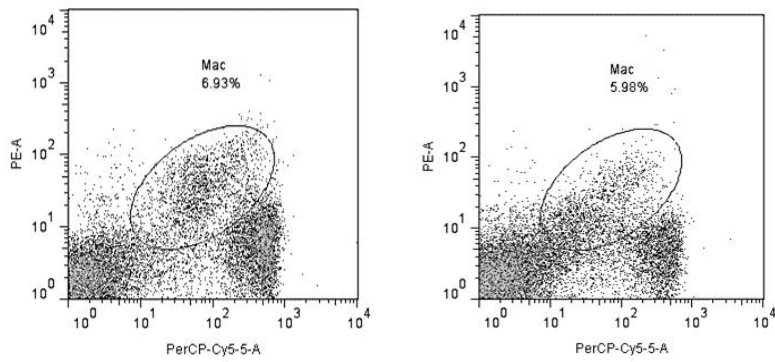
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533 **Fig. 5(a)**

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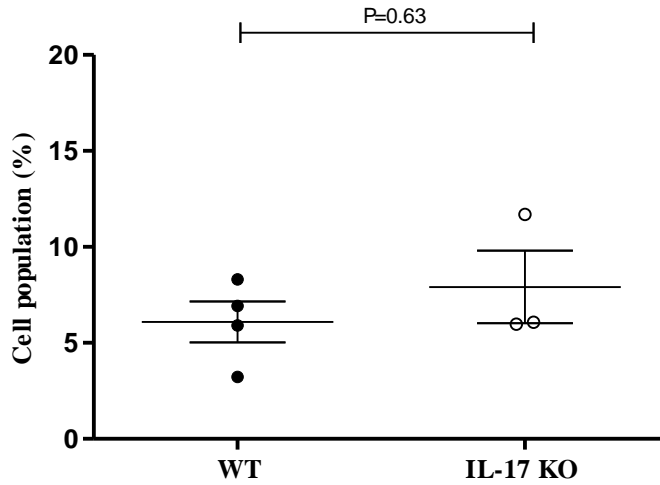
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536

WT

IL-17 KO

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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 \pm SEM. The asterisks denote significant differences (*, $P <$

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