Endogenous IL-17 as a factor determining the severity of Clostridium difficile infection in mice

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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. IL-17 is one of the cytokines that play critical roles in several inflammatory and immunological diseases through various actions, including promoting neutrophil recruitment. The aim of this study was to examine the role of this cytokine in CDI by employing IL-17 A and F double knockout (IL-17 KO) mice for the CDI model. We demonstrated that IL-17 KO mice were more resistant to CDI than WT mice using several factors, such as diarrhoea score, weight change and survival rate. Although the bacterial numbers of C. difficile in faeces 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 showed that infiltration of neutrophils, but not macrophages, in the large intestine was significantly decreased in IL-17 KO mice compared to WT mice. In conclusion, the data demonstrate that endogenous IL-17 may be a factor determining the severity of CDI in mice. Although the mechanism is totally unknown, IL-17-mediated inflammatory responses, such as cytokine/chemokine production and neutrophil accumulation, may be plausible targets for future investigations.

INTRODUCTION

Clostridium difficile infection (CDI) has become a serious problem in the world, especially in elderly and debilitated individuals, as a hospital- and healthcare-facility-associated infection (Miller et al., 2011). CDI issues have been complicated by the emergence and spreading of endemic strains, and also by the issue of relapse/recurrence (O’Connor et al., 2009). The primary virulence factors of C. difficile are its toxins, like toxin A, B and binary toxin. In addition to the direct toxin-mediated pathogenic effects on the target cells, these toxins have been shown to induce profound immunological and inflammatory responses (Hirota et al., 2012).

Neutrophils are key players in the pathogenesis of CDI, because a peripheral leukocytosis and massive infiltration of neutrophils into the sites of infection have been demonstrated to be a characteristic and a predictor of the 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, it is still controversial whether neutrophil influx is beneficial or detrimental (Kelly et al., 1994; Qiu et al., 1999; Jarchum et al., 2012; Hasegawa et al., 2011).

IL-17 is a proinflammatory cytokine, and plays a crucial role in bridging innate and adaptive immunity. Although there have been at least six molecules identified in the IL-17 family, IL-17 A and IL-17 F are the most studied subtypes. They share high homology in structure and use the same receptor on host cells. Contributions of IL-17 in host defence systems and the pathogenesis of diseases have been well characterized with respect to neutrophil recruitment and antimicrobial peptide production, especially in the skin, lungs and intestinal tract (Lochner et al., 2012).
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). However, there is growing evidence of the role of IL-17 in several infectious diseases (Ishigame et al., 2009). 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 indicated by the fact that IBD patients are more susceptible to CDI (Issa et al., 2007).

In this paper, we have investigated the roles of IL-17 in a mouse model of CDI. In particular, the survival data obtained from IL-17 A and F double knockout (IL-17 KO) mice experiments, along with diarrhoea score, macroscopic/microscopic findings and cytokine/chemokine responses, strongly suggested a role of endogenous IL-17 as a determining factor for inflammatory responses and the 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. Frozen stock of the bacteria were 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 an anaerobic chamber (Mitsubishi Gas Chemical). After 48 h, 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 0.045 mg vancomycin ml⁻¹, 850 U colistin ml⁻¹, 0.215 mg metronidazole ml⁻¹ and 0.045 mg vancomycin ml⁻¹ in the drinking water from 6 days to 3 days before the infection. One day before the infection, mice were injected with clindamycin (300 µg in 0.25 ml PBS) intra-peritoneally. Mice were intragastrically challenged with 5.0 × 10⁷ -1.0 × 10⁹ c.f.u. of the vegetative form of C. difficile (more than 95 % of the C. difficile were vegetative form in spore staining analysis; data not shown). After the infection, the survival rate and the weight change of the mice were monitored daily until 8 days post-infection. The stool condition was scored by day 3 post-infection as follows: 0, normal stool; 1, loose stool; 2, mushy stool; 3, watery stool. On day 3 post-infection, the mice were sacrificed by CO₂ asphyxia, and then the colon of each mouse was collected to measure the weight and length for evaluation of fluid accumulation and inflammation, as previously described by Inui et al. (2011). For measuring the bacterial count of C. difficile in faeces, 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, caecum was harvested on day 3 post-infection. The tissues were fixed and stained with haematoxylin/ezisin.

Gene expression and protein levels of inflammatory mediators. We evaluated inflammatory mediators in the colon using real-time quantitative PCR (qPCR). Total RNA was extracted by using a RiboPure kit (Ambion), according to the manufacturer’s instructions. A TURBO DNA-free kit (Ambion) was used to treat RNA with DNase, then the RNA was reverse transcribed by using a High Capacity cDNA reverse transcription kit (Applied Biosystems). Data analysis using Fast SYBR Green master mix (Applied Biosystems) was performed on a 7500 Fast real-time PCR system (Applied Biosystems). The PCR primers used are shown in Table 1. β-Actin was used as the comparator of each gene’s expression.

Scraped mucosa of the caecum was suspended in an eightfold 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 the supernatant. The concentrations of IL-1β, IL-6, interleukin-23, interleukin-25, and interleukin-27 were 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 caecum and colon were incubated in HBSS (Hank’s balanced salt solution) (Gibco) containing 1 mM EDTA (Wako Pure Chemical), 2 % fetal bovine serum (FBS) (Gibco) and 1 mM DTT (Wako Pure Chemical) for 20 min at 37 °C with 160 r.p.m. shaking. The tissue pieces were washed with HBSS and minced, then incubated in RPMI 1640 (Roswell Park Memorial Institute 1640 medium (Gibco) containing 2 % FBS, 0.5 mg collagenase D ml⁻¹ (Roche) and 150 µg DNase ml⁻¹ (Roche) for 45 min at 37 °C with 160 r.p.m. shaking. Suspensions were passed through a strainer and centrifuged at 400 g for 5 min. Pellets were washed with PBS and centrifuged. The samples were resuspended in 10 ml 40 % Percoll (GE Healthcare) diluted with RPMI 1640 and layered on 2 ml 80 % Percoll diluted with Tokyo, Tokyo, Japan (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 and pathogen protocols were approved by the institutional care and use committee (approval number 13-31-220, 14-52-58). The intestinal flora of WT and IL-17 KO mice were checked by the absence of specific pathogens in the faeces, and compared for composition and number of major culturable bacteria. WT and IL-17 mice were kept in the same room around 10 days before the infection. The murine CDI model was prepared according to the protocol described previously by Chen et al. (2008). Briefly, mice were administered an antibiotics cocktail (0.4 mg kanamycin ml⁻¹, 0.035 mg gentamicin ml⁻¹, 850 U colistin ml⁻¹, 0.215 mg metronidazole ml⁻¹ and 0.045 mg vancomycin ml⁻¹) in the drinking water from 6 days to 3 days before the infection. One day before the infection, mice were injected with clindamycin (300 µg in 0.25 ml PBS) intra-peritoneally. Mice were intragastrically challenged with 5.0 × 10⁷ -1.0 × 10⁹ c.f.u. of the vegetative form of C. difficile (more than 95 % of the C. difficile were vegetative form in spore staining analysis; data not shown). After the infection, the survival rate and the weight change of the mice were monitored daily until 8 days post-infection. The stool condition was scored by day 3 post-infection as follows: 0, normal stool; 1, loose stool; 2, mushy stool; 3, watery stool. On day 3 post-infection, the mice were sacrificed by CO₂ asphyxia, and then the colon of each mouse was collected to measure the weight and length for evaluation of fluid accumulation and inflammation, as previously described by Inui et al. (2011). For measuring the bacterial count of C. difficile in faeces, 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, caecum was harvested on day 3 post-infection. The tissues were fixed and stained with haematoxylin/ezisin.

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Table 1. Sequences of the primers used for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>F 5'-CAACCAACAAGTGTATATTCTCCAT-3' R 5'-GATCACACCTCCTAGGCTGA-3'</td>
</tr>
<tr>
<td>G-CSF</td>
<td>F 5'-AGATATTGCCAGCGGCTACT-3'</td>
</tr>
<tr>
<td>CXCL2</td>
<td>F 5'-TCGAGCCTGAGTGGTGAAGCG-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>F 5'-ACACAGTTCTCTCCTGGAGAATGT-3'</td>
</tr>
<tr>
<td>IL-17 A</td>
<td>F 5'-TTTACTGCTCCGGATTGACAC-3'</td>
</tr>
<tr>
<td>IL-17 F</td>
<td>F 5'-TGCTACTGCTGGTTGGGAC-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F 5'-AAATTGCTTGGTTGGCTGAC-3'</td>
</tr>
<tr>
<td></td>
<td>F 5'-CAATATGTATGACCTGGCCGTT-3'</td>
</tr>
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Role of IL-17 in C. difficile infection

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, diarrhoea score and weight changes between WT and IL-17 KO mice. As shown in Fig. 1(a), IL-17 KO mice demonstrated a significantly higher survival rate compared with that of WT mice. Although all WT mice showed watery diarrhoea within 3 days post-infection, only about 40% of IL-17 KO mice did. The mean diarrhoea score of the IL-17 KO mice group was significantly lower than that of the WT mice group (Fig. 1b). In addition, reduced weight loss was recorded in IL-17 KO mice compared to the WT mice group during the course of the 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 faeces 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 faeces 3 days after the challenge of 10³–10⁶ c.f.u. of C. difficile. Although more than 1000 times increase of C. difficile was observed in all faeces of mice, there were no significant differences in c.f.u. (g faeces)⁻¹ at this time point between WT mice and IL-17 KO mice (data not shown). However, WT and IL-17 KO mice demonstrated different stool scores and different water contents (Fig. 1b), so we could not exactly compare C. difficile number (g faeces)⁻¹ for these mice. Mice were kept in the same environment for 2 weeks, and we compared common commensal types of bacteria in their faeces (Escherichia coli, Bacteroides spp. and Clostridium spp.) 1 day before the infection. No differences were observed in the organisms examined at the time of testing between WT and IL-17 KO mice (data not shown).

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

In our mouse model of CDI, the predominant inflammation was observed in the large intestine, as reported previously by Sun et al. (2011). To compare the degree of inflammation, we evaluated the weights and lengths of the large intestines and carried out a macroscopic/microscopic examination of the gut on day 3 post-infection with C. difficile. As shown in Fig. 2(a), an increase of the weight/length ratio in the large intestines of WT mice was observed after the infection. Significantly, a lower response in the weight/length ratio was observed in IL-17 KO mice, compared to 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, compared to the uninfected WT mice (Fig. 2b). Interestingly, more reddish colour 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

Fig. 1. Susceptibility of IL-17 KO mice to CDI. The susceptibilities of IL-17 KO mice (n=8) to CDI were compared to those of WT mice (n=12) for several characteristics, such as survival rate (a), diarrhoea score (b) and weight change (c). The solid and dotted lines in (a) and (c) show WT and IL-17 KO mice, respectively. Values in (b) and (c) are presented as mean±SEM. The asterisks denote significant differences (⁎P<0.05; ⋆⋆⋆⋆P<0.005).
demonstrated remarkable inflammatory changes in the large intestines of *C. difficile*-infected WT mice, characterized with epithelial cell damage, submucosal oedema, fibrin synthesis and inflammatory cell infiltration (Fig. 2c). These results showed weaker inflammatory responses in the large intestines of IL-17 KO mice after the infection.

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

Next, we evaluated inflammatory mediator expression and production in the large intestine of the infected mice by qPCR and ELISA. The infection by *C. difficile* induced upregulation of all the genes examined in WT mice, as shown in Fig. 3(a). In IL-17 KO mice, clearly lower expression...
levels of these genes were demonstrated. These were substantially comparable to those of the uninfected WT mice. Protein levels were correlated with gene expression data. This suggests lower production of inflammatory mediators in IL-17 KO mice (Fig. 3b). In particular, the most prominent amount of production was observed for IL-1β, followed by G-CSF, CXCL2 and IL-6. We also examined IL-17A and IL-17F production by ELISA. The data were as follows: uninfected mice IL-17A, mean 0.47 ng ml⁻¹, and IL-17F, mean 1.11 ng ml⁻¹; infected WT mice IL-17 A, mean 0.95 ng ml⁻¹, and IL-17F, mean 2.23 ng.

**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 post-infection. As shown in Fig. 4(a), we found significant numbers of CD11b<sup>high</sup> Ly6G<sup>high</sup> cells (neutrophils) in the C. difficile-infected mice. In Fig. 4(a), the data clearly show a lower number of neutrophils in IL-17 KO mice, compared to WT mice. This difference was calculated to be statistically significant in neutrophils, but not in macrophages, as shown in Fig. 4(b). These results demonstrated a lower neutrophil accumulation into the site of infection in IL-17 KO mice at the early stage of CDI.

**DISCUSSION**

The present data clearly demonstrate that IL-17 KO mice are more resistant to the challenge of the epidemic strain of C. difficile BI/NAP1/027, as evidenced by diarrhoea score, weight change, macroscopic/microscopic findings and survival rates. These data are suggestive 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 the resistant phenotype in the IL-17-deficient situation. With regards 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-1β, IL-6, TNFa, 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 the 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-1β, IL-6, G-CSF and CXCL2 in the gut of IL-17 KO mice. The reduced production of inflammatory mediators was well correlated with lower numbers of neutrophils and a less severe course of CDI in IL-17-deficient situations. Although IL-17 is a cytokine responsible for the 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 a dual potential of IL-17-associated host responses in fungal infections. The previous data demonstrated induction of IL-17 and IL-23 (a crucial driver for the IL-17 axis) may be associated with activation of neutrophils (for example, matrix metalloprotease 9, myeloperoxidase),
which likely accounts for the high inflammatory pathology and tissue destruction in a mouse model of mucosal candidiasis. These data pointed to the detrimental effect for IL-17 on neutrophil function, and also suggested a possibility that IL-17 plays a different role in different types/sites of infection. In this respect, the decreased influx of neutrophils accounted for the high susceptibility of IL-17-receptor-deficient mice to systemic candidiasis (Huang et al., 2004), but not to mucosal candidiasis (Zelante et al., 2007). Buonomo et al. (2013) have reported increased IL-23 in human colon biopsy specimens from CDI patients, and mice lacking IL-23 signalling had a significant increase of survival in CDI. Recently, McDermott and colleagues (McDermott et al., 2016) have reported a limited role of IL-17A in a mouse model of CDI. They showed that inflammatory cytokine expression and neutrophilic inflammation were not reduced in IL-17A-deficient mice, whereas IL-23 deficiency was associated with significant defects in the recruitment of CD11b<sup>high</sup> Ly6G<sup>high</sup> neutrophils to the colon. The present data obtained from IL-17A and IL-17F double knock-out mice were similar to those of IL-23 KO mice, and are further suggestive of the role of IL-17F in CDI. Differential and combinational roles of IL-17-related factors may be a target for future investigations.

The source of IL-17 is believed to be restricted to haematopoietic cells, while the heterodimeric IL-17 receptor is widely expressed by haematopoietic and non-haematopoietic cells, such as endothelial and epithelial cells. The skin, lungs and intestines are the most investigated organs (Lochner et al., 2008), and high levels of IL-17 are induced in response to a range of infections, as well as during the development of several autoimmune diseases. IBD is one of the most investigated entities, and up-regulation of IL-17 was reported in active Crohn’s disease and ulcerative colitis (Pappu et al., 2010). In the gut, it seems likely that IL-17 has distinctive roles, because the Paneth cells of the crypts constitutively stored IL-17 inside cells and then rapid released this cytokine upon certain stimuli (Takahashi et al., 2008). A rapid local amplification mechanism needed for defence may turn out to be detrimental in certain inflammatory/immunological and infectious diseases, such as IBD and CDI. In summary, the present study demonstrated a role of endogenous IL-17 in a mouse model of CDI. IL-17-mediated inflammatory responses, such as cytokine/
chemokine production and neutrophil accumulation, may be plausible targets for future investigations.

REFERENCES


