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IL-17A contributes to reducing IFN-γ/IL-4 ratio and persistence of *Entamoeba histolytica* during intestinal amebiasis

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ABSTRACT

Amebiasis is an infectious disease caused by *Entamoeba histolytica*, an anaerobic protozoan parasite, and is a major public health problem worldwide, particularly in areas with inadequate sanitation and poor hygiene. Th1 responses, represented by interferon gamma (IFN-γ), play a protective role by clearing the amebae from the gut, whereas Th2 responses are responsible for chronic infection. Th17 responses preconditioned by vaccination or by modulating the intestinal microbiome protect mice from the settlement of *E. histolytica*. However, the role of interleukin-17A (IL-17A), which is upregulated during the natural course of intestinal amebiasis, has not been clarified. The aim of this study was to investigate the role of IL-17A during intestinal amebiasis in a mouse model. IL-17A knockout and wild-type CBA/J mice were challenged intracecally with 2×10^6 *E. histolytica* trophozoites, and their infection, pathology, and immune responses were monitored. Neither the initial settlement of *E. histolytica* nor the inflammation of the cecum was affected by the absence of IL-17A for week 1, but the infection rate and parasite burden declined in a late stage of infection, accompanied by an increased IFN-γ/IL-4 ratio. Therefore, IL-17A contributes to the persistence of *E. histolytica* and modulates the immune response, including the IFN-γ/IL-4 ratio, which may be responsible for the reduction of the parasite burden in the IL-17A knockout mice during the chronic phase of intestinal amebiasis.

1. Introduction

Amebiasis is an infectious disease caused by *Entamoeba histolytica*, an anaerobic protozoan parasite, and is a major public health problem worldwide, particularly in areas with inadequate sanitation and poor hygiene [1]. Although most cases of *E. histolytica* infection are asymptomatic and self-limiting, patients often develop severe complications, such as amebic colitis and liver abscess [2,3]. This is partly attributable to interindividual differences in genetic factors, immune responses, and nutritional status [4].

The IL-17 protein family consists of six members: IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F.

IL-17A, a potent inflammatory cytokine, is produced by activated T cells, γδ T cells, natural killer (NK) cells, NK T (NKT) cells, and innate lymphoid cells [5]. The receptor for IL-17A is expressed in various tissues and on various cell types, including lymphocytes, monocytes/macrophages, and epithelial cells. These cells produce diverse proinflammatory cytokines and chemokines in response to IL-17A stimulation. The effector role of IL-17A is mainly to accumulate neutrophils and to augment the production of proinflammatory cytokines and antimicrobial peptides [6–8].

Th1 cytokines, such as interferon γ (IFN-γ), play a protective role in clearing *E. histolytica* from the gut, whereas Th2 cytokines, such as IL-4, are responsible for chronic infections [9]. In addition to the Th1/Th2

cytokines, IL-17A was reported to play a protective role against bacterial and protozoan infections [10,11] and to act as a proinflammatory cytokine in inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease [12]. In a mouse model, IL-17A was significantly upregulated after inoculation with *E. histolytica* and remained upregulated throughout the course of the infection [9]. An increase in IL-17A was also observed when mice were vaccinated with a recombinant LecA fragment of the *E. histolytica* surface Gal/GalNAc lectin [13], and the blockade of IL-17A from 1 day before to 4 days after challenge abrogated the protective effect induced by the vaccine [14]. Although IL-17A augmentation before infection by either vaccination or the modulation of the intestinal microbiome made the host resistant to intestinal amebiasis, the roles of the IL-17A endogenously induced during the natural course of *E. histolytica* infection remain to be clarified.

In this study, we have investigated the role of IL-17A during *E. histolytica* infection using IL-17A knockout (KO) and wild-type (WT) mice in a CBA/J background as C57BL/6 mice are resistant to amebae infection [15]. We have monitored the kinetics of *E. histolytica* infection and the immunological cytokine profiles in the early (week 1) and late stages (week 4) of amebiasis in the mice. In the absence of IL-17A, the initial settlement of *E. histolytica* was unchanged, but the infection rate and parasite burden reduced in the late stage of infection. The cytokine profiles indicated that IFN-γ and IL-12p35 were greatly upregulated in the IL-17A KO mice, whereas the induction of IL-4 was more moderate than in the WT mice. These data suggest that IL-17A contributes to the persistence of *E. histolytica* by modulating IFN-γ and IL-4 production during intestinal amebiasis.

2. Materials and methods

2.1. Animals

Male CBA/J mice were purchased from Jackson Laboratories and maintained under pathogen-free conditions at the Animal Research Center for Tropical Infectious Diseases, Nagasaki University. IL-17A KO C57BL/6 mice were kindly gifted from Prof. Iwakura, Tokyo University of Science and backcrossed to CBA/J WT mice. We applied the speed congenic breeding strategies incorporating a marker-assisted selection of progeny with the highest percentage target background and successful backcross to CBA/J strain was confirmed by using > 100 of SNPs over the whole chromosomes.

For the confirmation of IL-17A KO mice, specific genes were checked by a conventional polymerase chain reaction (PCR). As following previous paper, primer sequences were: 5′ ACTCTTCATCCACCTCACACGA 3′ for IL-17A common; 5′ CAGCATCAGAGACTAGAAGGGA 3′ for IL-17A WT; and 5′ GCCATGATATAGACGTTGTGGC 3′ for IL-17A mutation [16]. PCR conditions were; 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s followed by 35 cycles. In the study, 6–8 weeks old, male littermate control mice were used for both WT and IL-17A KO mice. All experiments that involved mice were reviewed and approved by the Committee for Ethics on Animal Experiments in Nagasaki University (No. 1505181226-7, 1505181227-4). The study using recombinant DNA animal was also reviewed and approved by the Committee for Recombinant DNA Experiments (No. 1403041262-7). All of the study were conducted under the control of the Guidelines for Animal Experiments, Nagasaki University.

2.2. Parasite culture and infection

E. histolytica strain JPN51 was kindly gifted from Dr. Eric Houpt, University of Virginia. Trophozoites were serially passaged *in vivo* by intra-cecally inoculating *E. histolytica* to mice, collected from cecal contents and then cultured *in vitro* at 37 °C using Biostate-Iron-Serum-33 (BIS-33) media [17]. An intracecal inoculation procedure was extensively described in a previous publication [17,18]. In brief,

trophozoites were collected from tubes by cooling them on ice for 5–10 min. After mice were anesthetized with Dormitor (medetomidine hydrochloride: 0.1 mg/kg) (ZENOAQ, JAPAN) and Dormicum (midazolam: 0.1 mg/kg) (Astellas, Tokyo, JAPAN), each cecum was exteriorized from the peritoneum and 100 μl of 2×10^6 trophozoites was injected into the apical sites of cecum. Cecum was blotted and the peritoneum and the skin were sutured. Mice were kept on warm blankets at 37 °C throughout surgery time. Surgery survival rates were 100%.

2.3. Pathology of amebic colitis

Mice were sacrificed, and the cecum was fixed in 10% buffered formalin phosphate. After paraffin embedding, 4 μm slides were stained with hematoxylin and eosin (H & E) stain. The degree of inflammation was scored by 3–5 persons independently according to Houpt et al. [19] from 0 to 5. The scores 0: normal; 1: mucosal hyperplasia, mild to moderate increase of lymphocytes in mucosa and submucosa with no neutrophil infiltration; 2: increased chronic inflammation, with spotty infiltration of neutrophils, involving the entire thickness of the mucosa; 3: increased chronic inflammation with marked increase in neutrophil infiltration, involving full thickness of mucosa, 4: marked neutrophil infiltration of mucosa and submucosa, with tissue architecture intact; 5: complete destruction of cecal architecture by inflammation. The average score was used for analysis.

2.4. Quantification of E. histolytica

After sacrifice, 200 μl of cecal contents were collected and stored in − 20 °C. DNA was extracted using QIAamp DNA Stool Kits (Qiagen, GmbH, Hilden, FRG) according to the manufacturer's instructions. RT-qPCR was performed with 3 μl DNA in a 20 μl total reaction volume using SYBR® Green Supermix (Life Technologies, TA, CA, USA) in the Quant Studio™ 7 Flex Real-Time PCR System (Applied Biosystems®, Life Technologies™, CA, USA). The primer sequences used were as follows: for EntaF, 5′-ATG CAC GAG AGC GAA AGC AT-3′; for EhR, 5′-GAT CTA GAA ACA ATG CTT CTC T-3′ [20]. For a standard curve, tubes containing 2×10^5 trophozoites were prepared and storaged at − 30 °C until use. In extracting DNA from cecal content using QIAamp DNA Stool Kits, we also prepared trophozoites with 10 fold dilution (10^5–10^0), extracted DNA from the preparation, and used them to make a standard curve.

2.5. RNA extraction and quantitative RT-PCR

The mRNA expression of Th1, Th2, and Th17 cytokines in naïve and infected ceca was analyzed by quantitative RT-PCR. Briefly, total RNA was extracted from < 30 mg cecum tissue (> 30 mg) using the RNeasy Mini Kit (Qiagen, GmbH, Hilden, FRG) following the manufacturer's instructions. For reverse transcription, 1 μg of DNase-treated total RNA was transcribed using a PrimeScript™ RT reagent kit (Takara, Bio INC, JAPAN) in a 10 μl total reaction volume. Transcribed cDNA was ten times (10 ×) diluted. RT-qPCR was performed with 2 μl of reverse-transcribed cDNA in a 12 μl total reaction volume using an iQ SYBR® Green Supermix (BIO-RAD, CA, USA) in the iCycler iQ system (BIO-RAD, CA, USA). Samples without prior RT reaction were set up as negative controls. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control. The thermal cycling conditions comprised 10 min at 95 °C, followed by 30 cycles at 95 °C for 15 s, annealing temperature (varied per cytokine) for 60 s, and 72 °C for 40 s. Primer sequences (published in different paper previously [9,21]) were written in the Supplementary Table 1.

2.6. Immune cells isolation and flow cytometry

Lamina propria (LP) cells were isolated from the cecum and large

intestine. Cecum and large intestine tissues were washed by EDTA (2 mM) for removal of the epithelial layer. Tissues were digested using collagenase (400 U/mL) and DNase (20 µg/mL) into 20% FBS-containing RPMI medium and incubated for 2 h at 37 °C. LP cells were isolated by Percoll gradient separation and prepared single cell suspensions. 5×10^5 cells were cultured with soluble ameba antigen (SAE) (50 µg/ml) and PMA (10 ng/ml)/Ionomycin (1 µg/ml) for 2–3 h and were then treated with brefeldin-A (10 µg/ml) followed by overnight incubation. After surface staining using anti-MHCII, anti-F4/80, anti-Ly6g, anti-CD11b, anti-CD11c, anti-CD44, anti-CD4, anti-CD8 (eBioscience, Affymetrix, CA, USA) mAbs, cells were fixed using fixation and permeabilization solution (eBioscience, Affymetrix, CA, USA) according to manufacturer protocol. For intracellular staining, anti-IFN-γ mAbs (eBioscience, Affymetrix, CA, USA) were used. After staining, cells were suspended in FACS buffer and analyzed by FACS flow (BD FACSVerse™, BD Bioscience, NJ, USA).

2.7. Preparation of soluble ameba antigen

Soluble ameba antigens (SAE) were prepared as previously described by Haque et al. [22], with some minor modifications. In short, axenic *E. histolytica* JPN51 trophozoites (3×10^7) were harvested by centrifugation at 1500 rpm for 5 min at 4 °C. The pellet was washed by resuspension in PBS and centrifugation three times. The final cell pellet was frozen at −80 °C. After freeze-thawing, lysate was centrifuged at $10{,}000 \times g$ for 10 min at 4 °C and the supernatant was incubated with a half volume of polymyxin B agarose at room temperature for 1 h. The resultant absorbed supernatant was defined as SAE. Endotoxin levels were checked by Limulus Amebocyte Lysate test (LAL test).

2.8. Myeloperoxidase assay

The cecal neutrophil content was quantified by myeloperoxidase (MPO) assay as previously described by Asgharpour et al. with slight modifications [23]. In brief, 50–100 mg ceca were collected, rinsed to remove the luminal contents, frozen at −80 °C until experiment performed. Tissues were homogenized for 30 s with gentleMACS™ Octo Dissociator (Biotec, JAP), using hexadecyltrimethyl ammonium bromide (HATB) buffer (50 mM potassium phosphate buffer (pH 6.0) with 0.005% HATB). Supernatant was collected and 7 µl ofsupernatant was added to 200 µl of 5 mM potassium phosphate buffer containing 33 µg *o*-dianisidine and 0.005% H_2O_2. The results were read sequentially upto 40 min with a microplate reader at 450 nm. The change in the optical density was normalized by per milligram of tissue protein.

2.9. Bone marrow-derived dendritic cell (BMDC) culture and cytokine measurement

Bone marrow cells were prepared from naïve and infected mice. Cells were cultured in RPMI with 10% FBS, supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/ml, PEPROTECH, NJ, USA) on days 0 and 3 and harvested on day 5. For *in vitro* experiments, 4×10^5 cells were plated per well of a flat-bottom 96-well dish and stimulated with soluble ameba antigen (100 µg/ml), lipopolysaccharide (LPS) (100 ng/ml) as a positive control for 48 h. IL-12p70 in the supernatant was determined by ELISA (R & D systems).

2.10. Statistical analysis

All statistical analyses were performed using GraphPad Prism for Windows, version 7.0 (GraphPad Software, CA, USA). Either χ^2 test or Student's *t*-test were used for comparisons between two groups. *P* values of < 0.05 were considered significant. Results of representative experiments were shown; all experiments were replicated 2 to 3 times, with 3 to 5 individuals per group.
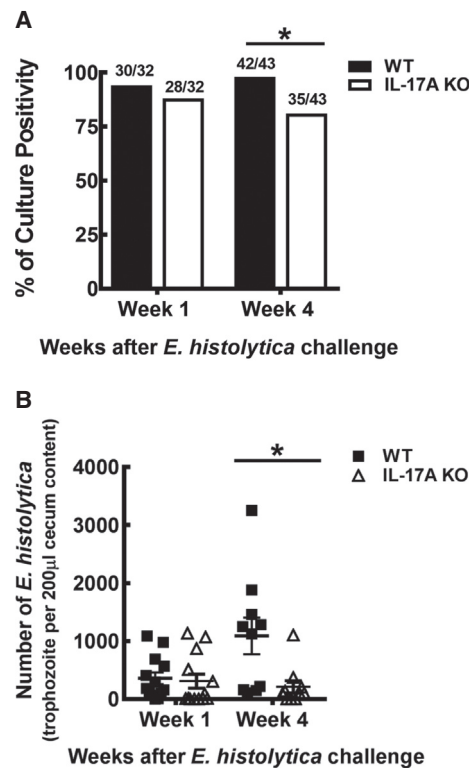

Fig. 1. Infection rates and burdens of the parasite *Entamoeba histolytica* were measured in wild-type (WT) and IL-17A knockout (KO) CBA/J mice in weeks 1 and 4 after their intracecal challenge with 2×10^6 trophozoites. The infection rate of *E. histolytica* was assessed by culturing cecal contents *ex vivo* (A). The indicated number of mice were used for the assessment of the infection rate of *Entamoeba histolytica* in ceca: week 1, $n = 32$ and week 4, $n = 43$ (A). At week 1, 30 out of 32 WT were infected, while 28 out of 32 KO were infected, respectively. At week 4, 42 out of 43 WT were infected, while 35 out of 43 KO were infected. *$P < 0.0137$ by χ^2 test. The number of *E. histolytica* was measured by quantitative real-time PCR (B). For parasite quantification the indicated number of mice was used in each group: week 1, $n = 13$ and week 4, $n = 10$. *$P < 0.05$.

3. Results

3.1. Entamoeba histolytica numbers reduced in IL-17A KO mice in week 4, but not in week 1 post-infection

To clarify the role of IL-17A during intestinal amebiasis, WT and IL-17A KO CBA/J mice were challenged intracecally with 2×10^6 trophozoites and sacrificed at week 1 or 4 post-infection. The infection rate was assessed with *in vitro* culture, and qPCR was used to quantify the parasite numbers. Both the WT and KO mice showed a high infection rate and no significant difference was observed in either the infection rate or the parasite numbers in week 1. However, in week 4, both the infection rate and the number of parasites were significantly reduced in the KO mice, but not in the WT mice (Fig. 1A, B). The ceca of the WT and KO mice were inflamed in week 1, and no significant difference was apparent in the inflammation scores of the WT and KO mice (Fig. 2C–D). However, in week 4, the WT ceca were highly inflamed, whereas the inflammation score was significantly lower in the IL-17A KO mice (Fig. 2E, F, and G).

3.2. IFN-γ expression was significantly higher in KO mice than in WT, but induction of IL-4 expression was much lower in KO mice in week 4 after infection with E. histolytica

To investigate why the IL-17A KO mice displayed fewer *E. histolytica* than the WT in week 4, we evaluated the expression levels of cytokine mRNAs. In the WT mice, a significant induction of the Th2 cytokines IL-4 and IL-13 was observed, as reported elsewhere [9], together with the
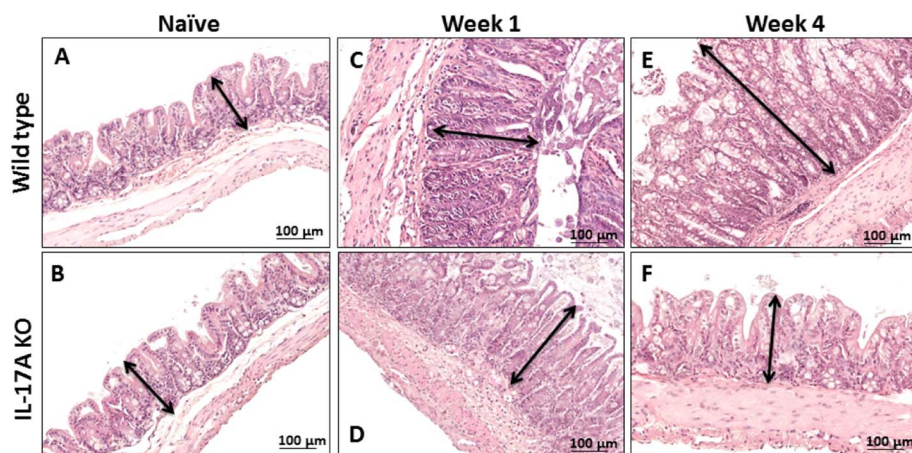
Fig. 2. Histopathology of ceca from WT and IL-17A KO CBA/J mice after intracecal challenge with 2×10^6 *E. histolytica*. Ceca of naïve mice are shown (A–B). Ceca of both WT and KO mice in week 1 (C–D) and week 4 post-infection (E–F) are shown (hematoxylin–eosin staining, $\times 20$). Black arrows indicate the thickness of the ceca. Inflammation scores were plotted on the graph (G). Naïve mice are used for week 0 ($n = 3$), week 1 ($n = 9$), and week 4 ($n = 7$). *$P < 0.05$.
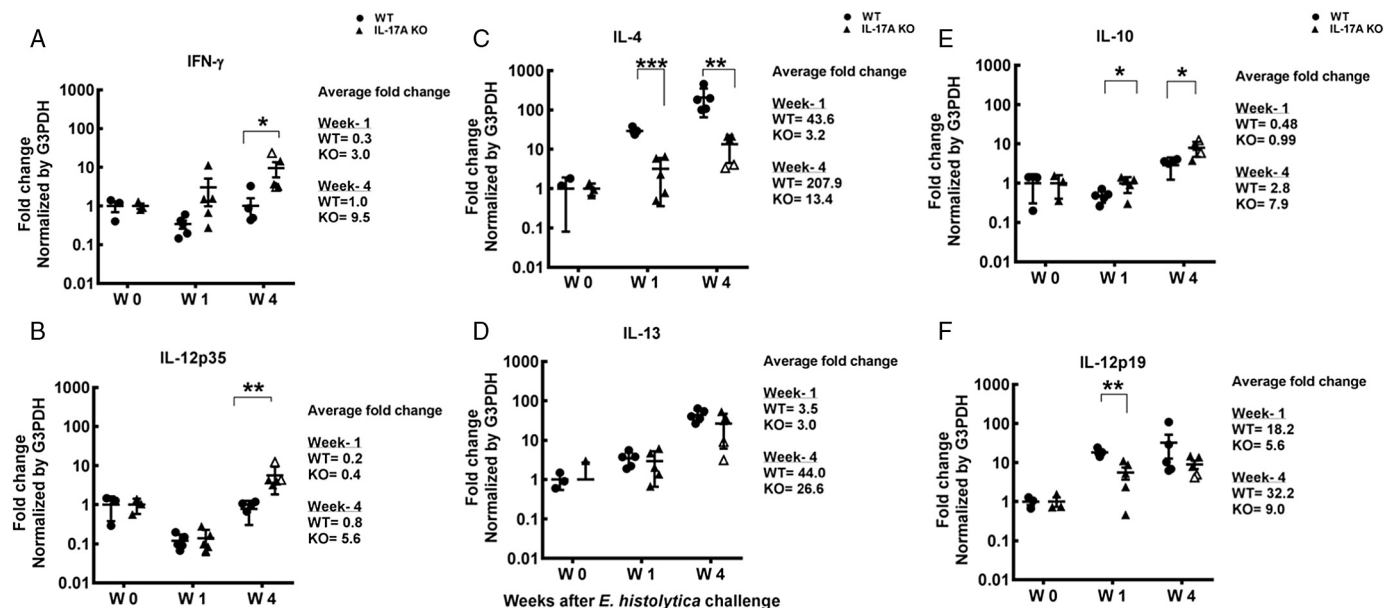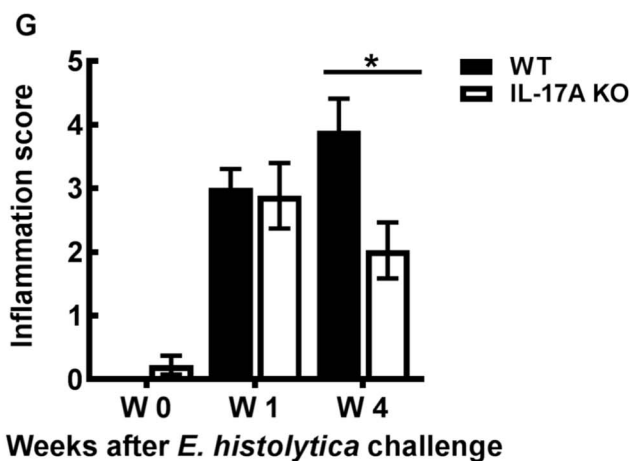

Fig. 3. Th1 and Th2 cytokine profiles were evaluated in the ceca, at the sites of infection, of naïve and infected WT and IL-17A KO mice at weeks 1 and 4 post-infection. Total RNA was extracted from the ceca. The expression level of each cytokine mRNA was determined by quantitative reverse-transcription PCR. IFN-γ, IL-12p35, IL-4, IL-13, IL-10, and IL-12p19 were shown (A–F). Closed triangles indicate the data of the infected KO and open triangles indicate the data of uninfected KO mice. Closed circles indicate WT mice. Data represent one of two independent experiments (naïve, $n = 3$ and infected $n = 5$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

induction of IL-12p19 and IL-10, but neither IFN-γ nor IL-12p35 was significantly induced. In the KO mice, the induction of IFN-γ expression was 3.0-fold and 9.5-fold higher than in naïve mice in weeks 1 and 4,

respectively, whereas, in the WT mice, it was 0.3-fold and 1.0-fold higher than in the naïve mice in weeks 1 and 4, respectively. In week 4, IFN-γ expression was significantly upregulated in the KO mice

compared with that in the WT (Fig. 3A), and IL-12p35 expression showed a similar trend. In week 4, IL-12p35 expression was 5.6-fold upregulated in the KO mice compared with that in the naïve mice and was higher than in the WT (Fig. 3B). IL-4 expression was 43.7-fold and 207.9-fold higher in the WT mice than in the naïve mice, but was weak in the KO mice, at only 3.2-fold and 13.4-fold higher than the naïve mouse levels in weeks 1 and 4, respectively (Fig. 3C). In both weeks 1 and 4, IL-4 was significantly downregulated in the KO mice compared with that in the WT (Fig. 3C), and IL-13 expression showed a similar trend. In week 4, IL-13 expression was 44.0-fold upregulated in the WT mice compared with the naïve mice, and was also 26.6-fold higher in the KO mice than in the naïve mice in week 4. No significant difference was observed between the WT and KO mice (Fig. 3D). The expression of IL-10 was moderately upregulated in week 4 compared with that in the naïve mice in both the WT (2.8-fold) and KO mice (7.9-fold) (Fig. 3E). In both weeks 1 and 4, IL-10 was more significantly upregulated in the KO mice than in the WT. In week 1, the induction of IL-12p19 was significantly lower in the IL-17A KO mice (5.6-fold) than in the WT (18.1-fold) (Fig. 3F). It was also lower in the KO mice (9.0-fold) than in the WT (32.3-fold) in week 4, but the difference was not statistically significant.

In week 4, two IL-17A KO mice were uninfected or recovered from the infection. Comparison of the infected and uninfected mice was not possible because the sample size was too small (closed and open triangles, respectively, in Fig. 3A–F).

In summary, Th1 cytokines, especially IFN-γ and IL-12p35, were significantly upregulated in the IL-17A KO mice in week 4 compared with their expression in WT. After *E. histolytica* challenge, the induction of IL-4 expression was significantly lower in the KO mice than in the WT in both weeks 1 and 4, but the expression of another Th2 cytokine, IL-13, did not differ between the two groups. IL-10 was significantly upregulated in the KO mice in both weeks 1 and 4. Significantly lower IL-12p19 expression was only observed in the KO mice in week 1, whereas in week 4, its expression in the two groups did not differ significantly. These results indicate that in the absence of IL-17A, the IFN-γ/IL-4 ratio increased.

3.3. Alteration in the infiltrating cells in the IL-17A KO mice

Because IL-17A is a chemoattractant for neutrophils, the numbers of neutrophils, monocytes, and dendritic cells were determined in the lamina propria of each mouse. Both neutrophils and dendritic cells were significantly reduced in the KO mice in week 4, consistent with our expectation, whereas their levels did not differ between the WT and KO mice in week 1 (Supplementary Fig. 1). Myeloperoxidase (MPO) activity significantly reduced at week 4 in KO mice compared to week 1, whereas WT mice showed no reduction with significant difference. At week 4, no difference was observed in MPO activity between WT and KO mice (Supplementary Fig. 2). We then examined γδ T cells, which did not differ between the two groups of mice during the course of infection (data not shown). The CD8$^+$ populations were also measured, but did not differ in number between the two groups of mice at either time point (Supplementary Fig. 1). The number of CD4$^+$ T cells continued to increase in the WT mice, but declined in the KO mice in week 4, becoming lower than in the WT (Fig. 4A, Supplementary Fig. 3). Because IFN-γ expression was higher in the KO mice than in the WT in week 4, we examined whether the IL-17A KO CD4$^+$ T cells were sufficiently activated to produce IFN-γ. The proportion of IFN-γ$^+$CD4$^+$ cells was significantly higher in the KO mice than in the WT in week 4 (Fig. 4B, Supplementary Fig. 4). The proportion of IFN-γ$^+$CD4$^+$ cells were also monitored in naïve mice at week 1 (9 weeks old mice) and week 4 (12 weeks old mice) (Supplementary Fig. 5). In the naïve stage, no difference was observed to produce IFN-γ between two groups of mice.

As IL-12p35 (IL-12) mRNA expression was higher in KO mice, we investigated IL-12 (IL-12p70) productions by ELISA also. Bone marrow

dendritic cells were harvested from naïve mice and stimulated with soluble ameba antigens and lipopolysaccharides. IL-12 production tendency was higher in KO mice compare to WT mice (Supplementary Fig. 6).

4. Discussion

This study demonstrates that a lack of IL-17A reduced the number of *E. histolytica* in the late stage of infection, but did not affect the initial settlement of the parasite in the cecum. IL-17A deficient mice also showed an increased Th1 response, including higher IFN-γ and IL-12p35, and lower IL-4 expression than WT in the cecum in the late stage of infection.

IL-17A expression is reported to be robustly induced by *E. histolytica* infection [13], but its role in the natural course of the infection remained to be clarified. In this study, we demonstrated that the number of *E. histolytica* that can establish an infection in the gut in naïve CBA/J mice was not affected by endogenous IL-17A. A previous study reported that the vaccination of CBA mice with the recombinant LecA fragment of Gal/GalNAc lectin and alum protected the mice against subsequent *E. histolytica* infection, and that the sensitization of the mice before infection, which caused them to produce IL-17A, was a crucial step in conferring this protection [14]. Another study reported that the alteration of the gut microbiota with segmented filamentous bacteria also conferred resistance to subsequent *E. histolytica* infection on CBA mice through the induction of IL-23 production by subsets of dendritic cells, which promoted downstream IL-17A production [8] and the accumulation of neutrophils at the site of infection. Therefore, the augmentation of IL-17A before infection confers resistance to intestinal *E. histolytica* infection. Considering the protective roles of neutrophils in the early stage of *E. histolytica* infection [23] and the impact of IL-17A on the accumulation of neutrophils, we expected that a lack of IL-17A would increase the number of *E. histolytica* in the gut by reducing the accumulation of neutrophils. However, no significant difference was observed in the numbers of *E. histolytica* in the WT and KO mice, which is consistent with the observation that neutrophils and monocytes accumulated to the same levels in the WT and KO mice in the early stage of infection (Supplementary Fig. 1). MPO (myeloperoxidase) assay also showed the similar phenomena, as the early stages neutrophil activity was not hampered by the lack of IL-17A (Supplementary Fig. 2). In the late stages (week 4), MPO activity was reduced in the KO mice comparing with week 1. This phenomenon indicated that neutrophil activity was reduced in the KO mice in the late stages. This is not clear whether this phenomenon is the cause or effect of IL-17A deficiency but it might be related with reduced infection rate in the KO mice in the late stages. This finding also suggests that the initial recruitment of neutrophils to the site of infection was not affected by the lack of IL-17A in the natural course of infection, which is supported by the findings of Terrazas et al. [7].

In our model, the mRNA expression of the Th1 cytokines, especially IFN-γ and IL-12p35, was upregulated in the IL-17A KO mice during infection. In an animal model of *Leishmania donovani* infection, IFN-γ production by T cells reportedly enhanced in the absence of IL-17A [7]. Moreover, during *Toxoplasma gondii* infection, the absence of IL-17RA, a receptor for both IL-17A and IL-17F, which binds to IL-17A with higher affinity, enhanced the IFN-γ response, causing exaggerated inflammation in the infected organs and increased mortality [24]. An IL-23-dependent Th17 response also impaired fungal clearance by downregulating IFN-γ production [25]. These data indicate a role for IL-17A in the negative regulation of IFN-γ production. Studies of an animal model and ameba-associated diarrhea in humans also suggested that a Th1 response, with high levels of IFN-γ production, allows the host to reduce the parasite burden [9,22]. The reduction in the number of *E. histolytica* and the inflammation score observed in the KO mice in the late stage of infection may be at least partly attributable to the modulation of the cytokine microenvironment, represented by the increase
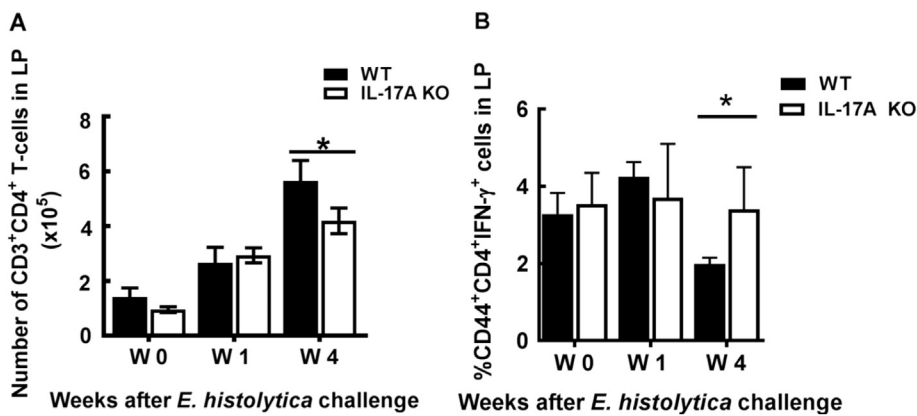
Fig. 4. T cells were quantified at the sites of infection in the WT and IL-17A-KO mice (A). Cells were isolated from the lamina propria of each cecum and large intestine. CD4⁺ T cells were isolated at week 1 and week 4 post infection, and also from naïve mice (Supplementary Fig. 1). IFN-γ production was determined in the WT and IL-17A KO mice during active intestinal amebiasis (B). Lamina propria cell populations were stimulated with soluble ameba antigen (SAE; 50 μg/ml) for 18 h, and IFN-γ production was measured (Supplementary Fig. 2). Naïve, $n = 3$; infected, $n = 3–5$. *$P < 0.05$.

in the IFN-γ.

The antagonistic relationship between IL-4 and IFN-γ is well known [26]. In fact, the depletion of CD4⁺ T cells, which predominantly produce Th2 cytokines such as IL-4 during intestinal amebiasis, reduces the number of *E. histolytica* and the accompanying inflammation in mice [19]. In our study, IL-4 expression was lower in the IL-17A KO mice than in the WT mice, which may also be associated with the lower parasite burden and disease severity in the IL-17A KO mice in week 4. However, the factors that regulate the IFN-γ/IL-4 ratio are unclear, and the direct or indirect relationship between IL-4 and IL-17A remains to be clarified.

In the absence of IL-17A, the number of CD4⁺ T cells was reduced at 4 weeks after infection. This seems to have accompanied the reduction of the number of *E. histolytica*. If so, the decline in the number of CD4⁺ T cells can be explained simply by the clearance of *E. histolytica* by the IL-17A KO mice in week 4. However, it is unclear whether this was the cause or the effect of the clearance of *E. histolytica*. Because CD4⁺ T cells are one of the main sources of Th2 cytokines, it is possible that the reduction in the number of CD4⁺ T cells partly modulated the cytokine microenvironment, represented by the balance between IFN-γ and IL-4, which may have reduced the number of *E. histolytica*.

Although the CD4⁺ T cell population was smaller in the IL-17A KO mice than in the WT in week 4, this population was well equipped to produce IFN-γ. We acknowledge that there may have been other sources of IFN-γ, such as NK cells, NKT cells, γδ T cells, innate lymphoid cells, and CD8⁺ T cells, in this model and this possibility requires further investigation. Furthermore, dendritic cells were also well equipped to produce high amount of IL-12 in the KO mice which indicate that this cytokine might plays a pivotal role to induce IFN-γ and helps to reduce parasite burden (Supplementary Fig. 6).

In the natural course of *E. histolytica* infection, endogenously induced IL-17A did not affect the initial steps of infection, but did affect the parasite burden and disease severity in the chronic stage, when the adaptive responses were fully activated. In conclusion, IL-17A plays an important role in reducing the IFN-γ/IL-4 ratio and in the persistence of *E. histolytica* during intestinal amebiasis.

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