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Immunology

Cytokine elevation in the mouse small intestine at the early stage of infection with the gastrointestinal parasite *Heligmosomoides polygyrus*

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ABSTRACT. To eliminate pathogens, the initiation of an appropriate immune response is critical. When the gastrointestinal nematode, *Heligmosomoides polygyrus* (Hp), invades the small intestine, a type-2 cytokine response is initiated; however, this response is not sufficient to clear the infection, and chronic infection can ensue. In this study, the host defense against Hp was investigated in mice with a focus on the role of CD4+ T cells. To this end, tissues from the small intestine and mesenteric lymph node (MLN) were collected every day from just after infection until Day 5 because many previous studies have described the later stages of infection from Day 8 to Day 12, during which Hp returns to the lumen and Th2 cytokine expression reaches its peak. In this study, we focused on investigating the initiation of the type-2 immune response. Our results indicated that the larvae encysted by Day 3. Increased type-2 cytokine gene expression started in the small intestine before Day 2 and increased again on Day 5. Interferon (IFN) γ increased significantly on the second day. Flow cytometry and gene expression analysis of MLN cells revealed that CD4+ T cells were not activated until Day 4. These results suggested that innate immune cells in submucosa are activated immediately after infection, but CD4+ T cells accumulate in the cyst zone later. In addition, IFNγ may have an important role in converting type-2 cytokine-producing cells from innate cells to CD4+ T cells.

KEY WORDS: CD4+ T cell, mouse, nematode, small intestine, type-2 cytokine

In the immune response against infection, the rapid initiation of an appropriate immune response and the induction of specific immune cells and molecules are important processes necessary for the elimination of pathogens. The gastrointestinal nematode, *Heligmosomoides polygyrus* (Hp), is considered a useful model for studying functional Th2 responses in the host. In mice, primary inoculation with Hp is associated with chronic infection because the initial elevation in type-2 cytokine production is not enough to eliminate the worms. However, after the parasite is cleared from the mouse with an antihelminthic drug such as pyrantel pamoate, subsequent reinfection with Hp results in a quick and strong memory response, with worm expulsion observed by about 12 days after reinfection [1, 2, 13]. In a previous study, our results indicated that the quick migration of CD4+ T cells to the host–parasite interface under the mucosa results in a strong Th2 response at the early stage after a challenge [14]. Thus, we considered that the initial strong elevation in type-2 cytokine production from CD4+ T cells was important for eliminating the worms. However, the important role of group 2 innate lymphoid cells (ILC2s) against gastrointestinal parasite infection was recently reported, and the cellular network and orchestration of cytokines in the early stages after parasite infection have been attracting increased attention. ILC2s are activated by interleukin (IL)-33 and IL-25 in the epithelium just after the worms arrive to the intestine and serve to produce enormous amounts of type-2 cytokines [8, 9]. The role of ILC2s in this process has been demonstrated in mouse models primarily using *Nippostrongylus brasiliensis* (Nb) infection [6, 16]. Briefly, Nb infection induced IL-33 production by epithelial cells and myeloid lineage cells in the lung and small intestine, and IL-33 activates ILC2s to produce IL-13 and initiate type-2 immune responses. However, Nb infection is quite different from Hp infection, and it can be cleared acutely within 2 weeks after primary inoculation [2]. Indeed, the host immune response against various nematodes varies depending on the life cycle and infection style of the respective parasite. For example, in contrast to Hp, Nb does not penetrate the epithelium and invade the submucosa. In addition, it has been clarified that ILC2s contribute to the initiation and development of adaptive type-2 immune responses using an Nb infection mouse model [7, 16]. At the same time, the crosstalk between ILC2s and CD4+ T cells through the

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major histocompatibility complex (MHC) II-dependent activation is also important for maintaining the survival of ILC2s [17]. Thus, the role of CD4+ T cells is considered important for the transition from an innate immune response to an adaptive response in order to expel Nb. On the other hand, previous studies have been limited regarding the early stage of host defense against Hp infection. Pelly *et al*. reported an elevation in type-2 cytokines from Day 5 after Hp infection [18]. Our previous results also indicated that the level of type-2 cytokine gene expression through Day 4 after infection remained unchanged, with an increase observed after Day 4 [13, 14]. From this previous study, we confirmed that CD4+ T cells had become activated and migrated to the cyst area by Day 8 after infection, but these processes had not yet occurred at Day 4. The importance of the cooperation between cells in the transition from the natural immune system to the adaptive immune system is clear for Hp infection; however, the detailed mechanisms remain unknown. Our research has previously shown that CD4+ cells have a critical role in the elimination of Hp because administration of the anti-CD4 antibody during primary infection increases eggs per gram of faeces (EPG), due primarily to an increase in worm fecundity, thus preventing worm clearance in the secondary infection [21]. In addition, unlike Nb, Hp directly arrives at the intestine within 24 hr after infection. Hence, the timing of the onset of CD4+ T cell activation may differ between Nb and Hp infection. Moreover, the reason for type-1 responses being characterized by the production of interferon (IFN) γ during the early stages of Hp infection remains unknown. Commonly, Hp induces strong type-2 responses but not type-1 to expel the worms. Hp-induced elevation of type-2 cytokines peak after the 8th day of infection. However, Molofsky *et al*. [12] demonstrated that IFNγ counter regulates ILC2 for host protection. If this is the case, then IFNγ gene expression might change during the early stage after Hp infection. Therefore, in this study, we sought to elucidate the timing of the different processes involved in Hp infection, including when the larvae encyst in the muscle layer of the small intestine, the timing of the elevation in type-2 cytokines (IL-4, IL-13 and IL-10) and IFNγ expression, the arrival of CD4+ T cells to the cyst zone, and the activation of cells in the mesenteric lymph node (MLN).

MATERIALS AND METHODS

Mice

Female Balb/c mice (8 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used for all experiments. Animals were housed under conventional conditions with a 12/12-hr light/dark cycle and provided food and water *ad libitum*. The environment for the animals was maintained at 22°C ± 1.5°C, with a relative humidity of 55% ± 5%. All mice were healthy and randomly divided into seven groups: control, Day 0 (6 hr after inoculation), 1, 2, 3, 4, and 5 after infection (n=5 each). All experiments were performed twice. The experimental protocol was approved by the Institutional Ethics Commission for Animal Research of Miyagi University (approval number: 2014-10, 2015-25).

Parasites

Hp was kindly provided by Dr. Kenji Ishiwata and maintained in ICR mice. Mice were orally inoculated with 200 infective Hp larvae (third stage larvae, L3). Control mice received oral saline during the same period. On Days 0 (6 hr after infection), 1, 2, 3, 4, and 5 after inoculation, mice were anesthetized by intraperitoneal injection of a cocktail of three anesthetic agents (medetomidine at 0.3 mg/kg, midazolam at 4 mg/kg, and butorphanol at 5 mg/kg), euthanized by cervical dislocation, and tissue samples from the small intestine (duodenum) and MLN were collected.

Gene expression analysis

RNA extraction, reverse transcription, and real-time PCR were performed. Total RNA extraction from whole tissue was performed using the TRIzol Reagent (Life Technologies, Inc., Frederic, MD, USA) following the manufacturer's instructions. RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and cDNA was synthesized from 5 µg of total RNA with random primers and SuperScript II (Life Technologies, Inc.). The primer sequences for IL-4, IL-13 [15], IFNγ [25], IL-10 [4], and IL-25 [25] were described previously. Primer sequences of IL-33 were designed using pick primers from the National Center for Biotechnology Information (NCBI) database: forward 5′-GGGCTCACTGCAGGAAAGTA-3′, reverse 5′-TTTGCCGGGGAAATCTTGGA-3′.

Real-time PCR was performed using the Brilliant SYBR Green QPCR Master Mix III (Stratagene, La Jolla, CA, USA) in conjunction with an MX3000P system (Stratagene). The amplification conditions were as follows: 95°C for 3 min, 40 cycles of 95°C for 5 sec, and 60°C for 20 sec . Fluorescence signals measured during the amplification were processed afterward. Ribosomal RNA primers were used as internal controls, and all data were normalized to constitutive rRNA values. Quantitative differences between groups were calculated following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

Preparation of frozen blocks and sectioning

After the tissues were harvested from Days 2 to 4 after infection, small pieces of the small intestine (duodenum) were slit longitudinally, laid flat with the mucosal surface facing down, rolled around a wood stick (Swiss roll), and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc., Torrance, CA, USA) using a cryomold. Tissue samples were frozen in dry ice–acetone, then removed from the cryomold, and stored at −80°C in an airtight container until sectioning. Tissue sections (4 µm) for immunohistochemistry and hematoxylin and eosin (HE) staining were obtained from frozen blocks using plain coated slides and an HM560 cryostat (Carl Zeiss, Oberkochen, Germany). Slides were immediately placed on dry ice and stored at −80°C until further analysis.

Immunofluorescent staining

Tissue sections (4 µm) were fixed in cold acetone for 20 min, followed by incubation in 10% normal rat serum with the addition of 1 µg/ml (in phosphate buffered saline (PBS)) of an affinity-purified rat anti-mouse CD16/CD32 antibody (2.4G2:BD Biosciences, San Jose, CA, USA) for 20 min at room temperature. After washing with PBS, tissue samples were incubated at room temperature for 45 min with 10 µg/ml of an Alexa647-conjugated rat anti-CD4 antibody (RM4-5:BD Biosciences) and 5 µg/ml of an Alexa488-conjugated rat anti-Gr-1 antibody (RB6-8C5: BioLegend, San Diego, CA, USA) in tandem. Antibodies were dissolved in PBS containing 0.1% BSA. Tissue samples were then washed in PBS, coverslipped with Vectorshield (Vector Laboratories, Burlingame, CA, USA), examined, and digitally photographed using an Axio Imager microscope with Axio Vision 4.6 software (Carl Zeiss). Gr-1 is variously expressed during the development and maturation of cells of the bone marrow cell lineage. Our previous study demonstrated that areas of cysts are observed as GR-1+ cells infiltrate them [14]. Staining with anti-Gr-1 antibody can visualize the location of the cyst. The number of CD4+ cells around cyst was recorded.

Cell preparation and flow cytometry

MLNs were isolated at 0 (6 hr), 1, 2, 3, 4, and 5 days after infection. After isolation, MLNs were pressed through a nylon cell strainer to obtain single cells. The cells were then resuspended in RPMI (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Thermo Fisher Scientific, Waltham, MA, NY, USA). After centrifugation and washing with PBS containing 2% FBS, cells were enumerated. To examine the T cell activation after Hp infection, the numbers of CD25+ cells, along with the CD4+/CD25+ ratio, were measured by flow cytometry. In total, 1×10^6 cells were incubated with 0.1 µg of Alexa488-conjugated rat anti-CD4 antibody (RM4-5: Biolegend) and APC-conjugated rat anti-CD25 antibody (PC61:BD Biosciences) for 30 min. Flow cytometric analysis was performed by FACS Calibur using the CellQuest software (BD Biosciences). Lymphocytes were initially gated by forward and side scatter properties. Dead cells, debris, and doublets were excluded using forward scatter (FSC) and side scatter (SSC). CD4+ T cells and CD25+ T cells were then gated and assessed for surface expression of CD4 and CD25. Overall, 10,000 cells were analyzed per sample.

Cell culture and measurement of IL-4 concentrations

MLNs were isolated at 1, 2, 3, and 5 days after infection. After isolation, MLNs were pressed through a nylon cell strainer to produce a single cell separation. The cells were then washed and resuspended in RPMI with L-glutamine (Wako Pure Chemical Industries, Ltd.) supplemented with 10% fetal bovine serum (GIBCO, Thermo Fisher Scientific), 5×10^{-5} M 2-mercaptoethanol, 20 U/ml of penicillin, and 20 µg/ml of streptomycin. The cells were then cultured in 24-well flat-bottom plates at a final concentration of 2×10^5 cells/ml in a volume of 1 ml per well and a final concentration of 4 µg/ml concanavalin A (ConA) for 24 hr at 37°C with 5% CO_2. The supernatants were frozen at −80°C until the cytokine levels were measured.

Mouse IL-4 concentrations in the supernatant after 24 hr culture were measured using a DuoSet ELISA Development System (R&D Systems, Inc., MN, USA) following the manufacturer's instructions.

Statistical analysis

All data are presented as mean ± SE for each treatment group. Differences in mRNA expression among the groups were determined using one-way ANOVA followed by the Tukey test. Differences with $P<0.05$ were considered statistically significant. Appropriate time- and age-matched controls were used with infection groups (n=5). Statistical significance of the difference in the number of CD4+ cells surrounding the cyst was determined using a *t*-test.

RESULTS

Hp infection induced cytokine gene expression in the small intestine

In nematode infection, type-2 cytokine-induced physiological alterations of the small intestine, including increased smooth muscle contractility and epithelium permeability, are needed to expel the worms from the intestine. To determine when the expression of type-2 cytokines begins to increase after infection, the small intestines from the mice were collected daily from Day 0 (6 hr after infection) to Day 5. Figure 1 demonstrates that IL-4 gene expression tended to rise even on Day 0, with a significant difference at Day 1 when compare to the uninfected control. However, IL-4 gene expression declined thereafter, at which point it rose again around Days 4 to 5. IL-13 gene expression was similar to IL-4, but individual differences were observed at Day 0, with half of the mice displaying increased gene expression, while the other half remained at control levels. IL-10 gene expression also started to rise at Day 0, then declined, and rose again around Days 4 to 5. These results demonstrated that cytokine production started in the small intestine at the very early stage just after Hp infection and then was downregulated at Day 3, followed by another strong increase. Interestingly, IFNγ gene expression increased at Day 2 after infection (Fig. 1). A significant increase was also observed at Day 4 for this cytokine, but expression levels remained at uninfected levels after that.

Hp larvae penetrated the muscle layers by Day 3 after infection

Hematoxylin and eosin staining revealed that the worm penetrated into the smooth muscle of the small intestine by Day 3 after infection (Fig. 2A), but no evident worm cyst was found at Day 2. Previous reports have also described Hp larvae embedded in the muscle layers of the small intestine by Day 3 after infection, but the larvae were shown to have penetrated the mucosa and submucosa during the first 24 hr [20]. Together, these findings indicate that the larvae move to the muscle layer and are encysted by

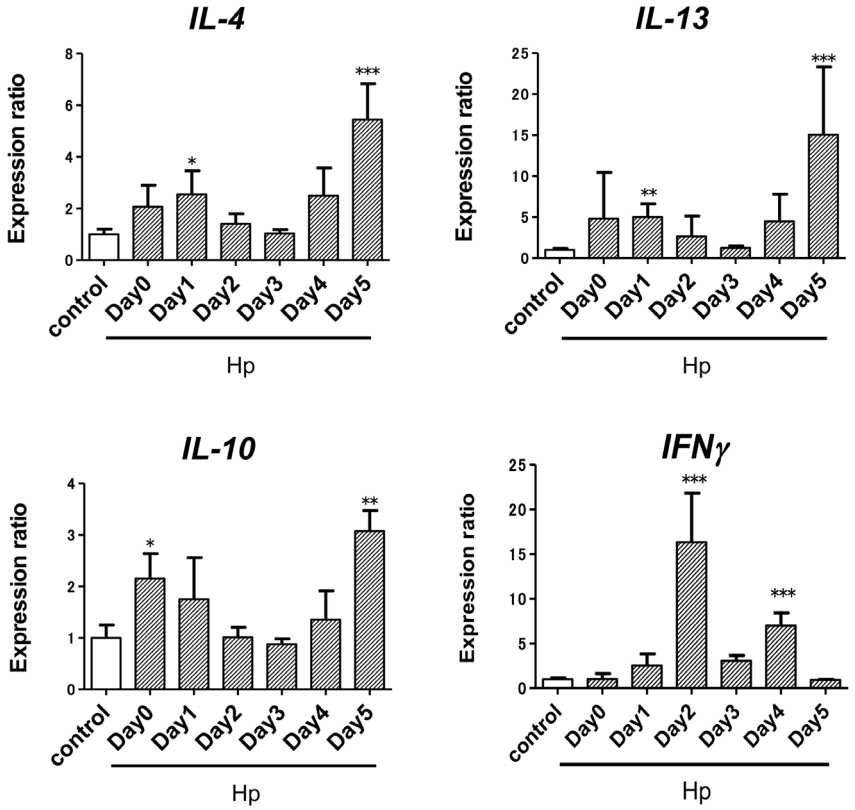

Fig. 1. Real-time PCR analysis for interleukin (IL)-4, IL-13, IL-10, and Interferon (IFN) γ gene expression in whole intestinal tissues from uninfected control mice and 0 (6 hr), 1, 2, 3, 4, and 5 days after *Heligmosomoides polygyrus* (Hp) infection in 3-month-old Balb/c mice (n=5). Results are expressed as relative units compared with those from uninfected mice (control). **P*<0.05, ***P*<0.01, and ****P*<0.001; infected mice compared with control mice. Results are shown as the mean ± SE. Data are representative of two independent experiments.

the third day of infection.

Immunofluorescent staining revealed that the number of CD4+ T cells surrounding the small cyst increased from Day 3 to Day 4 (Fig. 2B–C). However, the accumulation of CD4+ T cells was less than the number of CD4+ T cells at Day 8, which was demonstrated in a previous report [13]. No cysts were found on the second day; therefore, no cell accumulation was observed (data not shown), suggesting that the CD4+ T cells started to accumulate because the larvae were embedded in the muscle layer.

IL-33 and IL-25 gene expression at the early stage of infection

Epithelial cell-derived IL-33 and IL-25 are well-known important initiators of the type-2 immune response. These cytokines activate ILC2, which is a powerful producer of IL-13, before CD4 T cells migrate to the host–parasite interface. However, our results indicated that IL-33 gene expression tended to increase just after infection in the small intestine but then remained at uninfected levels until Day 4 (Fig. 3). IL-25 also tended to increase from Day 0 to Day1 and down until Day 4.

Activation of T cell and cytokine production in the MLN

The MLN is the most important site for the activation of T cells. To investigate when the immune response in the MLN starts after Hp infection, we analyzed the expression of relevant genes in the MLN. Our results indicated that IL-4 began to increase from Day 3 after infection and was strongly elevated after Day 4. The gene expression of IL-13 was similar to IL-4 and consistent with our previous report [14]. Although IL-10 gene expression did not show much elevation, it still significantly increased from Day 2 after infection. In addition, IFNγ gene expression also increased since Day 3, but the increase was not remarkable (Fig. 4A). These data suggested that the onset of Th2 gene expression in the MLN occurred somewhat later than that observed in the small intestine, and IFNγ did not show big change compared with the small intestine.

To investigate when the T cells in the MLN obtained the ability to produce Th2 cytokines after nematode infection, cells were collected from the MLN at Day1, 2, 3, and 5 after Hp inoculation and cultured for 24 hr with ConA. IL-4 was not detected until Day 5 (Fig. 4B).

Next, CD4+ T cells in the MLN were also analyzed by FACS. Our results indicated that the number of T cells expressing the activation marker CD25 started to increase at Day 5 after infection (Fig. 5), suggesting that the activation of T cells started late in the middle stage of infection. CD25 is also expressed on Tregs. However, previous research confirmed that the Foxp3^{-} T cells also

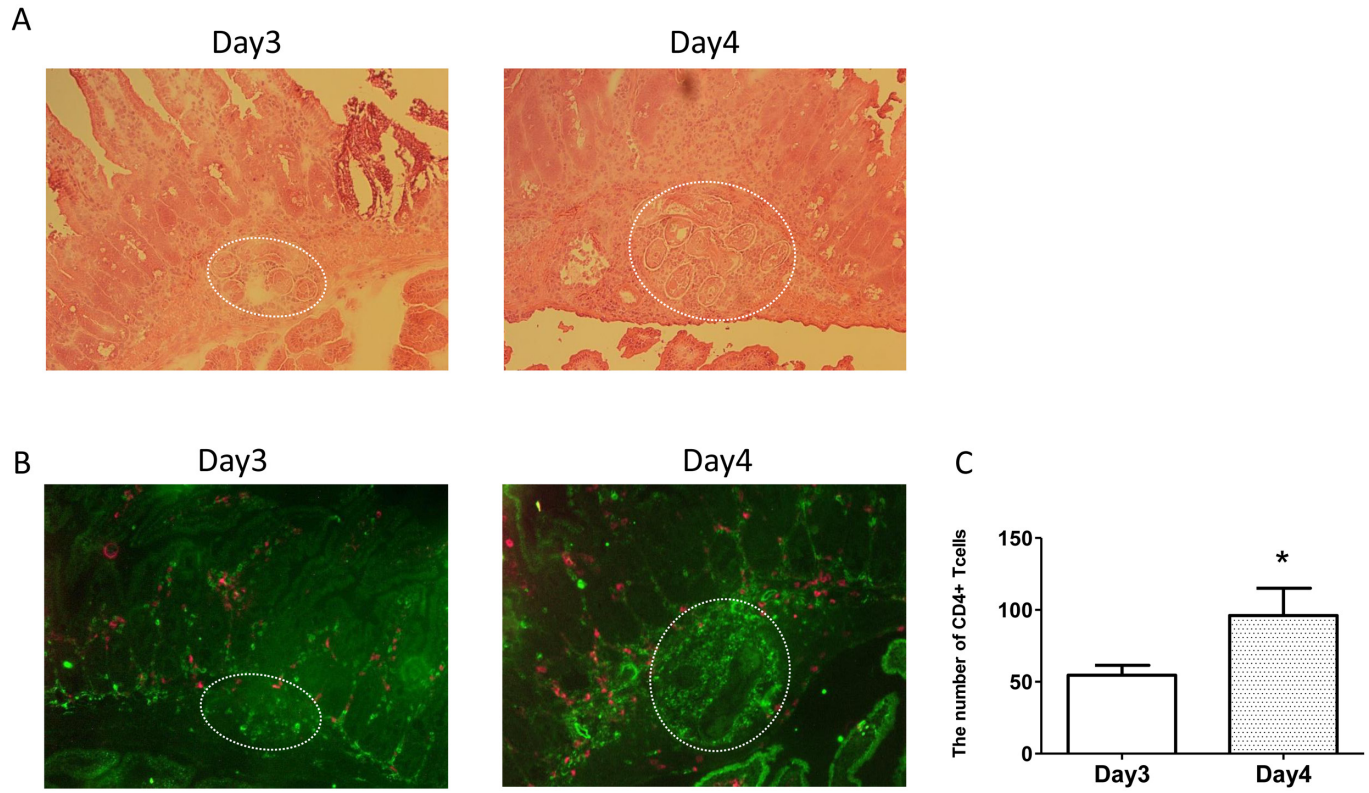

Fig. 2. A: Hematoxylin and eosin staining of the small intestine in infected mice (Days 3 and 4 after infection). B: Immunofluorescent staining in the cyst zone shown as dashed circles with CD4 (red) and Gr-1 (green). Day 3: A limited number of CD4+ cells surround the small cyst. Day 4: A larger number of CD4+ cells are accumulated around the cyst. C: Numbers of CD4+ cells around cysts (Day 3: n=5, Day 4: n=3). Results are shown as the mean ± SE. **P*<0.05

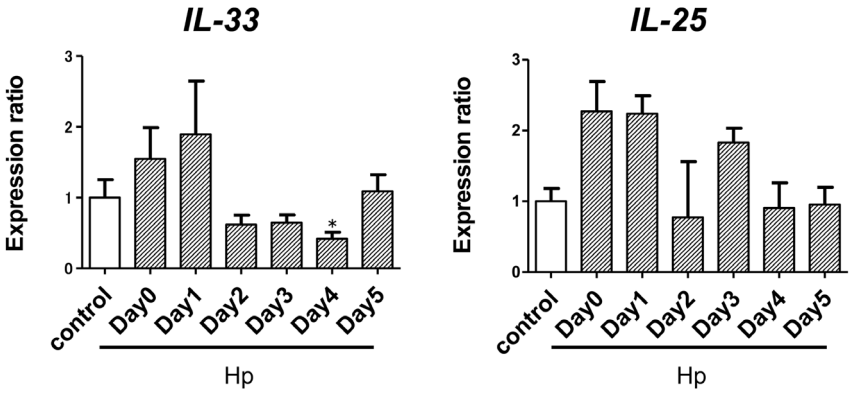

Fig. 3. Real-time PCR analysis for interleukin (IL)-33 and IL-25 gene expression in whole intestinal tissues from uninfected control mice and 0 (6 hr), 1, 2, 3, 4, and 5 days after primary *Heligmosomoides polygyrus* (Hp) infection in Balb/c mice (n=5). Results are expressed as relative units compared to those of uninfected mice (control). **P*<0.05. Results are shown as the mean ± SE. Data are representative of two independent experiments.

increased in MLN at Day 5 after Hp infection [19].

Collectively, our findings suggest that, while the general immune response against Hp begins soon after infection in the small intestine, T cells in the MLN are not activated until Day 4 or later.

DISCUSSION

The role of the innate immune system has been well reported, and the early stage of the immune response against helminth appears to occur in the local tissue [10]. Type-2 immune responses against nematodes have been investigated primarily with Nb and Hp. However, these two nematodes have different styles of infection. In Nb infections, the nematode has been shown to penetrate through the skin and move to the lung, where it is swallowed in order to arrive at the small intestine. In this infection, the host develops a strong type-2 immune response, and the worms are eliminated by two weeks. IL-13, but not IL-4, has been shown to be a key cytokine for the elimination of Nb [23]. In contrast, Hp is not removed at the time of primary infection and,

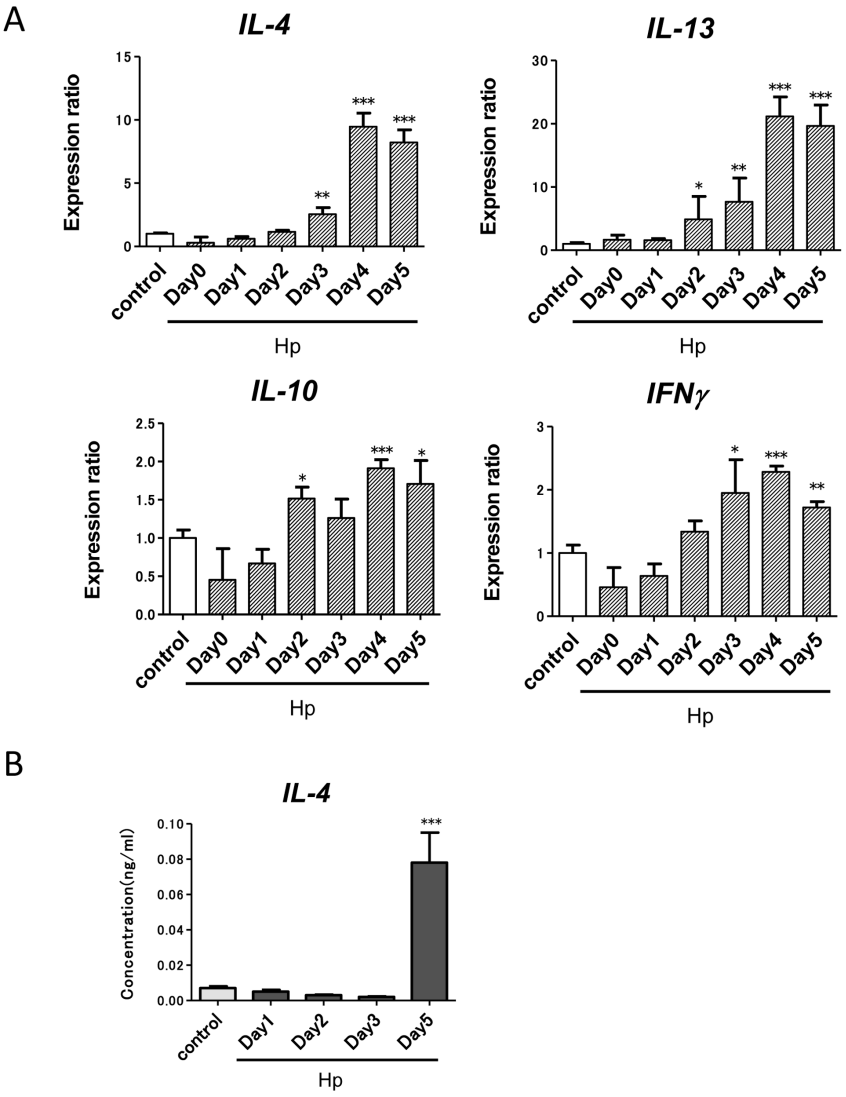

Fig. 4. A: Real-time PCR analysis for interleukin (IL)-4, IL-13, IL-10 and interferon (IFN) γ gene expression in the mesenteric lymph nodes from uninfected control mice and 0 (6 hr), 1, 2, 3, 4, and 5 days after Hp infection in Balb/c mice (n=5). Results are expressed as relative units compared with those for uninfected mice (control). **P*<0.05, ***P*<0.01, and ****P*<0.001. The data presented are the mean ± SE. Data are representative of two independent experiments. B: IL-4 production in cultured cells from the mesenteric lymph nodes. The culture supernatant was analyzed using an enzyme-linked immunosorbent assay (n≥4). ****P*<0.001 compared with the uninfected control.

rather, proceeds to a chronic infection in Balb/c mice. With oral exposure to Hp, the nematode arrives directly at the intestines within several hours, and type-2 cytokines increase similarly to Nb infection; however, the Hp worms are not eliminated. Instead, the Hp larvae penetrate into the submucosa and stay for several days to mature. Therefore, the time course of cytokine production in the host looks similar, but the local immune responses against these two kinds of nematode are different. The early stage of the immune response against Nb has been well documented, particularly the role of ILC2 in fat-associated lymphoid clusters as a trigger of the type-2 response [16]. While the early response against Hp infection remains largely unclear, Hp infection appears to induce migration and activation of both immune and nonimmune cells in the submucosa and represents a good model for understanding immune responses at the host–parasite interface. We previously reported that type-2 cytokine production peaks at Day 8 after Hp infection. The larvae first penetrate the epithelium and submucosa and then embed in the smooth muscle layer before leaving for the lumen at Day 8. In primary Hp infection, even though there is an elevation of type-2 cytokine production, along with an increased production of mucus and a hypercontractility of the smooth muscle, the worms are not expelled but, rather, stay in the small intestine. However, with secondary Hp infection, the elevation of type-2 cytokines is quick and strong, resulting in the effective removal of the worms [2, 22]. Our previous report demonstrated that this elevation of cytokines and the resulting expulsion of worms were impaired by the administration of the anti-CD4 antibody [14], suggesting that CD4+ T cells are essential for the elimination of Hp. Thus, the role of CD4+ T cells in the intestine is of interest; however, to our knowledge, it has not been investigated in terms of the early stages immediately after infection. In this study, we analyzed the type-2 response day by day and

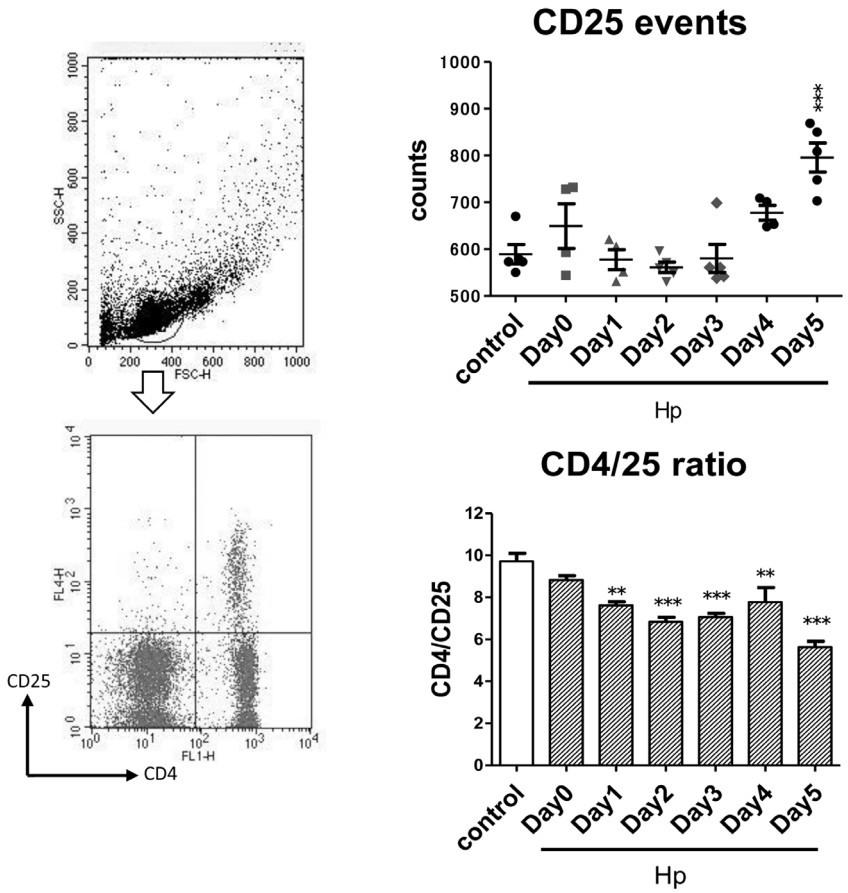

Fig. 5. Flow cytometry (FACS) analysis of the T cell subset at 0 (6 hr), 1, 2, 3, 4, and 5 days after *Heligmosomoides polygyrus* (Hp) infection in the mesenteric lymph nodes (MLN). Lymphocytes were initially gated by forward and side scatter properties (upper left panel). CD4+ T cells and CD25+ T cells were then gated and assessed for surface expression of CD4 and CD25 (lower left panel). The number of CD25+ cells (upper right panel) and CD4/CD25 ratio (lower right panel) in 10,000 cells. n≥4; **P<0.01 and ***P<0.001 vs. uninfected control.

found that CD4+ T cells are not activated until about Day 4 or 5 after Hp inoculation. Although type-2 cytokine production was shown to start from just after infection, it appears this process is not due to activated CD4+ T cells. Based on our prior findings, both innate immune cells and nonimmune cells appear to primarily work at the early stage of host defense against Hp and Nb infections. Our results in the current study demonstrated that cytokine elevation in the beginning continued until Day 3 after Hp infection but then went down (Fig. 1). At the same time, the elevation of IFNγ was observed from Days 2 to 4 in the small intestine after Hp infection (Fig. 1). A recent study also reported increased IFNγ gene expression in the early stage of Hp infection, which is consistent with our results [3]. It has been previously demonstrated that IFNγ is a key factor in the regulation of ILC2 [12]. Thus, it is suggested that the innate immune cells that were activated in the early stage declined owing to increasing IFNγ, and CD4+ T cells start to activate and migrate to the cyst zone around Day 3 or 4 after Hp infection, at which point they start to strongly produce type-2 cytokines. However, this host response during the early stage of Hp primary infection, involving the innate immune cells, is not enough to eliminate Hp. Indeed, a strong and quick response, as observed with secondary Hp infections, is needed to remove the worms, and CD4+ T cells appear to play a critical role in this process.

Innate immune cells and CD4+ T cell crosstalk is crucial to expel the worms [5, 17]. ILC2s function as a trigger for type-2 responses and also act as antigen presenting cells to CD4+ T cells [11].Therefore, it is considered that type-2 immune responses against nematodes start with ILC2s producing type-2 cytokines early and convert to activate CD4+ T cells later. Most studies were conducted with Nb; hence, the immune responses against Hp remains to be elucidated. However, after larvae migrate to the rumen in the small intestine, both Nb and Hp cause damage to the epithelium and activate myeloid lineage cells to induce the release of IL-25 [25] and IL-33 [24]. As mentioned above, Th2 differentiation relies on ILC2s, and ILC2s require CD4+ T cells to survive. Cells that produce IFNγ, which is believed to be important for switching the role from ILC2 to CD4, are still unidentified, but NK cells accumulate before ILC2 in the early stages of infection, and IFNγ is involved [3]. We could not analyze which cell population produces IFNγ; therefore, further study is needed.

Collectively, these results suggested that innate immune cells may become activated immediately after infection, with the later activation of CD4+ T cells at Day 4 or 5. Further, IFNγ may have an important role in converting type-2 cytokine-producing cells from innate cells to CD4+ T cells.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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