Interleukin (IL)-33 is dispensable for Schistosoma mansoni worm maturation, egg excretion, and the maintenance of egg-induced pathology in intestines of mice

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Research

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Abstract

Background: Schistosomes are trematode worms that dwell in their definitive host’s blood vessels, where females lay eggs that need to be eliminated in the environment with host excreta to maintain their life cycle. Both worms and eggs require type 2 immunity for their maturation and excretion, respectively. However, immune molecules that orchestrate such immunity remain unclear. IL-33 is one of the epithelium-derived cytokines that induce type 2 immunity in tissues. This study aimed at determining its role in the maturation, reproduction, and excretion of *S. mansoni* eggs, and in the maintenance of egg-induced pathology in the intestines of mice.

Methods: Using *S. mansoni*-infected IL-33-deficient (IL-33−/−) and wild-type (WT) mice, worm morphology, reproduction, and egg excretion were studied at different time points of infection. IL-5 and IL-13 production in spleens and mesenteric lymph nodes were measured. Tissue histology was performed on the terminal ilea of non- and infected mice.

Results: Morphology-wise, worms from IL-33−/− and WT mice at the fourth and sixth weeks of infection did not differ. The worms’ reproduction, expressed as eggs per worm pair, as well as the excretion of eggs, expressed as the number of eggs in intestinal tissues, did not differ between IL-33−/− and WT mice. In the sixth week of infection, IL-33−/− mice presented impaired type 2 immunity in intestines, characterized by decreased production of IL-5 and IL-13 in mesenteric lymph nodes and fewer inflammatory infiltrates with fewer eosinophils in the ilea. Besides, there was no difference between IL-33−/− and WT mice in the levels of IL-25 and TSLP in intestinal tissues.

Conclusions: Despite its ability to initiate type 2 immunity in tissues, IL-33 alone seems dispensable for *S. mansoni* maturation, reproduction, and egg excretion. The transient impairment of type 2 immunity observed in the intestines, but not spleens, highlights the importance of IL-33 over IL-25 and TSLP in initiating, but not maintaining, locally induced type 2 immunity in intestinal tissues in schistosome infection. Further studies are needed to decipher the role of each of them in schistosomiasis and clarify the possible interactions that might exist between them.

Background

Schistosomes are blood-dwelling trematode worms that affect over 250 million people in the world, of which 201.5 million live in sub-Saharan Africa (1). Of several schistosome species that exist, three, namely *Schistosoma haematobium*, *S. japonicum* and *S. mansoni*, are the main cause of schistosomiasis in humans (2). They cause urogenital and hepato-splenic schistosomiasis, respectively (2). *S. haematobium* worms live in perivesical vein plexuses, and *S. japonicum* and *S. mansoni* live in mesenteric veins where females lay hundreds to thousands of eggs per day (3). Half of the eggs are washed away to the liver by blood flow from the intestines, and most of the remaining are retained in intestinal tissues, of which only a few succeed in reaching the intestinal lumen (4, 5) to be eliminated in the environment with host’s feces.
By their excretory-secretory products (ESP) such as interleukin-4 (IL-4)-inducing principle of *S. mansoni* eggs (IPSE/α1) (6, 7) and omega-1 (ω1) (8) from *S. mansoni* and their homologs from *S. haematobium* (9) and *S. japonicum* (10), tissue-trapped eggs elicit strong and vigorous type 2 cell-mediated immunity that induces perioval granuloma formation and leads to fibrosis (11, 12), pathological characteristics of the patent schistosomae infection. While this immune response is beneficial for the host, especially in the liver where it protects hepatic cells from toxic effects of egg-derived ESP, it also plays a major role in the development of liver pathology (13). In contrast, in addition to being protective for and yet smiting the host with the pathology, granulomas in intestines play a beneficial role for the parasite, as they were found to favor the escape of eggs from the host through the intestinal wall (14).

Eggs are not the sole inducers of type 2 immunity in schistosomiasis, as studies have reported type 2 immune responses during prepatent schistosomiasis infection before egg deposition by female worms begins (15, 16). The type 2 immunity during the prepatent schistosome infection was later found to be essential for the maturation of the worms, as injection of IL-4, the T helper 2 (Th2) polarizing cytokine, in schistosome-infected recombination activating gene (RAG)-deficient mice, in which schistosome worms fail to mature and reproduce due to the lack of functional CD4$^+$ T cells, restored their maturation and egg deposition (17).

Emerging evidence indicates that the induction of type 2 immunity in tissues is not solely dependent on IL-4 as through activation of group 2 innate lymphoid cells (ILC2), epithelium-derived cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) also induce type 2 immunity. Activated ILC2, in turn, produce abundant amounts of type 2 effector cytokines IL-4, IL-5, IL-9 and IL-13 (18–21) and, through the expression of class II major histocompatibility complex (MHC II) (22) and OX40L (23), interact with CD4$^+$ T cells to potentiate such type 2 immune responses. Also, ILC2 were found to initiate the adaptive type 2 immunity in an IL-4-independent manner by inducing IL-13-dependent activation and migration of DCs to the draining lymph nodes where they polarize naïve CD4$^+$ T cells into Th2 cells (24).

However, the role of ILC2-activating cytokines IL-25, IL-33 and TSLP in schistosomiasis remains less understood. Focusing on the liver pathogenesis during *S. japonicum* infection, two studies showed that IL-33 contributes to the pathology development via induction of type 2 immune responses in infected mice (25, 26). Indeed, studies have shown that IL-33 plays a critical role in the development of liver pathology through ILC2-derived IL-13-induced activation of hepatic stellate cells (HSC) (27) and alternative activation of macrophages (M2) (26). Moreover, Yu et al. (25) found that injection of exogenous IL-33 into *S. japonicum*-infected mice led to increased worm burden at the sixth week of infection without affecting their fecundity, suggesting that IL-33 might play a role in the migration and maturation of schistosome worms. Whether endogenous IL-33 plays a role in schistosome maturation and reproduction is not known.

Because IL-33 is known to induce and/or amplify M2 polarization of macrophages (26, 28–30), known to be essential for the excretion of schistosome eggs (14), we thought that in addition to contributing to the maturation of schistosome worms through induction of type 2 immunity during prepatent schistosome...
infection, IL-33 may also play a critical role in the excretion of S. mansoni eggs across the intestinal tissues. We hypothesized that IL-33 deficiency will impair the maturation and reproduction of S. mansoni worms, and the expulsion of their eggs across the intestinal wall, leading to accumulation of eggs in intestinal tissues of IL-33−/− mice (31, 32), and that type 2 immunity would be impaired in the absence of IL-33. Here we show that IL-33 is dispensable for the maturation and reproduction of S. mansoni worms, as well as for the excretion of their eggs across the intestinal wall of infected mice. Also, our findings support the idea that IL-33 might be most potent in initiating, but not maintaining, type 2 immunity in tissues. To maintain initiated type 2 immunity, IL-33 might need the synergy of IL-25 and TSLP and/or of CD4+ Th2-derived effector cytokines.

**Methods**

**Parasite, mice, and infection**

A Puerto Rican strain of S. mansoni was maintained in the laboratory by passage between Biomphalaria glabrata snails and ICR mice. BALB/cCrSlc (hereinafter referred to as BALB/c) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan) and maintained in specific pathogen-free conditions at Nagasaki University animal facilities. Kindly provided by Professor Satoshi Uematsu (Osaka City University Graduate School of Medicine, Osaka, Japan), IL-33−/− mice on BALB/c background were bred and maintained in the same conditions as for WT BALB/c at Nagasaki University animal facilities. All mice were provided with water and food *ad libitum*. Female mice aged 8–12 weeks were subcutaneously infected (33) with 50 and 35 freshly shed S. mansoni cercariae, respectively for nine and 12 weeks. Mice were sacrificed every three weeks from week six, except for worm morphology assessment where mice were also sacrificed at week four of infection. To assess the production of IL-25, IL-33 and TSLP in intestinal tissues during S. mansoni infection, WT BALB/c mice were infected with S. mansoni cercariae as described above and sacrificed every week from week zero to week four, then every two weeks to week 12 of infection.

**Worm Morphology And Number**

Adult S. mansoni worms were obtained by portal vein perfusion, fixed with 4% neutral buffered formalin (NBF) (34), and their morphology was assessed under the inverted light microscope at 40x magnification and their number was counted. Briefly, the portal vein was cut at its base under the liver, then the left cardiac ventricle was perfused with 30 mL of saline citrate (7.5 g of sodium citrate and 8.5 g of sodium chloride in milliQ) (33), followed by perfusion with 30 mL of PBS. The mesenteric veins were thoroughly checked for manual retrieval of worms that failed to wash out during perfusion.

**Tissue Egg And Eggs Per Worm Pair Numbers**

Livers and intestines were harvested and digested with 4% potassium hydroxide (KOH) at 37 °C for 14 hours. Briefly, livers were weighed and digested with 10 mL of 4% KOH, and intestines were cleansed of fecal matters, opened longitudinally, thoroughly washed with PBS, weighed then digested as for livers.
After digestion, samples were centrifuged for 5 minutes at 2000 rpm and room temperature. Eggs were counted in 50 µL of thoroughly mixed pellet suspension under the light microscope at 40x magnification and related to the organ weight. The number of eggs per worm pair was obtained by dividing the total number of tissue eggs per mouse by the number of worm pairs from the same mouse.

Production of soluble egg antigen (Sm SEA)

Frozen eggs were thawed on ice, resuspended in ice-cold PBS containing 10 µg/mL of Leupeptin, then homogenized on ice by using handheld sterile glass Teflon homogenizer. The homogenate was subjected to five cycles of freeze (-80 °C) and thaw (on ice), incubated at 4 °C overnight with rotation, then centrifuged for 1 h at 30,000 g and 4 °C. The supernatant was collected in new tubes on ice, dialyzed in PBS three times at 4 °C, respectively for 2 h, 4 h and overnight, by using Slide-A-Lyzer Dialysis Cassette (Thermo Scientific, Rockford Illinois, USA) per manufacturer's instructions. The protein concentration was determined by the Bicinchoninic acid (BCA) method (Pierce BCA Protein Assay, Thermo Scientific, Rockford Illinois, USA). The solution was filter-sterilized with a 0.2 µm filter, aliquoted, and stored at -30 °C until use.

Cell Stimulation And Cytokine Measurement

Immune cells were isolated from spleens and mesenteric lymph nodes (MLN) of non- and infected WT and IL-33−/− mice and stimulated with SmSEA. Briefly, spleens were smashed and filtered through 70 µm cell strainer, washed with HBSS then resuspended in complete RPMI medium (containing 10% FBS, 100 units/mL of penicillin and 100 µg/mL of streptomycin, 55 µM of 2-mercaptoethanol, HEPES, and L-glutamine). MLN were filtered through a 40 µm cell strainer and processed as above. 1.0 × 10^6 cells per well were plated in a flat bottomed 96-well plate, stimulated with 50 µg/mL of SmSEA in a 5% CO₂ incubator at 37 °C for 72 hours. The plates were stored at -30 °C until use. The concentrations of IL-5 and IL-13 were measured in the culture supernatants by ELISA per the manufacturer's instructions (DuoSet ELISA, R&D Systems, Minneapolis, Minnesota, USA).

Tissue Cytokines

Small intestines were harvested in ice-cold PBS. After removal of fecal matters, intestines were opened along their axis, abundantly washed with ice-cold PBS, cut into small pieces and homogenized in 3 mL of ice-cold HBSS (containing 10 µg/mL of leupeptin and 0.1 mM/mL of PMSF) with gentleMACS Octo Dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) using the Protein_01 program. The homogenates were centrifuged for 20 minutes at 20,000 g and 0 °C (35). The supernatants were aliquoted and stored at -30 °C until use. The concentrations of IL-25, IL-33, and TSLP were measured in the supernatants by ELISA according to the manufacturer's instructions (DuoSet ELISA, R&D Systems, Inc., Minneapolis, USA).

Histology
A one cm-long fragment of terminal ileum was cut from each mouse, close to the secum of both infected and non-infected mice, cleaned from fecal matters and fixed in 10% NBF until use. Samples were sent to the Division of Cell Function Research Support, Biomedical Research Support Center at Nagasaki University School of Medicine, for tissue processing. Slide-embedded Hematoxylin and Eosin (H&E)-stained tissue sections were scanned at 40x magnification using Aperio CS2 Scanner (Leica Biosystems Imaging, Vista, California, USA), and digital images were analyzed using Aperio ImageScope version 12.4.3 software (Leica Biosystems Imaging, Vista, California, USA). The number and size of granuloma areas were, respectively, counted and measured. The intestinal wall thickness was measured at three different places. The abundance of eosinophils in inflammatory infiltrates was visually appreciated. All the measurement results were compared between IL-33−/− and WT.

**Statistical analysis**

Data normality was determined by the Shapiro-Wilk test. By using GraphPad Prism version 8.4.2 for Windows (GraphPad Software, San Diego, California, USA), Welch's *t*-test or Mann-Whitney test were performed for the comparison of IL-33−/− mice to WT. Set at *p* < 0.05, statistically significant *p* values are indicated on the figures above the connector bars between appropriate groups. Unless otherwise stated, all data are presented as mean with standard error of the mean (SEM) and are representative of at least two independent experiments with similar results.

**Results**

**IL-33 deficiency does not affect *S. mansoni* worm maturation, reproduction, and egg excretion**

Schistosome worms are characterized by their dependence on the host immune system, particularly type 2 immunity, for their maturation, reproduction, and egg excretion (14, 17, 34, 36), pointing to the importance of type 2 immunity in the biology of schistosomes. Because IL-33 is known to induce type 2 immunity independently of IL-4 (37), and that a recent study (25) reported increased *S. japonicum* worm numbers after injection of exogenous IL-33 into infected mice, we thought that its deficiency might compromise the maturation and reproduction of *S. mansoni* worms. Thus, we compared the morphology and number of worm pairs between IL-33−/− and WT mice. We infected IL-33−/− and WT BALB/c mice with *S. mansoni* cercariae and sacrificed them at indicated post-infection time points (Fig. 1a). As shown in Fig. 1b, morphology-wise, there was no difference between worms recovered from IL-33−/− and WT mice. Although the number of worm pairs was significantly higher in IL-33−/− mice at the ninth week of infection (Fig. 1c), their fecundity, expressed by the number of eggs per worm pair, did not differ between IL-33−/− and WT mice during the whole infection course (Fig. 1d).

It is known that IL-33 induces M2 polarization of macrophages (26, 28–30) and that M2 are essential for the excretion of schistosome eggs across intestinal tissues (14). It has also been reported that due to the impairment of their expulsion across the intestinal wall, more schistosome eggs accumulate in intestinal tissues (31, 32). To verify whether IL-33 plays a critical role in the excretion of eggs, we compared the
number of eggs in intestinal tissues between IL-33−/− and WT every three weeks from week six to week 12 of infection. No statistical difference was found between both mouse genotypes in the number of tissue eggs in the small intestines (Fig. 1e), indicating that IL-33 may be dispensable for the excretion of schistosome eggs. Because several studies reported a pathogenic role for IL-33 in egg-induced liver pathology by an increased number of liver tissue eggs (25, 26), we sought to see whether IL-33 deficiency would be associated with a decreased number of eggs in liver tissues of IL-33−/− compared to WT mice. Unexpectedly, we found no difference in liver egg numbers between both mouse genotypes (Fig. S1). Together, these data indicate that IL-33 is dispensable for the maturation and reproduction of S. mansoni worms, and the excretion of their eggs across the intestinal tissues.

**IL-33 deficiency is associated with transitory impairment of type 2 immunity in mesenteric lymph nodes of S. mansoni-infected mice**

Compared to IL-25 and TSLP, IL-33 is known to be stronger in inducing type 2 immunity through the activation of ILC2 and macrophages (38, 39). Therefore, we sought to assess whether IL-33 deficiency would impair type 2 immunity in intestines. We isolated immune cells from MLNs of S. mansoni-infected IL-33−/− and WT mice, stimulated them with SmSEA for 72 hours, and measured IL-5 and IL-13 cytokines by ELISA. As expected, IL-33 deficiency impaired the production of IL-5 and IL-13 in MLNs of infected mice in response to stimulation with SmSEA at six weeks of infection. However, this impairment was not sustained during the infection course as it disappeared in subsequent infection time points (Fig. 2a,b). To verify whether this impairment was limited to intestines or was systemic, we isolated immune cells from spleens of infected mice and measured IL-5 and IL-13 in the supernatants after 72 hours of stimulation with SmSEA. Surprisingly, we found that while IL-33 deficiency did not affect the production of IL-5 in spleens during the infection course (Fig. 2c), it was rather associated with increased production of IL-13 at six weeks of infection (Fig. 2d).

**IL-33 deficiency transiently attenuated egg-induced pathology in intestines of S. mansoni-infected mice**

Although schistosome worms also induce type 2 immunity (15, 16), eggs remain the most potent inducers of type 2 immunity and the main cause of the pathology in the liver and intestines of infected definitive hosts (11–13). Studies have reported a pathogenic role for IL-33 in liver pathology during schistosome infections (25–27). While none of them reported on the role of IL-33 in the intestinal pathology development, studies related to inflammatory bowel diseases reported controversial roles for IL-33 in the development and/or exacerbation of these diseases. Some of these studies reported a protective role for IL-33 (40), others incriminated it in the development or exacerbation of these diseases (41, 42). Thus, we thought to see whether IL-33 deficiency would compromise the development of egg-induced pathology in intestinal tissues of infected mice. As shown in Fig. 3a and b, IL-33 deficiency was transiently associated with attenuated type 2 inflammatory responses in terminal ilea of IL-33−/− mice compared to WT mice, characterized by less infiltration of intestinal tissues by inflammatory cells and wall thickness similar to naïve mice at the sixth week of infection. Moreover, the inflammatory infiltrates contained fewer eosinophils in IL-33−/− mice than in WT in the sixth week of infection (Fig. 3a). Both
mouse genotypes did not differ in the granulomas number and areas (Fig. 3c,d). Together, these data suggest that IL-33 may be important in initiating but not maintaining type 2 immunity at mucosal barriers than systemically and that it is dispensable for the maintenance of schistosome egg-induced pathology in intestines, as due to persistence of egg-derived ESP which strongly induce type 2 immunity, alternative compensatory mechanisms might have been triggered to compensate its absence. These results prompted us to speculate that IL-25 and TSLP expression might be upregulated in this setting, to compensate for the absence of IL-33 in later infection time points in intestines.

There is no change of IL-25 and TSLP production in the absence of IL-33 in intestines of infected mice

Individually or synergistically, IL-25, IL-33 and TSLP are known to induce tissue type 2 immune responses in different homeostatic and pathologic conditions (38, 39, 43, 44). Besides, the existence of possible interactions between these cytokines was raised (39, 44). We, therefore, reasoned that, due to IL-33 deficiency, there might be compensatory changes of IL-25 and/or TSLP production in S. mansoni-infected IL-33−/− mice compared to WT mice. As shown in Fig. 4, there was no statistically significant difference in the levels of IL-25 and TSLP in the small intestinal tissues between IL-33−/− and WT mice, indicating that there is no compensatory changes of IL-25 and TSLP production in the absence of IL-33. Although the levels of IL-25 and TSLP expression in intestinal tissue homogenates tended to increase with S. mansoni infection, compared to naïve mice, infected mice did not produce that much of these cytokines to reach a statistically significant difference (Fig. 4a,b).

Studies have reported an increase in IL-33 levels in the sera of individuals with S. japonicum infection compared to non-infected (26). In mice, this increase, which starts around week four of infection, reaches its peak around week eight (25), corresponding with the oviposition period. This may indicate that schistosome eggs are the major inducers of IL-33 release in schistosome infection settings. At the same time, because of its functional redundancy with IL-25 and TSLP (45), we sought to know the kinetics of these cytokines production during an S. mansoni infection. Thus, we infected only WT BALB/c mice with S. mansoni cercariae and checked for the release of IL-25, IL-33 and TSLP in their intestinal tissues. While the levels of IL-33 remained constantly higher even in naïve mice, levels of IL-25 and TSLP tended to increase with oviposition (Fig. S2), indicating that S. mansoni eggs may induce the release of IL-25 and TSLP but not of IL-33 in the intestines of infected mice.

Discussion

Studies have shown that both schistosome worms and eggs induce type 2 immunity, which is essential for their maturation, reproduction, and egg excretion (14–17). Deficiency in CD4+ Th2 cells and their effector cytokines IL-4 and IL-13 was shown to substantially decrease or completely abrogate egg excretion as a consequence of impaired worm maturation or failed signaling by type 2 effector cytokines (14, 46–48). Moreover, accumulating evidence suggests that ILC2 and their activating cytokines IL-25, IL-33 and TSLP induce adaptive type 2 immunity independently of IL-4 (22–24). Besides, while there is no report on the induction of IL-25, IL-33 and TSLP release by migrating schistosomula, it has been reported
that schistosome eggs induce and/or enhance the production of IL-25 and IL-33 (49, 50), indicating that schistosome eggs may be orchestrating their excretion by inducing the production of alarmin cytokines IL-25, IL-33 and TSLP to trigger type 2 immunity, essential to their excretion. In this study we report that IL-33 deficiency does not affect the maturation of worms as on both early (four weeks after infection) and late (six weeks after infection) worm maturation, the morphology did not differ between worms recovered from IL-33−/− and WT mice, suggesting that IL-33 may not be required for schistosome worm maturation. Consequently, despite a higher number of worm pairs at the ninth week of infection in IL-33−/− mice, the number of eggs per worm pair, as well as the number of tissue eggs did not differ between the mouse genotypes. Although we did not determine the worms’ lengths, the proportion of females in pairs (17, 51), and the number of eggs in feces (14), based on the morphology of worms (34, 52) and the intestinal tissue egg numbers (31, 32) as indications for worm maturation and egg excretion, respectively; to the best of our knowledge, this is the first study that has attempted to look at the role of IL-33, as a potent initiator of type 2 immunity necessary for schistosome worm maturation, in the maturation of *S. mansoni* worms and the excretion of their eggs.

In a study by Yu et al. (25), it was reported that the injection of *S. japonicum*-infected mice with exogenous IL-33 increased the number of worms recovered at the sixth week of infection, and exacerbated the liver pathology by increasing the number and size of liver granulomas. This may simply mean that as endogenous IL-33 plays a role in the development of egg-induced liver pathology (25–27), injecting exogenous IL-33 would exacerbate its pathogenic effects. In the present study, we could not find any statistically significant difference in the number of eggs per worm pair between IL-33−/− and WT mice. These results corroborate the ones reported by Yu et al. (25) as they did not find a difference in the number of eggs per female worm. This indicates that IL-33 alone may have a negligible role to play in worm maturation, reproduction, and egg excretion.

While studies related to inflammatory bowel diseases reported controversial roles for IL-33 in the development and/or exacerbation of these diseases, with some reporting a protective role for IL-33 (30, 40), and others incriminating it in the development or exacerbation of these diseases (41, 42), to the best of our knowledge, no report had been made on the role of this cytokine in the intestinal pathology during schistosomiasis. Although IL-33 seemed dispensable for *S. mansoni* worm maturation and the excretion of their eggs, we sought to know whether it may play a significant role in the development of egg-induced pathology in the intestines of infected mice as it does in the liver (25–27). We found that the absence of IL-33 transiently impaired type 2 immunity in small intestines of IL-33−/− mice, but not in their spleens, characterized by impaired production of IL-5 and IL-13 cytokines in MLNs in response to stimulation with SmSEA and attenuated egg-induced inflammation in IL-33−/− mice ilea at the sixth week of infection. These results are in line with findings by Vannella et al. (45) who, although focused on the role of alarmin cytokines IL-25, IL-33 and TSLP in the development and maintenance of type 2 cytokine-driven inflammation and fibrosis in lungs and liver, found that single ablation of these cytokines had no significant ameliorating effect on the liver pathology. However, when all three cytokines were ablated, a significant improvement of the pathology could be observed in the early phase of the infection, pointing
towards the existence of functional redundancy between these cytokines. Findings from the present study tread in the same direction as the absence of IL-33 did not affect the pathology development, nor the number and size of granulomas in the intestines of IL-33−/− mice in time points beyond the sixth week of infection. However, the difference between the study by Vannella et al. (45) and ours is that we started our observation at the sixth week of infection, when the egg-induced type 2 immunity is still at its start, while Vannella et al. (45) started their observation at the ninth week of infection when the egg-induced type 2 immunity has already reached its peak. We think that it could have been possible for them to notice a significant difference between IL-33 deficient mice and WT at an earlier stage of the infection, as observed in our study.

Despite its sharing of functional redundancy with IL-25 and TSLP (45), IL-33 remains the most potent of all three in inducing type 2 immunity (38, 39, 53). In addition to inducing type 2 immunity by itself, IL-33 can also potentiate the type 2 immunity induced by the two other cytokines, IL-25 and TSLP (54). Of all the cells that respond to IL-33, ILC2 and Th2 are the most important as through their production of abundant amounts of type 2 cytokines IL-4, IL-5 and IL-13, they play the most important role in cell-mediated effector type 2 immunity (55, 56), characterized by, among others, accumulation of M2 macrophages and eosinophils in affected tissues. Although dispersed in all tissues, ILC2 are more abundant in lungs and intestinal tissues (57), where they are the first to be activated by IL-33 and migrate to local draining lymph nodes to initiate the adaptive type 2 immunity (24, 58). Thus, it is understandable that the absence of IL-33 in IL-33−/− mice at the early stage of the patent infection might have left them inactivated, leading to impaired type 2 immunity (58) as seen in the present study. In addition to acting through ILC2 and Th2 cells, IL-33 also acts directly on eosinophils, inducing their expansion and activation (59, 60). Therefore, its absence in IL-33−/− mice can explain the small number of eosinophils in the inflammatory infiltrates in the sixth week of infection (61). However, due to the persistence of egg-derived ESP (6–10) as eggs keep accumulating in the tissues, and to the fact that IL-25 and TSLP can induce type 2 immunity independently of IL-33 (50, 62–64), alternative mechanisms leading to activation of both innate and adaptive type 2 immunity, including taking over of ILC2 activation by IL-25 and TSLP and Th2-dependent effector pathways, might have been activated to compensate the absence of IL-33. Together, these alternative mechanisms may have led to improved type 2 immunity in time points beyond the sixth week of infection.

Studies have pointed to the existence of possible interactions between IL-25, IL-33 and TSLP (39, 44). In one study, anti-IL-33 treatment and TSLP receptor deficiency blocked the infection-induced expression of IL-25 in lung epithelial cells, and ex vivo treatment of ILC2 with TSLP increased their expression of IL-25 and IL-33 receptors (44). In another, it was noted that IL-25 shared with IL-33 many activities on macrophages without having additive effects, pointing toward the possible existence of common downstream signaling pathways for their biological activities (39). Therefore, we thought that IL-33 deficiency might be associated with a modified production of IL-25 and TSLP in the intestines of S. mansoni-infected IL-33−/− mice. Our results showed no modification of intestinal production of IL-25 and TSLP as their levels in intestinal tissue homogenates did not differ between mouse genotypes, meaning
that although they can, individually or synergistically, induce type 2 immunity, the absence of one may not affect the others in the schistosome infection settings or intestines. The nature and conditions of occurrence of the interactions between IL-25, IL-33 and TSLP pointed out by the above-mentioned studies (39, 44) remain to be clarified.

Studies in humans and mice have reported the increase of IL-33 levels in the sera of individuals and animals infected with *S. japonicum* infection (26). Also, these increased levels of IL-33 in serum peaked around the eighth week of infection in mice (25), relatively corresponding to the peak of egg-induced immune responses, suggesting that through their ESP, eggs may be the main inducers of IL-33 release in schistosome infections. Indeed, Hams et al. (49, 50) reported that injection of *S. mansoni* eggs or recombinant form of their derived components, namely ω1, induced the production of IL-25 and IL-33, respectively in the lungs and fat tissue. Whether eggs in intestinal tissues induce the production of IL-33, IL-25 and TSLP is not known. By measuring the levels of these alarmin cytokines in the intestinal tissue homogenates during *S. mansoni* infection in WT BALB/c mice, we found that IL-33 levels remained constantly higher, even in non-infected mice. In contrast, IL-25 and TSLP levels fluctuated over the infection course, peaking around the tenth week of infection, with TSLP having much lower levels than IL-25. The start of an increase in levels of IL-25 and TSLP tended to correspond to the oviposition, suggesting that this latter might be inducing the release of IL-25 and TSLP, but not of IL-33. In their study, Flamar et al. (58) recently reported that IL-33 expression was high in small intestines of naïve mice, corroborating our findings. This indicates that IL-33 is constantly expressed in high amounts in mouse intestinal tissues.

**Conclusion**

To the best of our knowledge, this is the first study that looked at the role of IL-33 in the maturation and reproduction of *S. mansoni* worms, as well as in the excretion of their eggs across intestinal tissues toward the lumen. It is also the first study to report on the role that IL-33 may play in the maintenance of egg-induced type 2 immunity in intestines. It showed that IL-33 is dispensable for the maturation and reproduction of *S. mansoni* and the excretion of their eggs. Furthermore, due to transient impairment of type 2 immunity observed in the intestines but not spleens, this study highlights the importance of IL-33 over IL-25 and TSLP in initiating, but not maintaining, locally induced type 2 immunity in intestinal tissues in schistosome infections. These results corroborate previously reported findings that IL-25, IL-33 and TSLP might be sharing a partial functional redundancy in their ability to maintain the tissue-induced type 2 immunity. Their combined or sequential ablation might be the best option to decipher the role of each of them in schistosomiasis and clarify the possible interactions that might exist between them.

**Abbreviations**

BCA: Bicinchoninic acid; CO₂: Carbon dioxide; DC: Dendritic cell; ELISA: Enzyme-Linked Immunosorbent Assay; ESP: Excretory-secretory product; FBS: Fetal bovine serum; H&E: Hematoxylin and Eosin; HBSS: Hank’s balanced salt solution; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSC: Hepatic
stellate cell; IL: Interleukin; ILC2: Group 2 innate lymphoid cell; IPSE/α1: Interleukin-4 (IL-4)- inducing principle of *S. mansoni* eggs; KOH: Potassium hydroxide; M2: Alternatively activated macrophage; MHC II: Class II major histocompatibility complex; MLN: Mesenteric lymph node; NBF: Neutral buffered formalin; PBS: Phosphate buffered saline; PMSF: Phenylmethylsulfonyl fluoride; RAG: Recombination activating gene; RPMI: Roswell Park Memorial Institute; SEM: Standard error of the mean; *Sm*SEA: *Schistosoma mansoni* soluble egg antigens; Th2: T helper 2 cell; TSLP: Thymic stromal lymphopoietin; wpi: week post infection; WT: Wild-type; ω1: Omega-1.

**Declarations**

**Ethical considerations**

The study protocol was approved by the Nagasaki University Committee for Ethics on Animal Experiments (approval numbers: 1505181226; 1612081349) and Recombinant DNA experiments (approval numbers: 1403041262; 1902201550). All the experiments followed the Nagasaki University institutional guidelines for animal experiments and the Japanese Law for Humane Treatment and Management of Animals (Law No. 105 dated 19 October 1973, modified on 2 June 2006).

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

**Competing interests**

There are no existing competing interests to declare.

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**Authors’ contributions**

JPKM and SH conceived the study; JPKM and RN designed the experiments; JPKM performed the experiments, analyzed data and wrote the manuscript; SU provided the critical materials; RN and SH supervised the study; SH acquired the fund. All the authors read and approved the final manuscript.

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Consent for publication

Not applicable

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Figures
IL-33 deficiency does not affect S. mansoni worm maturation, reproduction, and egg excretion. (a) Female IL-33-/- and WT BALB/c mice (4-8 animals per group) were subcutaneously infected with 50 and 35 S. mansoni cercariae, respectively for nine and 12 weeks and sacrificed at four, six, nine and 12 weeks of infection to assess the morphology of worms and count the number of worm pairs and tissue eggs. (b) The morphology of worms recovered from both mouse genotypes at weeks four (upper panels) and six (lower panels) was assessed under the microscope at 40x magnification. Representative photographs are presented here. (c) The number of worm pairs from both IL-33-/- and WT mice was compared between the mouse genotypes. (d) The number of eggs per worm pair was determined as an indication of worm fecundity. Groups were compared using unpaired two-tail t-test with Welch’s correction. (e) The number of eggs in intestinal tissue was used to indicate the egg excretion status (14,32). Experiments were...
replicated at least three times. Data are representative of 2 independent experiments with similar results and are presented as mean with SEM. Mann-Whitney test was used for the comparison of mouse groups at p<0.05 significance. wpi=week post-infection.

**Figure 2**

IL-33 deficiency is associated with transitory impairment of type 2 immunity in MLNs of infected mice. Female IL-33−/− and WT BALB/c mice (4-8 animals per group) were subcutaneously infected with 50 and 35 S. mansoni cercariae, respectively for nine and 12 weeks, and sacrificed at six, nine and 12 weeks of infection. Immune cells were isolated from mesenteric lymph nodes (MLN) and spleens of naïve (control) and infected mice and stimulated with SmSEA for 72 hours at 37 °C, 5% CO2. IL-5 and IL-13 cytokines were measured in cell culture supernatants by ELISA. (a) IL-5 from MLNs. Comparison by unpaired two-tail t-test with Welch’s correction for 6wpi. (b) IL-13 from MLNs. (c) IL-5 from spleens. (d) IL-13 from spleens. Data are representative of two independent experiments with similar results and are presented...
as mean with SEM. The comparison of mouse groups was made by the Mann-Whitney test at p< 0.05 significance. SmSEA: S. mansoni soluble egg antigen.

Figure 3

IL-33 deficiency transiently attenuated egg-induced pathology in the intestines of infected mice. Female IL-33−/− and WT BALB/c mice were subcutaneously infected with 50 and 35 S. mansoni cercariae, respectively for nine and 12 weeks, and sacrificed at six, nine, and 12 weeks of infection. Terminal ileum fragment was obtained from each non and infected mouse (3 animals per group), fixed in 10% NBF, and processed for histology. (a) Representative H&E-stained histological sections of terminal ileum from naïve and infected WT (upper panels) and IL-33−/− (lower panels) mice. Scale bar: 200 µm for controls, 60 µm for 6 wpi with arrows indicating scarce eosinophils in inflammatory infiltrate, 100 µm for 9- and 12 wpi. (b) Intestinal wall thickness (mucosal layer excluded) in µm. Unpaired two-tails t-test with Welch’s correction was used for the comparison of mouse genotypes in controls and infected mouse groups at sixth and ninth weeks of infection, and Mann-Whitney test for infected mouse groups at the twelfth week of infection. (c) The number of granulomas per tissue section. (d) Granuloma area (mm2). Data are presented as mean with SEM. p< 0.05.
There is no change of IL-25 and TSLP production in the intestines of infected mice. Female IL-33⁻/⁻ and WT BALB/c mice (3 animals per group) were subcutaneously infected with 50 S. mansoni cercariae for nine weeks and sacrificed at six and nine weeks of infection. Small intestines were homogenized with gentleMACS Octo Dissociator and the cytokines were measured in the homogenate supernatants by ELISA. (a) IL-25 from intestinal tissues of naïve and infected mice. (b) TSLP from intestinal tissues of naïve and infected mice. Experiments were replicated twice with similar results. Data are presented as mean with SEM. TSLP: thymic stromal lymphopoietin. The comparison of mouse genotypes was made by the Mann-Whitney test for naïve mice and by unpaired two-tail t-test with Welch’s correction for infected mice.

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