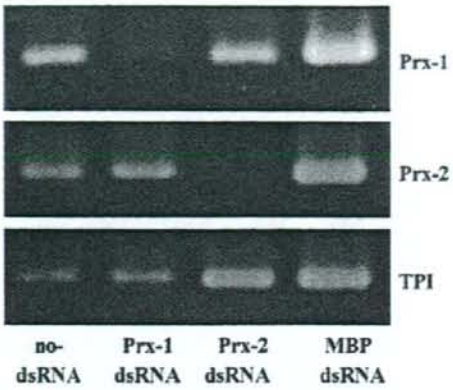

Fig. 1. Specifically down-regulated transcripts of each Prx by RNA interference. Total RNAs were recovered from about 30 schistosomula after treatment with 100 nM dsRNA coding Prx-1, Prx-2, and maltose-binding protein (MBP) of *E. coli* as a negative control. Reverse-transcription PCR was performed with specific primers amplifying Prx-1, Prx-2, and triose-phosphate isomerase (TPI) as an internal control.

3.2. Survival rate of the schistosomula treated by RNAi

To assess the influence of Prxs on the survival of schistosomula, the worms were incubated for 14 days after RNAi. Based on the surviving worm counts, there was no significant difference in the survival rate among the Prx-treated, the MBP-treated schistosomula, and no-treatment group (Fig. 2).

3.3. Killing assay against the dsRNA-treated schistosomula with hydrogen peroxide

The schistosomula that underwent RNAi treatment using each Prx dsRNA were exposed to hydrogen peroxide with the expectation that the Prxs would function as scavengers against hydrogen peroxide. As shown in Fig. 3A, only the group of schistosomula treated with Prx-1–dsRNA was killed by hydrogen peroxide at a concentration (10 μM) that was confirmed to be moderately toxic against normal schistosomula in preliminary experiments. Moreover, this susceptibility to hydrogen peroxide in the treated group depended on the concentration of Prx-1–dsRNA (Fig. 3B). By contrast, both the Prx-2-treated and the MBP-treated groups were resistant to hydrogen peroxide (Fig. 3A).

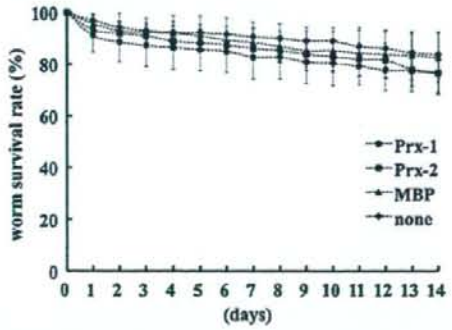

Fig. 2. The survival of schistsomules exposed to various dsRNA *in vitro*. Approximately 50 schistosomula treated with each long-dsRNA (100 nM) in triplicate were cultured in the medium at 37 °C. The number of surviving schistosomula was counted under microscopy daily for 2 weeks following RNAi. The results represent the average of combined experiments 1st with 2nd.

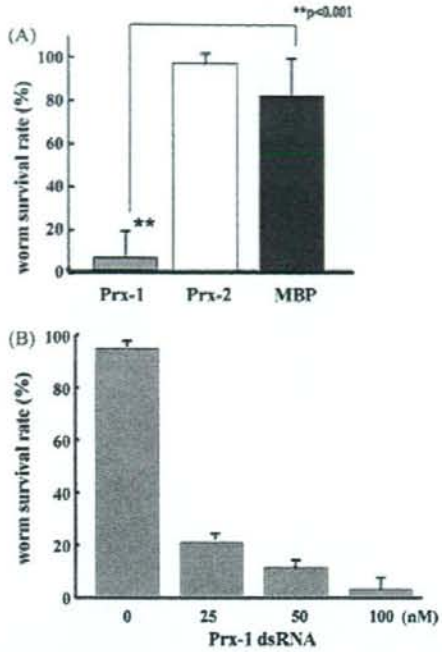

Fig. 3. Susceptibility to peroxide in the schistosomula treated with Prx-1 dsRNA. (A) Approximately 30 schistosomula treated with dsRNA (100 nM) for 6 days were incubated with hydrogen peroxide (10 μM) in triplicate. After 48 h, the number of surviving schistosomula was counted, and the worm survival rate was calculated. (B) The concentration dependency of the susceptibility to hydrogen peroxide (10 μM) was investigated using Prx-1 dsRNA. The results represent the average of all experiments repeated three times.

3.4. Killing assay against dsRNA-treated schistosomula with t-butyl hydroperoxide, cumene-hydroperoxide

To investigate whether Prx-1 scavenged the other hydroperoxide species, including the alkyl hydroperoxide and the lipid hydroperoxide, the schistosomula treated with dsRNA were exposed to *t*-butyl hydroperoxide or cumene-hydroperoxide, respectively. From Fig. 4A and B, Prx-1 dsRNA treatment showed significantly reduced survival rate following exposure to the hydroperoxide compounds, compared with the other treated groups.

3.5. Killing assay against two types of schistosomula, and dsRNA-treated schistosomula with nitric oxide

Because the 3 h schistosomula of *S. mansoni* were impaired by nitric oxide, as reported previously [19], we performed NO-killing test against schistosomula of *S. japonicum*. Similar to *S. mansoni*, 3 h m-schistosomula (and cercaria) were susceptible to NO generated from DETA/NO, compared with 24 h m-schistosomula cultured for 24 h after mechanical-transformation (Fig. 5A). On the other hand, the skin-penetrated schistosomula just collected from the mouse skin (s-schistosomula) were susceptible to NO; however, s-schistosomula became to be resistance to NO after the incubation for 24 h (Fig. 5A). This result showed that schistosomula (both mechanical-transformed and skin-penetrated) acquired resistance against NO by the culture in RPMI 1640 medium for 24 h.

Next, dsRNA-treated schistosomula were performed to the killing assay with DETA/NO. By contrast with Figs. 3 and 4, Fig. 5B shows that the nitric oxide had no effect on any of the treated groups.

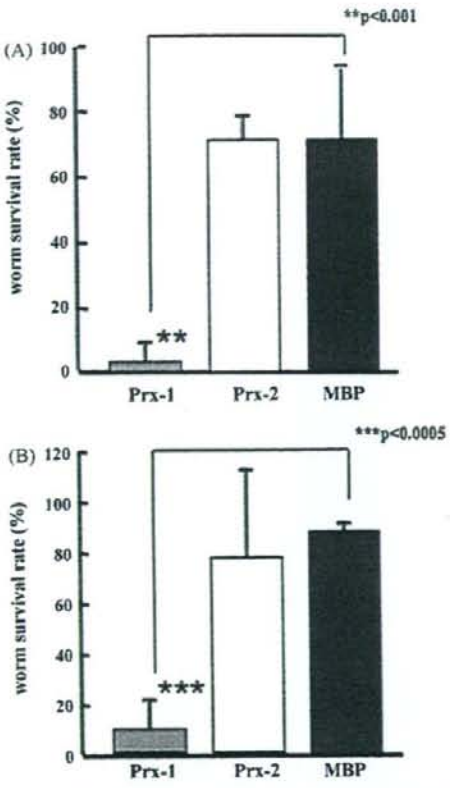

Fig. 4. Susceptibility to alkyl hydroperoxide in Prx-1 dsRNA-treated schistosomula. Approximately 30 schistosomula treated with dsRNA (100 nM) for 6 days were incubated with 10 μM *t*-butyl hydroperoxide (A) or 1 μM cumene-hydroperoxide (B) in triplicate. After 48 h, the number of surviving schistosomula was counted, and the worm survival rate was calculated.

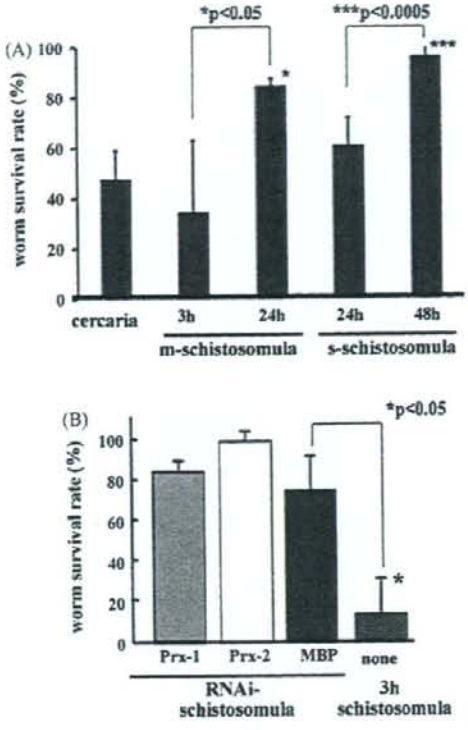

Fig. 5. Resistance to NO in the cultured schistosomula, and NO killing assay against dsRNA-treated schistosomula. (A) NO killing assay using DETA/NO (0.5 mM) were performed with cercaria, schistosomula passed for 3 h after newly mechanical-transformed (3 h m-schistosomula), m-schistosomula after culture in medium for 24 h (24 h m-schistosomula), skin-penetrated schistosomula just collected from skin passed for 24 h after the penetration (24 h s-schistosomula), s-schistosomula after additional culture in medium for 24 h (48 h s-schistosomula), respectively. After 48 h with NO donor, the number of surviving schistosomula was counted, and the worm survival rate was calculated in triplicate. (B) Following RNAi treatment with procedure similar to Fig. 4, each group of schistosomula was incubated with DETA/NO (0.5 mM) for 48 h. The controls consisted of schistosomula cultured for 3 h after mechanical transformation. The results represent the average of all experiments repeated three times.

4. Discussion

The 2-Cys Prx has been shown to reduce hydrogen peroxide and alkylhydroperoxide, using thioredoxin as an electron donor [20]. Thioredoxin is regenerated by the system of thioredoxin reductase and NADPH [21]. Although the enzyme activities of Prxs have been extensively investigated in several helminthes [22–24], their functions remain largely unknown. However, in *Fasciola hepatica*, a Prx induced the recruitment and alternative activation of macrophages [25]. Moreover, only Prx-1 of *Schistosoma mansoni* was reported to be an essential protein and to work as a scavenger of hydrogen peroxide [16]. In *S. japonicum*, we have shown that Prx-1 and Prx-2 had distinctly different tissue localizations in the adult worms and schistosomula [11]. Prx-1 was secreted from the bodies, and also localized in the tegument, while Prx-2 was found in the parenchyma, vitelline glands, and gut epithelium. Given these findings, Prx-1 may work as a scavenger against ROS generated outside of the worms. To test this hypothesis, we performed gene suppression of each Prx by RNAi.

The RNAi technique has recently been applied to the study of schistosomes, and the soaking method, which is the direct treatment of the organisms with long-dsRNA, has been very effective in producing gene suppression [12]. We performed this method against *S. japonicum* to suppress the expression of each Prx. As a result of RNAi against the skin-penetrated schistosomula of *S. japonicum*, specific gene suppression was observed in schistosomula treated with the long-dsRNA coding each Prx. The results show that the dsRNA including the full-length coding regions of each Prx could down-regulate each expression successfully, without affecting another gene (TPI) or the other Prx. However, the expression levels of each Prx returned to normal at 2 weeks after the RNAi treatment (data not shown). Thus, RNAi by the soaking method may have only transient effects against the lung stage and the post-lung stage of schistosomula. To confirm the direct effect against the schistosomula by RNAi, we counted the number of surviving larvae for 2 weeks after RNAi. None of the groups treated with any type of dsRNA showed a reduction of the survival rate compared with the non-treatment group. This result was contradictory to a previous report, which showed that Prx-1 was the essential protein for *S. mansoni* [16]. This may reflect differences between schistosoma species because the Prxs expression in *S. japonicum* differed from that in *S. mansoni* throughout all developmental stages [11,16]. Furthermore, they reported that all Prx family members were silenced as they used a Prx-1 dsRNA in RNAi silencing [16]. Taken together with our Prx-specific silencing results, both Prx-1 and Prx-2 silencing may be lethal against schistosomes.

Only the larvae treated with Prx-1 dsRNA were susceptible to 10 μM hydrogen peroxide; the group treated using Prx-2 dsRNA remained resistant. Furthermore, this effect of the dsRNA coding Prx-1 was dose-dependent in the schistosomula. This phenomenon was consistent with the findings in *S. mansoni*, which indicated that the Prx-1-treated schistosomula were highly susceptible to hydrogen peroxide (100 μM) [16].

Prxs are responsible for the essential scavenging function in trypanosomes [26] and for the enzyme activity in mammals [21], via their role in schistosomes [9] against organic hydroperoxide. The Prx-knockdown schistosomula were exposed to two organic hydroperoxides: *t*-butyl hydroperoxide and cumene-hydroperoxide, and only those treated with Prx-1 dsRNA were susceptible to both alkyl hydroperoxides. Thus, Prx-1, but not Prx-2, appeared to protect *S. japonicum* against not only hydrogen peroxide but also organic hydroperoxide, which has functions via secondary oxidation in the manner of lipid peroxidation [27]. Lipid hydroperoxide generated by lipid peroxidation can elicit iron-mediated cytotoxicity to the cell membrane [28]. Prx, alkyl hydroperoxide reductase (ahpC) from *Helicobacter pylori* and *H. hepaticus* is resistant to the organic hydroperoxide and prevents the accumulation of lipid hydrogen peroxide [29,30]. We suggest that Prx-1 produced from the bodies of *S. japonicum* may prevent membrane oxidation from organic hydroperoxide.

A previous study showed that Prx-2 had enzyme activity against organic hydroperoxide using glutathione as an electron donor [9]. Based on several observations, we propose that Prx-2 was not the essential scavenger against ROS generated in the outer environment, because this molecule was not localized in the tegument [11]. On the other hand, Prx-2 but not Prx-1 has YF motif in its C-terminal, which is known to be the signal transducer using hydrogen peroxide [31–33]. Based on the reports of 2-Cys Prx from the other organisms, upon severe H_2O_2 doses, the peroxidatic Cys of eukaryotic 2-Cys Prxs is selectively hyper-oxidized to Cys-sulfinic acid (SO_2H) during catalysis, and this modification inactivates the peroxidase [34,35]. The sulfinic form of several eukaryotic Prxs can be enzymatically reduced to the active thiol form through the action of sulfiredoxins [36] and/or sestrins [37]. This substrate-mediated reversible inactivation has been suggested to allow eukaryotic Prxs to act as floodgates, permitting high levels of H_2O_2 to trigger signal transduction [38]. This means that Prx-2 of schistosome may work as a scavenger at low H_2O_2 doses, and Prx-2 may be inactive and work in signaling pathway at severe H_2O_2 such our experiments. We propose that only Prx-1 primary functions to protect the schistosomes from ROS generated outer environment because Prx-1 of schistosome without YF motif was resistant to hyper-oxidations by hydrogen peroxide [9,39].

On the other hand, the Prxs of bacteria, plasmodium, mosquitoes, and other organisms showed resistance against both ROS and reactive nitrogen species (RNS) [40–42]. It was previously reported that the lung schistosomula, but not the skin-stage schistosomula, could acquire resistance against RNS in *S. mansoni* [19]. Moreover, we followed these results using the schistosomula of *S. japonicum*. Both the mechanical-transformed and the skin-penetrated schistosomula acquired tolerance to NO through the incubation in medium for 24 h. To our reglet, we found that neither Prxs of *S. japonicum* were essential for the resistance of schistosomula against nitric oxide generated from DETA/NO. Furthermore, the previous report showed that nitric oxide was not a major agent causing challenge parasite elimination, though NO mediated the cytotoxic killing of newly transformed schistosomula *in vitro* [43]. Their report demonstrated that the addition of erythrocytes to these larvicidal assays abolishes its effects [43]. However, our result showed 7d-cultured schistosomula of *S. japonicum* could inhibit nitric oxide without the erythrocytes by themselves. These results suggest that molecules other than the Prxs may play a role as scavengers against RNS, although the essential molecules have yet to be identified.

In summary, we performed RNAi against the schistosomula of *S. japonicum* using the soaking method with dsRNA encoding Prx-1 and Prx-2. Neither Prx was essential for the survival of the schistosomula, however, only Prx-1 played a role in the resistance against hydrogen peroxide and organic peroxide, but not against nitric oxide. These results showed that Prx-1, localized in the tegument, may function as scavenger against ROS generated in the outer environment, and may also protect the worms from oxidative stress for a long time in the bloodstream. Our observations in this paper have strong implications for the potential of this molecule as a future target of chemotherapy.

Acknowledgements

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Prolactin evokes lactational transmission of larvae in mice infected with *Toxocara canis*

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ABSTRACT

We investigated the trans-lactational maternal–neonatal transmission of *Toxocara canis* larvae in mice, with particular interest in the role of prolactin in their migration to the mammary gland. Two female mice were infected with 300 *T. canis* eggs soon after delivery of 27 offspring. After 1 week of breast-feeding, seven larvae were recovered from 4 of 13 offspring. After 2 weeks of lactation, 101 larvae were recovered from all the remaining offspring. Daily prolactin administration (5 μg) was performed 2 weeks before *T. canis* infection and continued until 2 weeks after infection in six non-pregnant female mice, which resulted in larval accumulation in the mammary gland. Furthermore, prolactin administration in female mice that had been infected with *T. canis* 4 weeks prior to prolactin treatment induced migration of larvae into the mammary gland. These findings suggest that prolactin is a promoting factor contributing to lactational transmission of *T. canis* larvae in mice.

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1. Introduction

Human larval toxocariasis is a serious public health problem in many countries [1]. Adult worms of *Toxocara canis* parasitize the intestines of domestic dogs and wild carnivores, and the larval stage of the parasite opportunistically invades undefinitive hosts including humans, resulting in human larval toxocariasis [2]. The migration behavior of the larvae in undefinitive hosts has been well documented [3–5]. In mice, *T. canis* larvae begin to accumulate in the liver 2 days post-infection, and they continue to migrate via systemic circulation. Beyond the 10th day of infection, most have settled in the brain and muscle tissue [6–8]. The larvae found in skeletal muscle are encapsulated in granulomatous inflammatory tissue and can survive for a long period [4,8]; those in the brain tissue elicit minimal inflammatory response [4].

Furthermore, it has been established that trans-placental transmission is the major route for *T. canis* larvae migration from infected female dogs to puppies [9–13]. In mice, it has also been regarded that *T. canis* larvae are transmissible via placenta [14–16], although no previous studies demonstrated larvae from offspring. Recently, Reiterova et al. [17] observed that *T. canis* larvae in offspring from infected mother mice were recovered at the beginning of the 5th day post-delivery. Thus, lactational transmission rather than trans-placental migration was certainly a possible route of maternal–neonatal infection with *T. canis*. After infection, migrating larvae settle in skeletal muscle tissue, in which they are then arrested in granulomatous inflammatory tissue. A re-emergence mechanism for these arrested larvae during pregnancy, however, has yet to be identified. In the present study, we demonstrate that *T. canis* larvae are able to transmit from mother to neonate via the mammary gland, and that prolactin evokes lactational transmission of the arrested larvae.

2. Materials and methods

2.1. Animals

Conventional ICR mice and an inbred strain of BALB/c mice were purchased from CLEA Japan Inc., Tokyo. All experimental procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

2.2. Infections

T. canis eggs were obtained from the uteri of adult worms collected from naturally infected puppies after the administration of anthelmintics. Mature embryonated eggs were prepared following the method of Ohsima [5], and 300 eggs were inoculated into each mouse via a Teflon tube with a siliconized glass syringe [18].

2.3. Recovery of larvae

Each of the mammary glands and whole body of newborn mice were digested with artificial gastric juice (0.5% of 1:10,000 pepsin and 0.7% hydrochloric acid, pH 1.5) for 3 to 4 h with vigorous agitation. After centrifugation, the larvae in the sediment were counted using a stereoscopic microscopy on a microscope slide (7×14 cm). Examination

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Table 1
Numbers of larvae recovered from neonates

Mother mouse	7th day after birth				14th say after birth			
	Number of neonates examined	Number of neonates larvae recovered	Number of larvae/neonate	Total number of larvae recovered	Number of neonates examined	Number of neonates larvae recovered	Number of larvae/neonate[a]	Total number of larvae recovered
#1	5	4	1.4±0.5 (1–3)[a]	7	6	6	9.5±0.8 (8–13)[a]	57
#2	8	0	0	0	8	8	5.5±0.9 (2–10)[a]	44

Neonates were allowed to breast-feed from the mother mice, which were infected with 300 eggs of *T. canis* immediately after delivery.
[a] Mean±SD (range).

of the brain was performed according to the method of Cho et al. [18]. In this experiment, we attempted to recover the larvae from skeletal muscle tissue by using the digestion method described above. However, the results were inconsistent in the number of larvae recovered from adult mice, because a large amount of sediments remained after digestion, making the counting of larvae using stereoscopic microscopy difficult. Therefore, we omitted the data on the muscle-stage larvae of the adult mice in this experiment.

2.4. Pathology of the mammary gland

Mammary glands of female mice were removed and fixed in 10% neutral formalin solution. Serial sections were then prepared and stained with haematoxylin and eosin. The degree of eosinophil infiltration around the mammary gland was estimated by the number of cells per square millimeter. We randomly selected 10 fields with a microscope of 100-fold magnification. To confirm cell identification, we observed at high magnification and counted the number of eosinophils. A careful attention was paid not to shift the original position.

2.5. Experimental design for trans-mammary transmission of larvae

Two pairs of 8-week-old ICR mice were mated in separate cages until the female mice became pregnant. Within 12 h after delivery, each of two female mice was infected with 300 eggs of *T. canis*, and then allowed to breast-feed their offspring for 2 weeks. The offspring were divided into two groups: one was killed on day 7 after delivery, the other was killed on day 14 after delivery. The number of larvae in the offspring was counted using the digestion method described above.

2.6. Effect of prolactin treatment in non-pregnant, infected mice

To investigate the effect of prolactin on the stimulation of larval migration from skeletal muscle or brain tissue, eight BALB/c female mice, at 8 weeks of age, were intraperitoneally injected with 5 μg of prolactin (100 mg/mL, Sigma, St. Louis, USA) in physiological saline everyday for 14 days, and were then infected with 300 *T. canis* eggs orally. Prolactin treatment was then continued for another 14 days. After treatment, the mammary glands were removed and the larvae were recovered. Two mice were used for histological purposes. As a control, seven additional mice were administered 0.5 mL of saline instead of prolactin.

Table 2
Effect of prolactin treatment in non-pregnant infected mice

Treatment	Number of mice used	Number of mice larvae identified in mammary glands	Number of mice larvae identified in the brain	Number of larvae in mammary glands of identified mice	Number of larvae in the brain of identified mice
Prolactin	6	6	6	9.8±3.5[a]	36±16.3[a]
Saline	5	0	5	0	34.4±24.2[a]

[a] Mean±SD.

2.7. Effect of prolactin treatment in chronically infected mice

Six BALB/c female mice, at 4 weeks of age, were infected with 300 *T. canis* eggs. Four weeks later, 5 μg of prolactin was intraperitoneally administered everyday for 14 days. The mammary glands were then examined as described above. As a control, equal numbers of BALB/c mice were employed, and 0.5 mL of saline was injected into the peritoneal cavity everyday for 14 days.

2.8. Statistics

Statistical analysis was performed using Student's *t* test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Larval transmission to neonates via mammary gland after birth

Two mother mice delivered 11 and 16 offspring, respectively. The offspring from each infected mother mouse, which were infected with *T. canis* within 12 h after delivery, were randomly selected and sacrificed on day 7 or day 14 after delivery. Table 1 presents the number of offspring infected and the number of larvae recovered on each of these days. The rate of infection in the offspring and the average number of larvae recovered were higher in the group sacrificed on day 14 compared with that sacrificed on day 7. Additionally, the total number of larvae recovered was significantly higher in the day-14 group ($P<0.05$).

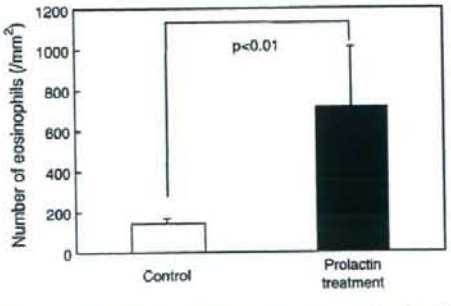

Fig. 1. Eosinophil counts around the capsules of mammary glands in mice. Solid bar, eosinophil count of prolactin-treated mice; open bar, that of untreated control mice. The mean number of eosinophils was 713.6±293.6 cells/mm² in the prolactin-treated group, and 144±21.3 cells/mm² in the saline-treated group. We randomly selected 10 fields with a microscope of 100-fold magnification. To confirm the cell identification, we observed at high magnification (×400) and counted the number of eosinophils. A careful attention was paid not to shift the original position.

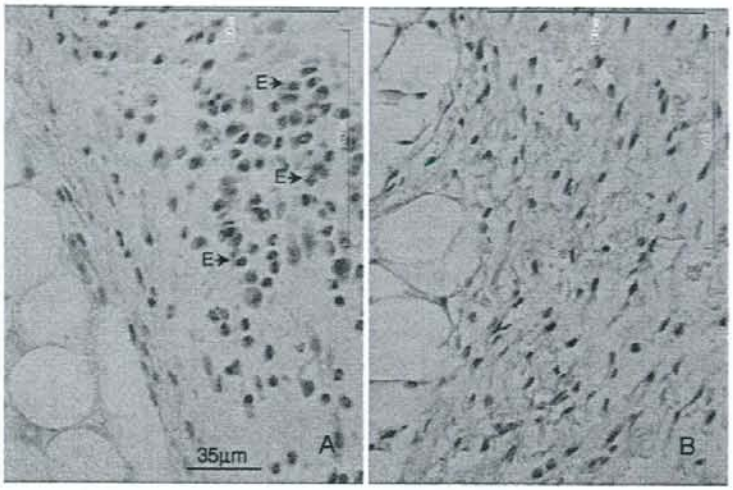

Fig. 2. Histopathological findings of mammary glands around the connective tissue in mice. Serial sections of mammary glands of female mice were stained with haematoxylin and eosin. Markedly higher eosinophilic (E) infiltrations around the connective tissue of the mammary gland were observed in the prolactin-treated mice (A) compared with the saline-treated mice (B).

3.2. Effect of prolactin on migration of larvae to the mammary gland

T. canis larvae were identified in the mammary glands of all infected mice, which were treated with 0.5 μg prolactin once a day intraperitoneally for 14 days before infection and 14 days after infection, although no larva was found in the control mice (Table 2). No significant difference in the number of larvae in the brain was observed between the prolactin-treated and saline-treated mice. These data suggest that prolactin might stimulate migration of larvae from skeletal muscle, the brain, or other organs to the mammary gland. Based on histological examination of 10 randomly selected fields, the eosinophil infiltrations around the capsule of the mammary gland were significantly increased in number in the prolactin-treated mice (713.6±293.6 6 cells/mm^2) compared with the saline-treated control mice (144±21.3 cells/mm^2, Figs. 1 and 2), suggesting that the inflammatory response against *T. canis* larvae was strong in the treated mice.

3.3. Effect of prolactin on chronically infected mice

Since administration of prolactin elicited a migration of larvae to the mammary gland, we next studied whether prolactin stimulates larval migration to the mammary glands from chronically infected mother mice in the absence of pregnancy. For this investigation, non-pregnant female mice, which had been infected with *T. canis* eggs 28 days previously, were administered prolactin for 14 days. Table 3 shows that larvae were recovered from the mammary glands in three of the four mice treated with prolactin, but no larva was found in the control mice. The number of eosinophils infiltrated in the mammary tissue was also significantly higher in the prolactin-treated group (Fig. 3).

In the prolactin-treated mice, glandular epithelial proliferation and dilatation of the ducts were observed, indicating a direct effect of prolactin against the mammary gland.

Table 3
Effect of prolactin treatment in chronically infected mice

Treatment	Number of mice used	Number of mice larvae identified in mammary glands	Number of mice larvae identified in the brain	Number of larvae in mammary glands of identified mice	Number of larvae in the brain of identified mice
Prolactin	4	3	4	3.8±1.9[a]	51.3±15.1[a]
Saline	4	0	4	0	49.8±5.7[a]

[a] Mean±SD.

4. Discussion

In this study, we demonstrate that *T. canis* larvae are able to migrate from the mother to neonates through suckling behavior, and that this migration can be induced by the administration of prolactin. While trans-placental migration of the larvae from female dogs to puppies has been established [9–13], few studies have investigated maternal–fetal transmission of the larvae in mice. Lee et al. [16] found that the larvae migrated in the uterus and placenta from the 9th day of pregnancy, and in the fetus from the 11th day of pregnancy when mother mice were infected during pregnancy. In addition, they

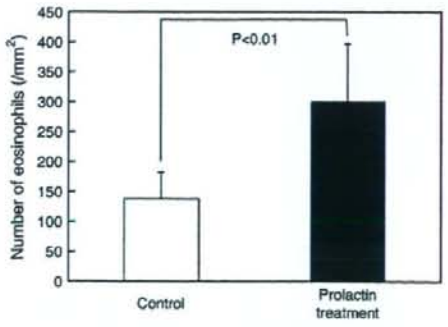

Fig. 3. Eosinophil counts around the capsules of mammary glands in chronically infected mice. Solid bar, eosinophil count of prolactin-treated mice; open bar, that of untreated control mice. The mean number of eosinophils was 300.8±95.6 cells/mm^2 in the prolactin-treated group, and 137.6±44.1 cells/mm^2 in the saline-treated group. Ten randomly selected fields at 100-fold magnification were observed via microscopy under a high magnification (400×).

identified larvae in the placenta and fetal blood vessels, histopathologically. They concluded that *T. canis* larvae were able to migrate through the placenta during pregnancy. However, because they did not examine the neonates after birth, they could not eliminate the possibility of trans-lactational transmission of the larvae from mother to neonates after delivery.

It is well documented that malaria infection induces placental injury, resulting in fetal loss in both humans and mice [19,20]. In murine toxocariasis, the litter sizes from infected mice are smaller than those from uninfected controls [21,22]. These data suggest that *T. canis* infection in mice can lead to mechanical injury of the placenta and a resultant decrease of litter size when the infection occurs during pregnancy.

Yet, in spite of these difficulties, newborns are still successfully delivered in most cases. In another previous study, larvae were found in offspring on day 5 after birth [15], suggesting suckling behavior might cause maternal–newborn transmission of *T. canis* larvae. In fact, our preliminary experiment revealed that larvae were first identified in offspring 11 days after birth (unpublished data). Thus, we hypothesized that larvae could migrate from mother to newborn mice through the mammary gland during suckling. The present findings support this hypothesis.

In general, *T. canis* larvae in mice settle in the brain and skeletal muscle after migration through the systemic circulation, and survive for a long period [4,8]. However, because we could not find any larvae in the mammary gland of non-pregnant infected mice, the larvae must be aroused by some sort of stimuli in order to migrate from those organs to the mammary gland. Prolactin, a lactogenic hormone, plays an essential role in the development of breast tissue. None of the non-pregnant mice not treated with prolactin showed the presence of larvae, in either the acute or chronic stage of infection, whereas prolactin-treated mice exhibited *T. canis* larvae infection in the mammary glands. One previous study discussed the relationship between *T. canis* infection and prolactin [23], reporting that the administration of prolactin led to a reduction in the number of larvae in infected mice. This may be related to the finding that prolactin acts as an immunomodulatory agent or proinflammatory cytokine in autoimmune diseases [24], and in several parasitic infections [25–28].

Eosinophil infiltration is a common feature in tissue-invading nematode infections, such as gnathostomiasis and trichinosis [29]. In toxocariasis, an eosinophilic granulomatous response is a typical pathological finding both in humans and in experimentally infected animals including mice [30,31]. Furthermore, eosinophil infiltration was demonstrated not only in the tissue adjacent to the larvae but also in that through which the larvae had passed [32]. These pathological changes are thought to be stimulated by the metabolic products from the larvae [29]. Therefore, we assumed that eosinophil infiltration around the capsule of the mammary gland in the prolactin-treated mice might be attributable to the migration of larvae into the mammary gland following stimulation of the tissue-arrested larvae.

The mechanism of this stimulation of tissue-arrested larvae during breast-feeding has yet to be elucidated. In hookworm infection, tissue-arrested larvae of *Ancylostoma caninum* were activated *in vitro* by TGF-β [33]. No such connection, however, has been demonstrated in *Toxocara* infection. The secretion of TGF-β is tightly regulated by the hormones estrogen and prolactin, and they are critical factors in the tissue-specific regulation of the local production of TGF-β in the mammary gland of the rat [34]. Therefore, we presumed that a similar cytokine reaction could be induced by prolactin, and may contribute to the reactivation of cryptic larvae in *Toxocara*-infected mice.

In the present study, we found clear evidence that prolactin is one of the factors in the lactational transmission of *T. canis* larvae from mother mice to offspring. Further investigation is needed to elucidate the precise mechanism of the stimulation of tissue-arrested larvae in mice.

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Schistosoma mansoni infection reduces severity of collagen-induced arthritis via down-regulation of pro-inflammatory mediators

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ABSTRACT

Various experimental and epidemiological studies have demonstrated that helminth infections affect outcomes of allergic or autoimmune disorders. Here, we examined the effects of *Schistosoma mansoni* infection on mouse collagen-induced arthritis, one of the most widely used animal models for rheumatoid arthritis. Male DBA/1 mice were infected with *S. mansoni* 2 weeks prior to being immunized with type II collagen (IIC). Cytokine mRNA expression in mouse paws, cytokine production by ConA-stimulated spleen cells, and anti-IIC antibodies were evaluated in addition to the severity of arthritis. *S. mansoni* infection significantly reduced the severity of arthritis. Anti-IIC IgG and IgG2a levels were lower in infected than uninfected mice. With regard to cytokine producing potentials in the infected mice, the down-regulation of Th1 (IFNγ) and pro-inflammatory cytokines (TNFα and IL-17A), and up-regulation of Th2 (IL-4) and an anti-inflammatory cytokine (IL-10) were observed. In addition, real-time PCR revealed that the augmentation of pro-inflammatory mediators such as IL-1β, IL-6 and receptor activator of NFκB in inflamed paws was abrogated by *S. mansoni* infection. In conclusion, schistosome infection reduced the severity of autoimmune arthritis via systemic and local suppression of pro-inflammatory mediators, suggesting the potential of parasite-derived materials as therapeutic agents against rheumatoid arthritis.

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1. Introduction

Schistosoma sp. is a genus of parasitic helminthes that affects humans globally. There are three main human-infecting species: *Schistosoma mansoni* (in Africa and South America), *Schistosoma haematobium* (in Middle-East and Africa) and *Schistosoma japonicum* (in East Asia and South-East Asia). Their infective larvae (cercariae) in fresh water bodies (rivers, lakes and ponds) infect humans percutaneously, then migrate into the bloodstream and finally become adult worms in the portal vein (*S. mansoni* and *S. japonicum*) or venous plexus of bladder (*S. haematobium*). The adult worms lay eggs in the portal vein and the eggs deposited in the liver robustly modulate host immune responses from Th1 to Th2 (Grzych et al., 1991; Pearce et al., 1991). Schistosome eggs are known to have various immunological effects on immune cells: e.g., activation of Treg cells (Mo et al., 2007; Yang et al., 2007) or expansion of B-1 cell numbers (Velupillai et al., 1997). Consequently, several studies have attempted to find immunomodulatory substances in this parasite (Velupillai and Harn, 1994; Faveeuw et al., 2003; Smith et al., 2005). However, the relative importance of each molecule in schistosome-induced immunomodulation is yet to be clarified.

According to the "hygiene hypothesis", microbial and/or parasitic infections protect against autoimmune and/or allergic disorders (Araújo et al., 2004; Zaccone et al., 2006). Although the debate continues (Christen and von Herrath, 2005), some epidemiological evidence supports the hypothesis. For instance, several autoimmune or allergic diseases are less prevalent in developing countries than in developed countries (Araújo et al., 2004; Zaccone et al., 2006). In Brazil, both skin reactions to mite allergen and asthmatic symptom levels were higher among people living in an area where *S. mansoni* is endemic than among people in a non-endemic area (Araújo et al., 2004). On the other hand, several animal experiments showed that schistosome infections or schistosome antigen (Ag) injections prevent autoimmune/allergic or inflammatory disorders, including Grave's hyperthyroidism (Nagayama et al., 2004), experimental colitis (Elliott et al., 2003; Mo et al., 2007; Smith et al., 2007), type-1 diabetes (Cooke et al., 1999; Zaccone et al., 2003), experimental allergic encephalomyelitis (La Flamme et al., 2003; Sewell et al., 2003) and airway hypersensitivity (Mangan et al., 2006; Yang et al., 2007). However, the mechanisms of the ameliorating effects of schistosome are still controversial. As possible mechanisms, an immunological skew from Th1 to Th2

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(Sewell et al., 2003), the proliferation of natural killer T (NKT) cells (Zaccone et al., 2003; Nagayama et al., 2004), and the involvement of regulatory T cells (Mo et al., 2007; Yang et al., 2007) have been suggested. To date, however, changes in Th17-related pro-inflammatory cytokines, especially IL-17 and TNFα, have received little attention in the study of *S. mansoni*-induced suppression of autoimmune/allergic disorders.

In this paper, we show prophylactic effects of schistosome infection on the development of a Th17-dependent animal model of human rheumatoid arthritis (RA): i.e., collagen-induced arthritis (CIA). Possible anti-inflammatory mechanisms of schistosome infection are discussed, mainly on the basis of cytokine expression profiles of the infected mice compared with uninfected controls.

2. Materials and methods

2.1. Maintenance of the parasite life cycle

The Puerto Rican strain of *S. mansoni* was maintained by using female ICR mice. As an intermediate host, the snail *Biomphalaria glabrata* (Puerto Rican strain) was used. Mice were infected percutaneously under anesthesia with pentobarbital sodium. Each animal was infected with 200–250 cercariae of *S. mansoni*. Seven to 8 weeks after infection, the portal system of the infected mice was perfused with physiological saline containing 0.45% trisodium citrate to harvest adult worms. Thereafter, the granulomatous livers of the mice were digested with 1 mg/mL collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1 mg/mL actinase E (Kaken Pharmaceutical Co., Ltd, Tokyo, Japan) in 1.8% NaCl for 6 h at 37 °C. Eggs were purified by mesh filtration. Miracidia were obtained by addition of aged tap water onto the eggs, and used for infection of the intermediate host snails.

2.2. Induction of collagen-induced arthritis (CIA)

Male DBA/1 mice (6 weeks old) were purchased from SLC Japan (Hamamatsu, Japan). The mice were infected with 40 cercariae by tail immersion in aged tap water containing the cercariae for 30 min. Two weeks later, the mice were immunized i.d. with 200 μg of bovine type II collagen (IIC) (Cosmo Bio Co., Ltd., Tokyo, Japan) emulsified with FCA (Difco Laboratories, Detroit, MI) at the base of their tails. From 4 weeks post-immunization, the severity of arthritis was evaluated in each mouse every 2 weeks, using both an arthritis score (graded 0–3 for each paw; 0, normal; 1, slight to moderate swelling; 2, marked swelling; 3, maximal swelling and/or ankylosis) and the number of "arthritic" paws, which had thickened more than or equal to 2 mm. At 12 weeks post-immunization, all mice were euthanized with diethyl ether and paws and spleens were prepared for histopathological examination or a cytokine-based analysis. At the same time, adult worms were recovered from each infected mouse by portal perfusion and enumerated. All the animal experiments were performed under the control of the Ethics Committee of Animal Care and Experimentation in accordance with The Guiding Principles for Animal Care Experimentation, The University of Occupational and Environmental Health, Japan and the Japanese Law for Animal Welfare and Care (No. 221).

2.3. Histopathology

At 12 weeks post-immunization, paws of each mouse were removed, fixed in 10% formalin and kept at room temperature until the preparation of tissue sections. The fixed samples were decalcified and subsequently embedded in paraffin. The tissue sections were stained with H & E.

2.4. Anti-IIC antibody measurement

Antibodies against bovine IIC were measured by microplate ELISA. The antigen (IIC) was diluted in 50 mM bicarbonate buffer (pH 9.6) to a final concentration of 1 μg/ml and used to coat wells of 96-well microplates. Fifty microliters of the diluted antigen solution was added to each well. The plates were kept at 4 °C overnight and afterwards washed twice with washing buffer (PBS containing 0.05% Tween 20). Blocking was performed using PBS containing 1% BSA and 0.05% NaN_3, for more than 2 h at room temperature. Plasma samples were diluted 1:500 with a dilution buffer (a washing buffer containing 1% BSA). Each sample was assayed in duplicate. Fifty microliters of diluted sample were added to the antigen-coated plates and incubated at room temperature for 1 h. The plates were then washed three times with the washing buffer. Peroxidase-conjugated anti-mouse IgG (Sigma, St. Louis, MO) or IgG2a (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was diluted with the dilution buffer at 1:1000 and 50 μL were added to the wells. After subsequent incubation at room temperature for 1 h, the plates were washed four times with the washing buffer. 2,2′-azino-bis[3-ethilbenzthiazoline-6-sulphonic acid], diammonium salt (Moss, Inc., Pasadena, MD) was used as a substrate. OD values were measured at 415 nm using a Model 550 microplate reader and the results were analyzed using Microplate Manager III for Macintosh (Bio-Rad laboratories, Hercules, CA).

2.5. Spleen cell culture and cytokine ELISA

At 12 weeks post-immunization, spleen cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin, at 6 million cells/mL in 48-well culture plates. During culture, the cells were stimulated with ConA (2 μg/mL) (Wako Pure Chemical Industries). The supernatants of the 48-well culture plates were collected at 48 h and kept frozen at −80 °C until used. T helper cytokines (IFNγ, IL-4, IL-10, IL-17A and TNFα) in the culture supernatants were measured with Ready-Set Go! Kits or recombinant cytokine/antibody sets from eBioscience (San Diego, CA) according to the supplier's instructions.

2.6. RNA extraction and real-time PCR analysis

At 12 weeks post-immunization, the four paws of each mouse were pooled. The paws were briefly minced with dissecting scissors and Trizol reagent (Invitrogen Ltd., Carlsbad, CA) was added. After homogenization in Trizol reagent, 0.2-fold volumes of chloroform were added and the samples were centrifuged at 10,000g for 15 min. The aqueous upper phase was collected and RNA was precipitated by addition of isopropanol. Precipitated RNA pellets were rinsed with 70% ethanol and then air-dried before being dissolved in diethylpyrocarbonate (DEPC)-treated water. Complementary DNA was synthesized using a High-Capacity reverse transcription kit (Applied Biosystems Japan, Ltd., Tokyo, Japan) after treatment with RNase-free DNase I (Qiagen K.K., Tokyo, Japan). The cDNA samples were subjected to a real-time quantitative PCR analysis in an ABI 7000 sequence detection system (Applied Biosystems Japan). TaqMan gene expression assays (Applied Biosystems) were used for each gene as pre-made primers/probe sets. The assay IDs are Mm00443258_m1 (TNFα), Mm00439619_m1 (IL-17A), Mm00439616_m1 (IL-10), Mm00446190_m1 (IL-6), Mm00439620_m1 (IL-1α), Mm00434228_m1 (IL-1β), Mm00441908_m1 (receptor activator of NFκB: RANKL), Mm00441724_m1 (TGFβ) and Mm00475156_m1 (Foxp3). Ex Taq Perfect Real-time (Takara Bio Inc., Otsu, Japan) was used as a master mix solution for the amplification. The PCR was performed in

duplicate for each sample. Highly expressed cDNA samples for each mediator were also prepared as standards, by stimulating mouse (BALB/c or C57BL/6) spleen cells with ConA or lipopolysaccharide (LPS) for 10 h. By using the standard samples, absolute quantification was performed. The expression levels of each inflammatory parameter were normalized to the expression levels of a housekeeping gene β-actin. These values are shown as relative expression levels (REL) in the figures. The primer/probe set for β-actin was Mouse ACTB Endogenous Control (Applied Biosystems Japan, Part No. 4352933E).

2.7. Statistical analyses

Differences in disease severity and immunological parameters between experimental groups were tested using a Mann–Whitney U test. Differences in anti-IIC antibody levels were analyzed with unpaired *t*-test. Correlations between parameters were tested with Nonparametric Spearman's Rank Correlation Test. Calculations for statistical analysis was performed with StatView5.0 software. *P* values lower than 0.05 were considered significant.

3. Results

3.1. Schistosoma mansoni infection reduces severity of CIA

As shown in Fig. 1A, *S. mansoni* infection prior to IIC immunization markedly reduced the severity of arthritis. Statistically significant prophylaxis was demonstrated by both the arthritis scores (a) and the numbers of arthritic paws (b). Non-immunized mice did not develop arthritis whether they were infected or not.

3.2. The anti-arthritic effect of S. mansoni depends on the numbers of infecting worm pairs

Schistosoma mansoni eggs may be essential to the anti-arthritic effect because they cause immunological modulation in mice (Velupillai and Harn, 1994; Smith et al., 2005). As *S. mansoni* is a gonochorist parasite which always forms male/female pairs in host blood vessels, the number of eggs produced is dependent on the number of pairs, not on the total number of worms. Therefore, we show correlation graphs between the harvested worm pair number (= the number of the fewer sex in each mouse) and arthritic index (Fig. 1B) in each mouse. As clearly demonstrated in the figure, both the arthritis score (a) and the number of arthritic paws (b) are negatively correlated with pair numbers. According to the graphs, infection by four to five pairs is enough to prevent the onset of arthritis.

3.3. Schistosoma mansoni infection prevents histopathological changes of CIA

Fig. 2 shows the histopathology of representative ankle joints of hind paws from an uninfected IIC-immunized mouse (Fig. 2A) or an infected IIC-immunized mouse (Fig. 2B). As indicated by arrowheads and arrows (Fig. 2Ab), the uninfected mouse joint showed marked synovial hyperplasia, inflammatory cell influx and destruction of bone and cartilage. In contrast, the infected mouse joint had no apparent inflammatory changes.

3.4. Anti-IIC IgG levels are lowered in S. mansoni-infected mice

It is well established that anti-IIC IgG is involved in the pathogenesis of collagen-induced arthritis (Terato et al., 1992; Nandakumar

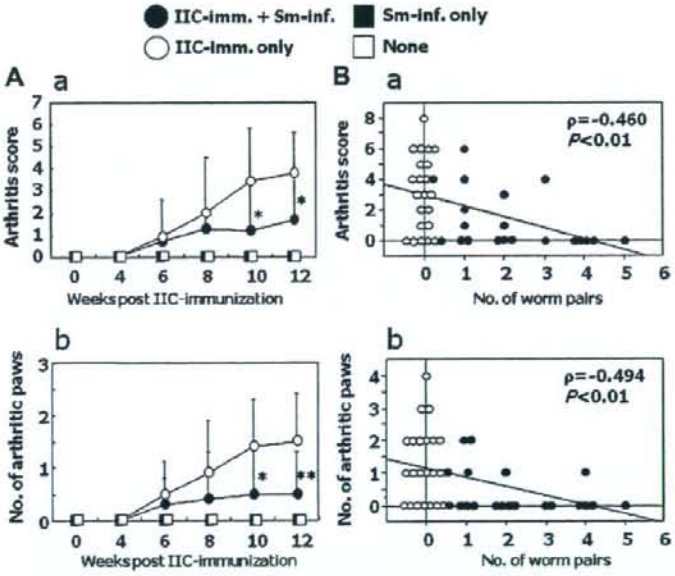

Fig. 1. Effects of *Schistosoma mansoni* (Sm) infection on development of collagen-induced arthritis (CIA) in DBA/1 mice. Mice were infected with Sm 2 weeks prior to type-II collagen (IIC) immunization. (A) Closed symbols and open symbols represent Sm-infected mice and uninfected mice, respectively. Circles and rectangles represent IIC-immunized mice and non-immunized mice, respectively. Data are expressed as means ± SD of total scores for four limbs (a) or total number of arthritic paws (≥2 mm) in each mouse (b). Asterisks * and ** represent $P < 0.05$ and $P < 0.01$, respectively (Mann–Whitney *U* test). Combined results of two independent experiments are shown. (B) Correlations between infecting worm pair numbers and arthritis severity indexes. Individual mouse data was from the IIC-immunized/Sm-infected group (closed circles) and the IIC-immunized/uninfected group (open circles). Data was analyzed by Spearman's Rank Correlation Test. Combined results of four independent experiments are shown. Sm, *Schistosoma mansoni*; IIC, type II collagen.

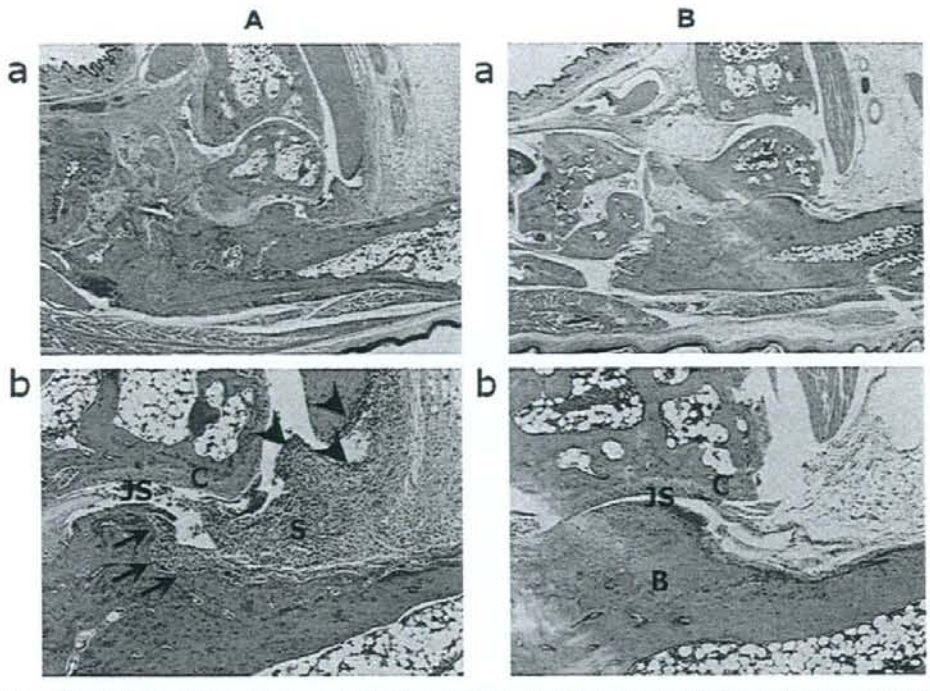

Fig. 2. Suppression of histopathology in the representative right ankle joints of infected mice at 12 weeks post-immunization. (A) Type-II collargen (IIC)-immunized/uninfected mouse. Note the presence of severe synovitis and bone erosion. Arrows indicate an area of cartilage and bone (calcaneus) destruction, and arrowheads indicate an area of synovial hyperplasia with a prominent influx of inflammatory cells. (B) IIC-immunized/*Schistosoma mansoni*-infected mouse. Note the marked reduction of cell influx. Neither severe synovitis nor synovial hyperplasia was observed. *Abbreviations:* B, bone (calcaneus); JS, joint space; C, cartilage; S, synovitis. Original magnification: 40× (a) and 100× (b).

et al., 2003). We compared IgG and IgG2a levels in the infected mice with those in uninfected control mice. As shown in Fig. 3, *S. mansoni* infection affected neither IgG nor IgG2a levels at 4 weeks p.i. However, at 8 and 12 weeks p.i., both IgG (Fig. 3A) and IgG2a (Fig. 3B) levels were lower in the infected mice than in the uninfected mice ($P < 0.05$ and $P < 0.01$, respectively). We did not observe anti-IIC IgG production in the non-immunized mice.

3.5. Cytokine pattern changes from "pro-inflammatory" to "anti-inflammatory" in the infected mice

To determine the systemic influence of *S. mansoni* infection on cytokine production potential, we compared cytokines in spleen cell culture supernatants from infected IIC-immunized mice with those from uninfected IIC-immunized mice. As shown in Fig. 4 (A

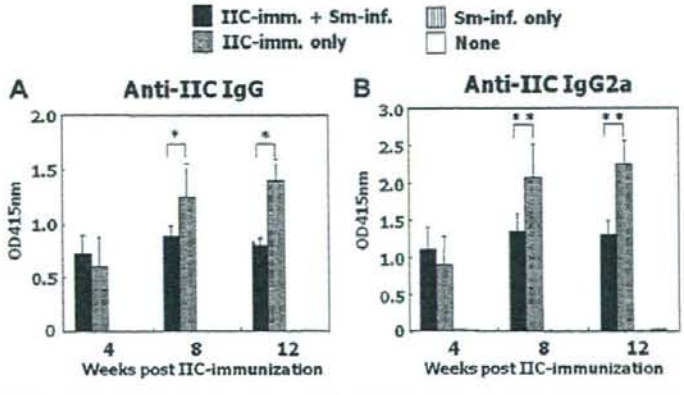

Fig. 3. Effects of *Schistosoma mansoni* (Sm) infection on anti-type-II collagen (anti-IIC) antibody production. Closed bars, gray bars, vertical lined bars and open bars represent IIC-immunized/Sm-infected mice, Sm-infected mice, IIC-immunized mice and untreated mice, respectively. Means ± SD (of OD_{415}) data for each mouse are shown. Asterisks * and ** represent $P < 0.05$ and $P < 0.01$, respectively (Student's *t*-test). Representative data of four repeated experiments is shown. Sm, *Schistosoma mansoni*; IIC, type II collagen.

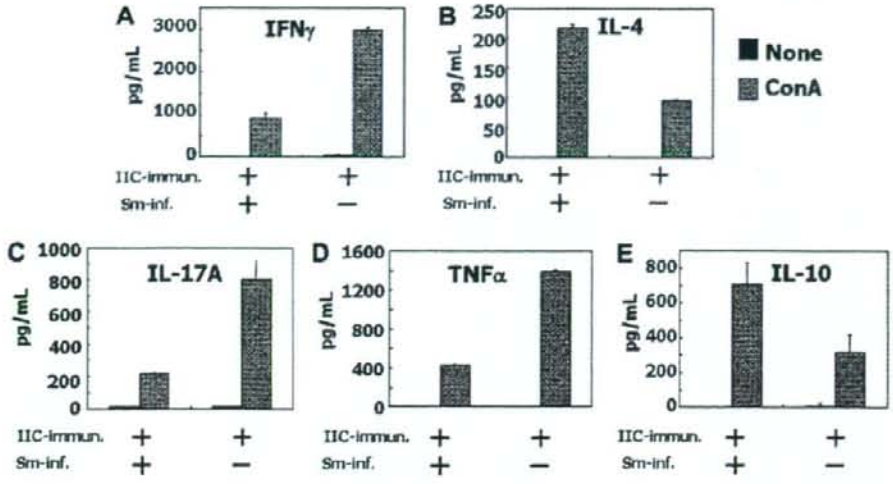

Fig. 4. Effects of *Schistosoma mansoni* (Sm) infection on cytokine production by spleen cells. Closed bars and gray bars represent culture supernatants with no stimulation and with ConA stimulation, respectively (A: IFNγ; B: IL-4; C: IL-17A; D: TNFα; E: IL-10). Data are expressed as means ± SD for duplicate cultures. Representative data of three repeated experiments is shown. Sm, *Schistosoma mansoni*; IIC, type II collagen.

and B), IFNγ production was lowered and IL-4 production was enhanced in *S. mansoni*-infected mice, indicating an apparent shift from Th1 to Th2-type responses. Here, moreover, we also observed an apparent shift in cytokine production from pro-inflammatory pattern to anti-inflammatory pattern. This is clearly demonstrated in Fig. 4 (C–E) which shows a reduction of IL-17A and TNFα, and an increase of IL-10 in the culture supernatant of the infected mice. Similar changes after *S. mansoni* infection were observed in non-immunized mice (data not shown).

3.6. *Schistosoma mansoni* infection abrogates augmentation of inflammatory mediators in IIC-immunized mouse paws

In addition to determining the cytokine profile of spleen cell cultures (= the systemic cytokine profile), it is also important to clarify changes of inflammatory mediators at the immediate sites of inflammation. Thus, we next measured the gene expression of pro/anti-inflammatory cytokines (IL1α, IL-1β, IL-6, TNFα, IL-10 and TGFβ), Foxp3 and RANKL in paws of IIC-immunized mice. As shown in Fig. 5B, D and E, IL-1β, IL-6 and RANKL levels were markedly increased in IIC-immunized uninfected mice compared with IIC-non-immunized uninfected control mice. Surprisingly, all of these increases were almost completely abrogated by *S. mansoni* infection. On the other hand, two other pro-inflammatory cytokines (IL1α and TNFα) were not augmented in their expression in the immunized mice and further *S. mansoni* infection did not influence their expression levels (Fig. 5A and C). In terms of suppressive factors, no enhancement of anti-inflammatory/regulatory cytokines (IL-10 and TGFβ) in *S. mansoni*-infected mice was observed (Fig. 5G and H). As for Foxp3, the key transcription factor of regulatory T cells, its expression was slightly increased in IIC-immunized mice and *S. mansoni* infection reversed the increase (Fig. 5F). Note that *S. mansoni* did not change the basal levels of any of the immune parameters in the non-immunized mice.

3.7. A single pair of worms is enough to abrogate cytokine induction in the paws

Having demonstrated the abrogation of pro-inflammatory cytokine up-regulation by *S. mansoni*, our next question was how many worm pairs were needed to suppress the up-regulation. Accordingly, we analyzed relationships between worm pair numbers and cytokine expression levels (Fig. 6). Surprisingly, as is clearly shown by the Fig. 6A–C, just one worm pair was enough to suppress IL-1β, IL-6 and RANKL.

4. Discussion

CIA is a classical but still widely used animal model of human RA (Stuart et al., 1982). Mouse CIA is usually induced by immunization of DBA/1 mice (H-2^q) with bovine IIC. The experimental model has been used to investigate the roles of various immune mediators in destructive arthritis. Here, we tested effects of *S. mansoni* infection on the progression of experimental arthritis.

We have demonstrated clear reduction of the severity of CIA by *S. mansoni* infection and negative correlations between the CIA severity indexes and worm pair numbers. An accidental single sex infection did not suppress arthritis in one of the repeated experiments (data not shown). Taken together, the number of eggs laid, which is proportional to worm pair numbers, seems to be important in the suppression of arthritis. An anti-arthritic effect of *S. mansoni* infection was also demonstrated by histopathological examination of the inflammatory paws, indicating that *S. mansoni* infection suppresses not only inflammation but also progression of the arthritis.

For the development of CIA, anti-IIC antibodies have been recognized as important pathogenic factors (Terato et al., 1992). Among anti-IIC antibodies, the IFNγ-dependent subclass IgG2a is arthritogenic as are other IgG subclasses (Terato et al., 1992; Nandakumar et al., 2003). As the Th1-type response is expected to be down-regulated in *S. mansoni*-infected mice (Grzych et al., 1991; Pearce et al., 1991), we measured levels of both IgG and IgG2a specific for IIC. At 8 and 12 weeks post-immunization, *S. mansoni* infection decreased the antibody levels. This reduction of IIC-specific antibodies may be at least partially related to the amelioration of arthritis by *S. mansoni* infection.

For a long time, CIA was thought to be a "Th1 type" autoimmunity (Mauri et al., 2003), however, the role of Th1-related cytokines (IL-12 and IFNγ) in the pathogenesis of CIA has been somewhat controversial. It was reported that IL-12 only exacerbated CIA early

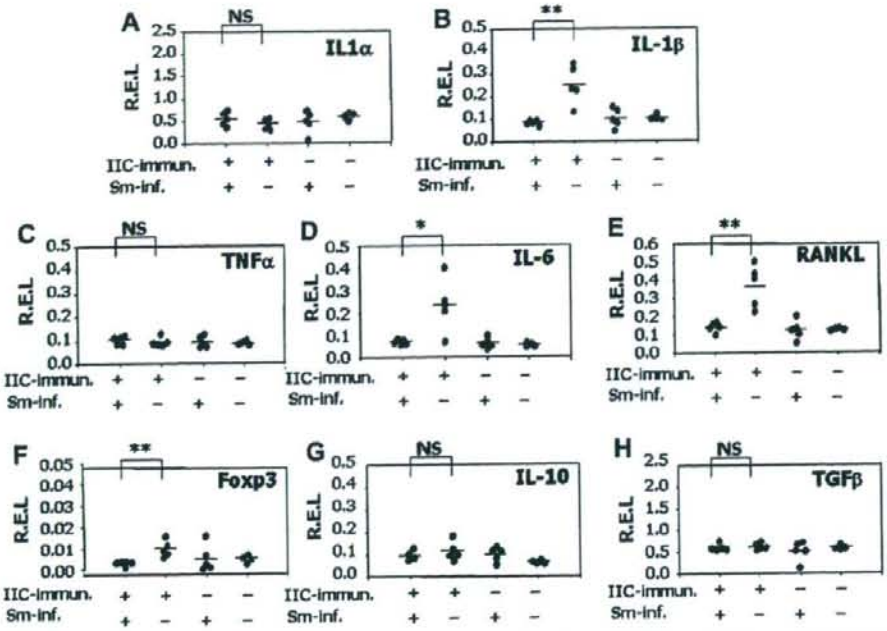

Fig. 5. Effects of *Schistosoma mansoni* (Sm) infection on immune mediator expression in the paws (A: IL-1α; B: IL-1β; C: TNFα; D: IL-6; E: RANKL; F: Foxp3; G: IL-10; H: TGFβ). Values along the vertical axis represent relative expression levels (REL) normalized with β-actin. Each dot represents an individual mouse. Means of each group are shown as short horizontal bars. Asterisks * and ** represent $P < 0.05$ and $P < 0.01$, respectively (Mann–Whitney U test). Representative data of three repeated experiments is shown. Sm, *Schistosoma mansoni*; IIC, type II collagen.

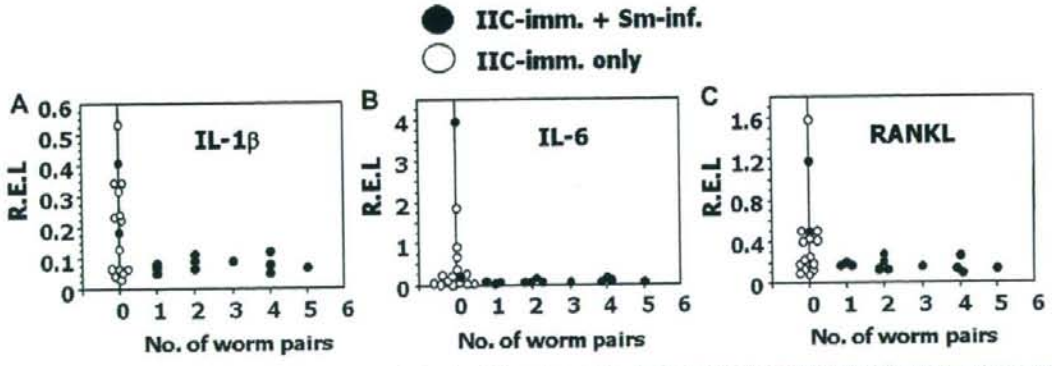

Fig. 6. Relationships between worm pair numbers and expression levels of pro-inflammatory mediators (A: IL-1β; B: IL-6; C: RANKL). Values along the vertical axis represent relative expression levels (REL) normalized with β-actin. Each dot represents an individual type-II collagen (IIC)-immunized mouse. Note that infection with only one pair of worms is enough to abrogate augmentation of cytokine expression. Combined results of three independent experiments are shown. Individual mouse data was from the IIC-immunized/Sm-infected group (closed circles) or the IIC-immunized/uninfected group (open circles). Sm, *Schistosoma mansoni*; IIC, type II collagen.

on and suppressed it at the established phase (Joosten et al., 1997). As for IFNγ, its role in CIA is more confusing (Rosloniec et al., 2002). Although there is a report of reduced severity in IFNγR gene-disrupted mice (Kageyama et al., 1998), another group reported that IFNγR gene-disrupted DBA/1 mice developed a more severe arthritis than wild-type controls (De Klerck et al., 2004). Moreover, C57BL/6 (usually a CIA-resistant strain) became susceptible to CIA when their IFNγ gene was disrupted (Chu et al., 2007). The regulatory role of IFNγ in CIA could be attributed to suppressive activity against osteoclast differentiation (De Klerck et al., 2004) and against IL-17 production (Chu et al., 2007), or to the up-regulation of regulatory T cell functions (Kelchtermans et al., 2005). Simultaneously, IFNγ can also act as an exacerbating factor through its pivotal role in pathogenic antibody production; e.g. anti-IIC IgG2a (Kageyama et al., 1998). In contrast, IL-4 is known to be an ameliorating factor for CIA (Joosten et al., 1999) as is IL-10 (Finnegan et al., 2003). Our observation of IFNγ and IL-4 from ConA-stimulated spleen cells is consistent with previous reports that showed a "Th1 to Th2" shift by schistosomes (Grzych et al., 1991; Pearce et al., 1991). As noted above, IFNγ can act as either an ameliorating

factor or an exacerbating factor for CIA. To determine overall effects of the parasite-induced down-regulation of IFNγ on CIA, additional experiments using IFNγ KO mice (DBA/1 strain) may be necessary. On the other hand, IL-4 up-regulation with the infection is expected to support the amelioration of CIA.

Recently, the importance of a newly identified IL-17-producing pathogenic T cell subset (Th17) (Harrington et al., 2005) has been emphasized in the pathogenesis of various autoimmune diseases (Langrish et al., 2005; Komiyama et al., 2006; Sato et al., 2006). This novel T helper subset is characterized by the production of certain cytokines: i.e., IL-17, TNFα, IL-6, etc. (Langrish et al., 2005). Based on these background studies, evidence of pivotal roles of IL-17 in the pathogenesis of autoimmune arthritis has accumulated (Kotake et al., 1999; Nakae et al., 2003; Lubberts et al., 2003, 2004). Now, taken together with the paradoxical roles of IL-12 and IFNγ, CIA as a model of RA should be considered as "Th17 disease" rather than "Th1 disease".

In RA and CIA, TNFα, IL-1 and IL-6 are important pro-inflammatory cytokines (Joosten et al., 1996; Takagi et al., 1998; Williams et al., 2000; Lee et al., 2004; Kavanaugh, 2007) which accelerate osteoclastogenesis. At present, anti-TNFα monoclonal antibody (infliximab), which has anti-osteoclastogenic effects, is in use for the clinical treatment of RA (Lee et al., 2004) and anti-IL-6R monoclonal antibody (tocilizumab) is under clinical development (Kavanaugh, 2007). In our study, it is reasonable to speculate that the *S. mansoni*-induced down-regulation of pro-inflammatory cytokines (IL-17 and TNFα) and up-regulation of IL-10 crucially affects the cytokine microenvironment in peripheral inflammation.

Based on the observation of cytokine patterns in spleen, we next examined the effects of *S. mansoni* infection on the local expression of cytokines. IL-1β and IL-6 expression were markedly augmented in IIC-immunized mice and the augmentation was abrogated by *S. mansoni* infection. Moreover, a similarly augmented expression of RANKL (Lubberts et al., 2002), essential for osteoclast development, was also reduced in *S. mansoni*-infected mice. In the same experiment, unexpectedly, we did not observe an increase of TNFα and IL-1α levels in IIC-immunized mouse paws. However, this result is consistent with a previous report showing that TNFα production was slight compared with IL-1β and IL-6 production in inflammatory joints of CIA (Rioja et al., 2004). As TNFα is an important cytokine for induction of local IL-1 expression (Joosten et al., 1996; Williams et al., 2000), it is highly probable that *S. mansoni*-induced systemic down-regulation of TNFα-producing potential of T cells affected the local expression of IL-1β, which is the dominant isoform in mice (Joosten et al., 1996; Williams et al., 2000).

As for main Th cytokines (IFNγ, IL-4 and IL-17), their expressions were under detectable levels in the paws. This is probably because of a small number of T cells in the peripheral tissue. Further experiments (T cell isolation from the tissue etc.) may be necessary to know the change of local Th cytokine expression.

The severity of CIA and RA is under the control of $CD4^+CD25^+$ regulatory T cells (Treg) (Kelchtermans et al., 2005; Morgan et al., 2005; van Amelsfort et al., 2007). In addition, TGFβ is involved in regulation of the disease (Marinova-Mutafchieva et al., 2006). Our study results on TGFβ and Foxp3 expression in the paws implied Treg cells did not accumulate or proliferate in local inflamed tissue. As schistosome eggs are known to activate Treg cells (Mo et al., 2007; Yang et al., 2007), such activated Treg cells may exert their regulatory roles by changing the systemic immunological environment rather than through direct regulation at local inflammation sites.

It is also noteworthy that a single pair of worms is enough to abrogate local augmentation of IL-1β, IL-6 and RANKL. However, in our experiments some infected mice still showed arthritis symptoms. This discrepancy might be explained by the presence of reduced but significant amounts of (pathogenic) anti-IIC antibodies in the infected mouse sera.

There are several reports of inhibition of experimental arthritis or osteoclastogeneis by parasites. A filarial nematode-derived excretory secretory product ES-62 reduced the severity of CIA in mice (McInnes et al., 2003). An ES product from *Spirometra erinaceieuropaei* plerocercoids was reported to suppress RANKL-induced osteoclastogenesis (Kina et al., 2005). Protective effect of *Ascaris suum* extract in arthritis models was also reported recently (Rocha et al., 2008). As for protozoa parasites, *Trypanosoma brucei* infection was shown to suppress CIA in rats (Mattsson et al., 2000). Taken together with our results, parasites may be a promising source of anti-inflammatory biological drugs.

In conclusion, *S. mansoni* infection suppresses the IL-17-producing potential of spleen cells and abrogates RANKL expression in inflamed paws. To our knowledge, this is the first report of an anti-Th17 effect and RANKL-down-regulating effect of a parasite. Further investigations are needed to identify essential molecules causing these phenomena, for the future development of anti-arthritic drugs from parasite-derived substances.

Acknowledgements

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Malaria Parasites Require TLR9 Signaling for Immune Evasion by Activating Regulatory T Cells[1]

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Malaria is still a life-threatening infectious disease that continues to produce 2 million deaths annually. Malaria parasites have acquired immune escape mechanisms and prevent the development of sterile immunity. Regulatory T cells (Tregs) have been reported to contribute to immune evasion during malaria in mice and humans, suggesting that activating Tregs is one of the mechanisms by which malaria parasites subvert host immune systems. However, little is known about how these parasites activate Tregs. We herein show that TLR9 signaling to dendritic cells (DCs) is crucial for activation of Tregs. Infection of mice with the rodent malaria parasite *Plasmodium yoelii* activates Tregs, leading to enhancement of their suppressive function. In vitro activation of Tregs requires the interaction of DCs with parasites in a TLR9-dependent manner. Furthermore, TLR9$^{-/-}$ mice are partially resistant to lethal infection, and this is associated with impaired activation of Tregs and subsequent development of effector T cells. Thus, malaria parasites require TLR9 to activate Tregs for immune escape. *The Journal of Immunology*, **2008, 180: 2496–2503.**

Malaria caused by protozoan parasites of the genus *Plasmodium* is still one of the most life-threatening infectious diseases. Approximately 40% of people worldwide reside in areas at risk of malaria. Three hundred million people are infected every year and 2 million die. Generation of effector T cells is crucial for the development of protective immunity against malaria. Ingenious strategies for immune escape by malaria parasites, including antigenic diversity (1), clonal antigenic variation (2), and impairment of dendritic cell (DC)[4] maturation (3), prevent the development of sterile immunity, resulting in repeated symptomatic infections throughout the life of the host. Therefore, an understanding of this evasion mechanism is important for the effective control of malaria.

CD4$^+$CD25$^+$ regulatory T cells (Tregs) contribute to the maintenance of self-tolerance by suppressing autoreactive T cells in the periphery (4, 5). Recently, Tregs were reported to play pivotal roles in infectious diseases as well as in the suppression of autoimmunity. For instance, Tregs suppress harmful immune pathogenesis caused by infection (6), and they contribute to the establishment of chronic infection instead of the elimination of pathogens, thus maintaining exposure of memory T cells to microbial Ags (7). With use of a murine model, we and others have demonstrated that the immune escape of malaria parasites requires activation of Tregs (8, 9). Furthermore, Walther and colleagues found that up-regulation of Tregs correlates with rapid parasite growth during human malaria infection (10). These results suggest that activation of Tregs is a central mechanism by which malaria parasites subvert host immune systems. However, it remains to be elucidated how malaria parasites activate Tregs.

Several lines of evidence for the functional regulation of Tregs have been accumulated. IL-2 is crucial for maintaining Tregs in the peripheral pool of T cells (11). TGF-β maintains the suppressive functions of Tregs (12). DCs also contribute to controlling Treg cell functions by supporting the expansion of functional Tregs in an Ag-specific manner (13), and immature DCs selectively induce Tregs (14). TLR signaling in DCs blocks Treg-mediated suppression by affecting effector T cells (15) and reverses suppression of Tregs (16). Some TLRs are expressed on Tregs themselves, and these receptors are also involved in both positive and negative regulation of Treg functions (17–20). Thus, the involvement of TLRs expressed on DCs or Tregs in the regulation of Treg function appears to be controversial.

We herein show that the interaction of malaria parasites with DCs through TLR9 is required for the activation of Tregs in vitro. Additionally, infection of TLR9$^{-/-}$ mice with malaria parasites failed to activate Tregs, resulting in the activation of effector T cells. These mice showed partial resistance to infection. Our results suggest a novel mechanism for immune evasion by malaria parasites; that is, Treg activation by DCs stimulated via TLR9.

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[4] Abbreviations used in this paper: DC, dendritic cell; pDC, plasmacytoid DC; PDCA1, anti-plasmacytoid DC Ag-1; pRBC, parasitized RBC; PyL, *Plasmodium yoelii* 17XL strain; Treg, regulatory T cell; TRIF, Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β.

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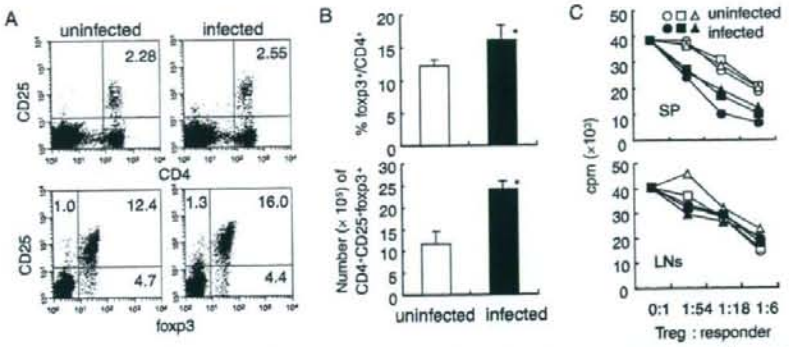

FIGURE 1. Activation of Tregs during *P. yoelii* infection. *A*, Flow cytometric analysis of Foxp3 expression in $CD4^+CD25^+$ T cells. Splenocytes obtained from uninfected (*left panels*) and PyL-infected (*right panels*) mice were stained with fluorescence-conjugated anti-CD4, anti-CD25, and anti-Foxp3 mAbs. Gated $CD4^+$ cells were separated on the basis of Foxp3 and CD25 expression (*bottom panels*). The numbers represent the percentages of all cells in each of the quadrants. The results are representative of five repeated experiments. *B*, The percentages of $Foxp3^+CD25^+$ cells among $CD4^+$ T cells (*upper panel*) and the absolute number of $CD4^+CD25^+Foxp3^+$ cells (*bottom panel*) in the spleens of uninfected (open bars) and PyL-infected (filled bars) mice were quantified. Values are means ± SD of six mice. Asterisks indicate statistical significance at $p < 0.05$ with the Student *t* test. *C*, Suppressive function of Tregs in mice infected with PyL. $CD4^+CD25^-$ T cells (1×10^5) purified from uninfected mice were stimulated with anti-CD3 mAb in the presence of T cell-depleted spleen cells as APCs, and they were mixed with splenic Tregs (*upper panel*) or Tregs from lymph nodes (*bottom panel*) obtained from uninfected (open symbols) or PyL-infected (filled symbols) mice at the indicated ratio. The proliferation of $CD4^+CD25^-$ T cells was measured by [^{3}H]thymidine incorporation. Values are means of triplicate cultures, and SD was <10% of the mean value. Each symbol represents results from an individual mouse. Splenic Tregs from infected mice suppressed significantly more than did those from uninfected mice ($p < 0.01$ with the unpaired Student *t* test). The results are representative of six repeated experiments.

Materials and Methods

Mice and parasites

C57BL/6 mice were purchased from Kyudo; *RAG2*$^{-/-}$ mice were from the Central Laboratory of Experimental Animals (Kawasaki, Japan); Ly5.1C57BL/6 mice were from the Sankyo Lab Service under permission of Dr. H. Nakauchi (Tokyo University), OT-II mice were provided by Dr. K. Yui (Nagasaki University); and TRIF-, MyD88-, TLR7-, and TLR9-deficient mice on C57BL/6 background were generated as previously described (21–24). TLR9-deficient mice had been backcrossed for at least 15 generations. Age- and sex-matched groups of wild-type and mutant mice were used for experiments. All experiments using mice were reviewed by the Committee for the Ethics on Animal Experiment in the Faculty of Medicine, and conducted under the control of the Guidelines for Animal Experiment in the Faculty of Medicine, Kyushu University, and the Law (no. 105) and Notification (no. 6) of the government of Japan. Blood-stage parasites of PyL were obtained after fresh passage through a donor mouse 2–3 days after inoculation with a frozen stock. Parasitized RBCs (pRBCs) were prepared as previously described (3) and used as a stimulant. Mice were infected with 10,000 to 15,000 pRBCs i.p.

Reagents

PE-anti-CD25 (PC61.5), allophycocyanin-anti-CD4 (RM4.4), and FITC-anti-Foxp3 (FJK-16s) staining kits, and FITC-anti-CD11c (N418), PE-anti-B220 (RA3–6B2), allophycocyanin-anti-CD86, PE-Cy5.5-anti-CD40, FITC-anti-CD69, FITC-anti-CD62L, PE-anti-IFN-γ, purified anti-CD3 (2C11), purified anti-CD16/32 (2.4G2), and purified anti-MHC class II (M5/114.15.2) Abs were obtained from eBioscience. The $CD4^+$ T cell separation kit, plasmacytoid DC (pDC) isolation kit, and anti-plasmacytoid DC Ag-1 (PDCA1), anti-PE, and anti-FITC microbeads (Miltenyi Biotec) were used for cell purification. mAbs to CD4 (GK1.5) or to IFN-γ (R4–6A2), purified from the ascites of hybridoma-injected athymic nude mice, were used for in vivo treatments.

Flow cytometry

For Treg cell analyses, cells in a single suspension were stained with allophycocyanin-anti-CD4 and PE-anti-CD25 followed by intracellular staining with FITC-anti-Foxp3 according to the manufacturer's protocol. Stained cells were analyzed by FACSCalibur (BD Biosciences) and the list data were analyzed using CellQuest Pro software (BD Biosciences).

Cell purification and cultures

Single-cell suspensions were prepared from spleens or lymph nodes. To purify Tregs, $CD4^+$ T cells were first negatively isolated using a $CD4^+$ T cell separation kit. Then, $CD4^+$ cells were stained with PE-anti-CD25 and labeled with anti-PE microbeads. Positively selected cells were used as Tregs, and others were used as $CD4^+CD25^-$ cells. For purification of DCs, splenic single-cell suspensions prepared using collagenase and DNase I were incubated with anti-CD16/32 and then stained with FITC-anti-CD11c, followed by staining with anti-FITC microbeads. pDCs were purified using a pDC isolation kit with slight modifications. After negative isolation of DCs (whole), pDCs were purified using anti-PDCA1 microbeads instead of PE-B220 Ab. The purity of the separated cell subset usually exceeded 92%. T cell-depleted spleen cells of uninfected mice after removal of $CD4^+$ and $CD8^+$ cells were used as APCs.

For activation of Tregs, typically 1.5×10^5 purified Tregs were cultured with 1.5×10^4 DCs and 2×10^5 pRBCs for 60 h. Then, Tregs were isolated as live cells. To analyze Treg function, purified $CD4^+CD25^-$ cells from uninfected mice stimulated with soluble anti-CD3 Ab or ConA (both 2.5 μg/ml), in the presence of APCs, were cultured with a variety of freshly isolated or cultured Tregs in 200 μl of media (for 72 h) and incubated with 1 μCi/well [^{3}H]thymidine for the last 6–8 h. Radioactivity was measured using a liquid scintillation counter.

In vivo depletion of $CD4^+$ T cells and neutralization of IFN-γ

To deplete $CD4^+$ T cells in vivo, mice were injected i.p. with 250 μg of anti-CD4 Ab 3 days and 1 day before infection. Depletion of $CD4^+$ T cells was evaluated using peripheral blood, from which >95% of $CD4^+$ T cells were depleted. To neutralize IFN-γ, mice were infected with 200 μg of anti-IFN-γ Ab 1 day before and 1 day after infection.

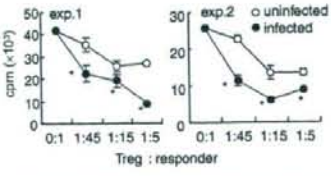

FIGURE 2. No lymphocytes other than Tregs are required for the activation of Tregs. $CD4^+$ cells purified 5 days after infection with PyL (●) from $RAG2^{-/-}$ mice that had received 1×10^6 Tregs a day before infection were analyzed for suppressive function as described in Fig. 1*C*. Those from uninfected recipients were also analyzed (○). The results of two separate experiments are shown.

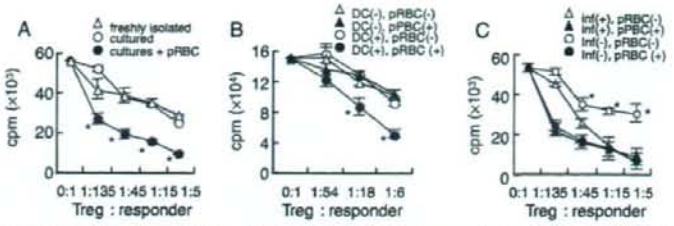

FIGURE 3. Requirement of an interaction between pRBC and DCs to activate Tregs. Suppressive functions of $CD4^+CD25^+$ cells from uninfected mice cultured with DCs were analyzed as in Fig. 1*A*, using ConA instead of anti-CD3. *A*, Tregs cultured with (●) or without (○) pRBC in the presence of DCs were analyzed for their suppressive function. Freshly isolated Tregs (△) were also used. *B*, Tregs cultured with (circles) or without (triangles) DCs in the absence (open symbols) or presence (filled symbols) of pRBCs were analyzed for their suppressive activity. *C*, Tregs cultured with DCs from uninfected (circles) or PyL-infected (triangles) mice in the absence (open symbols) or presence (filled symbols) of pRBCs were analyzed. DCs were collected 5 days after infection. Values are means ± SD of triplicate cultures. Asterisks indicate statistical significance at $p < 0.05$ with the Student *t* test. These in vitro experiments were repeated at least four times.

Statistical analyses

Differences between groups were analyzed for statistical significance using Excel software with two-tailed the unpaired Student *t* tests. For survival curves, Kaplan-Meier plots and χ^2 tests were performed. Probability below 0.05 was considered to be statistically significant.

Results

Infection with malaria parasites activates Tregs

Plasmodium yoelii 17XL strain (PyL), a rodent malaria parasite, is highly virulent in mice and causes lethal infection. We previously reported that high susceptibility to this parasite correlates with severe immune suppression induced by activation of Tregs, and that depletion of Tregs before infection made mice resistant to the otherwise lethal infection; this resistance was associated with a reversal of T cell unresponsiveness against the parasite (8). As early as 5 days after infection with PyL, when parasites began to be detected in the circulation, the proportion of $CD4^+CD25^+$ T cells increased in the spleen (Fig. 1*A*). Because CD25 is not a specific marker for Tregs and is expressed in activated non-Treg T cells, we analyzed the expression of Foxp3. Foxp3 is a forkhead/winged-helix transcription factor specifically expressed in Tregs, and its expression is associated with the development and function of Tregs (25, 26). At this time point, most $CD4^+CD25^+$ T cells in the spleens of both PyL-infected and uninfected mice were $Foxp3^+$ (Fig. 1*A*). The percentage of $CD25^-Foxp3^+$ cells among $CD4^+$ T cells and the total number of splenic $Foxp3^+CD4^+CD25^+$ cells in PyL-infected mice were significantly increased after infection (Fig. 1*B*). We next evaluated the suppressive function of Tregs by determining the degree of suppression of TCR-triggered T cell proliferation. Purified $CD4^+CD25^+$ T cells obtained from PyL-infected mice were mixed with $CD4^+CD25^-$ T cells obtained from uninfected mice stimulated by TCR engagement in the presence of APCs. $CD4^+CD25^+$ T cells from infected mice showed remarkable suppressive activity compared with those from uninfected mice (Fig. 1*C*). Such alterations in suppressive function were not observed in Tregs from mesenteric lymph nodes, in which no parasite was detected during infection (Fig. 1*C*), suggesting that Treg activation occurs after intimate contact with parasites.

Malaria parasites interact with DCs to activate Tregs

We next analyzed how Tregs are activated during malaria infection. Some cytokines secreted by lymphocytes are reported to be important for Treg activities. To determine the cellular requirements for Treg activation, $RAG2^{-/-}$ mice received 1×10^6 Tregs from syngeneic mice, and they were then infected with PyL. Because CD25 expressed by Tregs disappears in the inflammatory environment in lymphopenic hosts (7), $CD4^+$ cells were purified. Approximately 1×10^5 and 1.4×10^5 cells were recovered from uninfected and PyL-infected mice, respectively (not significant). Tregs recovered from uninfected mice still suppressed TCR-triggered T cell proliferation in a dose-dependent manner. Infection of recipient mice with PyL significantly enhanced this suppressive function, as observed in immunocompetent mice (Fig. 2), indicating that no lymphocytes other than Tregs are required for the activation of this function of Tregs. These results suggest that the interactions between Tregs, APCs, and pRBCs are sufficient for Treg activation.

To evaluate this possibility, we tried to reproduce Treg activation in vitro. Tregs from uninfected mice cultured with pRBCs and $CD11c^+$ splenic DCs, as APCs, were analyzed for a suppressive function. There was no evidence for Treg proliferation, even in the presence of pRBCs, as determined by the incorporation of [^{3}H]thymidine or CFSE dilution (data not shown). Recovered Tregs maintained their suppressive function at a comparable level to freshly isolated Tregs. The addition of pRBCs promoted the suppressive activity of Tregs (Fig. 3*A*). This enhancement was associated with parasites, because the addition of normal RBCs did not enhance Treg activities (data not shown). Thus, an in vitro system can

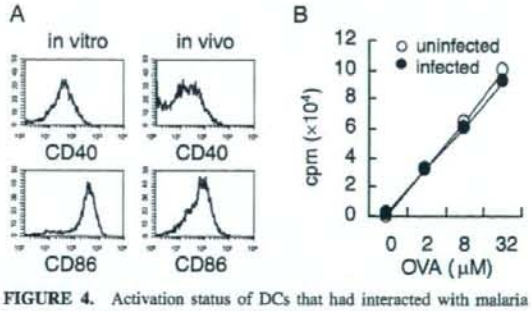

FIGURE 4. Activation status of DCs that had interacted with malaria parasites. *A*, DCs cultured with pRBCs (*left panels*) and splenic DCs from PyL-infected mice (*right panels*) were analyzed for the expression of CD40 and CD86. The expression profiles of DCs cultured with pRBCs or DCs from infected mice (solid lines) were plotted against that of DCs cultured without pRBCs or that of DCs from uninfected mice (shaded areas), respectively. *B*, $CD4^+CD25^-$ cells from OT-II mice were stimulated with the indicated amount of OVA in the presence of splenic DCs from mice infected with PyL (●) or with DCs from uninfected mice (○). The proliferation of OT-II T cells was measured by [^{3}H]thymidine incorporation. The results represent the means of triplicate cultures. SDs were <5% of the mean.

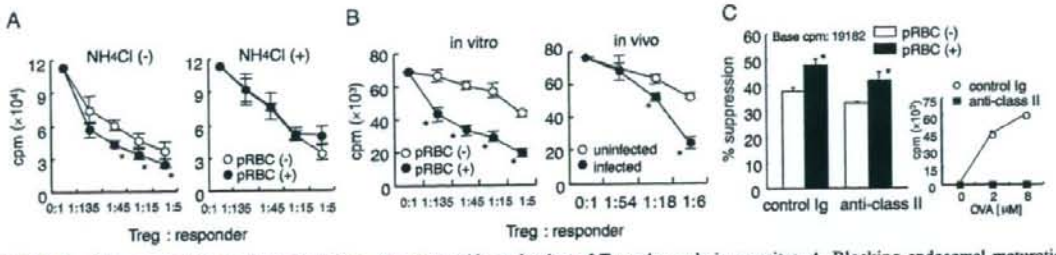

FIGURE 5. Phago-endocytic pathway-dependent, Ag-nonspecific activation of Tregs by malaria parasites. *A*, Blocking endosomal maturation inhibits Treg activation. Tregs and DCs obtained from uninfected mice were cultured in the absence (*left panel*) or presence (*right panel*) of NH_4Cl (250 μg/ml) and analyzed as in Fig. 3*A*. *B*, $CD4^+CD25^+$ cells from OT-II mice cultured with DCs and pRBCs (*left panel*) or those from OT-II mice infected with PyL (*right panel*) were analyzed for suppressive activity as in Fig. 3*A*. *C*, Treg activation with pRBCs was performed in the presence of anti-class II Ab (1 μg/ml). Suppressive activity was evaluated as the ratio of Tregs to $CD4^+CD25^-$ cells (1:5). Percentage suppression was calculated as (1 − experimental cpm/base cpm (cpm without Tregs)) × 100. Open and filled columns represent suppressive activity in the absence or presence of pRBCs, respectively. $CD4^+CD25^-$ cells from OT-II mice were cultured with OVA at the indicated concentration in the presence of anti-class II Ab (*inset*).

reproduce Treg activation during infection in vivo. Using this system, further analyses were performed. First, to pinpoint the cellular interactions of pRBCs with Tregs and DCs, Tregs were stimulated with pRBCs in the absence of DCs. The addition of pRBCs did not augment Treg function in this setting (Fig. 3*B*). Furthermore, DCs from PyL-infected mice activated Tregs, even in the absence of pRBCs (Fig. 3*C*). These results demonstrate that malaria parasites interact with DCs to activate Tregs. Malaria parasites have been reported to inhibit the maturation of DCs (3), and immature DCs preferentially activate Tregs (13), suggesting that PyL suppresses DC maturation. Thus, we checked the status of pRBC-interacting DCs capable of activating Tregs. The exposure of DCs to malaria parasites slightly enhanced the expression of CD40 and CD86, both in vitro and in vivo (Fig. 4*A*). Moreover, the Ag processing/presenting capacity of these DCs to activate OVA-specific OT-II $CD4^+$ T cells was identical with that of untreated DCs (Fig. 4*B*). These results exclude the possibility that malaria parasites down-regulate DC activities.

TLR9 signaling in DCs is required for Treg activation

Parasite-derived molecules usually contact DCs after being processed in phago-endosomes (27). In contrast, pRBCs express some molecules derived from the parasites on their surfaces, and this enables parasites to interact with the surfaces of DCs without phagocytosis (3). We next examined whether endocytic pathways are required to make DCs competent for Treg activation. Inhibition of endosomal maturation with ammonium chloride, a reagent that blocks endosomal acidification, precluded enhancement of the suppressive function of Tregs (Fig. 5*A*).

Foreign Ags phagocytosed by DCs are proteolytically processed and undergo Ag presentation to MHC class II molecules (28). The necessity for engulfment of pRBCs by DCs for Treg activation might be explained by the ability of DCs to activate Ag-specific Tregs. To address this issue, we used Tregs isolated from OVA-specific TCR-transgenic OT-II mice (29). Stimulation of these Tregs with pRBCs resulted in an enhancement of their suppressive function. Additionally, infection of OT-II mice with PyL also activated Tregs (Fig. 5*B*). We could not exclude the possibility of an involvement of remnant Tregs with non-OT-II TCR in the enhancement of Treg function, because RAG-deficient OT-II mice could not be used owing to requirement of TCR rearrangement for the development of Tregs (30). To further confirm Ag-nonspecific Treg activation, we used an anti-MHC class II Ab in Treg cultures with pRBCs. The enhancement of the suppressive function of Tregs by adding pRBCs was not altered, even in the presence of the anti-class II Ab, which completely blocked the OVA-specific proliferation of $CD4^+CD25^-$ cells isolated from OT-II mice (Fig. 5*C*).

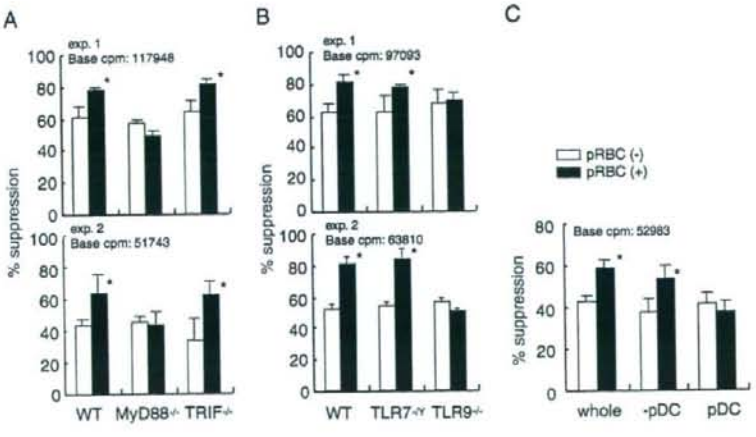

FIGURE 6. Essential role of TLR9 signaling in DCs for the activation of Tregs. $CD4^+CD25^+$ cells from uninfected WT mice were cultured with pRBCs in the presence of DCs from the indicated mutant mice (*A* and *B*) or with the indicated DCs from WT mice (*C*). These cells were then evaluated as in Fig. 5*C*. Values are means ± SD of triplicate cultures. Asterisks indicate statistical significance at $p < 0.05$ with the Student *t* test. The results of two separate experiments of four experiments are shown.