**Fig. 1** Amino sequence of HlSC-1 aligned with those of other members of the cystatin superfamily. Hlcyst-1 (GenBank accession no. ABZ89553), *H. longicornis* family 1 cystatin; Hlcyst-2 (ABC94582) and -3 (ABZ89554), *H. longicornis* family 2 cystatin; sialostatin L (AAM93646) and sialostatin L2 (AAY66685), *I. ricinus* family 2 cystatin; omcystat 1 (AAS01021) and omcystat 2 (AAS55948), *O. moubata* family 2 cystatin; cystatin C (NP_990831), *Gallus gallus*. The evolutionarily conserved papain binding sites (PI-III) are *boxed*. Similar amino acids are *shaded black*

```
                         P-I
HlSC-1          1   ----ARLVGGWR-HQNPLSNPRFFELAHFALSGENK---GHGIPGAAIKL   42
Hlcyst-1        1   ----MPMVGGLS-EEVKDADDTVREICEKVREEVEA---KLGKKFCEFTP   42
Hlcyst-2        1   -SHPKRLIGGWT-QHDPSSNPKYLELAHFAISQQTK---GLDVYHTVLKL   45
Hlcyst-3        1   -----SIPGGWR-DQDPQSSPKYKNLAHYAVAQRVE---GLDNYDTVLDL   41
sialostatin L   1   ----TGVFGGYSERANHQANPEFLNLAHYATSTWSAQQPGKTHFDTVAEV   46
sialostatin L2  1   ---ELALRGGYRERSN-QDDPEYLELAHYATSTWSAQQPGKTHFDTVVEV   46
omcystat 1      1   ---QRGFVGGWS-QVDPKIRPDLLELAHFAVASQTA---GLEYYHTVLEL   43
omcystat 2      1   ----TSIPGGWT-RQDP-TEARFLELAHFATSSQTE---GREFYDTVVTV   41
cystatin C      1   SEDRSRLLGAPVPVDE--NDEGLQRALQFAMAEYNRAS-NDKYSSRVVRV   47

                         P-II
HlSC-1          43  RSVATQVVAGIKYKIEFDLVPLVCSSNKVPRQLYDGSYRGYGSLLWVMGV   92
Hlcyst-1        43  LKYRTQLVNGVNYFIKVCVGES----EHVHVRAHKAFQG---------EIT   80
Hlcyst-2        46  VKVETQVVAGINYRVIFETAPTNCPVNE-KYSIENCKPT---------TNM   86
Hlcyst-3        42  TKVETQVVAGVNYRLTFTIAGSSCKIGEVEYTEESCPPT---------TTE   83
sialostatin L   47  VKVETQVVAGTNYRLTLKVAESTCELTS-TYNKDTCLPK---------ADA   87
sialostatin L2  47  LKVETQTVAGTNYRLTLKVAESTCELTS-TYNKDTCQAN---------ANA   87
omcystat 1      44  TKASCQVVAGVNYKLTLKVAPSKCKVSETVYSKELCQPQ---------LNA   85
omcystat 2      42  KEVETQVVAGMNYKLTIEISPSVCKIGEVQYSAEQCVPK---------DAQ   83
cystatin C      48  ISAKRQLVSGIKYILQVEIGRTTCPKSSGDLQSCEFHDE--------PEMA   90

                                P-III
HlSC-1          93  RMNTCTTVIYQVPWPAICKG---------   112
Hlcyst-1        81  FAAVQEGKALEEPLNHFQ-----------   98
Hlcyst-2        87  PSATCIATVYERPWENYRELTSFRCPR-   113
Hlcyst-3        84  AKATCTAVVYERPWENVRTLTSFDCV--   109
sialostatin L   88  AHRTCTTVVFEN-LQGDKSVSPFECEAA   114
sialostatin L2  88  AQRTCTTVIYRN-LQGEKSINSFECAAA   114
omcystat 1      86  APKDCEAQLYVVPWRNTKEVTSFECN--   111
omcystat 2      84  QKSTCVAVIYHVPWQNQKSVTSYRCE--   109
cystatin C      91  KYTTCTFVVYSIPWLNQIKLLESKCQ--   116
```

## Recombinant HlSC-1 is an active cystatin

To investigate the comparison of HlSC-1 with Hlcyst-1 and -2 for efficiency in inhibiting their overlapping target enzymes, purified recombinant cystatins were used to test the inhibitory activity against cysteine protease. The N-terminally (His)6-tagged version of HlSC-1 (62 kDa) was purified from *E. coli* lysate by resin. The (His)6-tag was selectively removed by thrombin treatment, resulting in a protein of 12 kDa (Fig. 4A). Hlcyst-1 effectively inhibited the enzyme activity of cathepsin L, cathepsin B, and papain. HlSC-1 and Hlcyst-2 also inhibited papain and cathepsin L, but inhibition of mammalian cathepsin B was not detected by our assays. The results are shown in Fig. 4B

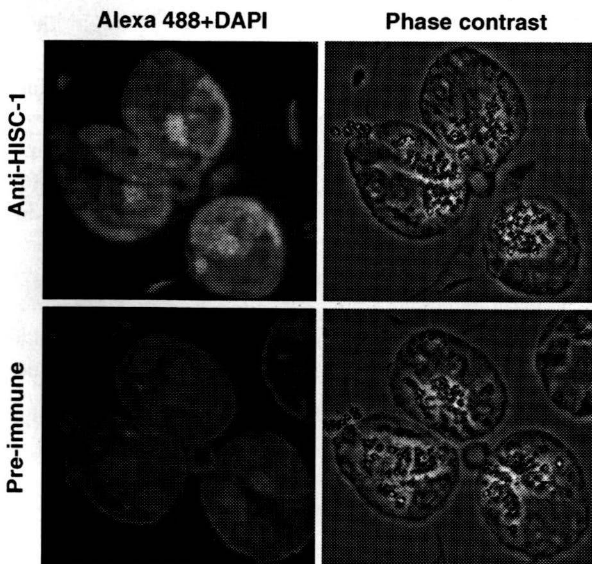


**Fig. 2** Localization of endogenous HlSC-1 immunofluorescent staining of salivary gland cells to detect intracellular localization of endogenous HlSC-1. The salivary gland cells of 48 h fed adult *H. longicornis* were fixed. The sections on glass slides of fixed salivary glands expressing HlSC-1 were treated with anti-HlSC-1 polyclonal antibody or pre-immune antibody and revealed with the secondary antibody Alexa Fluor 488 anti-mouse IgG (*green*). The nuclei were stained with 4′, 6-diamino-2-phenylindole (*blue*)

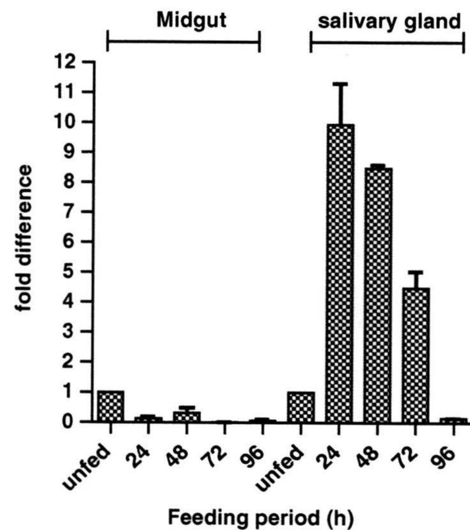


**Fig. 3** Quantitative RT-PCR analysis of *HlSC-1* regulation at different stages of blood feeding. Quantitative RT-PCR was performed on total RNA extracted from adult *H. longicornis* at different stages of feeding. *Bars* show the mean ± SD of relative mRNA copy numbers ($n$=4)

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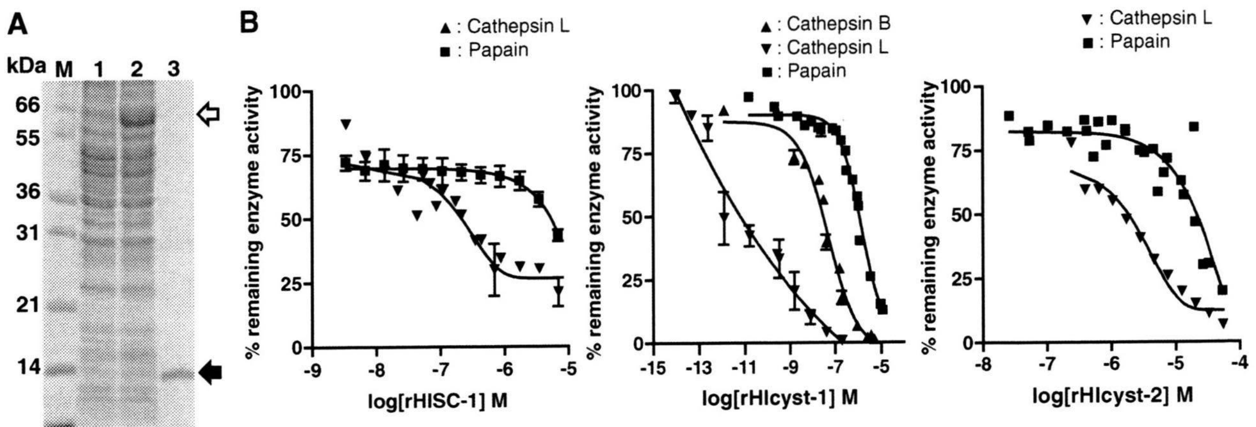


**Fig. 4** Expression and purification of the recombinant *H. longicornis* cystatin HlSC-1 and protease inhibition assays. **A** Protein samples were subjected to 12.5% SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue. *Lane 1* 5 μg of crude lysate of *E. coli* before induction. *Lane 2* 5 μg of crude lysate of *E. coli* incubated with 1 mM IPTG for 24 h. *Lane 3* 5 μg of purified recombinant HlSC-1. *White arrowhead* indicates recombinant His tag-HlSC-1, and the *black* one indicates purified recombinant HlSC-1. Positions and sizes of marker proteins are indicated on the *left*. **B** Inhibition of protease activities by HlSC-1. Purified cathepsin L, B, and papain were incubated with each of the substrates in the presence of different concentrations of HlSC-1. Incubation of cathepsins without HlSC-1 resulted in 100% enzyme activity

and are summarized in Table 1. HlSC-1 effectively inhibited the peptidase activity of mammalian cathepsin L in the nanomolar range ($IC_{50}$ values of 273.6±52.95 nM). The comparison assays of three inhibitors showed a distinct inhibition patterns.

## Discussion

The present localization and gene expression studies strongly suggested that HlSC-1 was secreted into the host's tissues during the early stage of blood feeding. Unlike blood-sucking insects, ticks make a blood pool by rupturing blood vessels under the host's skin and feed on this blood for a relatively long period, varying from several days to weeks, depending on the life stage, the host type, and the species of tick involved (Klompen 2005). We may therefore assume that HlSC-1 participates in the dynamic feeding process that occurs in tick and host interaction.

In the present study, we have shown that HlSC-1 strongly inhibits the proteolytic activity against a variety of cysteine proteases. Prior studies have shown that sialostatin expressed in the blacklegged tick *I. scapularis* may be secreted into the host's tissues (Kotsyfakis et al. 2007). Similar inhibition profiles were observed with HlSC-1, which inhibited a cathepsin L. Conserved function for successful blood feeding may exist in ixodid ticks. Recently, our group has identified two cystatins, an intracellular cystatin Hlcyst-1 and secreted Hlcyst-2, which had been characterized from the midgut of *H. longicornis* (Zhou et al. 2006, 2009). In vitro enzyme inhibition profiles revealed that HlSC-1-induced inhibitory pattern differs from that of midgut cystatins, suggesting that localization of these molecules may dictate their specific functions in the blood-feeding processes. We speculated that the two cystatin in the midgut of *H. longicornis* act to regulate hemoglobin degraded cascades as induced by the midgut cysteine proteases during the host blood meal digestion

**Table 1** Protease inhibition assays

| Enzyme | Enzyme conc. | HlSC-1 | Hlcyst-1 | Hlcyst-2 |
|---|---|---|---|---|
| Cysteine proteases | | | | |
| Cathepsin L | 0.6 μg/ml | 273.6±52.95 nM | 202.8±19.38 pM | 1.68±0.4913 μM |
| Cathepsin B | 15 mU/ml | NI | 91.49±13.99 nM | 55 μM< |
| Papain | 50 μg/ml | 5.3±0.6650 μM | 2.219±0.725 μM | 17.0±1.082 μM |
| Serine protease | | | | |
| Trypsin | 20 μg/ml | NI | NI | NI |

The concentration of *H. longicornis* cystatins at which 50% inhibition of the activity of the proteolytic enzymes is achieved ($IC_{50}$) were presented *NI* no inhibition (inhibition of the enzyme was not observed in the presence of 55 μM inhibitor)

(Alim et al. 2008; Tsuji et al. 2008; Yamaji et al. 2009; Zhou et al. 2006, 2009). To date, several bioactive molecules associated with tick blood-feeding processes are identified from the salivary glands of *H. longicornis* (Gong et al. 2009; Islam et al. 2009; Tsuji et al. 2001; You et al. 2003). Thus, HlSC-1 may participate in the regulation of the blood-feeding processes in *H. longicornis*.

Tick interacts in unique ways with both its hosts and the disease-causing microbes it transmits. It is believed that ticks have evolutionarily gained molecules that regulate tick biology and that tick's various organs expressing genes are highly correlated with their biological functions. As an alternative of toxic chemical acaricide usages, targeting specific molecules which play key roles in tick physiological processes and metabolic pathways, including blood-feeding processes, is one of the useful approaches to thwart tick survival (Tsuji et al. 2008). However, chemical acaricides, which are currently used for tick control, have disadvantages such as acaricidal resistance problems (Zaim and Guillet 2002) and chemical residues in food animals, which are a threat to human health. Consequently, novel approaches are sought to control tick populations based on tick-specific potential biochemical pathways (Nuttall and Labuda 2004). Therefore, functional characterization of HlSC-1 would help in knowing the prospects of their application as candidate molecules in the control of ticks and tick-borne diseases.

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**Original article**

# Schistosome eggs have a direct role in the induction of basophils capable of a high level of IL-4 production: Comparative study of single- and bisexual infection of *Schistosoma mansoni in vivo*

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**Abstract:** Immunobiological roles of schistosome eggs during murine experimental infection were investigated with special reference to the induction of basophilic leukocytes. After single- or bisexual infection with *Schistosoma mansoni* in BALB/c mice, splenomegaly and liver granulomas were observed only in bisexual infection in parallel with deposition of mature parasite eggs. Comparison of the kinetics of basophil response revealed a marked increase in number in the bone marrow of mice with bisexual infection at the 7$^{th}$ week post infection as opposed to a marginal increase in single- sex infections. In the spleen, bimodal response was observed in the basophil responses; a small but repeatable peak at the 4$^{th}$ week after infection, increasing again at the 8$^{th}$ week, which corresponded to the initiation and maturation of parasite eggs in the affected organs of infected mice. The same time course was observed for IL-4 production by the splenocytes from mice of bisexual infection. To obtain more concrete evidence of the role of eggs in the induction of basophils, we tested using the intravenous egg injection model. Injection of eggs induced basophilia, and it was accompanied by the up-regulation of IL-4 production in splenocytes from the 8$^{th}$ day. Basophils induced in this model showed a high level of IL-4 production confirmed by flow cytometry, while faint levels of IL-4 production were observed for CD4$^{+}$ T cells at this time point. In addition, we demonstrate that egg deposition is the trigger of basophil induction and activation in the murine experimental model of *S. mansoni* infection, which might play an essential role in the initiation of Th1/2 conversion during the course of *S. mansoni* infection *in vivo*.
**Key words:** *Schsitosoma mansoni*, Immunoregulation, basophils, Granuloma, Eggs antigens

## INTRODUCTION

*Schistosoma mansoni*, a blood dwelling dioecious helminth, is one of the three major schistosome species widely known to affect man in developing nations in Africa, South America and the Carribeans [1, 2]. Based on available information, it is estimated to be responsible for more than half of the over 200 million schistosome-affected people globally [1, 3]. Several reports indicate that the pathology of schistosome infection is CD4$^{+}$ T cell dependent [4, 5]. Two patterns of cytokine profile have been reported from CD4$^{+}$ T cells in host animals. Host T cells in early infection stages secrete Type 1 cytokines such as IFN$\gamma$ and IL-2. Later, hosts with egg-producing adult worms skew from Type 1 to Type 2 polarization with IL-4, IL-5, IL-6, IL-10 and IL13 production, among which IL-4 plays a central role in Th2 response [6-8]. Consistently elevated serum IgE and tissue eosinophilia which are under the control of Th2 cells, are acknowledged as dominant characteristics of the pathology and immune response [9].

Ironically, the shift from Th0 stage to Th2 cell polarization requires an initial IL-4, and there is controversy about its origin. For instance, CD4$^{+}$T cells are reported to generate the initial IL-4 [10], which is at variance to other research showing non-T, non-B cells as being the prime source of IL-4 [11, 12]. These differing opinions make the assertion of the origin of Th1/Th2 balance in helminth infections quite controversial. Nevertheless, accumulating

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evidence shows that basophil, which accounts for only about 0.5% of all leukocytes, is the early and major IL-4 producing potential [13-16] and appears to be responsible for inducing Th2-dominant response [17-21]. Basophils, as well as mast cells and eosinophils, are considered to originate from a common CD34$^+$ progenitor cell [22] and therefore to share many characteristics with mast cells [23]. In some cases, basophils were considered as "redundant mast cells" or at least a poor sister to mast cells and so were obscured [24]. Previously held notions, however, are rapidly being demystified by the advent of sophisticated tools for research on basophils [25-27].

In spite of recent works showing the presence of egg components inducing activation of basophils, details on the initiator and activator of basophils *in vivo* remain less characterized [28]. In the present study, we investigate the dynamics of basophil responses in infected mice with *S. mansoni* and attempt to determine whether it is under the control of schistosome eggs *in vivo*. Considering the probable role of matured eggs in basophil activation, we compared the phenomena of presence and absence of mature eggs in mice. For that purpose, we prepared bisexual (mixed-sex) and single-sex infection, the latter revealing no egg production in the host mice. In this study, we demonstrate the evidence of schistosome eggs responsible for the induction and activation of basophils *in vivo*, which might be related to immnoregulation during murine experimental *S. mansoni* infection.

## MATERIALS AND METHODS

*Animals and parasite infection:*
Female BALB/c mice of 4 weeks old were purchased from CLEA Japan (Tokyo, Japan). A Puerto Rican strain of *S. mansoni* cercariae maintained in our laboratory was infected to 30 mice at a dose of 100 cercariae/mouse by percutaneous exposure for an hour. In the single-sex male and female infection groups, 30 mice each were exposured to cercariae obtained from snails to which a single miracidium had been exposed. This indicates that cercariae shedding from snails are male or female only. Non-infected mice were maintained as controls.

To evaluate the direct role of schistosome eggs in the basophil response *in vivo*, 5,000 viable eggs were injected into the tail vein by the method described previously [29]. As controls, we injected 5,000 beads of the same size as the schistosome eggs (Dynabeads M-450 Dynal Biotech, Norway). In this mouse model, the eggs or beads were trapped in the lung capillary vein, and the immune response to egg antigen was induced in the absence of adult worms.

All experiments in the present study were conducted in conformity with the regulatory guidelines and approved by the committee of animal ethics of Tokyo Medical and Dental University.

*Eggs, worms and sera collection from mice:*
On a weekly basis starting from week 1, mice were euthanized and bled by cardiac puncture. From the 4$^{th}$ week, worms were perfused from the hepatic portal system of each mouse, and the worm burden was enumerated [30]. This was repeated every week up to the 12$^{th}$ week and the recovered worms were enumerated. The liver from each mouse was collected, weighed, and a third cut and placed in 10% formalin for histology to test for granuloma formation. The remaining liver samples from mice with bisexual infection were minced. Parasite eggs were obtained by filtering through a 38 μm mesh and purified by centrifugation and later quantified using a light microscope. Worms and eggs were washed and centrifuged 3 times with PBS and kept frozen at -80℃ until use.

*Cells preparation:*
Bone marrow (BM) and spleen cells were prepared under aseptic conditions from the hind leg bone and spleen respectively. Cells were resuspended in RPMI-1640 medium (Wako, Tokyo, Japan), and the viable cells were enumerated by the trypan blue dye exclusion method. Following subsequent washing, cells were resuspended and, for RNA extraction, a portion of the spleen cells was placed in TRIzol (Invitrogen, San Diego, USA) and stored at -80℃ until use. The remaining cells were used for FACS analysis (BD, San Jose, CA). In the case of mice injected with viable eggs intravenously, spleen, BM and lung cells were prepared on day 1, 4, 8, 12 and 16 post injection.

*Flow cytometry analysis:*
The antibodies used for FACS analyses were as follows: FITC-conjugated monoclonal antibodies (mAb) specific for CCR3 and CD4, Phycoerythrin (PE)-conjugated mAb specific for c-kit and IL-4, biotinylated Gr-1 (BD PharMingen, Franklin Lakes, USA). Allophycocyanin (APC)-conjugated strepavidin was used for detection of biotinylated reagents (BD PharMingen) FITC-conjugated mAb specific for CD49 b (DX5) and Gr-1, PE-conjugated mAb specific for Gr-1 and FcεRIα; and APC-conjugated mAb specific for IFN-γ and rat IgG1 was purchased from eBioscience (San Diego, USA). FITC-conjugated mAb CCR3 was purchased from R&D systems (Minneapolis, USA). Unlabelled anti-CD16/32 mAb (2.4G2) was purchased from BD PharMingen.

Surface staining was done by the method described elsewhere [25] with slight modification. In brief, 1 x 10$^6$ of BM or spleen cells were pre-incubated with 2.4G2 and nor-

Table 1: Parasite infectivity and maturation in single- or mixed-sex infection, and egg production and splenomegaly in mixed-sex infection

| Period (weeks) | Male only TWR (% mature) | Female only TWR (% mature) | Mixed-sex TWR (% mature) | Pair worms | Parasite eggs (x$10^5$) |
|---|---|---|---|---|---|
| 4 | 36.3 (52.3) | 18.3 (63.6) | 31.3 (54.8) | 3.3 | 0.00230 |
| 5 | 51.3 (81.2) | 27.3 (75.3) | 40.3 (96.3) | 8.7 | 0.129 |
| 6 | 20.0 (88.3) | 51.7 (93.5) | 52.0 (99.7) | 14.0 | 3.05 |
| 7 | 40.0 (99.0) | 33.0 (99.0) | 31.7 (100) | 13.3 | 9.57 |
| 8 | 37.3 (99.1) | 47.0 (100) | 31.7 (100) | 10.3 | 14.2 |
| 10 | 16.7 (100) | 65.0 (100) | 28.5 (100) | 12.0 | 20.7 |
| 12 | 9.7 (100) | 45.7 (100) | 23.0 (100) | 10.1 | 27.9 |

TWR: Total worms recovered

mal rat serum in FACS buffer (PBS with 1mg/ml of BSA and 0.5mg/ml of $NaN_3$) on ice for 15 min to eliminate non-specific binding of irrelevant antibodies. The cells were subsequently incubated on ice with the conjugated mAbs for 30 min, and then second antibodies were added for 10 min and finally propidium iodide was added as substrate. Stained cells were analyzed using FACS calibur (BD Bioscience) equipped with CellQuest software.

Cytoplasmic staining was done by the standard protocol [27]. Freshly prepared spleen cells were suspended at 2 x $10^6$ cells in 200 μl of complete medium (10% FCS added to RPMI-1640 with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 50 μM 2-mercapto ethanol). Cells were treated with 10 ng/ml phorbol myristate acetate (PMA) plus 500 ng/ml Ionomycin (Iono) and 2 μM monensin in 96 well plates for 4 hr at 37℃ in 5% $CO_2$. Cells were taken through all the steps of surface staining as described above, except for the addition of propidium iodide. Cells were then treated with cytofix/cytoperm and perm/wash solution (BD Bioscience) according to the manufacturer's instructions, and then stained for intracellular cytokines detected by FACS calibur with CellQuest software.

*Histopathology of the liver:*
Liver specimens from each week (1-12weeks) were fixed in 10% formalin and kept at room temperature until tissue section preparation. Each liver specimen was decalcified and then embedded in paraffin. The tissue sections of 4 μm were stained with haematoxylin and eosin for microscopic observation.

*Real-time reverse-transcriptase polymerase chain reaction:*
Total RNA was extracted from the spleen and BM cells using TRIzol reagent (Invitrogen), and cDNA synthesis was performed as described elsewhere [31]. We measured mRNA transcripts for IL-4 and IFNγ using the following primers: for IL-4
(forward 5'-CATCGGCATTTTGAACGA-3' reverse 5'-GTGGTGTCTCTCACTCGAGA-3'); for IFNγ (forward 5'-GGAGGAACTGGCAAAAGGAT-3', reverse 5'-GGAGTCTGAGAAACTTCAGAACTT-3'), relative to that of β-actin (forward 5'-GCTCTAGACTTCGAGCAGGAGA-3', reverse 5'-TCTTCCCGATACTCGACGGA-3'). We performed product amplification with the SYBR Green I kits (Roche, Mannheim, Germany) as described by the manufacturer using the LightCycler® (Roche) with the following profile: 95℃ for 5 min, 45 cycles at 95℃ for 10 sec, 60℃ for 10 sec, 72℃ for 4 sec and finally 50℃ for 30 sec. The relative expression levels were calculated based on ratio of target gene to house keeping gene.

*Statistical analyses:*
Statistical analyses were done using student *t*-test. *P* values of <0.05 were considered significant.

## RESULTS

*Parasite infectivity, pairing and eggs production in host:*
Parasite ability to infect host ranged between 9.7% and 65.0% in the single (male or female) and mixed sex (bisexual) infection (Table 1). More than 50% adult worms appeared by the 4th week, and by the 7th week all worms were morphologically matured in bisexual infection when maturation was determined by the presence of distinct suckers and worm shape, the single-sexes showing close to 99% morphologically matured worms. In the bisexual infection, marked egg production was realized from the 6th and 7th week and progressively increased through to the 12th week (Table 1). The spleen from single-sex infection revealed no significant increase in size as compared with controls, while bisexual infection showed enlarged spleen size corresponding closely with egg production seen from the 6th and 7th weeks (Fig.1a). In bisexual infections, eggs were found in

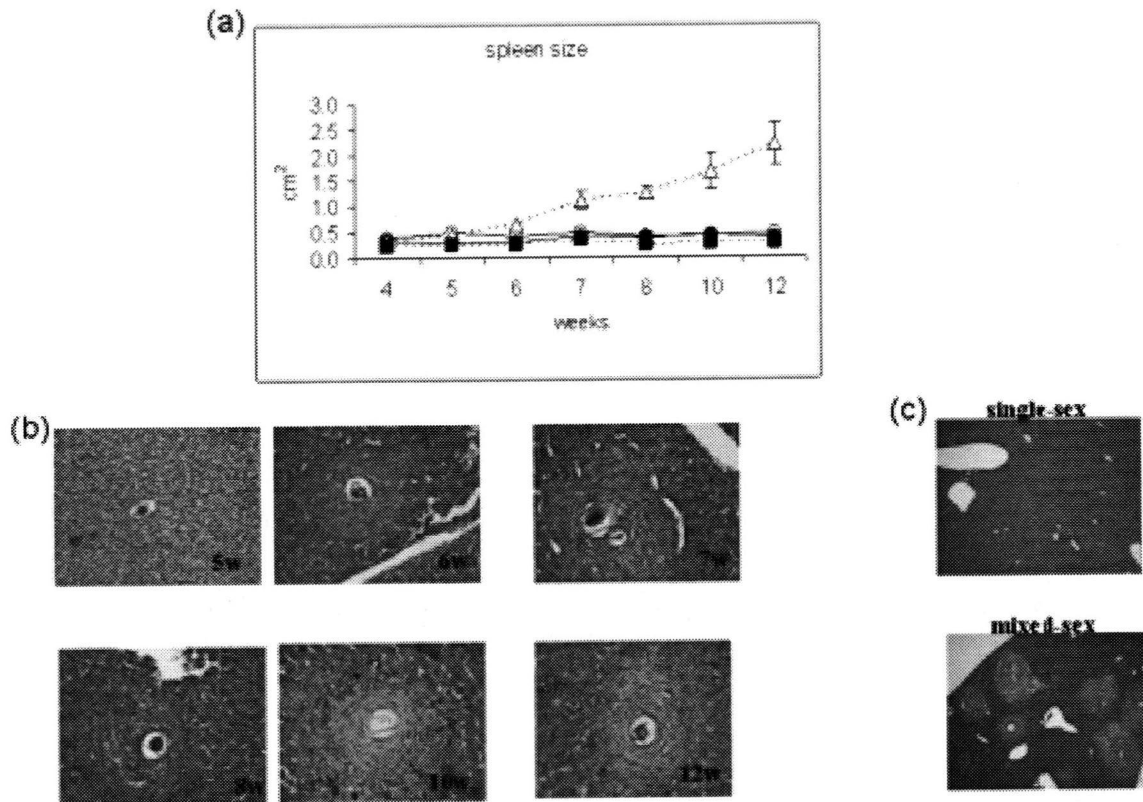


Fig. 1
Pathological parameters in bisexual and single-sex infection of *S. mansoni*. (a) Splenomegary of single-sex or bisexual infection is shown. ■: infection-free control,□: single sex male alone, ●: single sex female alone, and △: bisexual infection. (b) Schistosome eggs deposited in the liver and granuloma formation during bisexual infection are shown. All lesions are shown at x200 magnification. (c) Comparison of liver lesions between single-sex and bisexual infection is shown (x40).

the liver in the 4th and 5th weeks, but no granuloma formation was observed at those time points (Fig. 1b). Moreover, none of the single sex infected mice liver showed any granuloma formation (Fig. 1c).

*Dynamics of granulocyte response in BM and spleen during the course of* S. mansoni *infection:*

Basophils were identified in FACS by the use of antibodies to CD49b and FcεR1. It was possible to detect basophils as a double-positive stained population (Fig.2). FACS patterns for eosinophils and neutrophils are also shown. Basophils in BM from mixed sex infection began to show apparent but modest induction from the 3rd week with steady increase thereafter, all peaking by the 7th week (Fig. 3a). Contrary to the situation in the BM, the level of splenic basophils from mixed- and single-sex (female) infection reveal an equally modest increase at the 4th week (Fig. 3b). However, the splenic basophilia became significantly elevated after the 6th week and peaked at the 8th week, just at the time when BM basophils sharply return to the basal level. As shown in Table 1, egg deposition started around the 4th week, and egg maturation was confirmed from the 6th week after cercarial infection. The spleen neutrophils and eosinophils were concordant with the kinetics of spleen basophils. Both neutrophils and eosinophils showed progressive induction especially after the 6th week in only the mixed infection.

*Cytokines expression in spleen of infected mice by quantitative real-time PCR:*

Spleen cells from bisexual infection were tested for IL-4 and IFNγ expression by quantitative real-time PCR. As shown in Fig.4a, IFNγ expression reached a maximum level at the 7th week after infection. On the other hand, IL-4 expression showed a bimodal pattern during infection. The first peak was observed around the 4th and 5th weeks and the second peak at the 8th week or later, both peaks being con-

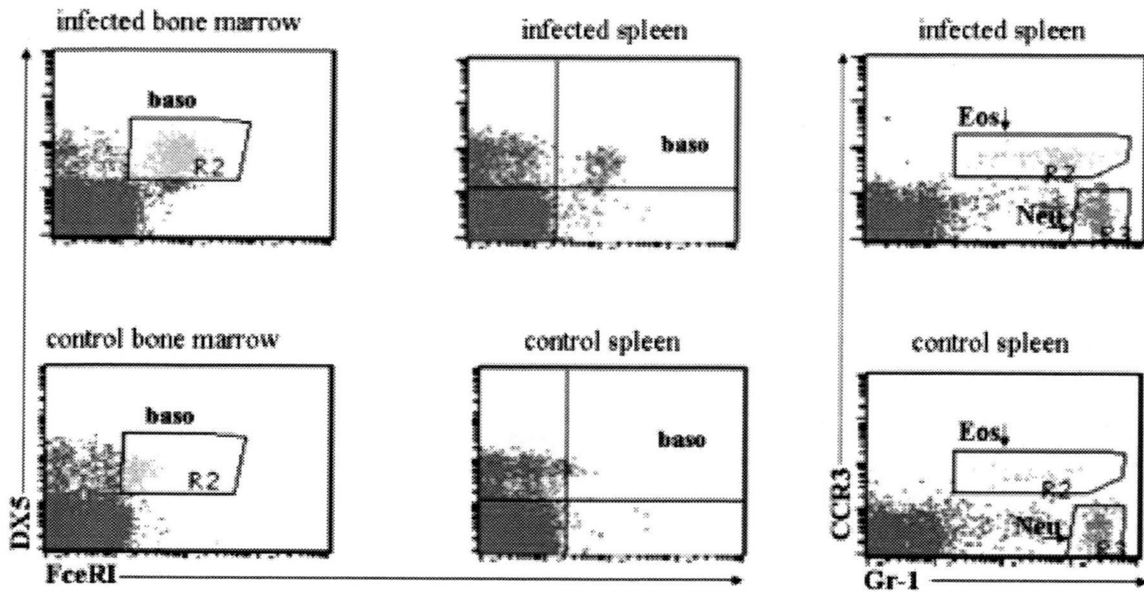


Fig. 2
FACS profiles of basophils, eosinophils and neutrophils. Basophils were identified as a cell population double positive for FceR1 and CD49b, and eosinophils were double positive for Gr-1 and CCR3. Neutrophils were of Gr-1-positive but CCR3-negative.

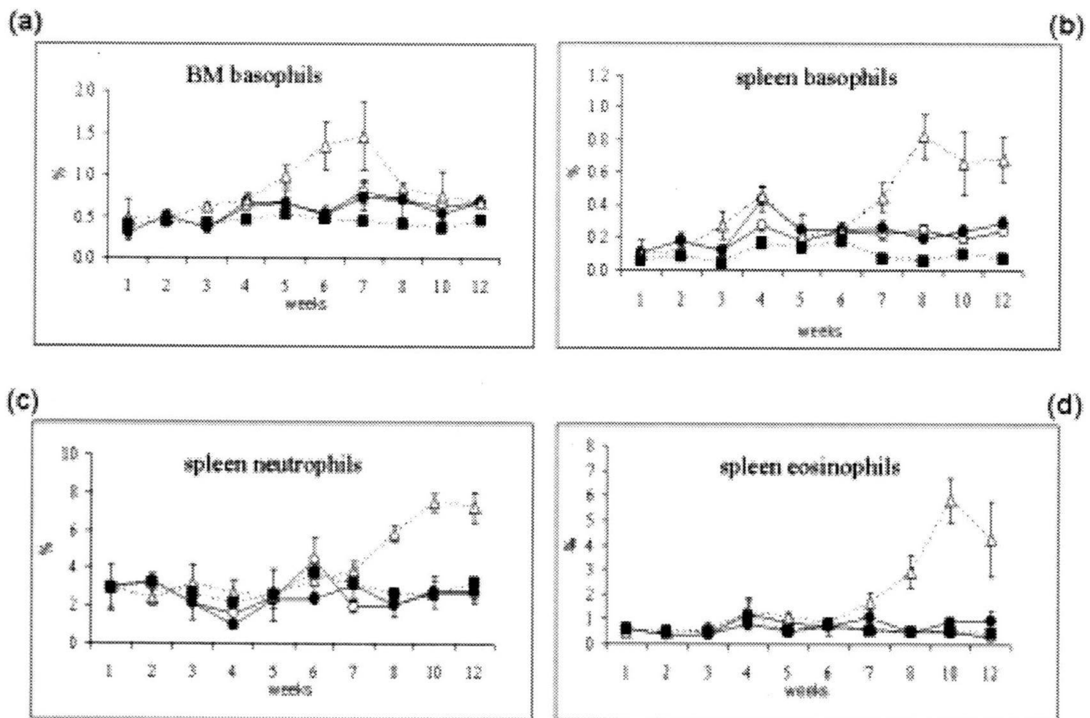


Fig. 3
Time course kinetics of basophils, eosinophils and neutrophils in (a) bone marrow and in (b)-(d) spleen during single-sex and bisexual infection are shown. ■: infection-free control, □: single sex male alone, ●: single sex female alone, and △: bisexual infection.

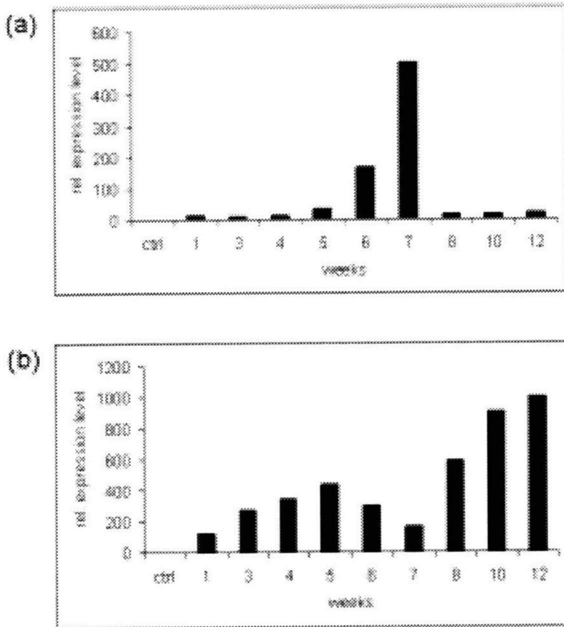


Fig. 4
IFNγ and IL-4 production measured by real-time PCR. Splenic cells from bisexual infection were tested for cytokine production by the method described in Materials and Methods. (a) IFNγ production and (b) IL-4 production are shown.

cordant with the two peaks of basophilia (Fig. 4b).

*Basophils in BM, lung, liver and spleen of mice intravenously injected with* S. mansoni *eggs:*
We tested to see whether eggs play a major role in the basophil induction observed in the model of intravenous injection of parasite eggs. As shown in Fig. 5, basophil induction was driven by eggs from the 8th day in the absence of adult worms and continued until the 12th day in the three organ tissues analyzed, although stimulation was still apparent beyond the 12th day in BM.

*Detection of cytokines produced in the spleen of mice intravenously injected with* S. mansoni *eggs:*
In view of the fact that the pattern of IL-4 expression closely resembled basophil kinetics in the spleen (Fig. 3b and Fig. 4b), we examined the role of splenic basophils in IL-4 production in the egg injection model by performing cytoplasmic staining. As shown in Fig. 6, splenic basophils expressed a high level of IL-4 production from the 8th day. Considering the expression level of IL-4 between basophils and $CD4^{+}$T cells, we observed an increase in the number of basophils with a higher intensity of IL-4 production than those in $CD4^{+}$ T cells (Fig. 6a-e). The ratio of IFNγ-

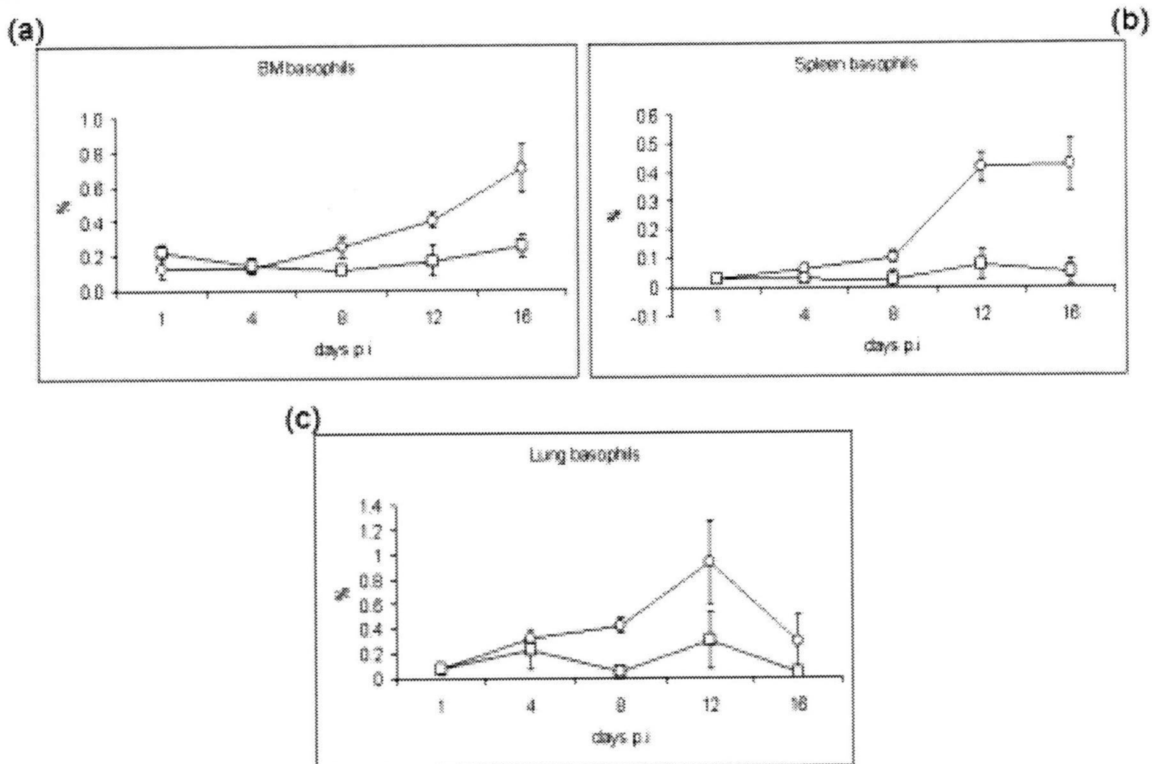


Fig. 5
Induction of basophils in the egg-injection model. Basophils were increased in number from the 8th day and later in three organs (bone marrow (a), spleen (b) and lung (c)) tested by FACS analyses. ○: Injection of *S. mansoni* eggs, □:beads-injection control.

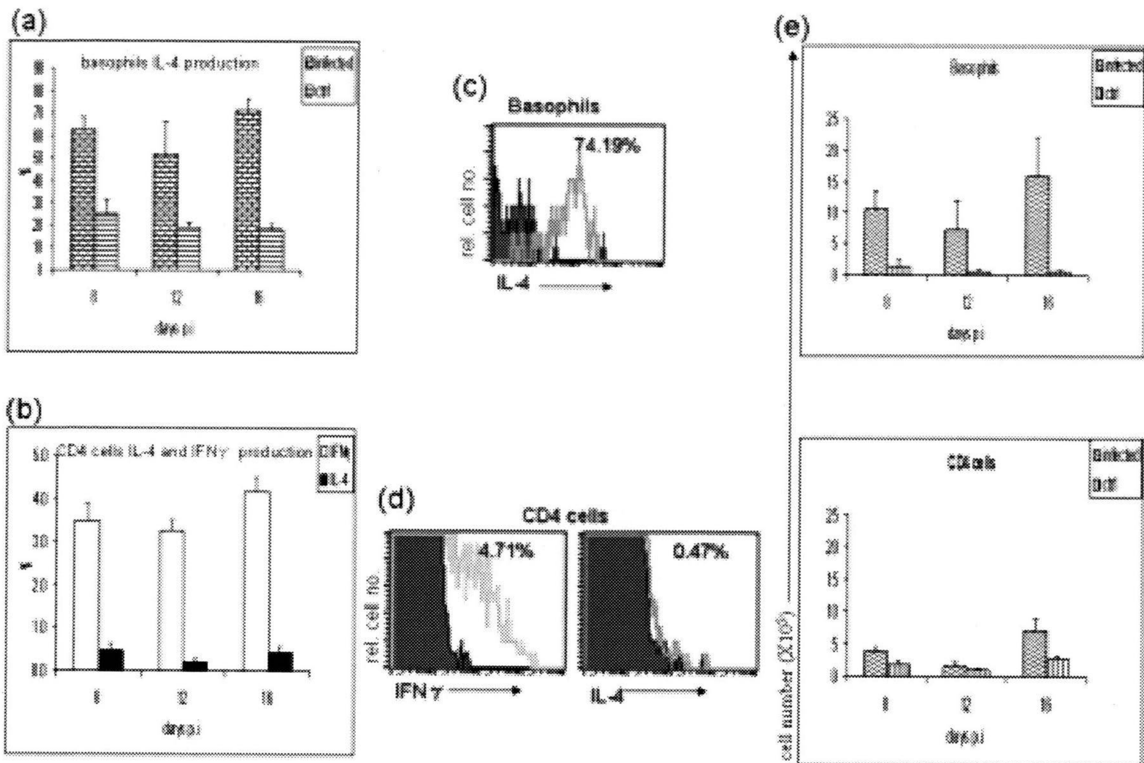


Fig. 6
Basophils induced in the egg-injection model produced a high level of IL-4 measured by cytoplasmic staining by FACS. (a) Splenic basophils with IL-4 production were increased in ratio in egg-injected mice at the 8th day and after. (b) CD4+T cells in the spleen of egg-injected mice produced dominantly IFNγ rather than IL-4. (c) Intensity of IL-4 production was evaluated by FACS. Representative results of basophils at the 16th day is shown. Activated basophils shifted to strong positive for cytoplasmic IL-4. (d) Intensities of cytoplasmic IFNγ and IL-4 of CD4+T cells from egg-injected mice at the 16th day are shown. (e) Actual cell number of IL-4-producing basophils and CD4+ T cells are shown. Basophils with IL-4 production were higher in number than CD4+ T cells with IL-4 production.

producing CD4+ T cells was higher compared with IL-4 at all time points tested (Fig. 6b). By calculating the number of basophils with a potentials for IL-4 production, it seems likely that basophils were dominant in IL-4 production in the egg-injection model (Fig. 6c).

## DISCUSSION

*Schistosoma mansoni* infection is prominently characterized by Th2 response accompanying elevated response of IgE and eosinophils [32, 33]. In recent times, basophils, quantitatively the least of the granulocytes, are rapidly being acknowledged to play a role in immunoregulation in some helminth infections [17]. Schistosome egg antigen (SEA) components in the presence of some reagents stimulate basophils to produce IL-4 *in vitro* [34, 35]. However, the mechanism of basophil stimulation *in vivo* has not yet been elucidated. The recent development of reagents and tools for identifying basophils [19, 25] enable researchers to explore whether schistosome eggs are responsible for the induction of basophilia during the course of *S. mansoni* infection as well as to determine whether basophils induced by the parasite egg play any key role in immunoregulation.

Considering the possibility that schistosome eggs are involved in the induction of basophilia, we conducted a comparative study using single- and mixed-sex infection. Adult worms were present in both situations, while only mixed-sex infection produced eggs. Although growth retardation of worms is suggested in single-sex infection [36], mice susceptibility to infection and worm maturation in the single-sex situation were comparable to those in mixed-sex infection in the present study. The lack of any apparent splenomegaly in the single-sex infection indicates that worms were not major players in the pathological lesion. Also, the failure of eggs produced at the 4th and 5th weeks in the mixed-sex infection mice to show any granuloma may

be consistent with reports that produced eggs require a substantial period to mature and to elicit an immune response. These findings were confirmed by the 6th week and beyond, as both splenomegaly and granuloma correlated well with egg maturation and production over the period, signifying the important role of parasite eggs in disease pathology [37].

The fact that basophils are the least of all the leukocytes is shown by our results. During the course of infection, basophils in BM and spleen were highly elevated at a specific time point in bisexual infection. Although the cell frequency increasing at this time point appears to be small, it was confirmed in our repeat experiment (data not shown). Interestingly, the modest basophil induction in BM and spleen appearing before and just at egg deposition suggests that adult worms could, in part, exert an effect on the induction of basophilia [38]. However, the highly significant BM basophilia from the mixed-sex infection as compared to the marginal elevation in single-sex infection ($p<0.05$), implies that eggs enhance induction to a higher degree than worms alone. This was clarified in the spleen where only mixed-sex infections showed marked basophilia after the modest 4th week peak. The marginal basophilia induced by worms is probably due to site inhabited and immune evasiveness in the host [6].

More concrete evidence for mature eggs being a direct trigger for basophilia was observed in the egg-injection model. After intravenous injection of parasite eggs, splenic basophils were highly induced. Those basophils showed high a level of IL-4 production, while $CD4^+$ T cells in the spleen produced only a faint level of IL-4 at the same time point. This confirms that mature eggs of *S. mansoni* are responsible for inducing basophilia with the potential for IL-4 production.

Conspicuously, the peak level of basophils was far lowerin the spleen than in the BM, which was, however, consistent with earlier reports [13, 17]. Furthermore, time points of induction were different as a drastic decline in BM basophils was concurrent with a rapid increase in spleen basophils, suggestive of a feed back mechanism to avoid overproduction probably fatal to the host. The splenic eosinophilia and neutrophilia coincided with the splenic basophilia. This gives further support to suggestions that BM basophils express or release factors which may have facilitated their stimulation [39] and recruitment [40]. Reports published elsewhere [22, 29, 38] indicate that basophils produce IL-4 which up-regulates expression of VCAM-1 on endothelial cells and eotaxin synthesis, thereby enhancing the influx or recruitment of other leukocytes into the affected tissues. This might be supported by another study showing that depletion of basophils diminished eosinophil infiltration at the site of inflammation [4]. It may also be consistent with a report by Wedemeyer et al. that basophils played a role in immunoregulation in both innate and adaptive responses [41]. Our observations strongly suggest that eggs are main inducers of basophilia and that the egg-driven basophilia may be involved in the regulation of pathology of schistosome infection.

It has already been reported that polarization of $CD4^+$ T cells to Th2 type profiles as well as B cell synthesis of IgE and IgG1 coincides with egg production [8] and that IL-4 plays a central role in all these events. The source of initial IL-4 continues to be a subject of debate. Williams et al. reported that IL-4 was first detected from spleen cells depleted of T cells at the egg production stage [38]. Despite the number of cells present in the spleen, our results suggest the involvement of spleen basophils in the Th1/Th2 conversion, because basophils were capable of a high level of IL-4 production in the initial phase of stimulation by the mature eggs (Fig. 6). This is consistent with earlier studies showing that the level of IL-4 production is much higher in basophils than in $CD4^+$ T cells [42]. The marked elevation of BM basophils at the 7th week in the mixed-sex infection model suggests that basophils are one of the major sources of IL-4, which is necessary to initiate the polarization of $CD4^+$ T cells to Th2 cells as well as the B cell class switch to IgE and IgG [20, 43].

In conclusion, the present study adds to earlier works postulating that stimulation of egg antigen to the host immune system is the main drive inducing basophils in the infected hosts. Even though the question of which cell population is responsible for providing IL-4 for Th2 polarization in the acute phase of infection has been controversial, our results point to basophils as the initiator and most probable candidate cell for immunoregulation. Further studies are underway to obtain additional evidence by preparing a new *in vivo* experimental model of *S. mansoni* infection.

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*Review Article*

# Parasitic Helminths: New Weapons against Immunological Disorders

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The prevalence of allergic and autoimmune diseases is increasing in developed countries, possibly due to reduced exposure to microorganisms in childhood (hygiene hypothesis). Epidemiological and experimental evidence in support of this hypothesis is accumulating. In this context, parasitic helminths are now important candidates for antiallergic/anti-inflammatory agents. Here we summarize antiallergic/anti-inflammatory effects of helminths together along with our own study of the effects of *Schistosoma mansoni* on Th17-dependent experimental arthritis. We also discuss possible mechanisms of helminth-induced suppression according to the recent advances of immunology.

## 1. Introduction: Autoimmunity, Allergy, and Helminth Infection

In 1989, the "hygiene hypothesis" was proposed by D.P. Strachan in an article that claimed an inverse relationship between the occurrence of hay fever and numbers of siblings [1]. According to the hypothesis, atopic disorders are due to reduced exposure to microorganisms in childhood. Nowadays, the concept is becoming more accepted with accumulating evidence not only in atopic diseases but also in autoimmune inflammatory diseases. For instance, the incidence of multiple sclerosis (MS) is higher in high latitude countries (= westernized developed countries) than in equatorial areas [2]. Not only residents of western countries but also immigrants from developing countries are at high risk of developing inflammatory bowel diseases (IBDs) and asthma [3]. In the case of type 1 diabetes (T1D), a similar geographical distribution to the diseases above and an inverse correlation to hygiene conditions are observed [4]. A population-based ecologic study in Canada showed that IBD, including ulcerative colitis (UC) and Crohn's disease (CD), correlated with a high socioeconomic status, low rate of enteric infection, and high rate of MS [5].

Many studies have demonstrated that helminth infections lower the risk of autoimmunity or allergy. For instance, an inverse correlation between autoimmune liver diseases and *Strongyloides stercoralis* infection was demonstrated in Okinawa, Japan [6]. Cross-sectional studies on the relationship between skin prick tests and helminth infections suggested a general protective effect on the atopic reaction [7]. The authors summarized effects of geohelminths on the risk of asthma according to previous studies; that is, hookworm lowered but *Ascaris* increased the risk of asthma and *Trichuris* had no effect. Collectively, it is concluded that at least some helminths seem to have anti-allergic or anti-inflammatory effects in humans.

Experimental studies have also shown protective effects of helminth infections in animal models of autoimmunity (e.g., colitis, arthritis, and diabetes) and allergy (e.g., airway hypersensitivity) [3, 8–10]. In this review, we discuss possible mechanisms of anti-allergic/anti-inflammatory effects of helminths in animal models including autoimmune arthritis. Possible clinical applications and future prospects are also discussed.

## 2. Helper T Cell Subset Dependence of Experimental Immunological Disorders

Based on T cell skewing patterns and their relative importance in the pathogenesis, disorders with excessive immune

responses had been briefly classified as "Th1 diseases" and "Th2 diseases" according to the Th1/Th2 paradigm. However, the recent discovery of a novel pathogenic T cell subset (Th17) [11] led investigators to the concept of "Th17 disease." While most atopic immune disorders (e.g., hay fever and bronchial asthma) can be classified as Th2 diseases, the classification of autoimmune diseases is relatively difficult. Experimental autoimmune encephalomyelitis (EAE) as a model of MS was long thought to be a Th1 disease; however, the recent studies using IL-12/23 subunit (p35, p19, or p40) deficient mice revealed the progression of the disease to be dependent on the IL-23/IL-17 axis (= Th17 response) rather than IL-12/IFN$\gamma$ axis (= Th1 response) [12]. The pathogenic role of IL-17 was shown directly by the finding that EAE development was significantly suppressed in IL-17-deficient mice [13]. The importance of the IL-23/IL-17 axis is supported also in human MS [12]. Regarding T1D, the diabetes observed in NOD mice (a model of T1D) has been classified as a Th1 disease despite the presence of some controversial study results [14–16]. Recent reports demonstrated that Th17 cells could also cause diabetes, but only after their conversion to Th1-type cells [17, 18]. This means that there is unknown plasticity of helper T subsets. Regarding IBD, the pathogenic roles of both Th1 and Th17 responses in TNBS-induced colitis (a model of IBD) are still controversial [19–23]. Collectively, some experimental autoimmune disorders cannot yet be distinctly classified as either Th1 or Th17 disease.

## 3. Protective Effects of Helminths against Immunological Disorders

The effects of schistosomes and other helminths on experimental autoimmunity/allergy are summarized in Table 1. Surprisingly, helminths have been shown to suppress all types (Th1, Th2, and Th17) of disease in the models described above [24–45]. Considering classical Th1/Th2 paradigm, it is reasonable to speculate that helminth-induced Th2-skewing with downregulation of Th1 immune responses results in an amelioration of Th1 diseases. As IL-4 is known to suppress Th17 development [46], Th17 response could also be suppressed as well as Th1 response in helminth-infected or helminth antigen-treated animals. In fact, STAT6-dependent IL-4/IL-13 signaling was shown to be essential in the suppression of TNBS-induced colitis [29] and EAE [25] by schistosome eggs, although the authors did not measure changes of IL-17. Given that the involvement of Th1 and Th17 in some forms of autoimmunity is still controversial, downregulation of both T helper responses may be beneficial for the amelioration of various kinds of autoimmunity. Along with other investigators, we recently found that schistosome-infected mice became resistant to experimental arthritis accompanying down-regulation of both Th1 and Th17 responses of splenocytes [47]. Likewise, Ruyssers et al. reported suppression of TNBS-induced colitis by schistosome antigens, accompanying down-regulation of IL-17 gene expression in the colon and mesenteric lymph node (MLN) [28]. An intestinal nematode (*Heligmosomoides polygyrus*) infection was also reported to suppress IL-17 production in MLN cells and lamina propria mononuclear cells [48]. The authors showed that the blocking of both IL-4 and IL-10 restored IL-17 production in vitro. Another study revealed that *Fasciola hepatica*-induced down-regulation of autoantigen-specific Th1 and Th17 responses (and protection from EAE) was dependent on TGF-$\beta$, not IL-10 [49]. Although the mechanisms of Th17's down-regulation by helminths are not yet established, some of the mechanisms might be common to those of Th1's down-regulation (e.g., through induction of IL-4 and IL-10, down-regulation of IL-12p40) and others might be distinct.

In our study on experimental arthritis in mice [47], we found no increase of Treg-related gene expression (Foxp3, TGF-$\beta$ and IL-10) in the paws of *S. mansoni*-infected mice compared to the paws of uninfected control mice. As Treg cell population was known to expand in schistosome-infected or egg-treated mice [31, 32, 35, 50], the cells might participate in the regulation of the disease systemically rather than locally. To confirm the essential involvement of Treg cells in the antiarthritic effects of schistosome, further studies (e.g., persistent Treg depletion experiments) are necessary. In contrast to our result, in the case of diabetes in NOD mice, schistosome egg antigens induce infiltration of Treg cells at a local inflamed site (pancreas) [32]. Likewise, filarial nematode (*Litomosoides sigmodontis*) infection induced Treg cells and protected mice from the diabetes [39]. *H. polygyrus* also protected mice from the diabetes; however, the protection was not dependent on Treg cells [40]. In addition to schistosome and nematodes, tapeworm (*Taenia crassiceps*) infection also has anti-diabetic effects in multiple low-dose streptozotocin-induced diabetes (MLDS) in mice [45, in this issue]. In the study, alternatively activated macrophages (AAM$\phi$) increased whereas Treg population did not increase. Taken together, these studies suggest that there may be various mechanisms in anti-diabetic effects of helminths.

We also found that schistosome-induced down-regulation of Th1 and Th17 occurred in the same period after the infection, corresponding to the beginning of egg-laying (unpublished observation). This result suggests that egg deposition is the major stimulus to lower Th17 responses (as well as Th1 response) in murine experimental schistosomiasis. Further studies using schsitosome eggs are currently in progress in our laboratory.

Some epidemiological studies support that helminth infections are protective against atopic reactions and/or symptoms [51–53]. The helminth-induced suppression of Th2 diseases (atopic disorders etc.) is difficult to explain in terms of the Th1/Th2 paradigm. In the paradigm, theoretically, helminth infections are expected to cause IgE overproduction and hypereosinophilia, followed by exacerbation of allergic reactions. Indeed, persistent bronchoalveolar eosinophilia, airway hyperresponsiveness [54], and exacerbation of allergic airway inflammation [55] were observed in *Toxocara*-infected mice. One interesting explanation of anti-allergic effects of helminths is introduced in a review by Fallon and Mangan [56], in which Th2 responses are subdivided to "allergic" and "modified", with helminth-induced Th2 responses corresponding to the latter

Table 1: Suppression of experimental immunological disorders by helminthes.

| Animal models | Th types | Helminths | Treatment | Proposed suppressive mechanisms | Refs |
|---|---|---|---|---|---|
| Collagen-induced arthritis (CIA) | Th17 | *Schistosoma mansoni* | Infection | IL-17 ↓, TNF-$\alpha$ ↓, IL-6 ↓, RANKL ↓, Anti-collagen IgG ↓ | [47] |
| | | *Ascaris suum* | Worm Ag | | [37] |
| | | *Acanthocheilonema viteae* | Purified Ag (ES-62) | IFN-$\gamma$ ↓, TNF-$\alpha$ ↓, IL-6↓, Anti-collagen IgG↓ | [78] |
| Experimental autoimmune encephalomyelitis (EAE) | Th17 | *Schistosoma mansoni* | Infection | IL-12p40↓, IFN-$\gamma$ ↓, TNF- $\alpha$ ↓, IL-4↑ | [24] |
| | | | Egg | IFN- $\gamma$ ↓, IL-4↑, TGF-$\beta$ ↑, IL-10↑ | [25] |
| | | *Schistosoma japonicum* | Egg Ag | IFN-$\gamma$ ↓, IL-4↑ | [26] |
| | | *Fasciola hepatica* | Infection | IFN-$\gamma$ ↓, IL-17↓, Dependent on TGF$\beta$ | [49] |
| | | *Trichinella spiralis* | Infection | | [27] |
| Type 1 diabetes in NOD mice | Th1? | *Schistosoma mansoni* | Infection, Eggs | Inhibition of Ab class switch (Anti-insulin IgG↓) | [81] |
| | | | Egg Ag | Treg | [32] |
| | | *Litomosoides sigmodontis* | Infection, Worm Ag | IL-4↑, IL-5↑, Treg | [39] |
| | | *Heligmosomoides polygyrus* | Infection | Independent of IL-10 and Treg | [40] |
| Streptozotocin-induced diabetes | Th1? | *Taenia crassiceps* | Infection | AAM$\phi$ | [45] |
| TNBS/DNBS-induced colitis | Th1? / Th17? | *Schistosoma mansoni* | Infection | IL-2 ↑, IL-4 ↑ | [30] |
| | | | Eggs | IFN-$\gamma$ ↓, IL-4↑ | [29] |
| | | | Worm Ag | IFN-$\gamma$ ↓, IL-17↓, TGF-$\beta$ ↑, IL-10↑ | [28] |
| | | *Schistosoma japonicum* | Egg Ag | IFN-$\gamma$ ↓, IL-4↑, IL-10↑, Treg | [31] |
| | | *Hymenolepis diminuta* | Infection | IL-10↑ | [41, 44] |
| Piroxicam-induced colitis | Th17? | *Heligmosomoides polygyrus* | Infection | IL-17↓, Independent of IL-10 | [48] |
| DSS-induced colitis | Th17? / Th2? | *Schistosoma mansoni* | Infection (male worm) | Macrophage infiltration | [64] |
| Systemic anaphylaxis | Th2 | *Schistosoma mansoni* | Infection | IL-10-producing Bcell | [34] |
| Asthma/Airway hypersensitivity or inflammation | Th2 | *Schistosoma mansoni* | Infection (male worm) | IL-5 ↓, IL-10 ↑ | [33] |
| | | *Schistosoma japonicum* | Egg Ag, Eggs | Treg | [35] |
| | | *Heligmosomoides polygyrus* | Infection | Treg | [36] |
| | | *Ascaris suum* | Purified Ag (PAS-1) | IL-4↓, IL-5↓, Eotaxin↓, RANTES ↓, IL-10↑ | [38] |
| | | | Worm Ag | IL-4↓, IL-5↓, Eotaxin↓, IgE↓ | [42] |
| | | *Litomosoides sigmodontis* | Infection | TGF$\beta$ ↑, Treg | [43] |

↓:downregulation, ↑:upregulation

type characterized by predominant Treg and IL-10 responses and a relatively weak IL-5 response. (The authors, however, describe that such modifications are localized in the lungs and different from systemic responses.) Along with this hypothesis, administration of *Ascaris* extract was shown to inhibit not only IL-5 production but also eosinophilic inflammation in a murine asthma model [42]. Filarial infection also suppressed airway hyperreactivity and pulmonary eosinophilia in a murine asthma model [43]. The suppressive effect of the filarial worms on hyperreactivity was dependent on Treg cells and TGF-$\beta$. As helminths seem to have both allergenic and immunomodulatory components [38] in their bodies, the balance of them may determine the outcomes (i.e., exacerbation or amelioration) of allergic disorders.

Helminthic infections often result in expansion and/or activation of Treg cells [31, 32, 35, 36, 50]. B cells are also suggested to be involved in disease suppression [34] through IL-10. However, the importance of IL-10 in helminth-induced suppression of atopic diseases is not fully established; that is, mesenteric lymph node cells from *Heligmosomoides*-infected IL-10-deficient mice could confer protection against allergic airway inflammation [36]. Likewise, in humans, anti-allergic effect of *Ascaris lumbricoides* is associated with Treg, but not with IL-10 [57]. In the case of mice, IL-35, a recently identified effecter molecule of Treg cells [58], might be involved in the suppression. Other than the regulation of lymphocytes, helminth-derived products have potential anti-allergic effects on various kinds of cells. For instance, the filarial product ES-62 was shown to suppress the release of mediators from bone marrow-derived mast cells [59]. In addition, helminths generally induce AAM$\phi$ [60–63]. Such macrophages not only suppress the pathogenesis by the parasites themselves [61] but also may suppress pathological immune responses to autoantigens or allergens. The involvement of AAM$\phi$ in anti-diabetic effects was suggested in experimental tapeworm infection [45]. On the other hand, schistosome-modulated macrophages (but NOT AAM$\phi$) were involved in suppression of dextran sodium sulfate-(DSS-) induced colitis [64]. These studies suggest the presence of various suppressive mechanisms by helminths. Immune cells and mediators possibly involved in the helminth-induced immunomodulation are illustrated in Figure 1.

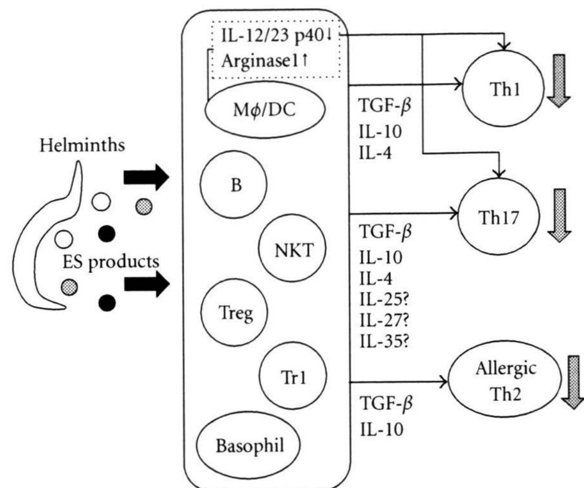


Figure 1: Possible involvement of immune cells and mediators in the helminth-induced immunomodulation on T helper subsets. Substances contained in the bodies of helminths or their excretory secretory (ES) products are recognized by innate immune cells via PAMPs receptors like TLRs. Thereafter, various changes occur in the immune cells, for example, down-modulation of IL-12/23p40 expression (DC), alternative activation (macrophages), proliferation and/or IL-10 production (Treg, Tr1, and B cells), and IL-4 production (basophils). We can observe suppression of immune disorders with down-regulation of pathogenic T helper subsets (Th1, Th17, and allergic Th2) as consequence of mixed effects of such immunological changes.

## 4. Protective Effects of Helminths against Experimental Autoimmune Arthritis

Collagen-induced arthritis (CIA) is an autoimmune arthritis in mice and rats [65] widely used as a model of rheumatoid arthritis (RA). Although the role of the IL-12/IFN$\gamma$ axis in CIA has been controversial [66, 67], recent findings suggest that IFN$\gamma$ has ameliorating rather than exacerbating effects [68–71]. Instead, the IL-23/IL-17 axis was recently shown to be important in the pathogenesis of CIA [71–76] as well as RA in humans [77].

Regarding anti-arthritic effects of helminths, a filarial ES product ES-62 [78] and porcine roundworm *Ascaris suum* extract [37] were shown to suppress CIA in mice. As described, we examined effects of *S. mansoni* infection on CIA in mice. In humans, schistosomiasis has been reported as "arthritogenic" rather than anti-arthritic [79, 80]. In our experiments, however, *S. mansoni* infection lowered arthritis scores and numbers of arthritic paws [47]. Histopathological examination revealed that cell infiltration and bone/cartilage destruction were diminished in the infected mice.

In CIA and RA, the pathogenic roles of IL-1 and IL-6 are well established [82–84]. We observed that the marked augmentation of IL-1$\beta$ and IL-6 gene expression in the paws was almost completely abrogated by *S. mansoni* infection [47]. It was especially noteworthy that receptor activator of NF$\kappa$B ligand (RANKL) gene expression in the inflamed paws was also abrogated by *S. mansoni* infection. As RANKL expression is induced by proinflammatory cytokines including IL-17, TNF-$\alpha$, and IL-1$\beta$ [85] and essential to osteoclast development followed by bone destruction [86–88], this result suggests that schistosome infection has anti-arthritic effects preventing bone destruction. Interestingly, we also found that intraperitoneally administered schistosome worm antigens (SWAP) or egg antigens (SEA) did not affect the progress of CIA (unpublished observation). In the antigen-administered mice, levels of IL-10, TNF$\alpha$, and IL-17 produced by splenocytes were comparable to those in antigen nonadministered control mice. Thus, regarding schistosome, there is a considerable difference in immunomodulating effects between infection and antigen administration.

## 5. Future Clinical Applications of Helminths and Their Products

As described above, our experiments with schistosome showed that anti-arthritic effects were observed only in the viable worm infected mice. Likewise, Hunter et al. showed that anticolitic effects of *Hymenolepis diminuta* were dependent on a viable infection [41], and surprisingly, Melon et al. showed that therapeutic efficacy of the viable tapeworm was superior to dexamethazone treatment [44, in this issue]. Because of these observations in experimental models, attenuated or non/lowly-pathogenic helminths are worth testing directly for therapeutic effects. In fact, porcine whipworm (*Trichuris suis*) eggs and *Necator americanus* infective larvae have been clinically tested for the treatment of chronic inflammatory or allergic diseases. Reddy and Fried [89] summarized the recent progress in clinical trials using these two intestinal nematodes. Summers et al. reported that the administration of *T. suis* eggs effectively ameliorated both UC and CD [90, 91] without adverse effects. *N. americanus* is also under clinical trials for asthmatic patients [92]. This worm is considered superior to porcine whipworm in that repeated administration is not needed.

Although it is not permissible to directly apply highly pathogenic helminths (e.g., schistosome) to clinical use, purified or synthetic immunomodulatory products from such worms can be considered for clinical purposes. Various immunomodulatory molecules (carbohydrates, proteins, and lipids) have been identified; for example, Lacto-*N*-fucopentaose III (LNFP III) contained in schsitosome eggs is an oligosaccalide as the molecule affecting B cells (especially B-1 cells) to induce IL-10 production [93]. LNFP III was also reported to alternatively activate macrophages [94] . A chemokine-binding protein (CBP) from schsitosome eggs inhibited the recruitment of neutrophils [95] to inflammatory foci. Peroxiredoxin (Prx) is an antioxidant protein found in various species including helminths [96, 97]. The molecules from *S. mansoni* and *F. hepatica* alternatively activate macrophages and are involved in the induction of a Th2-type immune response [97]. The IL-4-inducing principle of *S. mansoni* eggs (IPSE) is the molecule that induces "primary" IL-4 production from basophils [98]. Regarding anti-arthritic effects, a glycoprotein from *Spirometra erinaceieuropaei* was shown to suppress RANKL-induced osteoclastogenesis, suggesting that there may be more anti-arthritic substances in various helminths [99].

## 6. Concluding Remarks

Helminth-based immunotherapy for immnunological disorders is still in its infancy. It should be pointed out that helminths do not always suppress autoimmune/allergic disorders. There are epidemiological and experimental reports that helminths exacerbate such disorders [10]. Apart from Th2-biasing abilities of helminths, allergens contained in the worms may partially explain the mechanisms of exacerbation. *Ascaris* extract has cross-reactivity with domestic mite allergens [100]. Moreover, *Ascaris* was shown to have an allergenic component (APAS-3) [38]. Mice infected with *Toxocara canis* showed exacerbation of allergic airway inflammation [55] whereas hookworm (*Nippostrongylus brasiliensis*) infection persistently reduced airway responsiveness in mice [101], suggesting that there are considerable differences of outcomes even among lung-migratory nematodes. Thus, careful selection of "therapeutic" helminths and their target diseases is essential. Further studies on the mechanisms of immunomodulation are necessary for future human applications. The roles of Th1/Th17/Treg-related regulatory cytokines (IL-25, IL-27, IL-35, etc.) in the helminth-induced suppression of allergy/autoimmunity have not been sufficiently studied. It is well known that the roles of cytokines in human Th17 differentiation are very different from those in mice [12, 102]. For clinical applications in the future, we should ascertain changes in Th17-related cytokine patterns not only in animal models but also in patients with helminthiasis. It was reported that concurrent filarial infection suppressed both Th1 and Th17 responses to *Mycobacterium tuberculosis* [103]. This implies that helminths have down-regulating activity of both Th1 and Th17 in humans as well as in mice.

In human RA, the relative importance of Th1 and Th17 is still unclear, and therefore, suppression of both T helper responses is recommended [102]. Thus, at present we can conclude that regulatory effects on both Th1 and Th17 are promising characteristics of helminths as anti-inflammatory agents. However, the interpretation of experimental results of helminth-induced immunomodulation may change depending on changes in basic immunological knowledge. Indeed, it was recently reported that T-bet expression was more important in the pathogenesis of EAE than the Th1/Th17 balance [104]. Therefore, further studies of helminth-induced amelioration of immune disorders should strictly follow the studies of the diseases' pathogenesis.

As noted, in experimental animal studies, viable helminth infections seem to be superior to the administration of worm antigens or killed worms in the therapeutic effects. This might be due to that viable helminths can regulate secretion of immunomodulatory molecules in the most appropriate conditions for their survival. Taken together with successful treatment of UC and CD with viable porcine whipworms [90, 91], the optimal attenuation of human parasites by gene manipulation may be useful for the clinical application of parasitic helminths in the future.

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