Figure 4

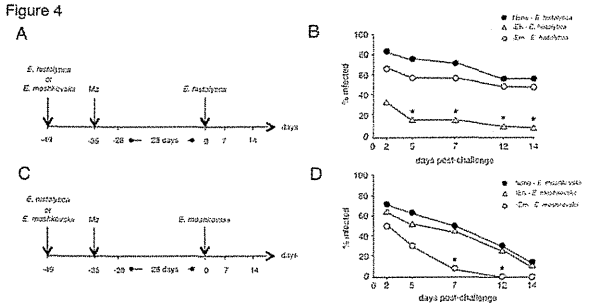

Figure 4. The protection induced by primary infection lasts more than four weeks. Mice were infected with 1 × 10^6 trophozoites of *Entamoeba histolytica* (open triangle) or *Entamoeba moshkovskii* (open circle) and treated with metronidazole (Mz) on day 14 following induction of the primary infection. The clearance of amoeba was confirmed seven days after treatment by PCR. Mice were kept without any intervention for 28 days, and re-challenged with 1 × 10^6 trophozoites of *E. histolytica* (A, B) or *E. moshkovskii* (C, D) at 35 days after treatment. The number of mice used was as follows: for naïve→*E. histolytica*, N=16: for *E. histolytica*→*E. histolytica*, N=20: for *E. moshkovskii* →*E. histolytica*, N=26 (A, B); for naïve→*E. moshkovskii*, N=15: for *E. histolytica*→*E. moshkovskii*, N=20: for *E. moshkovskii* →*E. moshkovskii*, N=20 (C, D). Asterisks indicate statistical significance with $p < 0.05$ by χ^2 test between mice infected with *E. histolytica* and *E. moshkovskii* in the primary infection.

doi: 10.1371/journal.pone.0082025.g004

Figure 5

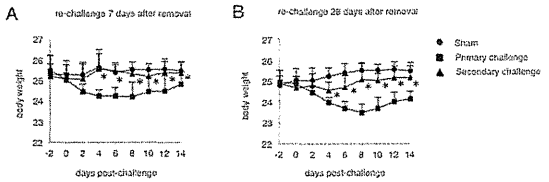

Figure 5. The change of body weight was monitored after re-challenging mice with *Entamoeba moshkovskii*. The naïve mice or mice exposed to primary *E. moshkovskii* infection were re-challenged with 1 × 10^6 trophozoites of *E. moshkovskii* at 7 or 28 days after confirming the clearance of primary infection. The studies were repeated 3 times with similar results. Asterisks indicate statistical significance with $p < 0.05$ between the groups of mice with primary and secondary infection using unpaired Student's *t*-test.

doi: 10.1371/journal.pone.0082025.g005

Despite the fact that it has long been suspected that people may acquire immunity against amoebae, as older children in endemic areas are infected less frequently than younger children [18], this work constitutes the first experimental proof of this phenomenon. The molecular and cellular mechanisms responsible for the observed protection have not been addressed in this work. Mucosal IgA has been reported to be associated with protection against intestinal amoebiasis in humans, mice and baboons [7,8,19]. Indeed, monoclonal IgA specific for the *E. histolytica* galactose inhibitable adherence (GalNAc) lectin heavy subunit (HgL) is thought to inhibit its interaction with a host sugar moiety in colonic mucins, resulting in the failure of amoebae to settle within the intestines [20]. Furthermore, we and Guo et al. recently reported that IFN-γ derived from amoeba-specific T cells plays a protective role against *E. moshkovskii* (unpublished data) and *E. histolytica* [21], respectively, suggesting that T cells as well as antibodies specific for amoebic antigens are involved in acquired resistance to intestinal amoebic infections.

The phenomenon of species-specific immunity against parasitic pathogens has been studied in a number of parasitic species, perhaps most comprehensively with the *Plasmodium* species responsible for malaria [22]. For this pathogen, which exhibits both species and strain specific immunity, antigenic variation of major parasite surface antigens such as the merozoite surface protein 1 (MSP1), induces antibody-mediated immune responses that are effective only against the inducing-strain [22,23]. Such highly polymorphic strain- and species-specific antigens are thought to evolve through the actions of positive diversifying selection, so that proteins that are targeted by the host immune response rapidly accumulate polymorphisms. Here we show, for the first time, that the phenomenon of species-specific immunity also exists for *Entamoeba spp*. It seems probable that this is due to

polymorphisms in major antigen target proteins between species. If so, then such antigens may be identified by comparative genomics. Of particular interest are the GalNAc-lectin HgL proteins previously implicated in antibody-mediated protection against *E. histolytica*. Nucleotide sequence comparisons of the genes encoding this protein in *E histolytica* and *E. moshkovskii* may shed further light on this.

We found that *E. moshkovskii* is susceptible to the anti-amoeba drug metronidazole both *in vitro* and *in vivo* to the same degree as *E. histolytica*. This finding supports the use of this drug in the treatment of pathogenic *E. moshkovskii*, and may ease concern of treatment failure following cases of misdiagnosis of *E. moshkovskii* as *E. histolytica*.

In summary, we show that exposure to a single drug cured amoebic infection confers resistance to re-challenge with the homologous, but not a heterologous species, for the first time, in which species-specific acquired immunity has been demonstrated for amoebic infections. This work paves the way, therefore, for the identification of novel amoebae antigens that may become the targets of vaccines.

Acknowledgements

We thank Fumie Hara, Masako Hayashida and Megumi Hamasaki for animal husbandry; Tomoko Takaya, Hiromi Oda and Rumiko Kosugi for administrative support; Richard Culleton for scientific editing; members of Institute of Tropical Medicine, Nagasaki University and Department of Parasitology, Graduate School of Medicine, Gunma University for technical assistance and precious comments.

Author Contributions

Conceived and designed the experiments: CS SH. Performed the experiments: CS TI KS MH TT SK. Analyzed the data: CS RC HH SH. Contributed reagents/materials/analysis tools: CS SK SH. Wrote the manuscript: CS RC SH.

References

1. Bryce J, Boschi-Pinto C, Shibuya K, Black RE (2005) WHO estimates of the causes of death in children. Lancet 365: 1147-1152. doi:10.1016/S0140-6736(05)71877-8. PubMed: 15794969.
2. Petri WA Jr., Miller M, Binder HJ, Levine MM, Dillingham R et al. (2008) Enteric infections, diarrhea, and their impact on function and development. J Clin Invest 118: 1277-1290. doi:10.1172/JCI34005. PubMed: 18382740.
3. Guerrant RL, Oriá RB, Moore SR, Oriá MO, Lima AA (2008) Malnutrition as an enteric infectious disease with long-term effects on child development. Nutr Rev 66: 487-505. doi:10.1111/j.1753-4887.2008.00082.x. PubMed: 18752473.
4. Irusen EM, Jackson TF, Simjee AE (1992) Asymptomatic intestinal colonization by pathogenic *Entamoeba histolytica* in amebic liver abscess: prevalence, response to therapy, and pathogenic potential. Clin Infect Dis 14: 889-893. doi:10.1093/clinids/14.4.889. PubMed: 1576284.
5. Blessmann J, Ali IK, Nu PA, Dinh BT, Viet TQ et al. (2003) Longitudinal study of intestinal *Entamoeba histolytica* infections in asymptomatic adult carriers. J Clin Microbiol 41: 4745-4750. doi:10.1128/JCM.41.10.4745-4750.2003. PubMed: 14532214.
6. Chadee K, Petri WA Jr., Innes DJ, Ravdin JI (1987) Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. J Clin Invest 80: 1245-1254. doi:10.1172/JCI113199. PubMed: 2890655.
7. Houpt E, Barroso L, Lockhart L, Wright R, Cramer C et al. (2004) Prevention of intestinal amebiasis by vaccination with the *Entamoeba histolytica* Gal/GalNac lectin. Vaccine 22: 611-617. doi:10.1016/j.vaccine.2003.09.003. PubMed: 14741152.
8. Haque R, Ali IM, Sack RB, Farr BM, Ramakrishnan G et al. (2001) Amebiasis and mucosal IgA antibody against the *Entamoeba histolytica* adherence lectin in Bangladeshi children. J Infect Dis 183: 1787-1793. doi:10.1086/320740. PubMed: 11372032.
9. Haque R, Duggal P, Ali IM, Hossain MB, Mondal D et al. (2002) Innate and acquired resistance to amebiasis in bangladeshi children. J Infect Dis 186: 547-552. doi:10.1086/341566. PubMed: 12195383.
10. Fotedar R, Stark D, Marriott D, Ellis J, Harkness J (2008) *Entamoeba moshkovskii* infections in Sydney, Australia. Eur J Clin Microbiol Infect Dis 27: 133-137. doi:10.1007/s10096-007-0399-9. PubMed: 17957394.
11. Shimokawa C, Kabir M, Taniuchi M, Mondal D, Kobayashi S et al. (2012) *Entamoeba moshkovskii* is associated with diarrhea in infants and causes diarrhea and colitis in mice. J Infect Dis 206: 744-751. doi:10.1093/infdis/jis414. PubMed: 22723640.
12. Hamano S, Asgharpour A, Stroup SE, Wynn TA, Leiter EH et al. (2006) Resistance of C57BL/6 mice to amoebiasis is mediated by nonhemopoietic cells but requires hemopoietic IL-10 production. J Immunol 177: 1208-1213. PubMed: 16818779.
13. Diamond LS, Harlow DR, Cunnick CC (1978) A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans R Soc Trop Med Hyg 72: 431-432. doi:10.1016/0035-9203(78)90144-X. PubMed: 212851.
14. Hamzah Z, Petmitr S, Mungthin M, Leelayoova S, Chavalitshewinkoon-Petmitr P (2006) Differential detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* by a single-round PCR assay. J Clin Microbiol 44: 3196-3200. doi:10.1128/JCM.00778-06. PubMed: 16954247.
15. Houpt ER, Glembocki DJ, Obrig TG, Moskaluk CA, Lockhart LA et al. (2002) The mouse model of amebic colitis reveals mouse strain susceptibility to infection and exacerbation of disease by CD4$^+$ T cells. J Immunol 169: 4496-4503. PubMed: 12370386.
16. Asgharpour A, Gilchrist C, Baba D, Hamano S, Houpt E (2005) Resistance to intestinal *Entamoeba histolytica* infection is conferred by innate immunity and Gr-1$^+$ cells. Infect Immun 73: 4522-4529. doi:10.1128/IAI.73.8.4522-4529.2005. PubMed: 16040963.
17. Hamano S, Becker S, Asgharpour A, Ocasio YP, Stroup SE et al. (2008) Gender and genetic control of resistance to intestinal amebiasis in inbred mice. Genes Immun 9: 452-461. doi:10.1038/gene.2008.37. PubMed: 18480826.
18. Haque R, Mondal D, Duggal P, Kabir M, Roy S et al. (2006) *Entamoeba histolytica* infection in children and protection from subsequent amebiasis. Infect Immun 74: 904-909. doi:10.1128/IAI.74.2.904-909.2006. PubMed: 16428733.
19. Abd Alla MD, Wolf R, White GL, Kosanke SD, Cary D et al. (2012) Efficacy of a Gal-lectin subunit vaccine against experimental *Entamoeba histolytica* infection and colitis in baboons (Papio sp.). Vaccine 30: 3068-3075. doi:10.1016/j.vaccine.2012.02.066. PubMed: 22406457.
20. Chadee K, Johnson ML, Orozco E, Petri WA Jr., Ravdin JI (1988) Binding and internalization of rat colonic mucins by the galactose/N-acetyl-D-galactosamine adherence lectin of *Entamoeba histolytica*. J Infect Dis 158: 398-406. doi:10.1093/infdis/158.2.398. PubMed: 2900266.
21. Guo X, Stroup SE, Houpt ER (2008) Persistence of *Entamoeba histolytica* infection in CBA mice owes to intestinal IL-4 production and inhibition of protective IFN-γ. Mucosal Immunol 1: 139-146. doi:10.1038/mi.2007.18. PubMed: 19079171.
22. Inoue M, Tang J, Miyakoda M, Kaneko O, Yui K et al. (2012) The species specificity of immunity generated by live whole organism immunisation with erythrocytic and pre-erythrocytic stages of rodent malaria parasites and implications for vaccine development. Int J Parasitol 42: 859-870. doi:10.1016/j.ijpara.2012.07.001. PubMed: 22846785.
23. Martinelli A, Cheesman S, Hunt P, Culleton R, Raza A et al. (2005) A genetic approach to the de novo identification of targets of strain-specific immunity in malaria parasites. Proc Natl Acad Sci U S A 102: 814-819. doi:10.1073/pnas.0405097102. PubMed: 15640359.

OPEN ACCESS Freely available online

PLOS ONE

Unique T Cells with Unconventional Cytokine Profiles Induced in the Livers of Mice during *Schistosoma mansoni* Infection

Keishi Adachi[1,2¤], Yoshio Osada[3], Risa Nakamura[1], Koji Tamada[4], Shinjiro Hamano[1,2*]

1 Department of Parasitology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan, **2** Global Center of Excellence Program, Nagasaki University, Nagasaki, Japan, **3** Department of Immunology and Parasitology, The University of Occupational and Environmental Health, Yahatanishi-ku, Kitakyushu, Japan, **4** Department of Immunology and Cell Signaling Analysis, Yamaguchi University Graduate School of Medicine, Ube, Japan

Abstract

During infection with *Schistosoma*, serious hepatic disorders are induced in the host. The liver possesses unique immune systems composed of specialized cells that differ from those of other immune competent organs or tissues. Host immune responses change dramatically during *Schistosoma mansoni* infection; in the early phase, Th1-related responses are induced, whereas during the late phase Th2 reactions dominate. Here, we describe unique T cell populations induced in the liver of mice during the period between Th1- and Th2-phases, which we term the transition phase. During this phase, varieties of immune cells including T lymphocytes increase in the liver. Subsets of $CD4^+$ T cells exhibit unique cytokine production profiles, simultaneously producing both IFN-γ and IL-13 or both IFN-γ and IL-4. Furthermore, cells triply positive for IFN-γ, IL-13 and IL-4 also expand in the *S. mansoni*-infected liver. The induction of these unique cell populations does not occur in the spleen, indicating it is a phenomenon specific to the liver. In single hepatic $CD4^+$ T cells showing the unique cytokine profiles, both T-bet and GATA-3 are expressed. Thus, our studies show that *S. mansoni* infection triggers the induction of hepatic T cell subsets with unique cytokine profiles.

Citation: Adachi K, Osada Y, Nakamura R, Tamada K, Hamano S (2013) Unique T Cells with Unconventional Cytokine Profiles Induced in the Livers of Mice during *Schistosoma mansoni* Infection. PLoS ONE 8(12): e82698. doi:10.1371/journal.pone.0082698

Editor: Mauricio Martins Rodrigues, Federal University of São Paulo, Brazil

Received August 2, 2013; **Accepted** October 26, 2013; **Published** December 16, 2013

Funding: This work was supported by JSPS KAKENHI Grants-in-Aid for Scientific Research Number 24790402 (to K.A.), 25670204 (to S.H.), (http://www.jsps.go.jp/english/e-grants/index.html), a Health Labour Sciences Research Grant (H20-Shinkoh-Ippan-016, H23-Shinkoh-Ippan-014 to S.H.) (http://www.mhlw.go.jp/english/), and the Global COE Program, Nagasaki University, supported by MEXT (to S.H.) (http://www-sdc.med.nagasaki-u.ac.jp/gcoe/activities/publication/200901_e.html, http://www.jsps.go.jp/english/e-globalcoe/index.html). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: shinjiro@nagasaki-u.ac.jp

¤ Current address: Department of Immunology and Cell Signaling Analysis, Yamaguchi University Graduate School of Medicine, Ube, Japan

Introduction

The liver is characterized by a unique micro-anatomical and immunological environment [1–3]. It contains Kupffer cells and a large number of resident lymphocytes, including NK cells and NKT cells, whose immunological environment unlike that of any other organs or tissues [2,4]. Although a large amount of enteric and systemic blood-borne antigens constitutively enter into, are trapped and accumulated in the liver, immune responses are tightly regulated in a homeostatic state, and many hepatic lymphocytes show 'activated-yet-resting' phenotypes. Important pathogens, for example, the hepatitis C virus and malaria parasites, take advantage of the liver's immune condition, circumvent immunity, and establish chronic infections [5,6]. In contrast, some microorganisms such as the hepatitis B virus induce severe immune reactions in a liver, resulting in fulminant hepatitis [6,7]. Why liver-specific immune competent cells show such uncommon and inconsistent features remains unresolved.

Parasitic worms are important pathogens, affecting the health of roughly 2 billion people living mostly in tropical and subtropical environments [8]. One specific genus within Platyhelminths, the *Schistosoma*, constitutes a major health burden for human populations in many parts of the world. In 2009, 239 million people were infected with schistosomes, 85% of them in sub-Saharan Africa, where approximately 150,000 deaths per year were attributable to the worms [9–11].

Following skin penetration by cercariae, schistosomes migrate via the blood to the hepatic portal vein, where they rapidly mature and mate. Egg production begins 4–6 weeks following infection. A single female parasite is estimated to produce 300 eggs per day, many of which enter the liver via the blood stream. The liver, therefore, is a primary organ of pathogenic injury and subsequent granulomatous tissue damage, and pathogenesis in liver is the most important for etiology, although chronic inflammation is induced in several other organs [12–14].

Following infection with *Schistosoma mansoni* (*S. mansoni*) cercariae, the host immune responses progress through at least two phases. Th1-related responses are induced in the early phase (3–5 weeks postinfection, PI). As the parasites begin oviposition (4.5–6 weeks PI), the Th1 components are gradually down-regulated, and strong Th2 reactions are induced [12]. Intensive studies of the Th2 phase have been conducted, as it is during this period that hepatic pathology becomes prominent. However, the roles of immune reactions during early Th1 phase, especially in the liver, have been

little investigated [12,14–17]. It has been previously reported that the balance between Th1 and Th2 responses are important for the severity of schistosomiasis, and that 'smooth' phase transitions are observed in hosts not showing serious symptoms [12,13,18–20].

It is conventionally believed that Th1 and Th2 reciprocally inhibit their generations, and that one helper T cell does not normally produce both Th1- and Th2-related cytokines, particularly IFN-γ and IL-4, simultaneously, [21,22]. Therefore, we hypothesized that unknown cellular and/or molecular mechanisms 'bridging' Th1 and Th2 generation occur in the liver between Th1 and Th2 phase ('transition' phase) of *S. mansoni* infection. In order to test this hypothesis, we analyzed the immune responses induced in the liver following *S. mansoni* infection, using mouse cercarial infection models.

Here we show that unique $CD4^+$ T cell populations that simultaneously produce Th1- and Th2-cytokines, combinations of "IFN-γ and IL-13" and "IFN-γ and IL-4", accumulate in the liver, but not in the spleen, during the transition phase of *S. mansoni* infection. Moreover, some of these unique populations acquire the potential for secreting the three cytokines concomitantly. Our present observations provide new insights into the mechanisms underlying the pathogenesis of schistosomiasis. Furthermore, these findings point to a new concept in T cell biology; the antagonism between Th1 and Th2 responses can be resolved in some immunological conditions.

Materials and Methods

Mice

Female BALB/c mice (6–10 week-old) and C57BL/6 mice (6–10 week-old) were purchased from SLC (Shizuoka, Japan), and maintained under specific pathogen-free conditions. Experiments were conducted with BALB/c mice unless otherwise specified.

Maintenance of the parasite life cycle and infection of mice with *S. mansoni*

S. mansoni was maintained as previously described [23,24]. Mice were anesthetized and percutaneously infected with 25 *S. mansoni* cercariae as previously described [25]. Egg burden was microscopically observed in feces and the caudate lobe of the liver, and in most cases, began at 4–5 weeks PI (data not shown), as previously reported [12].

Intracellular cytokine staining (ICS)

ICS technology was used to monitor cytokine production [26]. In brief, hepatic lymphocytes and splenocytes were prepared from mice at indicated weeks after the infection as previously described [27–29]. In each group, hepatic lymphocytes isolated from 3 mice were pooled in order to obtain sufficient cell numbers. These were then stimulated with immobilized anti-mouse CD3 (17A2, BioLegend) and anti-CD28 (E18, BioLegend) for 5 hours in the presence of brefeldin A. Cell surface molecules were stained with PE-Cy5-, PE-Cy7-, or Allophycocyanin (APC)-Cy7-conjugated anti-CD4 (GK1.5, BioLegend), APC-conjugated anti-CD8α (53-6.7, BioLegend), APC-conjugated pan-NK cell (DX5, BioLegend), PE-Cy7-conjugated anti-CD62L (MEL-14, BioLegend), PerCP-Cy5.5-conjugated anti-CD44 (IM7, BioLegend), PerCP-Cy5.5-conjugated anti-CD27 (LG.3A10, BioLegend), PerCP-Cy5.5-conjugated anti-CD197 (CCR7, 4B12, BioLegend), PE-Cy7-conjugated anti-CXCR5 (2G8, BD Biosciences), or PerCP-Cy5.5-conjugated anti-CD278 (ICOS, C398.4A, BioLegend). Fixation and permeabilization of the cells were conducted with 2% formaldehyde and 0.5% saponin, respectively. For the detection of intracellular cytokines, FITC-, PE-, or APC-conjugated, corresponding monoclonal antibodies were used (IL-4; 11B11, IFN-γ; XMG1.2, IL-5; TRFK5, BioLegend; IL-13; eBio13A, eBioscience). Flowcytometric analysis was conducted with FACSCalibur, FACSCanto II, or FACSVerse (BD Biosciences), and the data were analyzed with CellQuest (BD Biosciences) or FlowJo software (Tree Star, Inc.). Culture medium was RPMI-1640 supplemented with 10 % FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mM of 2-mercaptoethanol and 2 mM L-glutamine.

Flowcytometric analysis of transcription factors

Flowcytometry was used for the analysis of transcription factors. Briefly, cell surface molecules were stained with fluorochrome-conjugated monoclonal antibodies as mentioned above. Fixation, permeabilization, and staining of the target transcription factors were conducted with FoxP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. For the detection of the transcription factors, PerCP-Cy5.5-conjugated anti-T-box expressed in T-cells (T-bet, 4B10, BioLegend), PE-Cy7-conjugated anti-Gata-binding protein 3 (GATA-3, L50-823, BD Biosciences), or Alexa Fluor® 647 anti-Bcl-6 were used.

Statistics

All data are shown as the mean values of more than three independent experiments. Significance between the control group and treated group was determined with Student's unpaired *t*-tests. *P* values less than 0.05 were considered significant.

Ethics Statement

All mouse experiments were conducted according to relevant national and international guidelines, and were approved by the Institutional Animal Care and Use Committee at Nagasaki University.

Results

Explosive increase of immune competent cells in the livers of *S. mansoni*-infected mice during the transition phase

It has been reported that the balance between Th1- and Th2-related immune responses in a host infected with *S. mansoni* is closely related to the severity of the disease [12,13,18–20]. The most serious form of intestinal schistosomiasis is hepatic disorders [12–14]. This prompted us to analyze the immunological events induced in the liver during *S. mansoni* infection. First, we investigated the kinetics of the number of hepatic cells recruited into and/or expanded in the liver following cercarial infection. Hepatic cells were isolated at 0, 2, 4, 5, 6, 7, and 8 weeks PI. As shown in *Figure 1A*, cell yields began to increase at 4 weeks PI and peaked at 5–7 weeks PI, suggesting that significant numbers of cells infiltrated into and/or expanded in the liver during the transition phase of *S. mansoni* infection.

Next, we investigated the components of the cell populations that increased in the liver during the transition phase by flowcytometry. Consistent with previous reports [12,14,30], $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, and eosinophils were found (Fig. 1B, S1). Notably, most (≥ 70%) of $CD4^+$ and $CD8^+$ T cells exhibited an activated phenotype (CD62L-negative) in the liver, but not in the spleen, irrespective of the infection (Fig. 1C). Since total cell yield was dramatically increased after the infection (Fig. 1A), the absolute number of the activated T cells also increased (data not shown). Taken together, *S. mansoni* infection induced a marked increase in cell number including

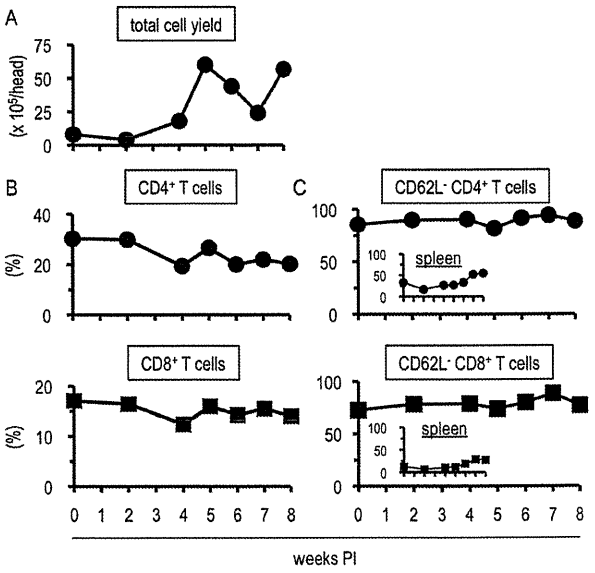

Figure 1. *Schistosoma mansoni* infection induced robust increase of immune competent cells in the liver. (A) Hepatic cells isolated from 3 mice were pooled and the cell number was calculated. (B and C) Flowcytometric analysis was conducted with the liver lymphocytes prepared in (A) or the splenocytes. The percentages in (B) represent the proportions in CD3-positive population, and those in (C) express the proportions in population of (B). (A-C) each shows one representative result of three independent experiments.
doi:10.1371/journal.pone.0082698.g001

activated T cells in the liver particularly during the transition phase.

Hepatic CD4$^+$ T cells induced during the transition phase of *S. mansoni* infection demonstrate a unique potential for cytokine production

As cytokine environments, especially those of CD4$^+$ helper T cells, play important roles in the generation of hepatic granulomatous lesions in intestinal schistosomiasis [12–14,31–33], the cytokine profiles of the hepatic CD4$^+$ T cells were analyzed. For this purpose, we conducted ICS experiments upon TCR-ligated cells, and found that *S. mansoni* infection elicited hepatic CD4$^+$ T cells to produce TNF-α, IL-5, or IL-10. In contrast, little IL-17 production was observed (Fig. S2). The production of all cytokines investigated started after 4 weeks PI, the early transition phase. It is noteworthy that the kinetics of the T cell population positive for each cytokine showed individually distinct time-courses (Fig. S2). This suggests that *S. mansoni* infection confers a wide variety of cytokine production to hepatic CD4$^+$ T cells, and that the local immune environment in the liver is closely related to fluke growth.

Next, we focused upon the typical Th1- and Th2-related cytokines, IFN-γ, IL-4, and IL-13. Similar to reports concerning systemic T cell reponses [12,13], the increase of IFN-γ-producing CD4$^+$ T cells began earlier than that of IL-4- or IL-13-producing cells in the liver (Fig. 2). Yet, unlike the systemic immune reactions previously reported [12,13], CD4$^+$ T cells secreting IFN-γ were not clearly down-regulated in the phase when Th2 cytokine-secreting T cells were increasing (Fig. 2).

Intriguingly, some hepatic CD4$^+$ T cells isolated from *S. mansoni*-infected mice exhibited unique cytokine profiles, producing both IFN-γ and IL-4 ("γ4 cells") or IFN-γ and IL-13 ("γ13 cells")

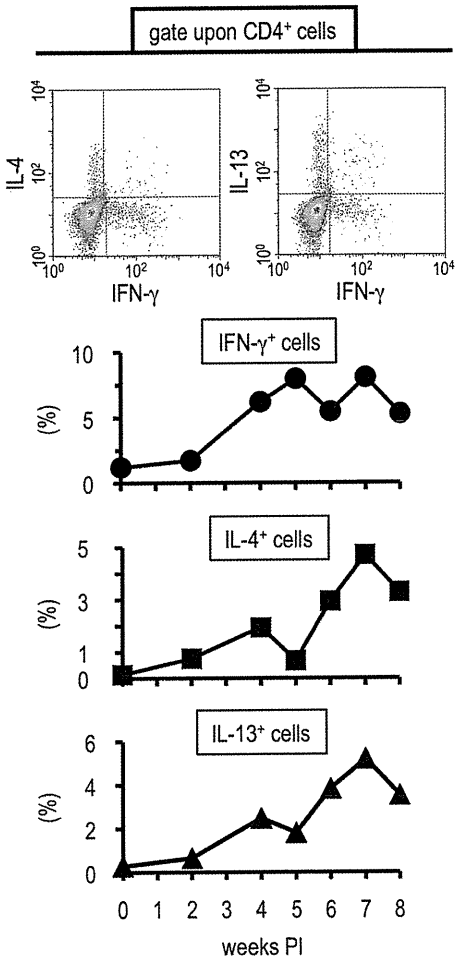

Figure 2. Th1 cells were induced in early and Th2 cells in late phase in the liver. Hepatic lymphocytes were isolated from *S. mansoni*-infected BALB/c mice at indicated time points, and their potential for producing IFN-γ, IL-4, or IL-13 was analyzed by ICS upon TCR ligation. Insets at the top represent one example using liver lymphocytes prepared at 4 weeks PI are shown. The percentages represent the proportions in CD4-positive population. Similar results were obtained in three independent experiments.
doi:10.1371/journal.pone.0082698.g002

(Fig. 3A). Both populations began to increase their proportion and absolute numbers during the transition phase of the infection (Fig. 3B). Then, in the late phase, when strong Th2 responses were induced systemically, both γ4 and γ13 cells showed a tendency to decrease (Fig. 3B). Hepatic CD8$^+$ T cells produced neither IL-4 nor IL-13 throughout the infection although obvious IFN-γ production was observed (Fig. S3, data not shown). Hence, populations exhibiting unique cytokine profiles were not observed within the CD8$^+$ T cell population. This suggests that hepatic CD4$^+$ T cells, but not CD8$^+$ T cells, acquired unconventional cytokine productivities during *S. mansoni* infection, especially during the transition phase. In the spleen, neither γ4 nor γ13 cells were induced throughout the infection (Fig. S4), indicating that the induction of these unusual cell populations was not ubiquitously observed in the body, but were liver-specific immunological events. As shown in *Figure S5*, both γ4 and γ13 cells were induced not only in the livers of BALB/c but also in

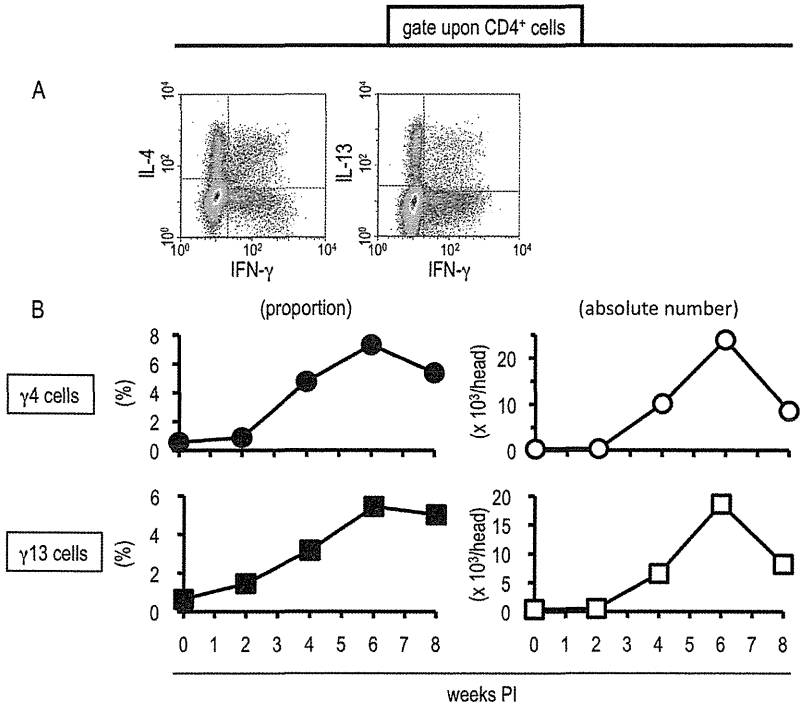

Figure 3. *S. mansoni* infection-induced hepatic T cells exhbited the potential to produce uncommon combinations of cytokines. Hepatic lymphocytes were isolated from *S. mansoni*-infected mice at indicated time points, and the proportions and absolute numbers of γ4 and γ13 cells were investigated by ICS. (A) One example using hepatic lymphocytes prepared at 6 weeks PI is displayed. (B) The percentages represent the proportions in CD4-positive population. This experiment is representative of three independent experiments.
doi:10.1371/journal.pone.0082698.g003

those of C57BL/6 mice during the transition phase of the infection. Therefore, the acquisition of the unique cytokine profiles was not restricted to a particular strain of congenic mice.

We next analyzed whether the unique hepatic T cell populations had the ability to produce IFN-γ, IL-4, and IL-13 simultaneouly. As shown in *Figure 4A*, γ4 cells were observed within the IL-13-producing $CD4^+$ T cell population. γ13 cells were also detected within the IL-4-secreting cells (Fig. 4B). And within the IFN-γ^+ population, $CD4^+$ cells doubly positive for IL-4 and IL-13 were also found (Fig. 4C). Taken together, some proportion of the hepatic $CD4^+$ T cells in the liver during the transition phase of the infection indeed acquired the capacity for producing IFN-γ, IL-13, and IL-4 simultaneously ("triple positive cells").

In the absence of TCR stimulation ex vivo, the freshly isolated hepatic lymphocytes produced little cytokine (Fig. S6). Moreover, incubation of the hepatic lymphocytes on the plates coated with isotype matched control antibodies, instead of anti-CD3 and anti-CD28, rarely induced cytokine secretion (data not shown).

All of the results, collectively, suggested that during the transition phase of *S. mansoni* infection, hepatic $CD4^+$ T cells acquire the potential to produce very unique combinations of cytokines; 'co-prime' Th1- and Th2-related cytokines.

The unique hepatic $CD4^+$ T cells demonstrate a production selectivity of Th2-related cytokines

Next, we investigated whether the unique liver T cells have the potential to produce Th2 cytokines other than IL-4 and IL-13. For this purpose, we selected IL-5 as a Th2-related cytokine other than IL-4 and IL-13, because infiltration of eosinophils into the liver and up-regulation of IL-5-producing $CD4^+$ T cells were induced after *S. mansoni* infection, particularly during the transition phase (Fig. S1 and S2). TCR ligation elicited the simultaneous production of IFN-γ and IL-4 or IFN-γ and IL-13 from the hepatic $CD4^+$ T cells isolated during the transition phase (Fig. 5A, Fig. 3). However, few cells produced IFN-γ and IL-5 concurrently although $CD4^+$ T cells that solely secrete either IFN-γ or IL-5 were unambigously induced in the liver (Fig. 5B). Taken together, *S. mansoni* infection confers selectivity for producing Th2-related cytokines as well as the unique cytokine profiles mentioned above on hepatic $CD4^+$ T cells.

αβ T cells, but not γδ T cells, exhibit uncommon cytokine profiles upon *S. mansoni* infection

We analyzed the precise characters of the hepatic $CD4^+$ T cell populations showing the unique cytokine secretion patterns. γδ T cells can act as producers of Th2- as well as Th1-related cytokines in some situations [34–37], and the liver is rich in γδ T cells [34–36,38]. Therefore, we first analyzed whether γδ T cells could be γ13 and γ4 cells. As shown in *Figure 6A*, the unique T cell populations little expressed γδ TCR. Moreover, neither γ13 nor γ4 cells were observed within γδ TCR-positive population throughout the infection (Fig. 6B). In contrast, after *S. mansoni* infection, the αβ TCR-expressing population displayed the unique cytokine production patterns during the transition phase (Fig. 6B). Taken together, this suggests that *S. mansoni* infection confers uncommon capacities for cytokine production upon αβ T cells but not on γδ T cells during the transition phase.

Then, we characterized the hepatic γ4 and γ13 cells more precisely with several cell surface molecules. As shown in *Figure S7*,

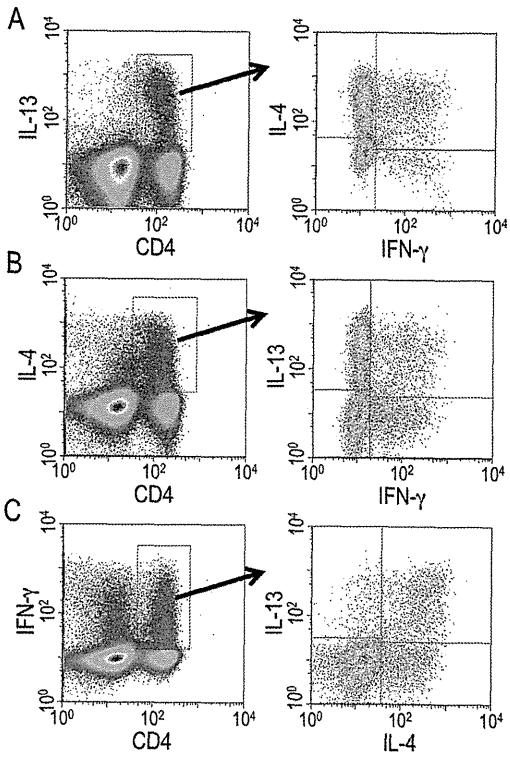

Figure 4. The unique hepatic T cells exhbited the ability to produce IFN-γ, IL-13, and IL-4 simultaneouly. Hepatic lymphocytes were isolated from *S. mansoni*-infected mice at 6 weeks PI and ICS was conducted after TCR stimulation. γ4 cells within IL-13-producing $CD4^+$ T cell population (A), γ13 cells within IL-4-producing $CD4^+$ T cell population (B), and IL-4- and IL-13-secreting cells within IFN-γ-producing $CD4^+$ T cell population (C) were analyzed. Data shown are a representative of five independent experiments.
doi:10.1371/journal.pone.0082698.g004

the majority of the both cell types exhibited CD62L-negative, CD44-positive, CD27-negative, and CCR7-negative phenotypes. This indicates that most of the hepatic γ4 and γ13 cells possesses the features resembling to effector memory T cells.

DX5-negative as well as –positive cells displayed unique cytokine production patterns

DX5, also known as integrin α2, is a mouse pan-NK cell marker. DX5-expressing T cells including classical iNKT cells, whose generation is restricted to CD1d and whose TCR is invariant, are abundant in the liver even in the homeostatic state [39–41] and rapidly increase in number in some circumstances, such as during *Plasmodium* spp. infection [27,42]. It is well-known that both NKT cells and activated T cells have a high-potential for producing cytokines. This prompted us to analyze whether DX5 is expressed upon hepatic γ4 and γ13 cells induced during the transition phase of *S. mansoni* infection, i.e., whether the γ4 and γ13 cells consist of a single population or not. As shown in *Figure 7A*, γ4 cells were observed within both DX5-negative and -positive cell populations. This is also the case for γ13 cells (Fig. 7B). The ratio of γ4 or γ13 cells within DX5-positive population was higher than that within DX5-negative population (Fig. 7C). These results suggest that the unique hepatic $CD4^+$ T cell populations could be divided into two sub-populations; DX5-negative 'conventional' T cells and DX5-positive T cells, including NKT cells.

T-bet and GATA-3 were co-expressed within a single γ4 or γ13 cell

T-bet and GATA-3 are the crucial transcription factors for Th1 and Th2, respectively. It has been believed that they counteract reciprocally and cannot be co-expressed within a T cell [43,44]. However, it was recently reported that the expression of GATA-3 could be induced in Th1 cells expressing T-bet, and that several kinds of Th2-related cytokines, including IL-13, can be released from the Th1 cells [45–47]. This prompted us to investigate the expression of T-bet and GATA-3 in a hepatic γ4 or γ13 cells induced during the transition phase of *S. mansoni* infection. As exhibited in *Figure 8*, large proportions of both γ4 and γ13 cells expressed T-bet and GATA-3 simultaneously. This strongly suggests that the combined expression of T-bet and GATA-3 within a single γ4 or γ13 cell should play a definitive role in the simultaneous production of IFN-γ and IL-4 or IFN-γ and IL-13, respectively.

Discussion

The generations of Th1 and Th2 immune responses are mutually repressed, and, in general, Th1- and Th2-related cytokines, especially IFN-γ and IL-4, the most typical Th1 and Th2 cytokines respectively, cannot be simultaneously secreted from one T cell [21,22]. However, according to several previous reports [12,13,18–20], Th1- and Th2-immune conditions coexist in a *S. mansoni*-infected host, particularly in the liver when symptoms are not severe. We interrogated the previously unidentified cellular and/or molecular mechanisms allowing the coexistence of Th1 and Th2 in the liver during the transition phase of *S. mansoni* infection, the period between early Th1- and late Th2-dominant phases.

We show that *S. mansoni* infection induces the accumulation of unique $CD4^+$ T cell populations in the liver of mice during the transition phase. These hepatic T cells produce uncommon combinations of cytokines, "IFN-γ and IL-13" and "IFN-γ and IL-4". It was notable that T cells triply positive for IL-13, IL-4, and IFN-γ were also induced in the liver. Furthermore, these hepatic $CD4^+$ T cells did not indiscriminately produce Th2-related cytokines, rather they preferentially produce a specific Th2 cytokines, as IL-5 was not produced.

Recently, it was reported that γδ T cells can acquire the potential to simultaneously produce IFN-γ and IL-4 [48]. As shown in *Figure 6*, both γ13 and γ4 cells that were induced during the transition phase of *S. mansoni* infection expressed αβ TCR, but not γδ TCR. DX5-negative T cells produced the combinations of "IFN-γ and IL-13" or "IFN-γ and IL-4" (Fig. 7). Not only DX5-negative T cells, but also DX5-positive T cells simultaneously produced these cytokines. As some NKT cells were reported to possess the ability to dually secrete IFN-γ and IL-4 [49,50], NKT cells may be involved in such hepatic γ13 and γ4 cells. ICS with α-galactosylceramide/CD1d tetramer upon the hepatic $CD4^+$ T cells or the usage of NKT cell-deficient mice, such as $cd1d^{-/-}$ or $j\alpha18^{-/-}$ mice, may elucidate it. The expression of DX5 can be induced and up-regulated upon conventional DX5-negative T cells after activation [51,52]. In addition, as shown in *Figure 1B*, most of the hepatic T cells were negative for CD62L and displayed an activated phenotype. Therefore, DX5-positive hepatic T cells, which showed unique cytokine profiles, would contain not only NKT cells but also conventional T cells upon which DX5 expression was induced. Collectively, conventional T cells are able to acquire the potential to produce uncommon combinations of cytokines during *S. mansoni* infection.

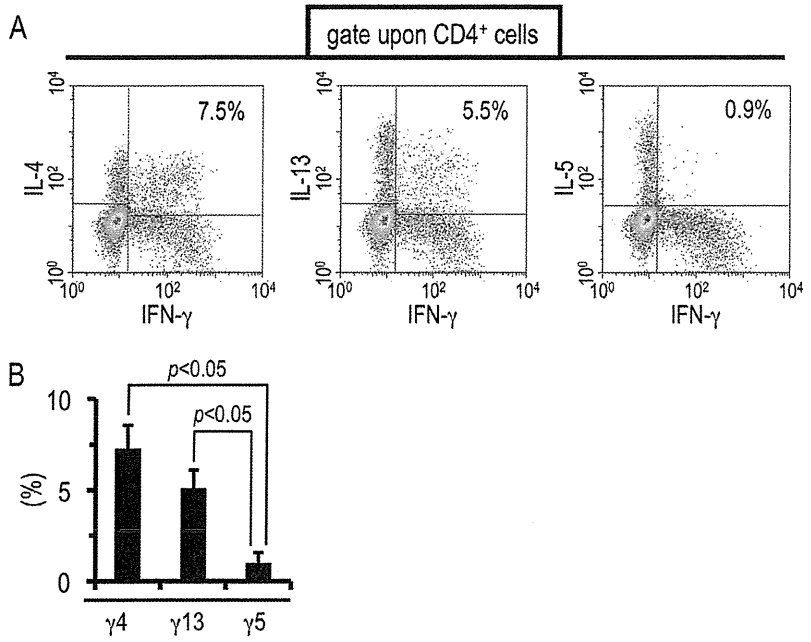

Figure 5. CD4$^+$ T cells doubly producing IFN-γ and IL-5 were rarely induced after *S. mansoni* infection. Hepatic lymphocytes were isolated from *S. mansoni*-infected mice at 6 weeks PI and ICS was conducted upon TCR ligation. (A) One representative result is shown. The numbers in the insets represent the percentages of γ4, γ13, or γ5 in CD4-positive population. (B) Data represent the mean values + SD of three independent experiments.
doi:10.1371/journal.pone.0082698.g005

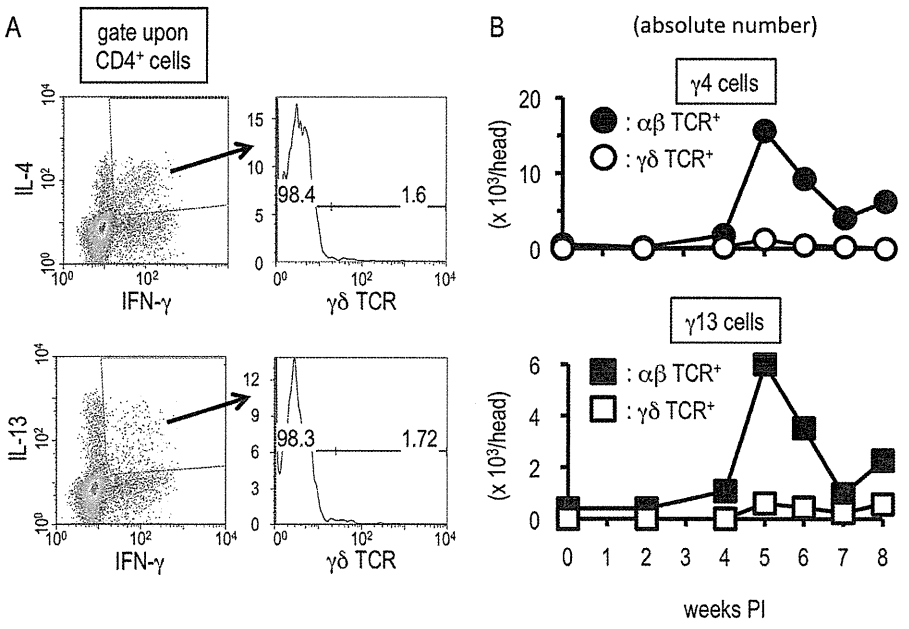

Figure 6. Hepatic αβ T cells are the responsible cells showing the unconventional cytokine profiles. Hepatic lymphocytes were isolated from *S. mansoni*-infected mice and flowcytometry was conducted after TCR ligation. (A) Expression levels of γδ TCR on CD4$^+$ γ4 cells or γ13 cells were analyzed with the hepatic lymphocytes isoleted at 6 weeks PI. The values in the right insets indicate percentages of γδ TCR-positive or –negative population in CD4$^+$ γ4 or γ13 cells. This experiment is a representative of four independent experiments. (B) The absolute numbers of γ4 cells (upper graph) or γ13 cells (lower graph) in αβ TCR- or γδ TCR-potitive population were investigated. Similar results were obtained in three independent experiments.
doi:10.1371/journal.pone.0082698.g006

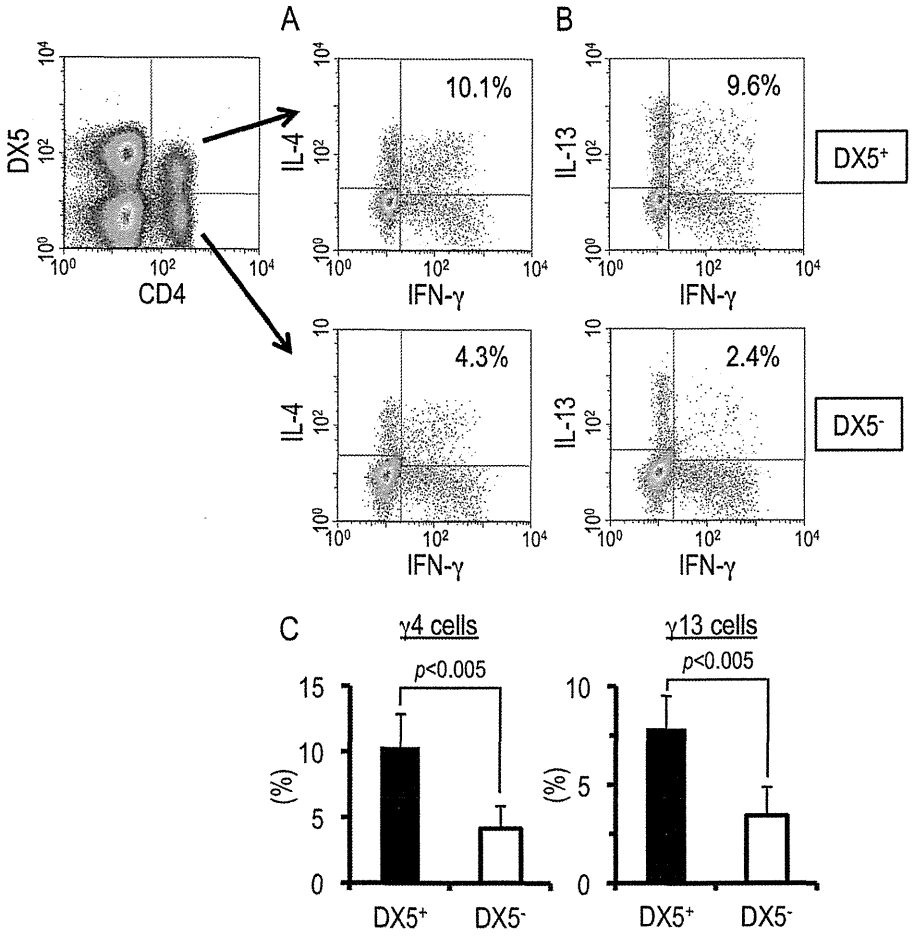

Figure 7. DX5-negative as well as -positive cells displayed the unique cytokine production patterns. (A and B) Hepatic lymphocytes were isolated from *S. mansoni*-infected mice and flowcytometric analysis was conducted upon TCR ligation at 6 weeks PI. The numbers in the insets represent percentages of γ4 (A) or γ13 (B) cell population in $CD4^+$ DX5-positive or -negative population. This experiment is representative of five independent experiments. (C) Data represent the mean + SD of five independent experiments.
doi:10.1371/journal.pone.0082698.g007

Although T-bet and GATA-3 should be important for the induction of the uncommon cytokine productivities upon the hepatic γ4 and γ13 cells (Figure 8), a possibility of the involvement of another transcription factor, promyelocytic leukemia zinc finger (PLZF), may not be excluded, as PLZF plays an important role in the exertion of the functions of γδ T cells and NKT cells that dually produced IFN-γ and IL-4 [48–50]. Furthermore, PLZF-transgenic T cells produced IFN-γ and IL-4 concomitantly upon TCR ligation [53]. As ectopic expression of PLZF seems to convert differentiated T cells into 'innate' type cells [53,54], PLZF might have a function to reset the commitment to Th1 and Th2 cells.

T follicular helper cells (Tfh) are another T cell subset that can produce IFN-γ and IL-4 [55–58]. However, it remains controversial whether Tfh can produce IFN-γ and IL-4 "simultaneously", and whether IFN-γ-producing Tfh cells and IL-4-producing Tfh cells are different sub-populations. Thus far, IL-13 production by Tfh has not been reported. Actually, the expression of the representative Tfh markers (CXCR5, ICOS, bcl-6) were rarely detected upon the hepatic γ4 and γ13 cells during the transition phase of *S. mansoni* infection (Fig. S8, data not shown). Moreover, no follicular structures were observed in the livers of *S. mansoni*-infected mice by histological analysis (data not shown). Therefore, it is unlikely that Tfh cells are the major population of the *S. mansoni*-induced hepatic γ4 and γ13 cells.

To our knowledge, this is the first report demonstrating conventional T cells simultaneously producing IFN-γ and IL-4, typical Th1 and Th2 cytokines, respectively. There are also very few other reports of T cells secreting triple cytokines IFN-γ, IL-13 and IL-4. Indeed, IL-18-elicited Th1 cells produced not only IFN-γ but also several Th2-related cytokines, including IL-13, and the Th2 cytokine-producing Th1 cells are termed as "super Th1 cells" [45,46,59]. Yet, super Th1 cells were reported not to secrete IL-4 [46]. Accordingly, the hepatic γ13 cells induced during *S. mansoni* infection may correspond to super Th1 cells, the γ4 cells and the triple positive cells were likely to be distinct from them.

Unfortunately, the roles of the unique $CD4^+$ T cells described here upon the pathology of schistosomiasis are still unclear, as a method to specifically deplete or isolate these cells has not been established. *In vitro* culture system for the generation of T cells with the uncommon cytokine profiles is also unestablished. One promising way to establish such a method is through the

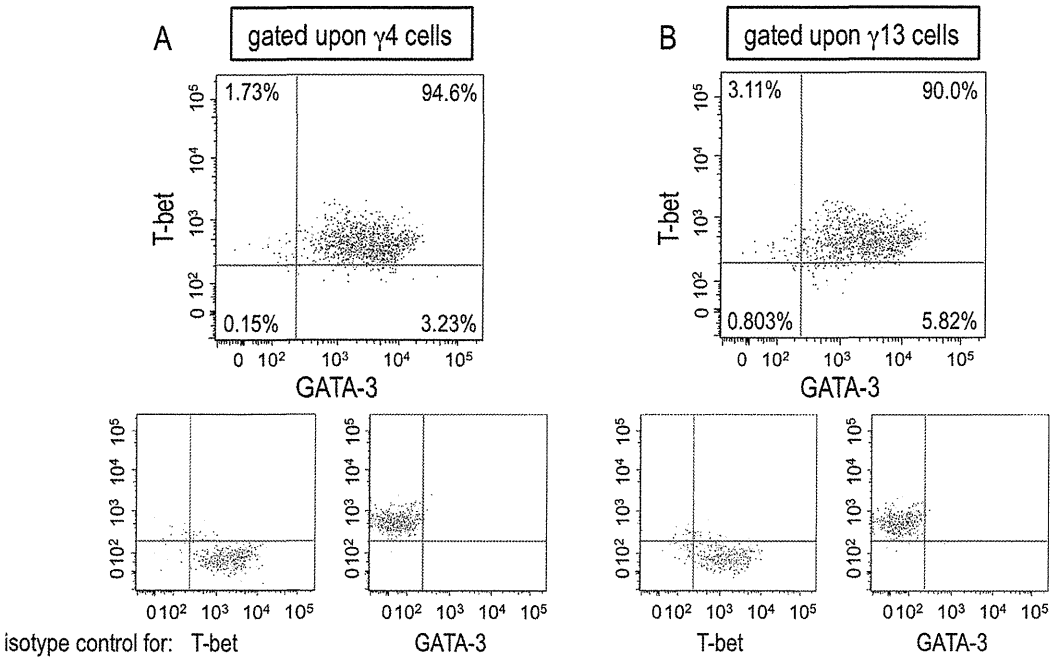

Figure 8. Both T-bet and GATA-3 were expressed in a single hepatic γ4 or γ13 cell. (A and B) Hepatic lymphocytes were isolated from *S. mansoni*-infected mice and the expressions of T-bet and GAT-3 were analyzed by flowcytometry after TCR stimulation at 6 weeks PI. The numbers in the upper, large insets represent percentages of each population divided by the expressions of T-bet and GATA-3 in γ4 (A) or γ13 (B) cells. The lower, small insets represent the data using isotype control antibodies. Similar results were obtained in three independent experiments.
doi:10.1371/journal.pone.0082698.g008

identification of unique marker(s) for the population. Other ways are to understand the mechanisms of differentiation to, or expansion of, the unique hepatic T cell populations, which remain to be elucidated. The machinery of the induction and regulation of PLZF in T cells is unknown. One attractive way is using IL-18-deficient mice since IL-18 stimulation converts Th1 cells to super Th1 cells, that express GATA-3 and secrete IL-13, as mentioned above. However, super Th1 cells are not IL-4-producing cells. Therefore, it is conceivable that factors other than IL-18 are essential for the differentiation to the γ4 and the triple positive cells induced after *S. mansoni* infection, although some other cytokines and/or liver-specific microenvironments are likely to be required. Further studies are necessary for clarifying the role of these uncommon hepatic T cells in schistosomiasis.

It remains unclear the origin of this unique cell population; naïve T cells, antigen-specific Th1 cells, or Th2 cells. As mentioned above, PLZF-transgenic T cells that are not committed to Th1 or Th2 turn to be γ4 cells [53]. Therefore, conventional naïve T cells might possess inherently the potential to acquire the unique cytokine profiles although it has not been reported whether PLZF is induced in conventional T cells under biological condition. Meanwhile, the uncommon T cells reported here proliferate in the liver, but not in the spleen, during transition phase of the infection, when oviposition begins (4–5 weeks PI, data not shown). This may indicate the importance of the antigens derived from the maturing worm and/or the egg. At present, it is ambiguous whether the unique T cells described here expanded mono-, oligo-, or poly-clonally. Recently, omega-1, a glycoprotein which is secreted from *S. mansoni* eggs and which is most abundantly present in soluble egg antigen, has been demonstrated to condition dendritic cells (DCs) to prime Th2 responses [60–62]. In these reports, naïve T cells were co-cultured with omega-1-immunized DCs and differentiation to Th2 cells was demonstrated. Since the unique hepatic T cells increased during the transition phase of *S. mansoni* infection, it would be worthwhile to analyze the fate of Th1 cells, not Th2 cells, stimulated with omega-1-immunized DCs, but we cannot exclude the possibility that Th2 cells changed their nature. In addition, the effect of omega-1 upon antigen-presenting cells in a liver, such as hepatic DCs, Kupffer cells, and hepatocytes [63], also warrants investigation.

In summary, these data shed light on the etiology of schistosomiasis. Furthermore, these results also put forward a novel concept in T cell biology; that the commitment to Th1 or Th2 might be reset in some immunological conditions.

Supporting Information

Figure S1 Varieties of immune competent cells were found in the liver after *S. mansoni* infection. (A-C) Hepatic cells were isolated at indicated time points from 3 BALB/c mice and were pooled for conducting following flowcytometric analysis. (A) The percentages represent the proportions of $B220^+$ $CD19^+$ cell population. (B) The percentages indicate the proportions of $DX5^+$ cells in CD3-negative population. (C) The percentages show the proportions of Siglec-F^+ population in the cells with $CD45^+$ $CD11c^{low/-}$ staining profile. (A-C) Similar results were obtained in three independent experiments.
(TIF)

Figure S2 *S. mansoni* infection elicited various cytokine production, except for IL-17 production, upon hepatic T cells. Hepatic lymphocytes were isolated from *S. mansoni*-infected

mice at indicated time points, and their potential for producing TNF-α, IL-10, IL-5, or IL-17A was analyzed by ICS upon TCR ligation. Insets at the top represent one example using liver lymphocytes prepared at 4 weeks PI. The values represent the percentages in CD4-positive population. This experiment is representative of three independent experiments.
(TIF)

Figure S3 *S. mansoni* infection induced production of IFN-γ but neither IL-4 nor IL-13 from hepatic CD8$^+$ T cells. Hepatic lymphocytes were isolated from *S. mansoni*-infected mice at 6 weeks PI, and ICS was conducted for investigating the potential of CD8$^+$ T cells to produce IFN-γ, IL-4, or IL-13. One example representative result is shown. The percentages in the insets represent the proportions in CD8-positive population. Similar results were obtained in five independent experiments.
(TIF)

Figure S4 Neither γ13 nor γ4 cells were induced in the spleens of *S. mansoni*-infected mice. (A and B) Hepatic lymphocytes and splenocytes were isolated from *S. mansoni*-infected mice at indicated time points, and ICS was conducted after TCR stimulation. (A) One example using hepatic lymphocytes prepared at 6 weeks PI is displayed. The percentages in the insets represent the proportions in CD4-positive population. (B) The proportions of γ4 cells (upper graph) or γ13 cells (lower graph) in CD4-positive hepatic or splenic lymphocytes were investigated. Similar results were obtained in three independent experiments.
(TIF)

Figure S5 The unique T cells were induced in the livers of *S. mansoni*-infected C57BL/6 mice. (A and B) Hepatic lymphocytes were isolated from *S. mansoni*-infected BALB/c or C57BL/6 mice at indicated time points, and ICS was conducted upon TCR ligation. (A) One example using liver lymphocytes prepared at 6 weeks PI is exhibited. (B) The proportions of γ4 cells (upper graph) or γ13 cells (lower graph) in CD4-positive hepatic lymphocytes were investigated. This experiment is representative of three independent experiments.
(TIF)

Figure S6 TCR ligation is required for the induction of cytokine production by the hepatic lymphocytes. In the absence of TCR stimulation, ICS was conducted using fresh hepatic lymphocytes isolated from *S. mansoni*-infected mice at 6 weeks PI. This experiment is representative of two independent experiments.
(TIF)

Figure S7 The hepatic γ4 and γ13 cells showed effector memory T cell-like surface phenotypes. Hepatic lymphocytes were isolated from *S. mansoni*-infected mice at 6 weeks PI, and ICS was conducted after TCR stimulation. The percentages in the insets represent the proportions in γ4 or γ13 cells. Similar results were obtained in two independent experiments.
(TIF)

Figure S8 Both γ4 and γ13 cells little expressed the surface markers of Tfh cells. Hepatic lymphocytes were isolated from *S. mansoni*-infected mice at 6 weeks PI and ICS was conducted upon TCR ligation. The percentages in the insets represent the proportions in γ4 or γ13 cells. One representative result of two independent experiments is shown.
(TIF)

Acknowledgments

We thank Mitsumasa Miura, Fumie Hara, Masako Hayashida and Megumi Hamasaki for great technical support, Tomoko Takaya, Hiromi Oda and for administrative support; Richard Culleton for scientific editing.

Author Contributions

Conceived and designed the experiments: KA KT SH. Performed the experiments: KA. Analyzed the data: KA. Contributed reagents/materials/analysis tools: KA YO RN. Wrote the paper: KA SH.

References

1. Tsutsui H, Matsui K, Okamura H, Nakanishi K (2000) Pathophysiological roles of interleukin-18 in inflammatory liver diseases. Immunol Rev 174: 192–209.
2. Crispe IN (2003) Hepatic T cells and liver tolerance. Nat Rev Immunol 3: 51–62.
3. Thomson AW, Knolle PA (2010) Antigen-presenting cell function in the tolerogenic liver environment. Nat Rev Immunol 10: 753–766.
4. Abo T, Kawamura T, Watanabe H (2000) Physiological responses of extrathymic T cells in the liver. Immunol Rev 174: 135–149.
5. Crispe IN (2009) The liver as a lymphoid organ. Annu Rev Immunol 27: 147–163.
6. Rehermann B, Nascimbeni M (2005) Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 5: 215–229.
7. Chisari FV, Ferrari C (1995) Hepatitis B virus immunopathogenesis. Annu Rev Immunol 13: 29–60.
8. Savioli L, Stansfield S, Bundy DA, Mitchell A, Bhatia R, et al. (2002) Schistosomiasis and soil-transmitted helminth infections: forging control efforts. Trans R Soc Trop Med Hyg 96: 577–579.
9. Maizels RM, Pearce EJ, Artis D, Yazdanbakhsh M, Wynn TA (2009) Regulation of pathogenesis and immunity in helminth infections. J Exp Med 206: 2059–2066.
10. Fitzpatrick JM, Fuentes JM, Chalmers IW, Wynn TA, Modolell M, et al. (2009) *Schistosoma mansoni* arginase shares functional similarities with human orthologs but depends upon disulphide bridges for enzymatic activity. Int J Parasitol 39: 267–279.
11. King CH, Olbrych SK, Soon M, Singer ME, Carter J, et al. (2011) Utility of repeated praziquantel dosing in the treatment of schistosomiasis in high-risk communities in Africa: a systematic review. PLoS Negl Trop Dis 5: e1321.
12. Pearce EJ, MacDonald AS (2002) The immunobiology of schistosomiasis. Nat Rev Immunol 2: 499–511.
13. Wynn TA, Thompson RW, Cheever AW, Mentink-Kane MM (2004) Immunopathogenesis of schistosomiasis. Immunol Rev 201: 156–167.
14. Andrade ZA (2009) Schistosomiasis and liver fibrosis. Parasite Immunol 31: 656–663.
15. Fallon PG (2000) Immunopathology of schistosomiasis: a cautionary tale of mice and men. Immunol Today 21: 29–35.
16. Cheever AW, Hoffmann KF, Wynn TA (2000) Immunopathology of schistosomiasis mansoni in mice and men. Immunol Today 21: 465–466.
17. Pearce EJ, C MK, Sun J, J JT, McKee AS, et al. (2004) Th2 response polarization during infection with the helminth parasite *Schistosoma mansoni*. Immunol Rev 201: 117–126.
18. Modolell M, Corraliza IM, Link F, Soler G, Eichmann K (1995) Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. Eur J Immunol 25: 1101–1104.
19. Hesse M, Cheever AW, Jankovic D, Wynn TA (2000) NOS-2 mediates the protective anti-inflammatory and antifibrotic effects of the Th1-inducing adjuvant, IL-12, in a Th2 model of granulomatous disease. Am J Pathol 157: 945–955.
20. Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, et al. (2001) Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. J Immunol 167: 6533–6544.
21. Constant SL, Bottomly K (1997) Induction of Th1 and Th2 CD4$^+$ T cell responses: the alternative approaches. Annu Rev Immunol 15: 297–322.
22. Mosmann TR, Coffman RL (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 7: 145–173.
23. Osada Y, Shimizu S, Kumagai T, Yamada S, Kanazawa T (2009) *Schistosoma mansoni* infection reduces severity of collagen-induced arthritis via down-regulation of pro-inflammatory mediators. Int J Parasitol 39: 457–464.
24. Osada Y, Kumagai T, Masuda K, Suzuki T, Kanazawa T (2005) Mutagenicity evaluation of *Schistosoma* spp. extracts by the umu-test and V79/HGPRT gene mutation assay. Parasitol Int 54: 29–34.
25. Hayashi N, Matsui K, Tsutsui H, Osada Y, Mohamed RT, et al. (1999) Kupffer cells from *Schistosoma mansoni*-infected mice participate in the prompt type 2

differentiation of hepatic T cells in response to worm antigens. J Immunol 163: 6702–6711.
26. Adachi K, Davis MM (2011) T-cell receptor ligation induces distinct signaling pathways in naive vs. antigen-experienced T cells. Proc Natl Acad Sci U S A 108: 1549–1554.
27. Adachi K, Tsutsui H, Seki E, Nakano H, Takeda K, et al. (2004) Contribution of CD1d-unrestricted hepatic DX5$^+$ NKT cells to liver injury in *Plasmodium berghei*-parasitized erythrocyte-injected mice. Int Immunol 16: 787–798.
28. Adachi K, Tsutsui H, Kashiwamura S, Seki E, Nakano H, et al. (2001) *Plasmodium berghei* infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism. J Immunol 167: 5928–5934.
29. Naka T, Tsutsui H, Fujimoto M, Kawazoe Y, Kohzaki H, et al. (2001) SOCS-1/SSI-1-deficient NKT cells participate in severe hepatitis through dysregulated cross-talk inhibition of IFN-gamma and IL-4 signaling in vivo. Immunity 14: 535–545.
30. Wilson MS, Mentink-Kane MM, Pesce JT, Ramalingam TR, Thompson R, et al. (2007) Immunopathology of schistosomiasis. Immunol Cell Biol 85: 148–154.
31. Jankovic D, Kullberg MC, Noben-Trauth N, Caspar P, Ward JM, et al. (1999) Schistosome-infected IL-4 receptor knockout (KO) mice, in contrast to IL-4 KO mice, fail to develop granulomatous pathology while maintaining the same lymphokine expression profile. J Immunol 163: 337–342.
32. Chiaramonte MG, Donaldson DD, Cheever AW, Wynn TA (1999) An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. J Clin Invest 104: 777–785.
33. Fallon PG, Richardson EJ, McKenzie GJ, McKenzie AN (2000) Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. J Immunol 164: 2585–2591.
34. O'Brien RL, Born WK (2010) gammadelta T cell subsets: a link between TCR and function? Semin Immunol 22: 193–198.
35. Azuara V, Levraud JP, Lembezat MP, Pereira P (1997) A novel subset of adult gamma delta thymocytes that secretes a distinct pattern of cytokines and expresses a very restricted T cell receptor repertoire. Eur J Immunol 27: 544–553.
36. Gerber DJ, Azuara V, Levraud JP, Huang SY, Lembezat MP, et al. (1999) IL-4-producing gamma delta T cells that express a very restricted TCR repertoire are preferentially localized in liver and spleen. J Immunol 163: 3076–3082.
37. Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, et al. (1995) Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. Nature 373: 255–257.
38. Klugewitz K, Adams DH, Emoto M, Eulenburg K, Hamann A (2004) The composition of intrahepatic lymphocytes: shaped by selective recruitment? Trends Immunol 25: 590–594.
39. Bendelac A, Rivera MN, Park SH, Roark JH (1997) Mouse CD1-specific NK1 T cells: development, specificity, and function. Annu Rev Immunol 15: 535–562.
40. Burdin N, Brossay L, Koezuka Y, Smiley ST, Grusby MJ, et al. (1998) Selective ability of mouse CD1 to present glycolipids: alpha-galactosylceramide specifically stimulates V alpha 14$^+$ NK T lymphocytes. J Immunol 161: 3271–3281.
41. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, et al. (1997) CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science 278: 1626–1629.
42. Soulard V, Roland J, Sellier C, Gruner AC, Leite-de-Moraes M, et al. (2007) Primary infection of C57BL/6 mice with *Plasmodium yoelii* induces a heterogeneous response of NKT cells. Infect Immun 75: 2511–2522.
43. Amsen D, Spilianakis CG, Flavell RA (2009) How are T(H)1 and T(H)2 effector cells made? Curr Opin Immunol 21: 153–160.
44. Grogan JL, Locksley RM (2002) T helper cell differentiation: on again, off again. Curr Opin Immunol 14: 366–372.
45. Nakahira M, Nakanishi K (2011) Requirement of GATA-binding protein 3 for *Il13* gene expression in IL-18-stimulated Th1 cells. Int Immunol 23: 761–772.
46. Sugimoto T, Ishikawa Y, Yoshimoto T, Hayashi N, Fujimoto J, et al. (2004) Interleukin 18 acts on memory T helper cells type 1 to induce airway inflammation and hyperresponsiveness in a naive host mouse. J Exp Med 199: 535–545.
47. Tsutsui H, Yoshimoto T, Hayashi N, Mizutani H, Nakanishi K (2004) Induction of allergic inflammation by interleukin-18 in experimental animal models. Immunol Rev 202: 115–138.
48. Kreslavsky T, Savage AK, Hobbs R, Gounari F, Bronson R, et al. (2009) TCR-inducible PLZF transcription factor required for innate phenotype of a subset of gammadelta T cells with restricted TCR diversity. Proc Natl Acad Sci U S A 106: 12453–12458.
49. Savage AK, Constantinides MG, Han J, Picard D, Martin E, et al. (2008) The transcription factor PLZF directs the effector program of the NKT cell lineage. Immunity 29: 391–403.
50. Kovalovsky D, Uche OU, Eladad S, Hobbs RM, Yi W, et al. (2008) The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. Nat Immunol 9: 1055–1064.
51. Kambayashi T, Assarsson E, Chambers BJ, Ljunggren HG (2001) Expression of the DX5 antigen on CD8$^+$ T cells is associated with activation and subsequent cell death or memory during influenza virus infection. Eur J Immunol 31: 1523–1530.
52. Charbonnier LM, van Duivenvoorde LM, Apparailly F, Cantos C, Han WG, et al. (2006) Immature dendritic cells suppress collagen-induced arthritis by in vivo expansion of CD49b$^+$ regulatory T cells. J Immunol 177: 3806–3813.
53. Kovalovsky D, Alonzo ES, Uche OU, Eidson M, Nichols KE, et al. (2010) PLZF induces the spontaneous acquisition of memory/effector functions in T cells independently of NKT cell-related signals. J Immunol 184: 6746–6755.
54. Alonzo ES, Sant'Angelo DB (2011) Development of PLZF-expressing innate T cells. Curr Opin Immunol 23: 220–227.
55. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, et al. (2008) Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity 29: 138–149.
56. Ramiscal RR, Vinuesa CG (2013) T-cell subsets in the germinal center. Immunol Rev 252: 146–155.
57. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, et al. (2010) Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). J Immunol 185: 190–202.
58. Craft JE (2012) Follicular helper T cells in immunity and systemic autoimmunity. Nat Rev Rheumatol 8: 337–347.
59. Terada M, Tsutsui H, Imai Y, Yasuda K, Mizutani H, et al. (2006) Contribution of IL-18 to atopic-dermatitis-like skin inflammation induced by *Staphylococcus aureus* product in mice. Proc Natl Acad Sci U S A 103: 8816–8821.
60. Steinfelder S, Andersen JF, Cannons JL, Feng CG, Joshi M, et al. (2009) The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). J Exp Med 206: 1681–1690.
61. Everts B, Hussaarts L, Driessen NN, Meevissen MH, Schramm G, et al. (2012) Schistosome-derived omega-1 drives Th2 polarization by suppressing protein synthesis following internalization by the mannose receptor. J Exp Med 209: 1753–1767, S1751.
62. Everts B, Perona-Wright G, Smits HH, Hokke CH, van der Ham AJ, et al. (2009) Omega-1, a glycoprotein secreted by *Schistosoma mansoni* eggs, drives Th2 responses. J Exp Med 206: 1673–1680.
63. Crispe IN (2011) Liver antigen-presenting cells. J Hepatol 54: 357–365.

Parasitology International 63 (2014) 327–331

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Comparison of PCR-based diagnoses for visceral leishmaniasis in Bangladesh

Md. Gulam Musawwir Khan [a], Khondaker Rifat Hasan Bhaskar [a], Mihoko Kikuchi [b], Md. Abdus Salam [c], Tania Akther [a], Rashidul Haque [a], Dinesh Mondal [a], Shinjiro Hamano [d,e,*]

[a] International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B), Mohakhali, Dhaka 1212, Bangladesh
[b] Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Japan
[c] Rajshahi Medical College, Rajshahi 6000, Bangladesh
[d] Department of Parasitology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Japan
[e] Global COE Program, Nagasaki University, Japan

ARTICLE INFO

Article history:
Received 15 July 2013
Received in revised form 1 December 2013
Accepted 4 December 2013
Available online 12 December 2013

Keywords:
Visceral leishmaniasis
Leishmania donovani
Diagnosis
Buffy coat
PCR
ITS1
ITS2
Mini-exon
Small subunit-rRNA (SSUrRNA)

ABSTRACT

The diagnosis of visceral leishmaniasis (VL) is performed using multiple methods encompassing parasitological, serological and nucleic acid-based diagnostic tools, each method with its own unique advantages and disadvantages. Conventional parasitological methods are risky for the patient and require skilled personnel to collect specimens from spleen or bone marrow, and hence they are not generally available in impoverished areas. Polymerase chain reaction (PCR) has been validated as an excellent alternative to microscopy in terms of sensitivity and specificity. Here, we evaluate four different PCR assays targeting ITS1, ITS2, mini-exon and small subunit-rRNA (SSUrRNA) using DNA extracted from peripheral blood buffy coat in order to avoid more invasive processes. A total of 61 VL patients and 75 non-VL infected control individuals were enrolled. The VL patients were confirmed to be positive for *Leishmania* amastigotes in splenic smears by microscopy. Sensitivities of the PCR targeting ITS1, ITS2, SSUrRNA and mini-exon were 96.7%, 91.8%, 88.5% and 34.4%, respectively, while the specificity was 98.7% for all methods. Nested PCR for ITS1 resulted in 100% sensitivity. The efficacy of each PCR was evaluated with various *Leishmania* amastigote parasite loads in each spleen smear, graded from 1+ to 5+. The PCR targeting ITS1 showed 100% sensitivity for the detection of *Leishmania donovani* in all samples from grades ≥3, ≥4, and ≥5, respectively. The restriction fragment length polymorphism observed in ITS1 amplicons digested by *Hae*III classified the parasite into *L. donovani* complex. The ITS1 PCR was found to be equal to conventional, but very invasive and risky parasitological diagnoses and superior to other PCR based methods in sensitivity and examination of genetic heterogeneity. We recommend the PCR targeting ITS1 using peripheral blood buffy coat DNA as an alternate, less invasive diagnostic choice for the confirmation of *L. donovani* infection.

1. Introduction

Visceral leishmaniasis (VL), also known as kala-azar, is a vector borne parasitic disease caused by the *Leishmania donovani* complex of protozoan parasites. The parasite multiplies in the reticulo-endothelial system causing prolonged fever, anemia, hepato-splenomegaly, weight loss, and death if left untreated [1–3]. Adequate investment in diagnosis, treatment and control is required in order to alleviate the burden of this disease, which disproportionately affects the poorest individuals in endemic countries [1,2]. The current standard diagnostic method for leishmaniasis is microscopic detection of amastigotes in Giemsa/Leishman stained smears prepared from spleen or bone marrow aspirates [3]. Microscopy sensitivity is typically 93–99% for smears prepared from the spleen, 53–86% for those prepared from bone marrow and only 53–65% for those prepared from lymph node aspirates [4,5]. These procedures are not only painful but also highly invasive with some risk of severe internal hemorrhage, and a high level of technical expertise is required, especially with spleen aspiration [4,6]. Recently, an alternative, minimally invasive buffy coat smear has been evaluated for microscopic identification of parasites [7]. However, the number of amastigotes in a buffy coat smear is low compared to those in a splenic smear. Due to the difficulty in identifying amastigotes in such smears, the technique requires further evaluation and field validation before being introduced as a new diagnostic tool. There are numerous serological tests available for VL such as the direct agglutination test (DAT), the rK39 dipstick test, and the indirect immunofluorescent antibody test (IFAT), all of which have been reported to be highly sensitive [3,8].

* Corresponding author at: Department of Parasitology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Sakamoto 1-12-4, Nagasaki, Nagasaki 852-8523, Japan. Tel.: +81 95 819 7822; fax: +81 95 819 7824.
E-mail address: shinjiro@nagasaki-u.ac.jp (S. Hamano).

1383-5769/$ – see front matter © 2013 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.parint.2013.12.001

Unfortunately, the major drawbacks of the serological tests are their inability to discriminate between past and present infections [3,6,9] as well as their inability to discriminate between active VL and asymptomatic infections [3,4,10].

Molecular biological approaches such as PCR have been introduced as alternative diagnostic tools [6,7,9,11], and various sequences from both genomic and extra-chromosomal regions have been exploited as targets of amplification by PCR [6,9,12].

The small subunit ribosomal RNA (SSUrRNA) gene of *Leishmania* is present in the genome in multiple copies (>100) arranged as tandem repeats, and is conserved within the *Leishmania* genus [7,9,11,13,14], making it an attractive target for PCR amplification based diagnostics. A PCR and restriction fragment length polymorphism (RFLP) based diagnostic method targeting the internal transcribed spacer (ITS) region, comprising ITS1 and ITS2, has proven very effective at assessing genetic heterogeneity within the genus [12,15,16]. ITS1 is a spacer sequence lying between the small subunit rRNA and the 5.8S rRNA genes, and ITS2 is the spacer sequence between the 5.8S rRNA and the large subunit rRNA genes [15,17]. The mini-exon (or spliced leader) gene is present on the genome as 100 to 200 tandem repeats in all kinetoplastida and is also a well-used PCR diagnostic target. Its absence from the vertebrate host and invertebrate vector makes it an appropriate target for molecular diagnostic and genotyping studies [18–20]. Here, we evaluate four different PCR assays for the diagnosis of VL using peripheral blood buffy coat.

2. Materials and methods

2.1. Patients and controls

A total of 61 VL patients, who were confirmed positive for *Leishmania* by splenic smear microscopy, were included in this study. Patients were admitted at the medical and pediatric wards of Rajshahi Medical College Hospital (RMCH), Bangladesh. We also included 75 controls: 25 healthy individuals from the Godagari subdistrict, a highly VL endemic area, 25 healthy individuals from different non-endemic regions of Rajshahi and 25 individuals admitted to the RMCH, who had been suffering from various forms of acute or chronic diseases with fever such as follows: acute lymphoblastic leukemia (2 patients), acute myeloid leukemia (1), aplastic anemia (1), chronic liver disease (3), chronic myeloid leukemia (3), enteric fever (2), febrile diseases on thalassemia (5), lupus vulgaris (1), liver abscess (1), pyrexia of unknown origin (3), rheumatoid fever (1), space occupying lesion in spleen (1), and viral hepatitis (1). Healthy individuals were carefully examined by a physician and confirmed to be healthy and free from any acute or chronic illness.

2.2. Splenic aspiration and microscopic examination for the presence of *Leishmania amastigotes*

After relevant laboratory evaluations [21], splenic aspiration was performed by an experienced doctor on those patients with suspected VL. Two smears were prepared for microscopic examination. Splenic aspiration was not performed on the control individuals. Two experienced microscopists independently examined spleen smears stained with Leishman stain under 10 × 100 magnification for the presence of *L. donovani* amastigotes. Positive smears were graded for parasite burden on a scale from 1+ to 6+. If the number of amastigotes counted per single field was >100, 10–100 or 1–10, the smear was graded as 6+, 5+ and 4+ respectively. Similarly, 1–10 amastigotes counted in this logarithmic scale of 10, 100 or 1000 fields were graded as 3+, 2+ and 1+ respectively [21].

2.3. Blood collection and rK39 dipstick test

With proper aseptic precautions, 3.0 mL of venous blood was collected in a vacuette (K3 EDTA tube) from all subjects. Immediately after collection, blood was transferred to a 15.0 mL falcon tube, overlaid with an equal volume of Histopaque-1119 (Sigma-Aldrich Co.) and centrifuged at 3000 *g* for 15 min. After centrifugation, the top layer of plasma was carefully transferred into a sterile 1.5 mL microcentrifuge tube and the buffy coat was transferred by a pasture pipette into a different sterile 1.5 mL microcentrifuge tube. The plasma and buffy coat thus separated were utilized for rapid test and for DNA extraction for different PCR methods respectively. All patients and controls were tested for the presence of anti rK39 antibody in their plasma by the rK39 dipstick test (Kalazar Detect™, InBios Inc., USA).

2.4. DNA extraction

DNA was extracted from buffy coat using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in a final volume of 0.2 mL of AE buffer. The purity of the DNA was measured in a Nanodrop and was found to be satisfactory as the A260/A280 OD ratio was always within the range of 1.7–1.9.

2.5. Small subunit ribosomal RNA (SSUrRNA) PCR

For detection of parasite DNA, *Leishmania* specific PCR with primers targeting the parasite's small subunit-rRNA region was used, as described by Cruz et al. [11]. Briefly each 25 μL reaction consisted of 4 μL of DNA, 2.5 μL of Takara 10× Ex Taq Buffer (Mg^{2+} plus), 2 μL of dNTP Mix (2.5 mM each), 1 M betain (Sigma-Aldrich Co., USA), 0.625 unit of Takara Ex Taq DNA polymerase (Takara Bio Inc., Japan), 1 μM of each of the forward and reverse primers: R221 (5′-GGTTCCTTTCCTGA TTTACG-3′) and R332 (5′-GGCCGGTAAAGGCCGAATAG-3′). The PCR cycle condition was as follows: an initial denaturation at 95 °C for 5 min; 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s; and a final extension at 72 °C for 10 min in a Thermal Cycler (ASTEC CO. LTD., Japan).

2.6. ITS1 PCR, ITS2 PCR and ITS1 PCR–RFLP

Two separate sets of primer pairs, L5.8S (5′-TGATACCACTTATCGCAC TT-3′)/LITSR (5′-CTGGATCATTT-TCCGATG-3′) and L5.8SR (5′-AAGT GCG-ATAAGTGGTA-3′)/LITSV (5′-ACACTCAGGTCTGTAAAC-3′) were used respectively for amplifying the ITS1 and ITS2 regions, according to el Tai et al. [15]. The PCR was carried out in a final volume of 25 μL reaction consisting of 4 μL of DNA, 2.5 μL of Takara 10× Ex Taq Buffer (Mg^{2+} plus), 2 μL of dNTP Mix (2.5 mM each), 1 M betain (Sigma-Aldrich Co., USA), 0.625 units of Takara Ex Taq DNA polymerase (Takara Bio Inc., Japan), and 1 μM of each of the forward and reverse primers.

The PCR cycle conditions for both ITS1 and ITS2 were as follows: an initial denaturation at 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 60 s; and a final extension at 72 °C for 10 min using a Thermal Cycler (ASTEC CO. LTD., Japan).

After amplification and visualization of the ITS1 PCR products, 12.5 μL of the amplicons were digested with 10 U of *Hae*III restriction endonuclease (New England Biolabs, UK) in a final volume of 20 μL and were kept overnight at 37 °C. The restriction products were then visualized on 2% agarose gels and compared with reference strains of *L. donovani* and *Leishmania tropica*.

2.7. Mini-exon PCR

The PCR was performed according to Marfrut et al. [19] with minor modifications. For each round of PCR, 4 μL of DNA was amplified in a final volume of 25 μL reaction consisting of 2.5 μL of Takara 10× Ex Taq Buffer, 2.5 mM $MgCl_2$, 2 μL of dNTP Mix (2.5 mM each), 0.5 M betain (Sigma-Aldrich Co., USA), 0.625 unit of Takara Ex Taq DNA polymerase (Takara Bio Inc., Japan), 1 μM of each of the forward primer Fme (5′-TATTGGTATGCGAAACTTCCG-3′) and reverse primer, Rme (5′-ACAG

AAACTGATACTTATATAGCG-3′). The PCR reaction was performed at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 45 s and followed by a final extension at 72 °C for 6 min in a Thermal Cycler (ASTEC CO. LTD., Japan).

2.8. Sensitivity test for PCR detection

DNA was extracted from 200 μL of EDTA blood from a non-infected donor mixed with 10^5 cultured *L. donovani* parasites originally isolated in Bangladesh, or 200 μL of EDTA blood from a non-infected donor, using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). For the sensitivity test, samples were prepared with ten-fold serial dilution; samples containing 10^5 to 10^{-1} parasites in 200 μL respectively. Each PCR was performed using 4 μL of extracted DNA as described above.

2.9. PCR product visualization

PCR products were separated by electrophoresis on 1.5% agarose gel with 100 bp DNA molecular size marker (Invitrogen, USA, Cat. No. 15628-019) and stained with ethidium bromide (0.1 mg/mL). Stained gels were visualized and photographed under UV light emission with a UV transilluminator (BioRad, Milan, Italy, S.N. 75S/03589). For each round of PCR amplification by all methods, molecular-grade water in duplicate was used as negative control and DNA from cultured promastigotes served as positive control. The products of ITS1, ITS2 and SSUrRNA are shown in Fig. 1.

2.10. Ethical considerations

Ethical issues relating to this research protocol were reviewed by the Institutional Review Board of Rajshahi Medical College, Bangladesh, which gave approval. Informed written consent or assent (where applicable) was obtained from each patient or from the legal guardian before splenic aspiration and venipuncture for the collection of blood samples.

3. Results

3.1. Characteristics of study population

Of the patients with VL, 64.4% were male and 35.6% were female. The mean age of the VL patients was 18.5 years, whereas that of the control group was 25.7 years. Eight patients had previously been diagnosed with VL (13.1%) and 10 patients had a family history of VL (16.4%).

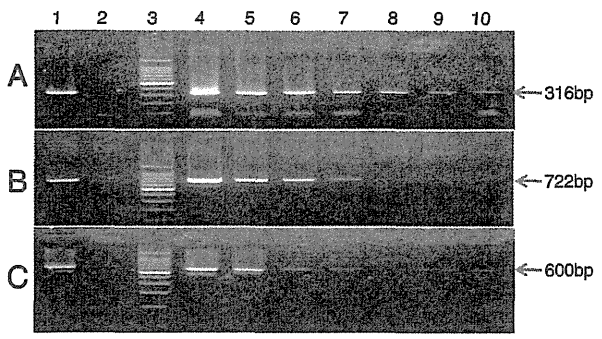

Fig. 1. Comparison of PCR-based diagnosis for *L. donovani* and visceral leishmaniasis. Each PCR amplicon shown was A) for ITS1, B) for ITS2 and C) for SSUrRNA. In lanes 1 and 2, the PCR products using DNA sample collected from a patient with visceral leishmaniasis and endemic negative control were shown, respectively. In lane 3, the 100 bp DNA marker (Takara Bio Inc., Japan) was shown. From lanes 4 to 10, the PCR products using *L. donovani* DNA ten-fold serially diluted with healthy donors' DNA were shown. PCR was run using 4 μL DNA prepared from 200 μL of non-contaminated human DNA, which was spiked with DNA fragments corresponding to 10^5 (lane 4: 2×10^3 in 4 μL), 10^4 (lane 5: 2×10^2 in 4 μL), 10^3 (lane 6: 2×10 in 4 μL), 10^2 (lane 7: 2 in 4 μL), 10^1 (lane 8: 0.2 in 4 μL), 10^0 (lane 9: 0.02 in 4 μL) and 10^{-1} (lane 10: 0.002 in 4 μL) *L. donovani*, respectively.

The clinical parameters observed in the VL patients were as follows: All patients showed typical symptoms for VL—like wasting (61 patients, 100.0%) and weight loss (61 patients, 100.0%), and most patients showed anemia (60 patients, 98.4%), blackening (60 patients, 98.4%) and splenomegaly (60 patients, 98.4%). Many VL patients showed hepatomegaly (46 patients, 75.4%) and pancytopenia (23 patients, 37.7%). Six patients showed bleeding (9.8%) and one patient had jaundice (1.6%). The mean erythrocyte sedimentation rate was 102.8 mm ranging from 25 to 175. The mean duration of fever was 20.5 weeks ranging from 4 to 52. The mean income of patients was 4057 TK per month ranging from 2000 to 20,000.

3.2. Parasitological and serological examination in patients and controls

All clinically suspected cases of VL were examined for *Leishmania* parasites in splenic smears. This resulted in 61 confirmed VL patients enrolling in the study. Using the rK39 dipstick test, 60 out of 61 VL patients were found to be positive, and 4 of the 25 non-symptomatic control individuals from an endemic region were also positive. Neither the control individuals from non-endemic regions nor those control individuals with other diseases were positive (Table 1). The majority of the patients had a parasite load with grade 3+ or less (Table 2). A major disadvantage of serological tests is their failure to discriminate between past and present infections [21–23]. We therefore tested four modern PCR methods to diagnose VL using buffy coat DNA from venous blood.

3.3. Comparison of PCR-based diagnosis for visceral leishmaniasis

As shown in Fig. 1, the PCR targeting ITS1 was able to detect one parasite in 200 μL of whole blood. The detection levels of PCR targeting ITS2 and SSUrRNA were 10^2 and 10^3 per 200 μL of whole blood, respectively. The PCR targeting the mini exon gene showed very low sensitivity and required over 10^6 parasites in 200 μL of whole blood for a positive result (data not shown). Thus, the PCR targeting ITS1 showed the highest sensitivity among the PCR techniques targeting various regions on the genome, including ITS2, SSUrRNA and mini exon. Then, we applied these PCR techniques to DNA purified from peripheral blood buffy coats collected from 61 VL patients and 75 controls. The sensitivities of the PCR targeting ITS1, ITS2, SSUrRNA and mini-exon were 96.7%, 91.8%, 88.5% and 34.4%, respectively, while the specificity was 98.7% for all methods. Nested PCR for ITS1 resulted in 100% sensitivity. The restriction fragment length polymorphism derived from ITS1 amplicons digested by *Hae*III confirmed that all parasites were part of the *L. donovani* complex (Fig. 2). The sensitivity of the PCR targeting ITS1 when applied to DNA extracted from the buffy coat of peripheral blood was found to be equal to conventional, but very invasive and risky parasitological diagnosis and superior to other PCR based methods.

Table 1
Sensitivity and specificity of different PCR based methods for diagnoses of visceral leishmaniasis.

Diagnostic method	Sensitivity, VL group (n = 61), % (n)	Specificity, control groups (n = 75), % (n)			
	% positive (n)	Endemic control (n = 25), % negative (n)	Non endemic control (n = 25), % negative (n)	Disease control (n = 25), % negative (n)	Total group (n = 75), % negative (n)
Splenic smear	100.0	ND	ND	ND	ND
rK39 ICT	98.4 (60)	84.0 (21)	100.0 (25)	100.0 (25)	94.7 (71)
ITS1	96.7 (59)	96.0 (24)	100.0 (25)	100.0 (25)	98.7 (74)
ITS2	91.8 (56)	96.0 (24)	100.0 (25)	100.0 (25)	98.7 (74)
SSUrRNA	88.5 (54)	96.0 (24)	100.0 (25)	100.0 (25)	98.7 (74)
Mini-exon	34.4 (21)	96.0 (24)	100.0 (25)	100.0 (25)	98.7 (74)

Table 2
Detection rate of different methods vs. splenic smear microscopy grading.

Grades	No. of patients	rK39	SSUrRNA	ITS1	ITS2	Mini-exon
1+	7	6 (85.7%)	7 (100.0%)	7 (100.0%)	7 (100.0%)	4 (57.1%)
2+	28	28 (100.0%)	23 (82.1%)	26 (92.9%)	24 (85.7%)	7 (25.0%)
3+	16	16 (100.0%)	14 (87.5%)	16 (100.0%)	15 (93.8%)	7 (43.8%)
4+	5	5 (100.0%)	5 (100.0%)	5 (100.0%)	5 (100.0%)	1 (20.0%)
5+	5	5 (100.0%)	5 (100.0%)	5 (100.0%)	5 (100.0%)	2 (40.0%)

4. Discussion

We demonstrate the usefulness of peripheral blood buffy coat for the diagnosis of VL. As shown in Fig. 1A, a PCR targeting the ITS1 region was able to detect one parasite in 200 μL of whole blood and was superior to other PCR techniques targeting ITS2, SSUrRNA and the mini-exon. When these PCR techniques were used to amplify parasite DNA extracted from the buffy coat of peripheral blood from VL patients and non-infected control individuals, the PCR targeting ITS1 region showed the highest sensitivity. In addition, all of the subjects from non-endemic areas and those patients with non-VL diseases associated with acute or chronic fever were negative for *Leishmania* by all four PCR methods, showing their excellent specificity. We note that there are other regions of the *Leishmania* genome that are potential targets for diagnostic PCR, and that they may offer opportunities for the design of assays that are as sensitive, or more so, than the ITS1 PCR described here.

When compared with microscopy, the current standard parasite detection technique, we found satisfactory results for ITS1 (sensitivity of 96.7%, and specificity of 98.7%) and ITS2 (sensitivity of 91.8% and specificity of 98.7%). Furthermore, nested PCR for ITS1 resulted in 100% sensitivity. Our results are consistent with other reports of the use of ITS1 PCR for diagnosis of VL using bone marrow and blood [24,25]. ITS1 PCR using DNA extracted from peripheral blood buffy coat should be the preferred choice for the diagnosis of VL, if it is available.

According to some publications, the emergence of *L. tropica* in the Indian subcontinent might be a cause for concern in the near future [26,27]. All the specimens positive for ITS1 by PCR were subjected to digestion by *Hae*III restriction endonuclease, and the restriction patterns were compared with reference strains of *L. donovani* and *L. tropica*. All VL samples, which were collected in Rajshahi, Bangladesh adjacent to India, were classified into the *L. donovani* complex, suggesting that *L. tropica* is not a problem in this region at least. ITS1 PCR–RFLP has the added advantage over other methods, in that species identification is possible [16,24].

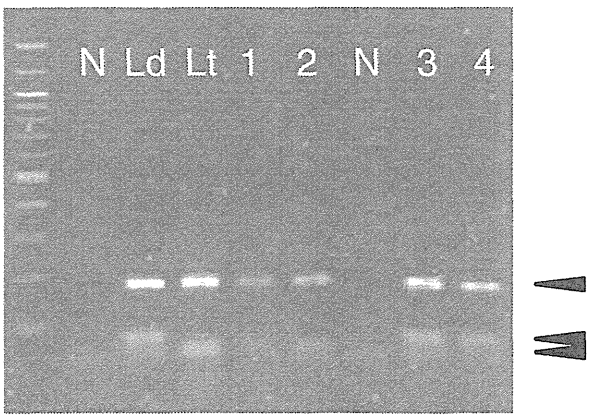

Fig. 2. The restriction fragment length polymorphism of ITS1 amplicons amplified by PCR using DNA extracted from buffy coat collected in Bangladesh corresponded with *L. donovani* complex based on the patterns of reference strains, *L. donovani* and *L. tropica*. The ITS1 amplicons were digested by restriction enzyme, *Hae*III, and then, electrophoresed on agarose gel. In the leftmost lane, the 100 bp DNA marker was shown. N = negative control, Ld = *L. donovani* reference strain showing RFLP with 54, 75, and 184 bp bands, Lt = *L. tropica* reference strain showing RFLP with 2 bands, numbers 1–2 = *L. tropica*, and numbers 3–4 = samples from VL patients.

Of the 25 control individuals from endemic regions, only one was found to be positive for *Leishmania* by all PCR assays. The rK39 dipstick test showed high sensitivity in comparison with PCR. However, 4 of the 25 endemic controls were positive by the rK39 dipstick test. One of these was shown to be asymptomatically infected based on the PCR data. However, it is impossible to discriminate between past and present infections in the three other positive cases. In addition, the rK39 dipstick test may give a positive result during the early stages of the infection, when PCR results are negative.

For its inherent unique biological features, the mini-exon gene has become a potential target for clinical observation in VL. We utilized this target gene for PCR amplification for the first time in Bangladesh to assess its diagnostic value for VL. The sensitivity and specificity of mini-exon PCR were found to be 34.4% and 98.7% respectively, in agreement with a previous report [19]. PCR targeting mini-exon gene resulted in false negatives for some confirmed VL cases, and these were obtained not only in specimens with low parasite burden but also in those with high parasite burden, perhaps indicating nucleotide polymorphism within the primer-binding regions [28,29]. These comparatively low sensitivities were also observed in a study performed in China, where even nested PCR targeting the mini-exon gene showed 66% sensitivity for buffy coat samples [30]. It is also possible that this low sensitivity was due the difference in the biological characteristics of parasite including parasite loads in the study area where *Leishmania infantum* was thought to be prevalent.

5. Conclusions

Based on extensive sensitivity tests, we recommend the ITS1 PCR applied to DNA extracted from peripheral blood buffy coat as an alternative, less invasive diagnostic choice for the confirmation of *L. donovani* infection.

Acknowledgments

We sincerely thank Tomoko Takaya and Hiromi Oda for administrative support; Richard Culleton and Todd Saunders for scientific editing; and members of the Institute of Tropical Medicine, Nagasaki University for discussion. This work was supported by the Global COE Program, Nagasaki University (to S. H.).

References

[1] Alvar J, Yactayo S, Bern C. Leishmaniasis and poverty. Trends Parasitol 2006;22:552–7.
[2] Bern C, Maguire JH, Alvar J. Complexities of assessing the disease burden attributable to leishmaniasis. PLoS Negl Trop Dis 2008;2:e313.
[3] Sundar S, Rai M. Laboratory diagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol 2002;9:951–8.
[4] Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol 2007;5:873–82.
[5] Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis 2004;27:305–18.
[6] Reithinger R, Dujardin JC. Molecular diagnosis of leishmaniasis: current status and future applications. J Clin Microbiol 2007;45:21–5.
[7] Salam MA, Khan MG, Bhaskar KR, Afrad MH, Huda MM, Mondal D. Peripheral blood buffy coat smear: a promising tool for diagnosis of visceral leishmaniasis. J Clin Microbiol 2012;50:837–40.
[8] Boelaert M, El-Safi S, Hailu A, Mukhtar M, Rijal S, Sundar S, et al. Diagnostic tests for kala-azar: a multi-centre study of the freeze-dried DAT, rK39 strip test and KAtex in East Africa and the Indian subcontinent. Trans R Soc Trop Med Hyg 2008;102:32–40.
[9] Antinori S, Calattini S, Longhi E, Bestetti G, Piolini R, Magni C, et al. Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature. Clin Infect Dis 2007;44:1602–10.
[10] Berman JD. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. Clin Infect Dis 1997;24:684–703.

[11] Cruz I, Canavate C, Rubio JM, Morales MA, Chicharro C, Laguna F, et al. A nested polymerase chain reaction (Ln-PCR) for diagnosing and monitoring *Leishmania infantum* infection in patients co-infected with human immunodeficiency virus. Trans R Soc Trop Med Hyg 2002;96(Suppl. 1):S185–9.
[12] Schallig HD, Oskam L. Molecular biological applications in the diagnosis and control of leishmaniasis and parasite identification. Trop Med Int Health 2002;7:641–51.
[13] Lachaud L, Dereure J, Chabbert E, Reynes J, Mauboussin JM, Oziol E, et al. Optimized PCR using patient blood samples for diagnosis and follow-up of visceral leishmaniasis, with special reference to AIDS patients. J Clin Microbiol 2000;38:236–40.
[14] van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol Biochem Parasitol 1992;51:133–42.
[15] el Tai NO, Osman OF, el Fari M, Presber W, Schonian G. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. Trans R Soc Trop Med Hyg 2000;94:575–9.
[16] Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W, et al. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis 2003;47:349–58.
[17] Cupolillo E, Grimaldi Junior G, Momen H, Beverley SM. Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of *Leishmania*. Mol Biochem Parasitol 1995;73:145–55.
[18] Fernandes O, Murthy VK, Kurath U, Degrave WM, Campbell DA. Mini-exon gene variation in human pathogenic *Leishmania* species. Mol Biochem Parasitol 1994;66:261–71.
[19] Marfurt J, Niederwieser I, Makia ND, Beck HP, Felger I. Diagnostic genotyping of old and new world *Leishmania* species by PCR–RFLP. Diagn Microbiol Infect Dis 2003;46:115–24.
[20] Smyth AJ, Ghosh A, Hassan MQ, Basu D, De Bruijn MH, Adhya S, et al. Rapid and sensitive detection of *Leishmania* kinetoplast DNA from spleen and blood samples of kala-azar patients. Parasitology 1992;105(Pt 2):183–92.
[21] Chulay JD, Bryceson AD. Quantitation of amastigotes of *Leishmania donovani* in smears of splenic aspirates from patients with visceral leishmaniasis. Am J Trop Med Hyg 1983;32:475–9.
[22] Lachaud L, Chabbert E, Dubessay P, Reynes J, Lamothe J, Bastien P. Comparison of various sample preparation methods for PCR diagnosis of visceral leishmaniasis using peripheral blood. J Clin Microbiol 2001;39:613–7.
[23] Osman OF, Kager PA, Zijlstra EE, el-Hassan AM, Oskam L. Use of PCR on lymph-node sample as test of cure of visceral leishmaniasis. Ann Trop Med Parasitol 1997; 91:845–50.
[24] Alam MZ, Shamsuzzaman AK, Kuhls K, Schonian G. PCR diagnosis of visceral leishmaniasis in an endemic region, Mymensingh district, Bangladesh. Trop Med Int Health 2009;14:499–503.
[25] Roelfsema JH, Nozari N, Herremans T, Kortbeek LM, Pinelli E. Evaluation and improvement of two PCR targets in molecular typing of clinical samples of *Leishmania* patients. Exp Parasitol 2011;127:36–41.
[26] Kumar R, Bumb RA, Ansari NA, Mehta RD, Salotra P. Cutaneous leishmaniasis caused by *Leishmania tropica* in Bikaner, India: parasite identification and characterization using molecular and immunologic tools. Am J Trop Med Hyg 2007;76:896–901.
[27] Sacks DL, Kenney RT, Kreutzer RD, Jaffe CL, Gupta AK, Sharma MC, et al. Indian kala-azar caused by *Leishmania tropica*. Lancet 1995;345:959–61.
[28] Fisa R, Riera C, Ribera E, Gallego M, Portus M. A nested polymerase chain reaction for diagnosis and follow-up of human visceral leishmaniasis patients using blood samples. Trans R Soc Trop Med Hyg 2002;96(Suppl. 1):S191–4.
[29] Saran R, Sharma MC, Gupta AK, Sinha SP, Kar SK. Diurnal periodicity of *Leishmania* amastigotes in peripheral blood of Indian kala-azar patients. Acta Trop 1997; 68:357–60.
[30] Katakura K, Kawazu S, Naya T, Nagakura K, Ito M, Aikawa M, et al. Diagnosis of kala-azar by nested PCR based on amplification of the *Leishmania* mini-exon gene. J Clin Microbiol 1998;36:2173–7.

ORIGINAL ARTICLE 10.1111/1469-0691.12421

Biomarkers for intracellular pathogens: establishing tools as vaccine and therapeutic endpoints for visceral leishmaniasis

A. C. Vallur[1], M. S. Duthie[1], C. Reinhart[1], Y. Tutterrow[1], S. Hamano[2,3], K. R. H. Bhaskar[4], R. N. Coler[1], D. Mondal[4] and S. G. Reed[1]

1) *Infectious Disease Research Institute, Seattle, WA, USA,* 2) *Department of Parasitology, Institute of Tropical Medicine Nagasaki University,* 3) *Global COE programme, Nagasaki University, Nagasaki, Japan and* 4) *Laboratory Sciences Division, International Centre for Diarrhoeal Diseases Research, Dhaka, Bangladesh*

Abstract

Visceral leishmaniasis in South Asia is a serious disease affecting children and adults. Acute visceral leishmaniasis develops in only a fraction of those infected individuals, the majority being asymptomatic with the potential to transmit infection and develop disease. We followed 56 individuals characterized as being asymptomatic by seropositivity with rk39 rapid diagnostic test in a hyperendemic district of Bangladesh to define the utility of *Leishmania*-specific antibodies and DNA in identifying infection. At baseline, 54 of the individuals were seropositive with one or more quantitative antibody assays and antibody levels persisted at follow up. Most seropositive individuals (47/54) tested positive by quantitative PCR at baseline, but only 16 tested positive at follow up. The discrepancies among the different tests may shed light on the dynamics of asymptomatic infections of *Leishmania donovani*, as well as underscore the need for standard diagnostic tools for active surveillance as well as assessing the effectiveness of prophylactic and therapeutic interventions.

Keywords: Asymptomatic infection, biomarkers, ELISA, intracellular pathogens, leishmania, PCR
Original Submission: 31 May 2013; **Revised Submission:** 20 September 2013; **Accepted:** 30 September 2013
Editor: E. Bottieau

Clin Microbiol Infect

Corresponding author: S. G. Reed, Infectious Disease Research Institute, 1616 Eastlake Avenue E, Suite 400, Seattle, WA 98102, USA
E-mail: steve.reed@idri.org

Introduction

One of the characteristics of neglected diseases is the lack of tools for successful disease management or prevention. As an example, it has only been recently that tools to detect infected individuals have begun to be applied in regions where *Leishmania donovani* is endemic. *Leishmania* are vector-borne protozoan parasites spread by the bite of the infected phlebotomine sand-fly [1,2]. Two *Leishmania* spp. cause visceral leishmaniasis (VL; also known as kala-azar), which is the most severe form, often fatal if not treated [3]. Half a million new VL cases are diagnosed every year and, with a mortality rate of 10%, VL is second only to malaria in terms of fatality [4].

In the Indian subcontinent VL is caused by *L. donovani*. An estimated 100 000 VL cases are reported annually in the endemic foci of northeastern India, Nepal and Bangladesh, costing 400 000 disability-adjusted life-years and putting 150 million people at risk for infection[5] The situation in this region, where humans are the only reservoir of the parasite, makes elimination feasible [3]. In 2005 these three countries committed to a kala-azar elimination programme, with the intent of decreasing incidence to 1 in 10 000 per year by 2015 [6]. Goals of the programme include early diagnosis and treatment, coordinated vector control and effective disease surveillance through passive and active case detection [7]. A pressing concern that could interfere with this goal is the presence of large populations who harbour infection with no overt signs of disease but who can potentially develop VL and probably spread infection [8,9]. Considerably more asymptomatic individuals than those with VL disease are identified in areas of endemicity [10,11].

Traditionally, both direct (detection of parasites) and indirect (detection of antibodies) tests have been used in the diagnosis of VL. The standard diagnostic method for VL has been microscopic visualization of amastigotes in splenic, lymph node or bone marrow aspirates, ethically and technically unsuitable for asymptomatic individuals [12]. The kala-azar elimination programme does not evaluate asymptomatic individuals because defined parameters to identify low levels of infection are lacking. A limited number of antibody detection tests, including the direct agglutination test (DAT), ELISA and immunochromatography-based rapid tests, have been standardized [12]. Given the endemic nature of *L. donovani* infection, serological tests lack the ability to discern between uninfected, antibody-positive individuals and asymptomatic infected individuals [13,14]. Unlike the situation in Africa and Brazil, *Leishmania*-specific antibodies persist for extended periods of time in the Indian subcontinent, emphasizing the importance of defining biomarkers that reflect the dynamics of asymptomatic infections [14]. PCR-based tests directly demonstrating the presence of parasite nucleic acids probably represent a more accurate tool for the assessment of asymptomatic individuals, with the advantage of being sensitive enough to detect very low levels of parasite DNA [15]. Serological and PCR-based techniques can complement each other to establish how these biomarkers reflect asymptomatic *L. donovani* infection. Given the potential availability of vaccines against *L. donovani*, these tests could be used to identify individuals or populations that will benefit most from vaccination.

To determine the utility of various tests, a group of asymptomatic infected individuals in a region where *L. donovani* is hyperendemic were examined over a period of 24 months with serological tests, DAT and ELISA, as well as a sensitive quantitative PCR test to define the dynamics of these biomarkers.

Patients and Methods

Study design, ethics and parameters

We defined an asymptomatic infected individual as a person from a VL endemic area with no past history of VL or post-kala-azar dermal leishmaniasis (PKDL), clinically healthy and positive by the rK39 rapid test (Kalazar Detect™; Inbios, Seattle, WA, USA) in the field using finger-prick blood. We recruited 3849 clinically healthy people in the Harirampur Union of subdistrict Trishal, Mymensingh district, where VL is hyperendemic with a reported incidence of 65 per 10 000 people in 2007 (Trishal Hospital). Initial consent was obtained from the head of the household to screen household members based on past VL or PKDL history, then individual written consent was obtained before enrolment in the study. Initial screening was conducted using the Kalazar Detect™ rk39 rapid diagnostic test (RDT) and 332 were found positive, with 200 deemed fit to participate in the study based on no prior history of VL or PKDL (50% were female and 35.5% were under 15 years). Fifty-six were then enrolled as study subjects based on the availability of matching serum and DNA samples when the study was initiated (baseline) and 12 months after initiation (follow up) (Fig. 1a). All serological and PCR tests were conducted on these 56 serum and DNA samples collected at baseline and 12-month follow up (Fig. 1b). The enrolees were monitored each month for clinical symptoms of VL during household visits up to 24 months after study initiation, with three of the 56 enrolees developing VL disease by 24 months. They were referred to the study clinic where a qualified medical officer examined them following the diagnostic criteria for VL of the National Guideline before referring them to the Trishal Hospital for treatment (Fig. 1b) [16].

Sample collection and storage

Blood specimens were collected at enrolment and then at 12 months. Blood specimens were collected by venepuncture. For DNA extraction, an aliquot of 2 mL was placed in an EDTA-containing vacutainer and centrifuged at 2200 g for 20 min for separation of buffy coat. Collected buffy coat was transported to the ICDDRB Parasitology Laboratory maintaining the cold chain and DNA was isolated using a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. For serum preparation, 2 mL of blood was placed in red-top vacutainers and allowed to clot at room temperature for an hour and centrifuged for 5 min for separation of serum. Collected serum was transported as above.

Serological tests

DAT was performed according to the manufacturer's instructions (KIT Biomedical Research, Amsterdam, the Netherlands) with minor modifications [17]. Briefly, in V-bottom 96-well plates, healthy US control and test serum samples were serially diluted two-fold starting at 1 : 400 in 0.9% sodium chloride added 0.8% β-mercaptoethanol at a final volume of 50 μL per well. Antigen re-suspended in 50 μL of 0.9% sodium chloride was then added per well. Each plate included at least two blank wells containing only sample diluent. After brief mixing, plates were covered and left undisturbed at room temperature for 18 h before reading. Samples were run in singlet per plate and duplicated by a second researcher. Results were independently scored by three readers. Each reader recorded the titre as the last sample dilution at which agglutination was apparent. The

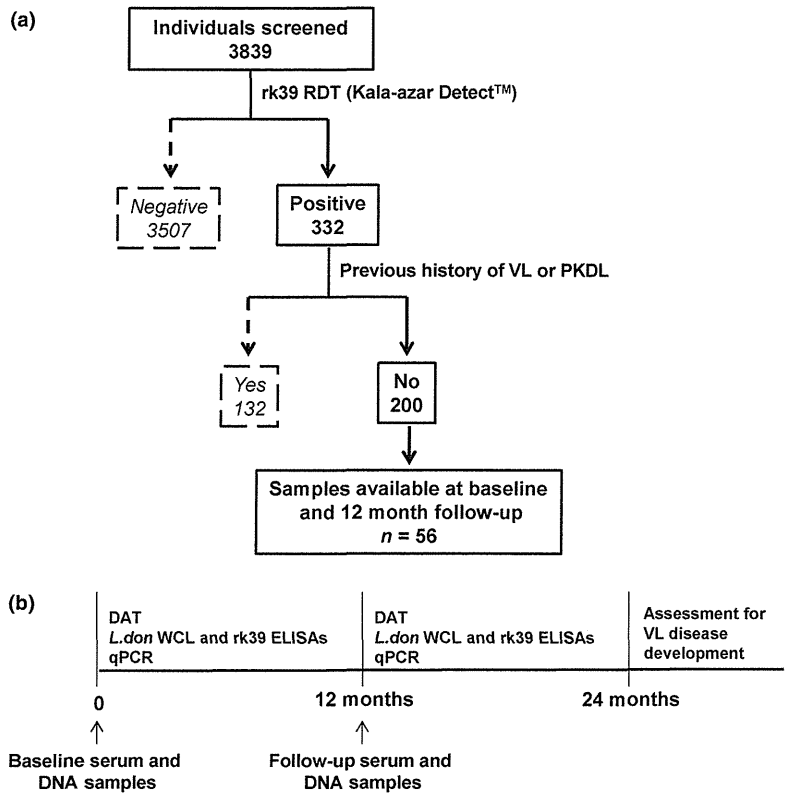

FIG. 1. Study enrolment criteria, timeline and tests performed on study subjects (a) Out of 3839 individuals screened in the Mymensingh district of Bangladesh, 332 tested positive by the rk39 rapid test; 200 of them were eligible for the study because they had no past history of visceral leishmaniasis (VL)/post-kala-azar dermal leishmaniasis (PKDL). Of these, 56 were enrolled on the basis of availability of matching serum and DNA samples at study initiation (baseline) and 12 months after initiation (follow up). (b) Timeline of sample collection and tests performed during the course of the study. Serum and DNA were extracted from the 56 study subjects at baseline and 12-month follow up. After that they were monitored for VL disease development according to the Bangladesh National Guidelines by monthly surveillance until 24 months.

titre for each sample was taken as the median score from the three readers, and the scores from the two plates were compiled to obtain a final titre. In the event of disagreement between duplicates, the higher of the two titres was chosen. Samples with a final titre of 1 : 1600 or higher were considered positive.

Serum antibodies against *L. donovani* whole cell lysate (WCL) and recombinant k39 were assessed by ELISA. In brief, 0.5 μg/well of WCL or 0.1 μg/well of antigens at 50 μL/well in 0.1 M PBS pH 7.2 were used to coat 384-microwell plates (Corning, Tewksbury, MA, USA) at 4°C overnight. After blocking with 1% bovine serum albumin in PBS/0.05% Tween-20, serum samples at 1 : 100 dilutions were added to wells in triplicate and allowed to incubate at room temperature with shaking for 1.5 h. After washing, 1 : 5000 diluted peroxidase-conjugated donkey anti-human IgG H + L chains (Jackson ImmunoResearch, Westgrove, PA, USA) were added for 1 h with shaking. Plates were developed with SureBlue TMB Microwell peroxidase (Thermofisher, Lafayette, CO, USA) for 5 min and stopped with 0.1 M H_2SO_4. Plates were read immediately at 450 and 570 nm (Synergy 2 plate reader, Biotek, Winooski, VT, USA) and final optical density (OD) was recorded as the difference between the two. Mean values from triplicate readings for each sample were calculated. A cut-off value for positive results was determined as the mean OD of non-endemic control sera from healthy US normal participants plus two standard deviations. Sera from eight confirmed VL patients from Bangladesh and Sudan served as positive controls.

DNA extraction and PCR tests

Genomic DNA was extracted from the buffy coat of blood samples using the QiaAMP DNA blood mini kit (Qiagen) in the ICDDRB Parasitology laboratory. An aliquot of DNA was shipped to the Infectious Disease Research Institute, Seattle. Quantitative detection of *Leishmania* DNA was performed on a