Increased Interleukin-4 and Interleukin-5 Production in Response to 
Schistosoma haematobium Adult Worm Antigens Correlates 
with Lack of Reinfection after Treatment

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Acquired immunity to human schistosomiasis correlates with increased serum levels of schistosome antigen–specific IgE. Since interleukin (IL)-4 stimulates IgE production, the hypothesis that Th2-associated cell-mediated immunity participates in protection to reinfection was studied in a cohort of adolescent boys 12–18 months after chemotherapeutic cure in Upper Egypt. Initial Schistosoma haematobium prevalence was 51% and posttreatment incidence was 44%. Water contact was similar between putatively resistant and susceptible patients. Resistant persons had a 3.5- to 14-fold greater frequency of schistosome adult worm antigen (SWAP)–specific lymphocytes secreting IL-5 or IL-4 (by ELISPOT) and IL-5 or IL-4 production in peripheral blood lymphocyte culture supernatants ($P < .05$ to $< .001, n = 48$) versus susceptible subjects ($n = 38$). In contrast, SWAP-induced interferon-γ and IL-10 production and lymphocyte proliferation were similar between the 2 groups. Schistosome egg antigen and streptolysin O each stimulated similar cytokine production in susceptible and resistant persons. Thus, enhanced SWAP-driven IL-4 and IL-5 production correlates with immunity to reinfection in adolescents exposed to urinary schistosomiasis.

Epidemiologic studies of human schistosomiasis suggest that acquired immunity gradually develops with age [1, 2]. The mechanisms of this acquired resistance remain poorly understood and could prove useful in identifying immune responses needed for an effective vaccine. What is known is that persons with increased resistance to reinfection after chemotherapeutic cure have elevated serum levels of schistosome adult worm antigen (SWAP)–specific IgE [3–5], IgA directed to the 28-kDa schistosome antigen glutathione-S-transferase [6], and peripheral blood eosinophilia [7, 8]. Conversely, increased serum levels of IgG4, IgG2, and IgM directed against adult worm antigens are associated with an increased susceptibility to reinfection [3, 9, 10]. Antigen-specific IgG4 and IgG2 have been postulated to block IgE-mediated killing of the parasite and thus enhance susceptibility to infection [10]. These studies, however, are only correlative, and direct experimental evidence that IgE participates in acquired immunity to helminth infections in animal models is surprisingly limited [11].

It is possible that elevated levels of IgE and peripheral blood eosinophilia are markers of anti-parasite cell-mediated immunity. Since interleukin (IL)-4 and IL-5 participate in the induction and regulation of IgE and eosinophilia, increased production of these cytokines may participate in the development of acquired immunity. Several studies indicate such an association may exist in Schistosoma mansoni infection. In Kenya, peripheral blood mononuclear cells (PBMC) from putatively resistant persons produced IL-5; however, this association was confounded by age, and cytokine production was directed to egg, not adult worm antigens [12].

T cell clones generated in response to adult worm antigens from putatively immune persons produced an increased ratio of IL-4 to interferon (IFN)-γ release compared with a susceptible individual; however, the study examined only 4 subjects [13]. Recently, a locus on the human chromosome 5q31-q33 has been linked to controlling the intensity of infection by S. mansoni infection [14]. This same region of chromosome 5 also contains genes that regulate IL-4, IL-5, and IL-13 levels [15], which further supports an association between increased IL-4 and IL-5 production with resistance. No studies, to our knowledge, have examined correlates of cell-mediated immunity to resistance in Schistosoma haematobium infections.

Most studies examining the mechanisms of acquired immunity have investigated cohorts of subjects with a broad range of age [9, 12, 16–18]. Because age and exposure to contaminated water affect the intensity of infection, they can act as confounding variables in assessing the role of acquired immunity and must be controlled for in population-based studies. Previous studies of acquired immunity also examined popula-
tions with moderate to heavy parasite exposure that may obscure development of partial immunity.

In such treatment and reinfection studies, resistance was often defined as persons with low (resistant) compared with high (susceptible) egg excretion. This poses a problem for studying urinary schistosomiasis, in which egg excretion is highly variable and correlates poorly with overall intensity of infection [19]. To address these issues, we examined a cohort of adolescent boys in Upper Egypt, where the incidence of reinfection remains high but the intensities of urinary schistosomiasis are low compared with those in East Africa, for example [20]. Infected persons were treated and reinfection was monitored over the following 18 months. Prior water contact studies in Upper Egypt show that adolescent boys consistently have the highest and most uniform exposure [21]. The present study examines the cell-mediated immunologic correlates in putatively resistant versus susceptible subjects as determined by whether they are reinfected or not after chemotherapeutic cure.

Materials and Methods

Study design. Study subjects resided in a village on the East Bank of the Nile River 30 km from Assiut, a city in Upper Egypt. The village population was ~4000, and the principal occupation is farming. In this village, a cohort of 145 male children aged 9–15 years were selected on the basis of their willingness to participate. Midday urine was obtained on 2 consecutive days at the beginning of the study and at 3, 6, and 12–18 months later. Any individual found infected with schistosomiasis was treated with 40 mg/kg praziquantel. Between 12 and 18 months after treatment, ~20 mL of peripheral venous blood was obtained to prepare PBMC.

Exclusion criteria from the final analysis comprised persons who (1) were infected before enrollment in the study but did not have a urine sample collected 3 or 6 months after treatment to determine the efficacy of therapy or (2) remained ova-positive at 3 months after treatment, indicating probable treatment failure.

Water contact studies. Transmission sites were identified on the basis of where people frequented the canals and the presence of S. haematobium–infected snails, Bulinus alexandria. The frequency of, duration of, and amount of the body in contact with the water (e.g., whole body [swimming], to waist, just arms and legs) were recorded for study subjects by trained local observers throughout the course of the transmission season (April through September) based on previously established protocols [22].

Criteria for classification of susceptible versus putatively resistant subjects. Susceptible persons were (1) ova-positive at the beginning of the study, cured with treatment (ova-negative at 3 months), and then ova-positive at 6 and/or 12 months after treatment or (2) ova-negative at the onset of the study and at 3 months and then ova-positive at 12 months after treatment. Water contact behavior was not used to exclude these subjects from the study, since the fact that they became infected demonstrated exposure.

Putatively resistant subjects were (1) ova-positive at the study beginning and became ova-negative after treatment and remained ova-negative, and had documentation of direct water contact at potential transmission sites at least twice a month during the observation period.

Antigen and mitogens. SWAP and soluble egg antigen (SEA) were prepared as a saline extract of adult-stage parasites or from freshly harvested ova [23]. Endotoxin in these preparations was <0.5 ng/mL; 5- to 50-fold less than that required for lipopolysaccharide stimulation of cytokines from human lymphocytes [24].

Isolation of PBMC and culture conditions for in vitro cytokine production. All studies were performed on fresh PBMC separated by density-gradient centrifugation on ficoll-hypaque from heparinized cord and venous blood and resuspended in C-RPMI: RPMI 1640 supplemented with 10% fetal calf serum (FCS), 4 mM t-glutamine, 25 mM HEPES, and 40 μg/mL gentamicin (BioWhitaker, Walkersville, MD). PBMC were cultured at 2 × 10^6 cells/mL in C-RPMI in a total volume of 1 mL. To duplicate cultures was added medium alone, SWAP (50 μg/mL), SEA (10 μg/mL), streptolysin O (SLO) (10 μg/mL), or phytohemagglutinin (PHA, 5 μg/mL). Cells were incubated at 37°C in 5% CO₂; supernatants were collected at 36 h (for IL-4) and 72 h (IL-5, IL-10, and IFN-γ) and immediately frozen at −70°C for subsequent determination of cytokine production.

Cytokine production by PBMC subsets. To assess cytokine production by lymphocyte subpopulations, PBMC were washed once in cold RPMI with 2% fetal bovine serum. CD4 cell depletion was performed using magnetic beads directly conjugated to anti-CD4 antibodies (Dynal, Lake Success, NY). For control data, immunomagnetic depletion was accomplished according to the manufacturer’s instructions with whole PBMC from 4 healthy North American adults, using the identical reagents and protocols used for the study subjects; >88% of CD4 cells were removed. The efficiency of CD4 cell depletion was not checked on individual samples in the study.

Cytokine assays. Cytokine levels in cell supernatants were measured by ELISA and expressed in picograms per milliliter by interpolation from standard curves based on recombinant lymphokines by using antibodies and methods previously described [25]. Antibody pairs for capture and detection (all biotinylated) for the cytokines studied were as follows: for IL-5, TRFK5 and 5D10 (PharMingen, San Diego); for IL-4, 8D4 and 25D2 (PharMingen); for IFN-γ, M-700 and M-701 (Endogen, Cambridge, MA); and for IL-10, 18551D and 18652D (PharMingen). The limits of detection for ELISA for each cytokine were 18 pg/mL for IL-5, 16 pg/mL for IL-4, 10 pg/mL for IFN-γ, and 16 pg/mL for IL-10.

The ELISPOT assays utilized T-spot plates (Athersys, Cleveland), which were coated with capture antibodies (the same as used in the ELISAs) in sterile PBS overnight at 4°C and blocked with C-RPMI. Plates were then washed three times with sterile PBS. Single cell suspensions were prepared, counted, transferred to the plate at 200 μL/well, and incubated at 37°C with 5% CO₂ for 24 h (IL-4 and IFN-γ) and 48 h (IL-5 and IL-10). For antigen-specific cytokine production, 0.5–1 × 10^6 cells were added per well. For mitogen-driven cytokine production, this was reduced to 0.1–1 × 10^5 cells/well. The wells were run in duplicate if there were enough cells. After incubation, plates were washed three times with PBS followed by three washes with PBS-Tween (0.05%). Detecting antibodies (the same as used in the ELISAs) were added and incubated overnight at 4°C. The resulting spots were initially enumerated with a dissecting microscope (×5–20),
Table 1. Population of adolescent boys studied for schistosomiasis.

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<th>Susceptible (n = 43)</th>
<th>Resistant (n = 58)</th>
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<tr>
<td>Median age, years (range)</td>
<td>11 (10–15)</td>
<td>12 (9–15)</td>
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<tr>
<td>% ova-positive</td>
<td>46.5</td>
<td>53.4</td>
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<tr>
<td>Geometric mean ova count (range)</td>
<td>2.6 (0–31)</td>
<td>2.7 (0–46)</td>
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<tr>
<td>Water contact</td>
<td></td>
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<tr>
<td>Frequency, per week*</td>
<td>2.7 (0–13)</td>
<td>2.2 (1–12)</td>
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<tr>
<td>Average duration (range)</td>
<td>65 (3–120)</td>
<td>57 (1–120)</td>
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* Mean (range) no. of water contacts per week based on 8 h of daily observation 6 days per week. Direct water contact observations were on alternating weeks during time of transmission (April to September).

and numbers were verified by an analyzer (T Spot Image Analyzer; Athersys) that is designed to detect the immunoplaques using predetermined criteria based on size, shape, and colorimetric density.

Statistics. Results are expressed as mean ± SD, and experimental conditions were compared using Student’s t test of log-transformed data.

**Results**

**Study population.** In the initial cohort of 145 persons within the study villages, 101 fulfilled the criteria as either putatively resistant or susceptible (table 1). Thirty persons were excluded because they did not provide urine samples in one or more of the follow-up periods, particularly at 3 months to verify treatment efficacy, or at 12 months. An additional 14 subjects remained ova-positive at 3 months after treatment and were excluded because of treatment failure. Of those remaining (table 1), susceptible and putatively resistant subjects were of similar age and similar prevalence and intensity of infection at study entry. Susceptible subjects had similar intensity and prevalence of infection compared with resistant subjects prior to infection. The geometric mean (GM) egg count among susceptible subjects obtained ~12 months after treatment (or 6 months if the 12-month specimen was unavailable) was 4.2/10 mL of urine.

Age profoundly affects the susceptibility to infection, and 11–13 years of age is perhaps the most critical time in which an individual begins to shift from susceptible to resistant. To examine whether the age range of susceptible versus resistant subjects differed over this critical period of age, the distribution of subjects was compared (figure 1). Most subjects in the present study were 10–12 years old, with similar age distribution between the 2 groups. Thus, age is unlikely to account for apparent resistance observed in some subjects in the present cohort.

Of the 101 persons classified as susceptible or resistant, PBMC were successfully obtained from 86 for cytokine studies, either by ELISPOT, if sufficient cells were available, or by detection of cytokine production in culture supernatants by ELISA.

**Relationship of water contact behavior with susceptibility or resistance to infection.** All the water contact data were condensed into two variables for each individual: the average duration of water contact in minutes and the frequency of contact. The amount of body-water contact showed little variability in adolescent boys. Most boys either swam or exposed more than half of their body in the water. Therefore, this variable was not considered further in the analysis. From the five major water contact sites, 6050 Bulinus truncatus snails were collected, of which 76 (1.3%) had S. haematobium infections. Although the number and proportion of infected snails varied considerably, infected snails were found at all sites. However, most water contact by study subjects occurred at two sites. Most of the infected snails were recovered from these two sites (combined): >4500 snails, 1.6% of which were infected with S. haematobium.

Therefore, we considered that the risk of infection was likely to be similar between these two sites, and no different weight was given for water contact at the various sites in the final analysis. We found no correlation between the frequency and duration of water contact with the intensity of infection or with levels of antigen-driven cytokine production. The mean frequency and duration of water contact were equivalent between susceptible and resistant subjects (table 1).

**Increased frequency of SWAP-induced IL-5 and IL-4 by PBMC from putatively resistant subjects.** The frequency of cytokine-secreting cells in response to adult worm antigens was examined using ELISPOT. Only some subjects (n = 27) were examined using this procedure because of the large number of lymphocytes required for the assay. The frequency of SWAP-induced IL-4 – and IL-5 – secreting cells from putatively
Acquired Immunity to Urinary Schistosomiasis

Figure 2. Frequency of cytokine-secreting cells in peripheral blood mononuclear cells (PBMC) from susceptible (Sus) and putatively resistant (Res) subjects. PBMC (0.5–1 \times 10^6/well) were stimulated with antigen for 24 or 48 h, depending on cytokine, as described in Materials and Methods. Each dot represents mean value of duplicate cultures from 1 patient 12–18 months after treatment. Unstimulated cultures usually showed rare or undetectable cytokine-secreting cells. Bars represent geometric mean for each group. P values were calculated by Student’s t test using log-transformed data. IFN, interferon.

immune subjects was significantly higher than that observed for susceptible subjects (figure 2). No difference in the frequency of SWAP-specific IFN-γ–secreting cells was observed between the 2 groups. Of note, the frequencies of detectable IL-5–secreting cells was 5- to 10-fold greater than that observed for IL-4– and IFN-γ–secreting cells. There was no correlation between the frequency of IL-5– and IL-4–secreting cells among either resistant or susceptible subjects (data not shown). There were fewer subjects with ELISPOT data for IL-4 and IFN-γ than for IL-5 because of a lack of sufficient lymphocytes for some assays.

The increased frequency of IL-4– and IL-5–secreting cells by PBMC from resistant compared with susceptible subjects was restricted to SWAP. The frequencies of SEA-reactive or SLO (the nonparasite antigen)-reactive PBMC were similar between the 2 groups (table 2). Numbers of mitogen (PHA)-induced IL-5–, IL-4–, and IFN-γ–secreting cells were also equivalent between susceptible and resistant subjects (table 2). In some conditions, the number of PHA-driven cytokine-secreting cells were fewer than was observed for SWAP-stimulated cytokine production because insufficient cells were available.

Increased levels of SWAP-induced IL-5 and IL-4 production in PBMC culture supernatants from putatively resistant versus susceptible subjects. Because there were insufficient cells from most study subjects for ELISPOT, PBMC cultures were done instead. Similar to the observations for cytokine-secreting cells, SWAP-induced IL-5 and IL-4 levels were significantly greater among putatively resistant subjects (figure 3). Mean IL-5 release was 14-fold greater (GM = 142 pg/mL) in resistant subjects than in susceptible subjects (GM = 10 pg/mL). A similar, but less striking difference was observed for IL-4 production. No difference in the levels of SWAP-induced IFN-γ and IL-10 was observed between the 2 groups (figure 3). Similarly, both SWAP- and SEA-induced lymphocyte proliferation did not differ between susceptible and resistant subjects (for

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<th>Antigen/mitogen cytokine</th>
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<td>Resistant</td>
</tr>
<tr>
<td>SEA</td>
<td>IL-5</td>
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<td>IL-4</td>
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<td>IFN-γ</td>
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NOTE. Data are geometric mean (range) no. of cytokine secreting cells per 10^6 (SEA and SLO) or 10^5 (PHA) peripheral blood mononuclear cells. IL, interleukin; IFN, interferon. No differences were statistically significant.
Figure 3. Cytokine production by culture supernatants between susceptible (Sus) and putatively resistant (Res) subjects. Values represent net schistosome adult worm antigen (SWAP)-induced cytokine production in culture supernatants of 2 × 10^6 peripheral blood mononuclear cells/mL in 0.5-mL cultures harvested 72 h (IL-5, IL-10, and interferon [IFN]-γ) or 24 h (IL-4) after addition of antigen. Each dot represents mean value of single or duplicate cultures from 1 patient. P values were calculated by Student’s t test using log-transformed data.

250). Similarly, there was no significant difference in the levels of SEA-induced IFN-γ (GM for resistant = 7 pg/mL vs. susceptible = 11 pg/mL, P = .45) or IL-10 (GM for resistant = 97 pg/mL vs. susceptible = 171 pg/mL, P = .22).

CD4 lymphocytes in PBMC are the major source of helminth antigen–driven cytokine production. To determine whether CD4 cells are the primary source of antigen-driven cytokine production, lymphocytes from subjects from whom sufficient samples were obtained underwent CD4 cell depletion. The ELISPOT test was performed because of its potentially greater sensitivity in detecting IL-4 release. Figure 4 shows 3 representatives of 15 patients in whom SWAP induced cytokine production and whose lymphocytes were subjected to CD4 depletion. Depletion of CD4 cells significantly reduced or abolished detectable cytokine-secreting cells in PBMC from most subjects studied, indicating CD4 cells were the predominant cell type for antigen-induced cytokine production. Depletion of IL-4 release by removing CD4 cells was less pronounced than that observed for SWAP-induced IL-5 and IFN-γ, indicating other cell subpopulations are important for IL-4 production.

Discussion

In the present study, many putatively resistant persons had an increased frequency of adult worm antigen–specific T cells that primarily secreted IL-4 and IL-5 compared with susceptible persons. Only adult worm antigens, not egg antigens or SLO, induced cytokine production that correlated with protection. This is not surprising, since protection against schistosomiasis is likely to be directed to antigens expressed by developing larvae and adults, which share many of the same molecules with SEA-induced IFN-γ [26]. We observed no difference in antigen-induced lymphocyte proliferation or IFN-γ or IL-10 production between the 2 groups, indicating that the protective effect was restricted to a Th2-associated cytokine response. One application of these findings suggests that adjuvants in a schistosome vaccine that augment antigen-specific IL-4 and IL-5 production may enhance its efficacy.

Study subjects were classified as resistant or susceptible on the basis of documented frequent exposure and whether they became infected or not over the year after chemotherapeutic cure with praziquantel. This epidemiologic design was based on previous studies examining the mechanisms of acquired resistance to human schistosome infections [9, 12, 16–18]. However, most of those studies and others found that the risk of infection was strongly associated with age [2, 27–29]. Although age may represent cumulative exposure to the parasite and thus development of immunity, the consistent drop in the intensity and frequency of infection among young adults in schistosomiasis-endemic areas, irrespective of intensity of exposure, suggests a component of age-related innate immunity [30].

To reduce the potentially confounding effect of age, the present study examined a cohort of adolescents who were 9–15 years old. However, even among adolescents, age-related differences in immunity may develop. First we found the same age distribution between resistant and susceptible subjects in our cohort. Since most in the study cohort were 10–12 years of age,
old, children may have been too young for age to have a large influence on susceptibility. To examine this possibility further, subjects were stratified into 9–11 and 12–15 years old and compared for differences in reinfection rates or levels of antigen-induced cell-mediated immunity. No differences were observed between the 2 age groups, demonstrating that the enhanced SWAP-induced IL-4 and IL-5 production in putatively resistant subjects occurred independent of age in our study population.

A danger of classifying persons as susceptible or resistant may reflect inaccuracies in determining exposure [31]. For example, putatively resistant persons may have shown high levels of water contact but fortuitously avoided exposure to cercariae, while a susceptible individual may have had relatively little water contact but had a brief exposure to water containing a large number of cercariae. To reduce this potential variation of exposure, our cohort included only boys. Previous detailed studies of water contact behavior in adolescent boys from similar villages in Upper Egypt show a uniformly high exposure in this group [21]. Moreover, we monitored water contact in our study population and excluded those in the putatively resistant group who did not have documented exposure to potential transmission sites.

Our use of crude schistosome antigen preparations has the advantage of maximizing possible immune responses between susceptible and immune persons. However, it also has the possible drawback that a mixture of antigens may produce a complex array of cytokines in vitro, with cross-modulatory properties that may obscure important responses to individual antigens. Immune sera [6, 32, 33] or T cell clones [34] from putatively resistant persons have been used to identify specific adult worm antigens that may participate in this resistance. Whether these same molecules or others participate in cell-mediated acquired resistance has not been addressed and is of potential importance in the development of a schistosome vaccine.

Some of the putatively resistant subjects had no detectable IL-4 and/or IL-5 production. This could reflect an insensitivity of the assay to detect these cytokines, or schistosome-reactive lymphocytes may not be adequately represented in a single sample if they do not consistently appear in the intravascular compartment. Indeed, we obtained multiple samples from some persons, and the patterns of immune response differed considerably from one time to the next in a few subjects. An alternative conclusion might be that IL-4 and IL-5 may not be critical for protection in all persons and that other effector mechanisms may participate in resistance. More likely, however, putatively resistant subjects may have been misclassified and are really susceptible subjects that escaped adequate exposure despite their documented water contact.

The mechanisms by which increased IL-4 and IL-5 production participates in elimination of the parasite remains uncertain. The enhanced Th2-associated cytokine response to adult worm antigens is consistent with previous studies indicating that human resistance is associated with IgE-mediated immunity to adult worm antigens [3–5, 9] and increased peripheral eosinophilia [7, 8]. In vivo studies show that IgE in rats [35] and eosinophils in mice [36] play critical roles in protection against reinfection in these animal models, and activated human eosinophils and platelets or macrophages armed by IgE kill schistosomula in vitro [37]. In addition, natural immunity to *S. mansoni* infection is reduced in IgE-deficient mice [11]. This
is also consistent with the role of parasite-specific IgG1 in protection in mice that is elicited by multiple immunizations with irradiated cercariae [38]. However, protection in mice immunized once with irradiated cercariae has been shown to be IFN-γ-dependent [39]. This suggests that multiple mechanisms may generate protection; however, repeated exposure to infective stages of the parasite, as is typical of natural infection, favors a more Th2-type protective response.

IL-4 and IL-5 may play a role in resistance apart from stimulating a protective IgE response. For example, in humans, elevated serum levels of parasite-specific IgG4 occur with active infection and are associated with increased susceptibility to reinfection [3, 9]. It has been postulated that this results from blocking the protective effect of IgE [3, 9]. Since IL-4 stimulates both IgE and IgG4 production in human helminth infections [40], this suggests that the role of IL-4 in protection may be more complex. IL-4 has an important role in controlling Heligmosomoides polygyrus and Trichuris muris and can contribute to the protection against Nippostrongylus brasiliensis and Trichinella spiralis infections in mice independent of IgE (reviewed in [41]). IL-4 also stimulates T cell growth and enhances expression of VCAM-1 (the endothelial cell receptor for the integrin VLA-4, which is involved in the migration of macrophages, lymphocytes, and eosinophils) [42]. In addition, IL-4 stimulates nitric oxide (NO) synthase, the enzyme that catalyzes macrophage production of NO [43]. IL-4, or TNF production and resistance. Infection and are associated with increased susceptibility to reinfection [3, 9]. Our findings indicate that Th2-mediated immunity participates in development of acquired immunity to human schistosomiasis, although multiple effector mechanisms are likely involved. To advance the study of acquired immunity to human schistosomiasis, additional studies of immune responses to selected antigens will be needed in larger population-based studies that carefully control for age and exposure. In this way, vaccine candidate molecules can be administered in a context that might enhance their efficacy. However, the complex nature of immunity that must develop in human schistosomiasis will likely require vaccines with several antigens delivered in a fashion to evoke multiple effector responses.

References


