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T-helper type-1-dominated lymph node responses induced in C57BL/6 mice by optimally irradiated cercariae of *Schistosoma mansoni* are down-regulated after challenge infection

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SUMMARY

Following a single percutaneous vaccination with optimally irradiated cercariae of *Schistosoma mansoni*, C57BL/6 mice mount a T-helper type-1 (Th1) lymphocyte-dominant immune response and are highly resistant to challenge infection. In this study, we show that, besides interferon-γ (IFN-γ), lymph node (LN) cells draining the site of vaccination produce significant amounts of interleukin (IL)-4 and IL-10 in culture with parasite antigen. After a challenge infection at the original site of vaccination, these LN cells did not generate an anamnestic Th1 response. Paradoxically, IFN-γ production and cell proliferation were profoundly down-regulated, whereas IL-4 production was enhanced and occurred earlier than in challenge control cultures. When challenge was applied to a site remote from vaccination, IFN-γ down-regulation was less evident, but the IL-4 response was consistently enhanced. Neutralization of IL-10 *in vitro* restored IFN-γ production by LN cells, whilst IL-4 levels were reduced. These data indicate that down-regulation of IFN-γ is controlled by IL-10 and/or IL-4. Mice showing down-regulated Th1 responses in the LN after *S. mansoni* challenge infection did not have a reduced ability to eliminate challenge parasites, indicating that the post-vaccination Th1 response had already armed the lungs with effector T cells before administration of challenge parasites. The observed phenomena of down-regulated Th1 and enhanced Th2 responses may be of relevance to other systems involving multiple infections or vaccination/boosting. Repeated applications to percutaneous sites having common lymphatic drainage would be expected to favour Th2 responses. Alternatively, in order to induce Th1-dominant responses and avoid unwanted IL-4/IL-10 induction, the use of remote sites is indicated.

INTRODUCTION

Murine T-helper (Th) cells have been classified into two subsets based on the cytokines they produce: Th1 cells are associated with delayed-type hypersensitivity (DTH) responses and secrete interleukin-2 (IL-2) and interferon-γ (IFN-γ), whereas Th2 cells produce IL-4, IL-5 and IL-10 and stimulate antibody production.[1] Differentiation into Th1 or Th2 subsets occurs through an intermediate Th0 stage, where both Th1 and Th2 cytokines are released from the same cell.[1] Th1 and Th2 responses show reciprocal negative regulation; while IFN-γ inhibits the proliferation of Th2 cells,[2] IL-4 down-regulates IL-2 and IFN-γ production,[3] and IL-10 inhibits IFN-γ release by, and proliferation of, Th1 cell clones *in vitro*.[4,5] Since protective immunity in a number of model infection systems results from domination of either a Th1 or a Th2 response, much interest has been focused on understanding the mechanism that regulates the Th1/Th2 bias. A key role is played by cytokines present in the external milieu when Th cells encounter antigen for the first time. Thus, early exposure to IL-4 will drive differentiation towards a Th2 phenotype, whereas IFN-γ promotes the emergence of Th1 cells.[6,7]

A high level of protection against infection with the parasitic helminth *Schistosoma mansoni* can be induced in C57Bl/6 mice by a single vaccination with radiation-attenuated cercariae.[8] The majority of challenge parasites are eliminated in the lungs as the result of a DTH response.[9] This resistance is largely abrogated by depletion of $CD4^+$ T cells[10,11] and is reduced by 90% following *in vivo* administration of neutralizing antibody to IFN-γ,[12] demonstrating the importance of a Th1-type response in this model.

Lymphadenectomy has revealed that the development of protective immunity is initiated in the lymph nodes (LN) draining the skin site of vaccination.[13] A combination of *ex vivo* and *in vivo* studies has indicated that, during the first 14 days after vaccination, B cells are retained preferentially over T cells in these LN, but that lymphocyte proliferation within the nodes can be attributed primarily to T cells.[14,15] Cells taken from LN draining the exposure site and cultured with larval antigen showed maximum proliferation *in vitro* between days 4 and 7

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postvaccination.[16,17] $CD4^+$ cells within this cell population released abundant amounts of IFN-γ, while the presence of IL-4 in culture supernatants as well indicated a Th0, or mixed Th1/Th2, profile.[16] By contrast with LN cells from protectively vaccinated animals, the equivalent cells from mice exposed either to normal or hyperirradiated cercariae, which do not confer protection, showed similar levels of proliferation, but IFN-γ production was less sustained and up to 24-fold lower between days 15 and 21.[16,17] Examination of the cytokines released by LN and spleen cells, beyond 4 weeks after vaccination, has revealed that in cultures from singly vaccinated mice Th1 responses (IL-2, IFN-γ) dominate, but that multiple vaccination favours an increase in production of the Th2 cytokines (IL-4 and IL-5).[18]

The present study set out to examine how LN cells respond during the first 3 weeks after exposure of singly vaccinated mice to a challenge infection, i.e. whether dominant Th1 responses are reinforced and IFN-γ is produced anamnestically, contributing to protective immunity. A challenge infection was applied either to naive mice or to mice that had earlier received vaccinating parasites at the same or a remote site. Cells from LN draining the challenge site were then examined for their ability to proliferate and release the cytokines IL-2, IFN-γ, IL-4 and IL-10 in response to antigen. The importance of IL-10 was investigated by *in vitro* neutralization and findings are interpreted in relation to the levels of immunity observed in the different groups of vaccinated mice.

MATERIALS AND METHODS

Parasites and experimental hosts

A Puerto Rican isolate of *S. mansoni* was maintained at York by passage through LACA mice and albino *Biomphalaria glabrata* snails. Experimental hosts were female C57BL/6 mice, bred at the University of York and weighing 17–20 g.

Vaccination and challenge infection

Mice were anaesthetized by intraperitoneal injection of 10% Sagatal (Rhone Merieux Ltd, Harlow, Essex, UK) in 10% ethanol (0·01 ml/g body weight). Vaccinations were performed percutaneously by exposing the shaved right or left flank (each with an independent lymphatic drainage) to 500 cercariae attenuated with 20 krads of γ irradiation from a ^{60}Co source at the Department of Radiobiology, Cookridge Hospital, Leeds. According to the site of exposure, groups of animals were designated as RF (right flank vaccination), LF (left flank vaccination) or NV (not vaccinated). Thirty-five days after vaccination, mice ($n = 5$) from each group received a challenge infection with 200 normal cercariae via the right flank.

Removal of LN and culture of lymphocytes

At the indicated times after vaccination or challenge infection, axillary and inguinal LN draining the right flank were removed from four mice within each of the RF, LF and NV groups, and weighed. For each group, LN were pooled and a single-cell suspension made.

As described in detail elsewhere,[16] 10^5 responder LN cells were incubated with 2×10^5 antigen-presenting cells (APC; 3000 rads γ-irradiated naive syngeneic splenocytes) in quadruplicate microwells. Soluble 18-hr schistosomular protein (SSP) was included as antigen at 50 μg/ml. Medium was RPMI-1640, containing 10% fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and antibiotics (RPMI/10). Supernatants were harvested after 72 hr of incubation, pooled from replicate wells and stored at −80° until analysis for cytokine activity. Identical cultures were incubated for 5 days, each well receiving 18·5 KBq [^{3}H]thymidine for the final 18 hr; cells were harvested and incorporation of radioactivity into DNA was determined by liquid scintillation counting. Mean values for proliferation, in counts per minute (c.p.m.), were derived from: (c.p.m. in the presence of SSP) − (c.p.m. in the absence of antigen), and expressed as mean Δc.p.m. (±SEM) for four replicate microwells.

Cytokine assays

Double antibody ELISAs were used to measure levels of IFN-γ, IL-2, IL-4 and IL-10 in culture supernatants. The IFN-γ assay is described elsewhere.[19] IL-2 was quantified using a rat anti-mouse coating monoclonal antibody (mAb) (Pharmingen, Cambridge, UK; cat. no. 18161D; clone JES6-1A12), followed by a biotinylated rat anti-mouse IL-2 detection mAb (Pharmingen; cat. no. 18172D; clone JES5-5H4). IL-4 was bound to plates with a rat anti-mouse IL-4 mAb (Endogen, Boston, MA; clone BVD4-11B11) and detected using a biotinylated rat anti-mouse IL-4 mAb (Pharmingen; clone BVD6-24G2). IL-10 was captured by a rat anti-mouse IL-10 coating mAb (Pharmingen; clone JES5-2A5) and detected with a biotinylated rat anti-mouse IL-10 mAb (Pharmingen; clone SXC-1).

IL-2 and IL-4 were quantified using commercially available (Pharmingen) murine recombinant cytokines as standards. Recombinant murine IL-10 was a generous gift from Dr S. Menon (DNAX Research Institute, Palo Alto, CA). Limits of detection were 1 pg/ml for IFN-γ, 4 pg/ml for IL-2, 4 pg/ml for IL-4 and 12 pg/ml for IL-10.

Neutralization of IL-10 in vitro

Anti-IL-10 mAb (rat IgM) was obtained as supernatant from cultured SXC-1 cells,[20] concentrated 50-fold in a stirred ultrafiltration cell, then filter-sterilized. At a final dilution of 1/12, the antibody concentrate was capable of neutralizing at least 5 ng/ml IL-10.

Axillary/inguinal LN cells draining the right flank were removed 4 days after challenge infection of RF, LF and NV mice. Cells were cultured in the presence or absence of SSP with or without anti-IL-10 mAb, added at the outset. Supernatants for cytokine assay were harvested after 72 hr of culture.

Resistance measurements

Thirty-five days after challenge, the worm burdens of five vaccinated (v), i.e. RF and LF, and control (c), i.e. NV, mice were determined by portal perfusion. Resistance (R) was calculated using the formula: $\%R = (C - V) \times 100/C$.

Statistical analysis

All statistical analyses were performed using the Student's *t*-test.

RESULTS

Events after vaccination: cytokine production by LN cells

LN cells were removed from the right flank of RF mice on days

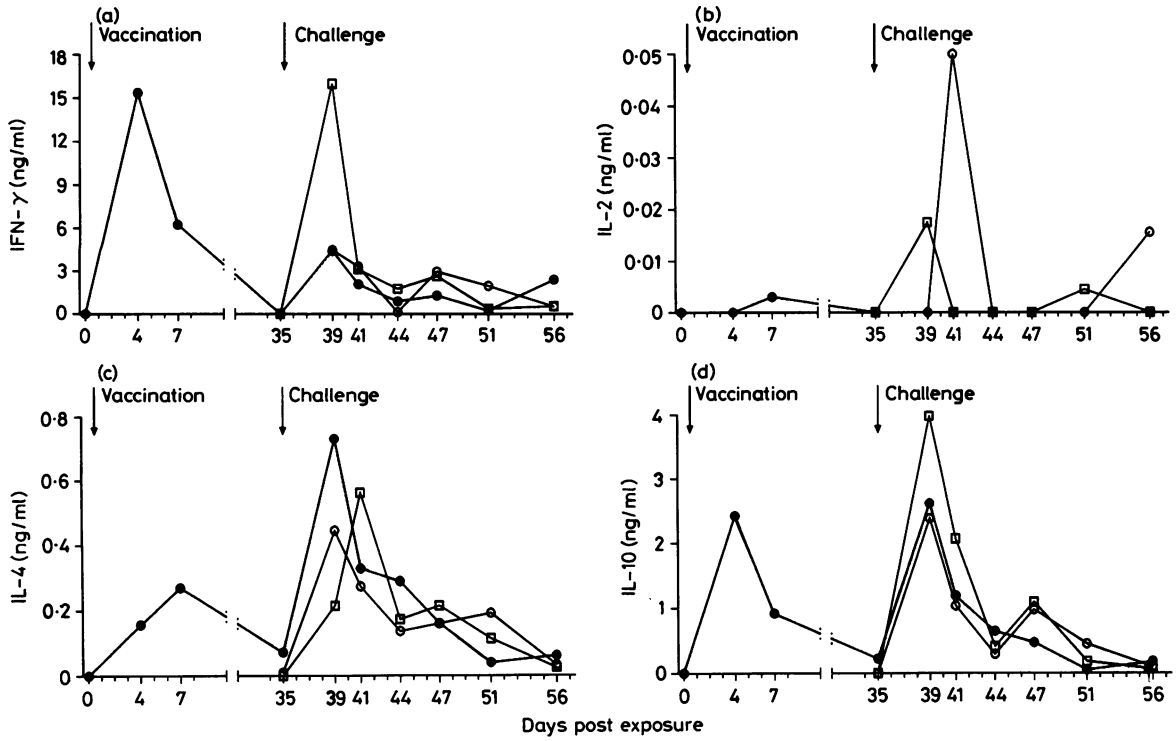

Figure 1. SSP-specific production of (a) IFN-γ, (b) IL-2, (c) IL-4 and (d) IL-10 by LN cells draining the right flank, after vaccination of RF mice and challenge of NV (□), RF (●) and LF (○) mice on the right flank. Maximum cytokine levels in the absence of SSP were: IFN-γ, 0·4 ng/ml; IL-2, 0·004 ng/ml; IL-4, 0·01 ng/ml; and IL-10, 0·186 ng/ml.

4 and 7 postvaccination. IFN-γ production was high (15 ng/ml) on day 4 and had declined by day 7 (Fig. 1a), whereas IL-2 production was negligible at both times (Fig. 1b). Significant levels of IL-4 were present, being higher at day 7 than at day 4 (Fig. 1c). In contrast to IL-4 release, the pattern of IL-10 production resembled that of IFN-γ, being high on day 4 (2·4 ng/ml) and in decline by day 7 (0·9 ng/ml; Fig. 1d).

Events after challenge: changes in LN weight

Figure 2 shows the change in weight of LN from RF, LF and NV mice, with time after challenge infection. Data shown are from one of two repeat experiments and demonstrate the significantly increased cell mass in the nodes of RF mice, particularly on day 6 ($p < 0{\cdot}005$), compared with nodes from the other two groups of animals (RF versus NV, $0{\cdot}002 > p > 0{\cdot}001$; RF versus LF, $0{\cdot}05 > p > 0{\cdot}02$). There was no significant difference between the weights of LF and NV nodes ($p > 0{\cdot}05$).

Events after challenge: antigen-specific LN cell proliferation

Culture of LN cells from RF, LF and NV mice revealed that antigen-specific proliferative responses were maximal on day 4 after challenge for cells from all three groups of mice (Fig. 3). However, the magnitude of the response differed between the groups, being highest for NV mice (45 000 c.p.m.) and marginally lower for LF mice (42 500 c.p.m.), but profoundly diminished for RF mice (16 000 c.p.m.). Figure 3 shows results for cells taken from the LN depicted in Fig. 2; a second experiment gave essentially the same result (data not shown).

Events after challenge: cytokine production by LN cells

Figure 1 illustrates IFN-γ, IL-2, IL-4 and IL-10 production by LN cells draining the site of challenge infection on the right flank of RF, LF and NV mice. RF mice receiving challenge infection were taken from the batch sampled after vaccination in Fig. 1; cells were from the same suspension as those illustrated in Fig. 3. Cells from NV mice produced maximum IFN-γ on day 4 after challenge (Fig. 1a). In marked contrast,

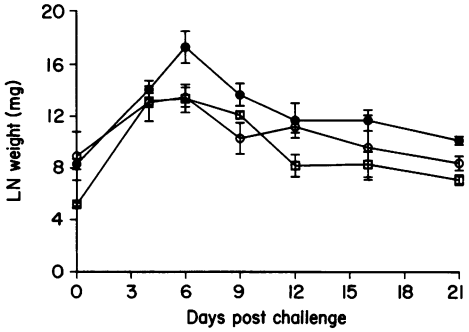

Figure 2. Changes in combined weights of right flank axillary and inguinal LN with time after challenge of NV (□), RF (●) and LF (○) mice on the right flank. Values shown are means ± SEM from four animals.

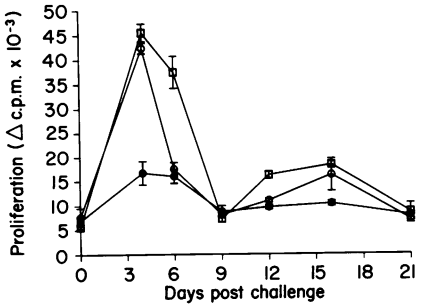

Figure 3. SSP-specific proliferation of draining LN cells from NV (□), RF (●) and LF (○) mice ($n = 4$), cultured at times after challenge infection on the right flank. Results are expressed as c.p.m. ± SEM for replicate microwells.

IFN-γ production by cells from RF mice postchallenge was reduced by 72·5% compared with the NV response. This result was reproduced in two repeat experiments (data not shown), with reductions of 96·2% (measured on day 4) and 95·7% (on day 6). IFN-γ release by cells from LF mice was variable between experiments. In the experiment shown in Fig. 1a (chosen to illustrate both postvaccination and postchallenge responses), the IFN-γ concentration in LF cultures was much reduced compared with NV values, whereas in two repeat experiments IFN-γ production by LF cells was equivalent to (96% and 100%) that of NV values.

Very little IL-2 was detected in any of the cultures (Fig. 1b). The highest level was found in LF cultures set up 6 days postchallenge. In NV cultures, production of this cytokine at a lower level was evident 2 days earlier (day 4), whilst no IL-2 was detected in RF cultures.

Peak IL-4 production by cells from NV mice was found 6 days after challenge (Fig. 1c). The timing of this response resembled the IL-4 response postvaccination (Fig. 1c and unpublished data), which clearly peaked later than day 4. In both groups of previously vaccinated mice (RF and LF), peak IL-4 release occurred earlier, at day 4. At this time, IL-4 production was greater for RF cells taken postchallenge than those taken postvaccination, indicating an anamnestic response. In repeat experiments, this peak IL-4 response in RF mice was consistently greater than that produced by NV cells. IL-4 production by cells from LF mice occurred consistently earlier than for NV cells, but the peak level was always below that obtained for RF mice, as shown in Fig. 1c.

IL-10 was produced maximally on day 4 postchallenge by cells from each group of mice (Fig. 1d). There was no evidence for an anamnestic response induced by previous vaccination in any of three repeat experiments. As shown in Fig. 1d, levels of IL-10 produced by RF cells were similar to those for LF cells and below those for NV cells.

Neutralization of IL-10 reduces IL-4 and restores IFN-γ production in RF LN cell cultures

Addition of anti-IL-10 mAb to cultures of LN cells taken 4 days after challenge resulted in a reduction of IL-10 levels to below 200 pg/ml in RF, LF and NV cultures (Fig. 4a). As seen in previous postchallenge experiments, IFN-γ production in the presence of antigen was greatly reduced in RF compared with

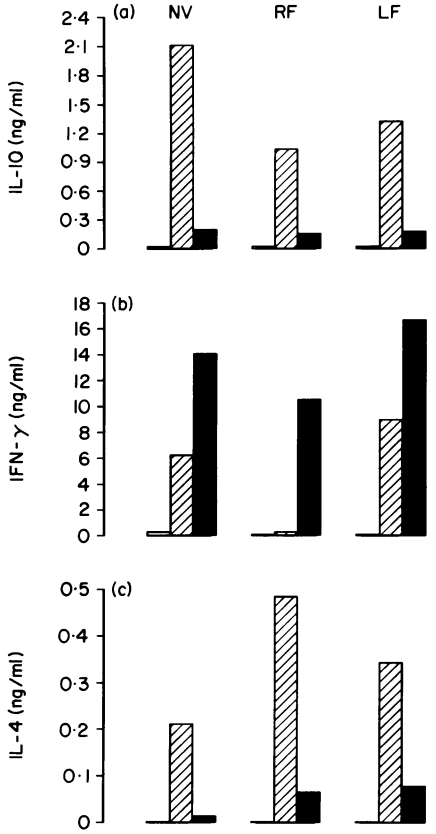

Figure 4. Production of (a) IL-10, (b) IFN-γ and (c) IL-4 by draining LN cells cultured 4 days after challenge infection of NV, RF and LF mice. Cells were incubated without antigen (□) or with 50 μg/ml SSP in the absence (□) or presence (■) of anti-IL-10 mAb.

NV cultures (Fig. 4b). In this experiment, LF cultures did not show a reduced concentration of IFN-γ compared with NV cultures. The effect of adding anti-IL-10 mAb was a striking increase in the level of IFN-γ in all three culture groups (Fig. 4b). Thus, in RF cultures, anti-IL-10 treatment restored the IFN-γ concentration from near-baseline to a level above that found in untreated NV cultures. Since inclusion of mAb also increased IFN-γ levels in NV and LF cultures, the restored IFN-γ level in RF cultures was still lower than that in the other two groups (Fig. 4b). A further effect of anti-IL-10 mAb was a reduction in the concentration of IL-4 to below 100 pg/ml in all culture groups (Fig. 4c). RF cultures showed the greatest reduction, since the level of IL-4 in untreated cultures was highest in this group (500 pg/ml; Fig. 4c).

Resistance of mice to challenge parasite infection

In the experiment illustrated in Fig. 1, where both RF and LF cells showed reduced IFN-γ production *in vitro*, both groups of mice were highly resistant to challenge infection: RF, 67·1% ($p < 0·001$); LF 66·8% ($p < 0·001$). In a second experiment, where IFN-γ production by RF cells was negligible but production by LF cells remained high (96% of the NV value), both groups of mice were again highly resistant: RF, 77·6% ($p < 0·001$); LF 79·3% ($p < 0·001$). In neither experiment was there a significant difference between the resistance of RF and LF mice ($p > 0·05$).

DISCUSSION

The initial observations made in this paper support and extend previous findings regarding events in draining LN following vaccination of C57BL/6 mice with irradiated cercariae of *S. mansoni*.[16,17] The results confirm that IFN-γ is produced abundantly during the first 7 days after vaccination. Previous studies have shown that this cytokine continues to be produced in significant quantities up to day 21[16,17] and plays a key role in the pulmonary effector response operating against challenge parasites.[12] Our finding that IL-4 and IL-10 are also produced during the first 7 days postvaccination suggests that, besides Th1 cells, significant numbers of Th2 cells are present in the LN, or that cytokine-secreting cells have a Th0 phenotype.

The co-existence of Th1 and Th2 responses raises the question of how or why the response in C57BL/6 mice becomes polarized towards Th1 domination. Following infection with *Leishmania major* it seems that, in C57BL/6 mice, a preferential down-regulation of IL-4 transcripts allows an IFN-γ/Th1 response to dominate.[21] Similar regulatory control may apply in response to *S. mansoni* cercariae in this mouse strain. Although in the present experiments IL-4 and IL-10 were produced by LN cells, their levels were presumably too low to depress IFN-γ production significantly *in vitro* or indeed *in vivo* (since the vaccinated mice were strongly immune to challenge).

It is worth noting that, by comparison with the vaccinating dose of 500 irradiated cercariae given to the RF group of mice, the challenge dose of only 200 normal cercariae given to the NV mice induced an equal level of IFN-γ, but greater production of IL-4 and IL-10. We have shown previously that 500 normal cercariae induce a similar peak level of IFN-γ production to equivalent numbers of irradiated cercariae.[16] These observations together suggest that irradiation may reduce Th2 antigenicity whilst preserving the Th1 antigenicity of living schistosomula and/or their proteins.

Given the dominant role of IFN-γ in the protective immune response induced by vaccination, an important question we wished to answer was whether the arrival of challenge parasites would boost IFN-γ levels, i.e. generate an anamnestic response. When previously vaccinated RF mice were challenged on the same site with normal cercariae, LN increased significantly in weight and, by inference, in cell number, compared with nodes from NV mice not previously vaccinated or LF mice vaccinated at a site remote from that of challenge. However, when cultured *in vitro*, LN cells from RF mice showed a profoundly lower level of proliferation and IFN-γ production than such cells from NV mice. Thus, far from inducing an anamnestic Th1 response to challenge parasites, the effect of vaccination was to anergize IFN-γ production, at least *in vitro*. Such T-cell unresponsiveness has been induced artificially in other *in vivo* and *in vitro* experimental systems. It is striking that both administration of high doses of soluble haptenated antigen *in vivo*[22] and stimulation of Th0 clones *in vitro* in the absence of costimulatory signals[23] result in a selective down-regulation of Th1 characteristics (IFN-γ, IL-2 production), but not of Th2 characteristics (IL-4 production). Thus, Th1 cells appear to be more easily anergized than Th2 cells.

In our study, neutralization of IL-10 *in vitro*, besides restoring IFN-γ production in RF cultures, enhanced IFN-γ production in NV and LF cultures. Thus, IL-10 was clearly a major cause of suppressed IFN-γ production in all three culture groups. A further effect of neutralizing IL-10, also observed in studies on Th phenotype development using the same antibody,[7] was a reduction of IL-4 levels, in tandem with restored production of IFN-γ. This observation, together with the finding that IL-4 production was enhanced in cultures of cells from vaccinated and challenged mice and, critically, peaked 3 days earlier than in control cultures, suggests an important role for IL-4 in inhibiting proliferation and IFN-γ production by Th1 cells. We know, however, that the levels of IL-4 produced by LN cells after challenge in C57BL/6 mice never approach those seen after vaccination in less resistant BALB/c mice (S. Anderson & R. A. Wilson, unpublished observations). Thus, IL-10 may be synergistic with IL-4 during the first 3 days after challenge infection in order to inhibit IFN-γ production. IL-4 and IL-10 are known to act in this way to inhibit *L. major*-specific DTH footpad swelling.[24]

An important question is whether the inhibitory effects seen in the present *in vitro* experiments also occur *in vivo* and are relevant to protective immunity. Hepatic portal perfusion revealed that resistance to challenge infection was similar in groups of RF and LF mice, even when IFN-γ production was down-regulated in LN cell cultures from RF but not LF animals. This result may imply that IFN-γ-producing memory T cells generated in the LN after challenge do not contribute to protective immunity. Th1 cells with the capability to secrete IFN-γ are clearly present in the lymph nodes, but the presence of IL-10/IL-4 inhibits the production of this cytokine. Since after IL-10 neutralization, RF cultures still produce less IFN-γ than NV cultures, there does not appear to be an anamnestic Th1 response in terms of proliferation and cytokine production. These observations could imply that the protective effector mechanism acting against lung schistosomula relies upon memory Th cells elicited by vaccination and is not much augmented by anamnestic responses to challenge. The fact that whole-body irradiation of mice just prior to challenge greatly reduces the circulating lymphocyte pool but has no effect on protection or pulmonary immune responses supports this idea.[25,26]

Alternatively, the anergy observed *in vitro* may not occur *in vivo*, once Th1 cells have left the LN and escaped the influence of Th2 cytokines. Since IL-10 acts on macrophages but not B cells,[27] the potency of this cytokine in controlling Th1 responses *in vivo* will be determined by the types of APC. For instance, if B cells were the predominant APC then the effect of IL-10 *in vivo* would be minimized, placing more emphasis on IL-4. There is evidence that B cells *per se*, acting as APC, drive proliferation of differentiated Th2, but not Th1 cells.[28] Furthermore, splenic $B220^+$ cells (B cells), primed by *S. mansoni* infection, can be potent producers of IL-10[29] and it will be essential, in future experiments, to determine which cells within the LN are contributing both to IL-10 and to IL-4 production. In this context, following exposure to irradiated cercariae, the B : T-cell ratio within LN increases fourfold for at least 35 days.[14] The pre-existence of abundant B cells may therefore bias the response stimulated by challenge parasite antigens towards a Th2 phenotype. In preliminary experiments (R. M. Pemberton & R. A. Wilson, unpublished data), we have observed that at day 6 postchallenge, the percentage of B cells in LN of RF mice is double that for NV mice. Further experiments are required to determine the exact timing of

this increase and to investigate the contribution of B cells in down-regulating Th1 responses after challenge infection.

In summary, results presented in this paper demonstrate that exposure of mice, via the same dermal site, to irradiated and then normal cercariae of *S. mansoni* results in a skewing of Th lymphocyte phenotype from a Th0/Th1 to a Th2 profile. In contrast, this phenomenon does not usually occur (only once in four experiments) when vaccination and challenge are applied to sites having independent lymphatic drainage. Although there appears to be no resulting impairment of resistance in our model, it will be important to determine if multiple vaccinations (>2) would generate Th2 responses to a degree that would lower resistance. The levels of resistance observed in the present paper were typical of both single and multiple vaccination studies in having an upper limit of around 75%. The restraining influence of IL-10 on IFN-γ production *in vivo* may be important in this respect, particularly after multiple exposures, a hypothesis testable in IL-10$-/-$ mice. In addition to the possible relevance for immunity following repeated natural exposure to *S. mansoni* cercariae, these results may have implications, generally, for multiple vaccination/boosting where repeated applications to dermal sites with common lymphatic drainage would be favoured in order to generate a Th2-type immunity. Alternatively, this practice would need to be avoided if a dominant Th1 response were the goal.

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